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Localization of Sarcolemmal Proteins to Lipid Rafts in the Myocardium

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Abstract

The localization of sarcolemmal proteins within the membrane can have a dramatic effect on excitation-contraction coupling. We examine the localization of the Na⁺-Ca²⁺ exchanger, the dihydropyridine receptor, and other proteins involved in excitation-contraction coupling in rat heart using biochemical and immunolocalization techniques. Specifically, we assess the distribution of proteins within the lipid raft fraction of the sarcolemma. We find that the distribution of proteins in lipid raft fractions is very dependent on the solubilization technique. A common technique using sodium carbonate/pH 11 to solubilize non-lipid raft proteins was inappropriate for use with sarcolemmal membranes. Use of Triton X-100 was more efficacious as a solubilization agent. A large majority of the Na⁺-Ca²⁺ exchanger, Na⁺/K⁺-ATPase, and plasma membrane Ca²⁺ pump are not present in lipid rafts. In contrast, most adenosine A₁ receptors and dihydropyridine receptors were in lipid raft fractions. Most of the adenosine A₁ receptors could be co-immunoprecipitated with caveolin indicating a localization to caveolae (a subclass of lipid rafts). In contrast, the dihydropyridine receptors could not be co-immunoprecipitated with caveolin. Most biochemical data were confirmed by high resolution immunolocalization studies. Using correlation analysis, only a small fraction of the Na⁺-Ca²⁺ exchangers colocalized with caveolin whereas a substantial fraction of dihydropyridine and adenosine A₁ receptors did colocalize with caveolin. The most pertinent findings are that the Na⁺-Ca²⁺ exchanger and the dihydropyridine receptor are in separate sarcolemmal subcompartments. These spatial relationships may be relevant for understanding excitation-contraction coupling.

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1. Introduction

Excitation-contraction (EC) coupling in cardiac muscle depends on precise communication among ion channels and transporters. EC coupling is initiated by depolarization of the sarcolemma. The depolarization induces a conformational change of the L type Ca^{2+} channel (or dihydropyridine receptor (DHPR)) leading to entry of Ca^{2+} from the extracellular environment. The elevation of Ca^{2+} triggers a release of sarcoplasmic reticulum (SR) Ca^{2+} through the ryanodine receptor of the junctional SR. The flux of Ca^{2+} into the cytosol induces contraction. Ca^{2+} is resequestered into the SR through an ATP-dependent Ca^{2+} pump of the longitudinal SR and simultaneously extruded from the cell through the Na^{+} - Ca^{2+} exchanger (NCX1). This allows for relaxation of the cardiomyocyte. These processes constitute the elementary Ca^{2+} flux events underlying cardiac E-C coupling [1].

The amplitudes and temporal relationships of Ca^{2+} fluxes are tightly controlled processes. Therefore, the spatial distribution of ion channels and transporters is essential to maintain efficient coupling. Thus, for example, the proximities of the DHPR, the ryanodine receptor, and the NCX1 are essential determinants of contractility.

At one time, lipids were thought to be homogeneously distributed in the membrane, and proteins were considered to be fluid within the membrane lipid environment. It is now clear, however, that the plasma membrane contains microdomains termed lipid rafts. These microdomains are enriched in cholesterol and sphingolipids [2]. Certain proteins reside in lipid rafts whereas others are excluded. This may serve to concentrate proteins at particular sites and may be significant in signaling processes.

Caveolae are a subclass of lipid rafts. Caveolae were first described by Palade in 1953 as flask-shaped uncoated invaginations on the surface of differentiated cells [3]. Caveolae are recognized to be plasma membrane compartments with distinct lipid and protein composition that regulate signal transduction [4]. Caveolae are present in most cell types including cardiomyocytes. Caveolae may comprise up to 25% of the total myocardial cell surface area [5]. A second subclass of lipid rafts is termed non-caveolar lipid rafts, based upon the absence of caveolin. Although less well characterized, non-caveolar lipid rafts are also present in the sarcolemma of cardiomyocytes. The co-existence of multiple raft populations within the cardiac sarcolemma provides a novel view of functional regulation based upon spatial organization.

Spatial and functional regulation of the Na^{+} - Ca^{2+} exchanger may be especially important because of the role of NCX1 in myocardial Ca^{2+} homeostasis. It has recently been suggested, based on biochemical methods, that NCX1 is localized to caveolae and specifically associates with caveolin-3, the main caveolin isoform of cardiomyocytes [6]. Because of the potential importance of this finding in understanding EC coupling, we re-investigated the membrane localization of NCX1.

Investigation of lipid rafts has benefited from the development of biochemical methods to isolate these membrane components. These methods take advantage of the distinctive lipid and protein compositions of lipid rafts. Lipid rafts are characterized by the presence of high levels of glycosphingolipids and cholesterol. These lipids tightly associate into a liquid-ordered phase and are intrinsically resistant to solubilization by non-ionic detergents such as Triton X-100. Therefore, a long-standing method for isolation of rafts uses solubilization with Triton X-100. In the presence of Triton X-100, lipid rafts remain intact and retain a low buoyant density. A more recent detergent-free method introduced by Song *et al.* [7] utilizes alkaline pH to disrupt membranes and has gained some popularity.

The present investigation utilizes both the detergent and detergent-free methods for the isolation of lipid rafts. We find that the apparent domain localization of membrane proteins is

highly dependent upon the solubilization method. In our hands, the detergent-free method is inappropriate for the isolation of lipid rafts from cardiac tissue. We also find that the Na⁺-Ca²⁺ exchanger is not associated with caveolae.

Lipid raft microdomains have been implicated as sites of assembly of signaling complexes. Therefore, an additional goal was to determine whether other sarcolemmal proteins involved in ion fluxes during EC coupling were associated with lipid rafts. Using biochemical and immunolocalization techniques, we find that the DHPR is localized to lipid rafts. The membrane localization pattern of sarcolemmal proteins could have important implications for cardiac EC coupling.

2. Materials and Methods

2.1. Materials

Sprague Dawley adult male rats were from Charles River. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Complete Mini EDTA-free protease inhibitors and protein A sepharose beads were from Roche and Sigma, respectively. Antibodies used for immunoblots were anti-caveolin from BD Transduction Laboratories, anti-adenosine A₁ receptor from Chemicon, anti-DHPR, anti-calsequestrin and anti-PMCA from Affinity Bioreagents and anti-Na/K-ATPase β 1 subunit from Upstate. The anti-Na/K-ATPase α 1 and α 2 antibodies were a kind gift from Dr. Alicia McDonough. The anti-NCX1 antibody was R3F1 [8].

2.2. Buffers

The Na₂CO₃ detergent-free buffer solutions contained 25 mM MES, 150 mM NaCl, 250 mM Na₂CO₃ and were adjusted to pH 11.0 with NaOH. The buffer for the detergent extraction with Triton X-100 contained 25 mM MES, 150 mM NaCl and 1% (w/v) Triton X-100 at a pH of 7.4. Immunoprecipitation (IP) buffer consisted of 10 mM Tris, 150 mM NaCl and 5 mM EDTA at pH 8.0. The IP lysis buffer consisted of the IP buffer with the addition of 1% Triton X-100 and 1% NP-40 (w/v). All solutions were made fresh, chilled on ice, and contained a cocktail of EDTA-free protease inhibitors.

2.3. Lipid raft isolation

Lipid rafts were isolated either by methods described by Song *et al.* [7], using Na₂CO₃ at pH 11 or by detergent solubilization with Triton X-100 as reviewed by Chamberlain [9]. Rats (180-200g) were anesthetized with Na-pentobarbital administered by intraperitoneal injection. Hearts were excised and rinsed in ice cold PBS with protease inhibitors. The cardiac tissue was finely diced and placed in 4 ml of the appropriate buffer according to the method utilized (either the Na₂CO₃ or Triton X-100 method). The tissue was then homogenized in three steps as follows: 1) By polytron (setting 5; Brinkman Instrument PCU-2-110), repeated 3 times for 20 s each, cooling 30 s between each round), 2) With a chilled dounce homogenizer (10 strokes), and 3) By sonication (Fisher 60 Dismembrator Sonicator), on ice, 3 times for 20 s with 30 s of cooling between each sonication round. The lysate was centrifuged at 1000 g for 10 min at 4° C. Supernatant was removed and adjusted to 40% (w/v) sucrose and placed at the bottom of a 12 ml ultracentrifuge tube. 4 ml of 30% sucrose were layered on top of the supernatant followed by 4 ml of 5% sucrose to form a discontinuous gradient. The gradient was then centrifuged at 125,000 g for 22 h at 4° C. Fractions (1 ml) were collected from the top of the gradient. Fractions were concentrated by precipitation with 10% TCA on ice for 30 min. Precipitates were collected by centrifugation for 15 min at 13,000 g and washed 3 times with ice-cold acetone. Pellets were dissolved in SDS-DTT sample buffer and sonicated. Samples

were then boiled for 5 min and analyzed by SDS-PAGE and immunoblot. All steps were performed at 4°C.

2.4. Immunoprecipitation

Protein A sepharose beads were washed in cold PBS and incubated with 1 µg of anti-caveolin antibody with rotation for 1 h at 4° C. The beads were washed three times with IP buffer and then incubated with canine cardiac sarcolemmal vesicles (100 µg/ml in 0.7 ml lysis solution) with rotation for 4 h at 4° C. Canine sarcolemmal vesicles were isolated as previously described [10]. After incubation, the beads were harvested and the supernatant was removed. The beads were washed three times with IP buffer and resuspended in SDS-DTT sample buffer. Samples were boiled for 5 min and analyzed by SDS-PAGE and immunoblot.

2.5. SDS-PAGE and immunoblots

Samples were separated on 7.5 or 12% polyacrylamide gels, transferred onto nitrocellulose, and stained for 5 min with Ponceau S dye to detect protein transfer. The nitrocellulose membranes were blocked in 5% non-fat milk in PBS for 1 h and incubated with primary antibody for 1 h at room temperature. Antibody dilutions were as follows: 1:10,000 for caveolin, 1:1,000 for NCX1, Na/K ATPase $\alpha 1$, $\alpha 2$ and β subunits, PMCA and calsequestrin, and 1:500 for DHPR. The blots were incubated for 1 h at room temperature with secondary anti-mouse or anti-rabbit antibodies diluted in 5% non-fat milk in PBS. The membranes were washed again and developed for chemiluminescence (Western Lightening Chemiluminescence, Pierce).

2.6. Cardiomyocyte isolation and fluorescence microscopy

Adult cardiomyocytes were isolated, fixed, and immunolabeled as previously described [14]. Cells were incubated with antibodies (Abs) against the following proteins: caveolin-1 (CAVg; BD Transduction Laboratories); caveolin-3 (CAV3; Santa Cruz); adenosine A_1 receptor (AD1R; Affinity Bioreagents); dihydropyridine receptors (DHPR; Alomone); and Na^+ - Ca^{2+} exchanger (NCX1; R3F1 [8]). Anti-CAV3 and -NCX1 Abs were anti-mouse monoclonal, while anti-CAVg, -AD1R and -DHPR Abs were anti-rabbit polyclonal. The specificity of Abs was tested by preadsorbing the antibodies with the corresponding antigenic peptides (10 µg antigen/µg antibody) overnight at 4°C prior to immunostaining. Secondary antibodies were Alexa 488 anti-rabbit IgG and Alexa 594 anti-mouse IgG₁ (2 µg/ml, Molecular Probes) or with Alexa 488 anti-mouse IgG (2 µg/ml, Molecular Probes) and Rhodamine Red™-X-conjugated affinity-purified donkey anti-rabbit IgG (3 µg/ml Jackson ImmunoResearch Laboratories, Inc.). As an additional control, samples were incubated with secondary antibodies alone which yielded no signals.

“Anti-caveolin-1 antibody” labels both CAV1 and CAV3 and we refer to it as anti-CAVgeneric (CAVg). Anti-caveolin-3 antibody was specific for CAV3 (Dr. L. Toro and Dr. A. Alioua, personal communication). As caveolin-1 is not thought to be present in cardiomyocytes, we attribute immunofluorescence generated by this antibody as being due to reactivity with caveolin-3. This is confirmed below by the identical fluorescence patterns obtained by the two antibodies. If any caveolin-1 is present in the myocytes, it co-localizes almost perfectly with caveolin-3.

2.7. Digital image processing and colocalization analysis

Image resolution was brought to the diffraction limit by deconvolution algorithms using 3D image stacks to restore the signal lost by diffraction to its original point source [12]. Images were 3-D blind deconvolved using Auto Deblur (AutoQuant) as previously described [13, 14]. To measure “colocalization” levels, 10-20 consecutive planes were analyzed in each cell.

The images were divided with a grid of equal squares ($2 \mu\text{m}^2$), and the correlation coefficient (CC) and $P_{\text{sign test}}$ were calculated for each square in the grid. The correlation coefficient (CC) between arrays A and B of images A and B can be calculated from $[\sum_N(A_i - A_{av}) \cdot (B_i - B_{av})] / [\sum_N(A_i - A_{av})^2 \cdot \sum_N(B_i - B_{av})^2]^{1/2}$, where: A_i and B_i are the intensities of pixel i in image A and B; A_{av} and B_{av} their average values; and N number of i . To quantify the degree of protein association, we divided the number of squares with positive CC values and $P < 0.05$ by the total number of squares. We refer to this quantity as Protein Proximity Index (PPI) as described by the following equation: $\text{PPI} = N_{(\text{CC} > 0; P < 0.05)} / N_{\text{total}}$, where $N_{(\text{CC} > 0; P < 0.05)}$ = number of squares with $\text{CC} > 0$ and $P_{\text{sign value}} < 0.05$ and N_{total} = total number of squares. Squares with a pixel value ≥ 0.25 of the maximum pixel value for the whole image, A or B, are selected giving a *vectorial* CC histogram and PPI. When squares of image A are selected, PPI is referred as $A \rightarrow B$, and vice versa; see details in the legend to Fig. 4 [14,15].

3. Results

3.1. Lipid raft isolation

Lipid rafts were isolated from fresh cardiac tissue using 5-40% sucrose gradient centrifugation. A hallmark of lipid rafts is high buoyancy due to a lipid composition rich in cholesterol and glycosphingolipids which confers a resistance to detergent solubilization. This provides the rationale for the common use of Triton X-100 in the isolation of lipid rafts. After detergent solubilization, the lipid rafts, with their associated proteins, will float up to a lower density sucrose (approximately 10-18% sucrose). Conversely, components of the solubilized membranes and other cellular constituents will remain in higher density sucrose (30-40% sucrose). Another popular method for isolation of lipid rafts uses Na_2CO_3 at pH 11 for membrane solubilization [7]. Although the rationale for this approach is not clear, we used the Na_2CO_3 /pH 11 approach in our initial experiments.

Figure 1A demonstrates that with both methods for isolating lipid rafts, a large majority of the protein (~98%) resided in the higher density fractions (fractions 9-12) of the sucrose gradient. The lower density fractions (fractions 1-8) contain only a small amount of protein. The putative lipid rafts (caveolae and non-caveolar lipid rafts) fractionate to the lower density fractions and exclude the majority of other cellular proteins. Fig. 1B shows the sucrose concentration of each fraction.

The left panel of Figure 2 depicts the relative migrations of membrane-bound protein markers obtained using the Na_2CO_3 /pH 11.0 method for the isolation of rafts. As expected, caveolin, a marker for caveolae, fractionates to the less dense fractions (centered in fraction 5). These lighter fractions are expected to be composed exclusively of lipid rafts. Surprisingly, however, all other integral membrane proteins, NCX1, DHPR, PMCA, Na^+/K^+ -ATPase subunits, and adenosine A_1 receptor, also appeared almost exclusively in the lower density fractions. The fractionation patterns would suggest that these proteins are all associated with caveolae or non-caveolar lipid rafts. The cardiac adenosine A_1 receptor is a known caveolae-associated protein [16]; migration with lipid rafts was expected. As a negative control, calsequestrin, a soluble protein that does not associate with membranes, remained at the bottom of the gradient. Three other membrane proteins (ankyrin, the SR Ca^{2+} pump, and the K^+ channel Kv4.3) also appeared exclusively in putative lipid raft fractions (data not shown). These unexpected results made us suspicious of the ability of Na_2CO_3 /pH 11 to solubilize membranes. We investigated another method to isolate lipid rafts.

We turned to a standard detergent-based method utilizing Triton X-100 for lipid raft isolation. The results with Triton X-100 (right panel of Figure 2) contrast markedly with migration patterns achieved utilizing the Na_2CO_3 /pH 11 method. As expected, caveolin and the adenosine A_1 receptor migrated to the lower density fractions. PMCA and the $\alpha 1$, $\alpha 2$ and β subunits of

the Na⁺/K⁺-ATPase, however, remained in the higher density fractions. This is in agreement with published data. With the apparently more selective Triton X-100 method, NCX1 remained in the higher density fractions while the DHPR fractionated to the lighter fractions. Importantly, this demonstrates that NCX1 is not localized to lipid rafts as previously reported [6] and suggests that the DHPR is localized to lipid rafts. Similar data were obtained when the detergent-to-protein ration was either decreased or increased by two-fold (data not shown) indicating that we were not using an inappropriate concentration of detergent.

3.2. NCX1 and DHPR are not localized in caveolae

To further investigate the possible association of NCX1 and DHPR with caveolae, we performed co-immunoprecipitation experiments. Caveolin antibody was bound to protein A sepharose beads and the beads were incubated with solubilized sarcolemmal vesicles. As demonstrated in Figure 3, the majority of the caveolin from the sarcolemmal vesicles bound to the anti-caveolin antibodies on the beads. There was no detectable protein binding to beads not conjugated with the caveolin antibody. The adenosine A₁ receptor, used as a positive control, also bound to the caveolin-antibody-bead complex. However, neither the NCX1 nor the DHPR protein co-immunoprecipitated with caveolin. This demonstrates that NCX1 and DHPR are not associated with caveolin and are not localized in caveolae (though some apparently conflicting immunolocalization data are presented below). As demonstrated in Figure 2, DHPR co-fractionates with lipid rafts but, since DHPR does not associate with caveolin, DHPR appears to be associated with non-caveolar lipid rafts. We have not carried out the reciprocal co-immunoprecipitation experiments in which NCX1 or DHPR antibodies were immobilized on the sepharose beads.

3.3. Quantification of protein proximity (colocalization)

We recently published on the use of a Protein Proximity Index (PPI) [14,15]. The PPI is based on the quantification of the correlation coefficient (CC) of discrete areas (2 μm²) of the whole image (Fig. 4), and their CC statistical significance (P_{sign test}) [17] as a measure of “colocalization” (see “Materials and Methods”). If two proteins associate, the intensity staining landscape of the two images will have a positive CC. If two proteins are localized in distinct compartments the result will be a negative CC, and if the proteins are labeled randomly (diffuse non-structured pattern), CC will tend to zero. CC values range between -1 and 1.

Figure 4 exemplifies this analysis. The images are of cardiomyocytes immunostained for caveolin-g (CAVg; panel A), caveolin-3 (CAV3; panel B), the overlay of CAV3 and CAVg (panel C), and after a x axis-shift of the CAVg plane (panel D). Both the CAV3 and CAVg antibodies will stain caveolin-3 present in cardiomyocytes. Regions depicted in squares are shown in (panels a-d) at higher magnification. A 2×2 μm grid was applied to the whole images in panels A and B to measure the CC in each square. 2D correlation pixel intensity plots are illustrated for the square marked with an asterisk before (panel E) and after a 6 pixel shift (panel F). CC values were 0.94 and -0.48 before and after the pixel shift. The frequency distribution of the CC and the relationship between the statistical significance (P_{sign value}) and CC of the individual squares in the grid for CAVg→CAV3 are shown in panels G and H). In panel H, each data point is the P_{sign value} and CC of each square; the highlighted area marks the squares with CC ≥ 0 and P_{sign value} < 0.05. PPI values were 0.84 and 0.87 for CAV3→CAVg and CAVg→CAV3, respectively. As an example of “non-colocalization”, we repeated the analysis after a 0.228 μm (6 pixels) x-shift of the CAVg plane (panels I and J). The CC histogram and P_{sign test}-CC plots for CAVg→CAV3 show a large increase in the number of squares with a negative CC and also fewer squares with CC > 0 and P_{sign value} < 0.05, with PPI = 0.02.

To validate the PPI measurement and to examine its spatial sensitivity, we measured the changes in PPI values as function of the pixel shift of one plane in the x direction (in this case

of CAVg). Figure 4K shows a dramatic reduction of PPI as a function of pixel shift. This provides a direct indication of the sensitivity of the method as a function of x,y resolution when using a pixel sample size much lower than the microscope resolution (i.e., 0.038 $\mu\text{m}/\text{pixel}$). The curve also shows that PPI measurements can resolve the degree of protein proximity with position precision values of $\sim 50\text{-}100\text{ nm}$. **3.4. The $\text{Na}^+\text{-Ca}^{2+}$ exchanger and caveolin do not colocalize.**

We next applied this analysis to isolated rat myocytes labeled with antibodies to NCX1 and caveolin (Fig. 5). Both NCX1 and caveolin strongly stained both surface and transverse tubular sarcolemma (Fig. 5 A, a, B, b). Superficially, the labeling patterns are similar. Merging of the staining, however, visually demonstrates that caveolin and NCX have quite distinct locations on the sarcolemma (Fig. 5 C, c). The lack of colocalization is quantitated with the mostly negative CC frequency distribution (Fig. 5D) and by the relationship of the P_{sign} value and CC of the individual squares (Fig. 5E). The difference between these data and those shown in Fig. 4G and H is striking. As before, the protein proximity index diminishes rapidly upon shifting one plane in the x axis (Fig. 5F). In this case though, the initial PPI value is 0.06 instead of 0.8 when CAVg and CAV3 are compared (Fig. 4K). This indicates that only a very small fraction of the NCX1 proteins may colocalize with caveolin. Another study has also examined the proximity of caveolin and NCX1. Scriven *et al.* [18] found no significant colocalization in the cell interior consistent with our results. These authors did observe some colocalization at the cell surface, and we have no explanation for this apparent discrepancy.

3.5. Colocalization of the adenosine receptor with caveolin

As a positive control, we applied our immunolocalization techniques to the adenosine A_1 receptor and caveolin (Fig. 6). The adenosine receptor is known to be present in caveolae [16] consistent with our biochemical measurements (Figs. 2 and 3). In this case, the adenosine A_1 receptor colocalized with caveolin by visual analysis (Fig. 6A-C, a-c) and by detailed correlation analysis. This is verified by the CC histogram and by the relationship of the statistical significance to the CC (Fig. 6D and E). The reduction of the PPI (0.7) by a small shift in the plane of the x axis validates the association of the adenosine A_1 receptor and caveolin. Similar data were obtained for DHPR and caveolin-3 (not shown; but see Fig. 7)

3.6. Colocalization summary

The results of our colocalization analyses are displayed in Fig. 7. Also included in this summary are data on the proximity of the DHPR to caveolin-3. Analysis indicates an intermediate level of colocalization; the implication of this result is discussed below.

4. Discussion

The function of cardiac sarcolemmal proteins will be strongly influenced by their specific location within the membrane and by the presence of other proteins. We have begun to address these issues for the $\text{Na}^+\text{-Ca}^{2+}$ exchanger. One impetus for our study was a report on the localization of NCX1 in lipid rafts and on a specific interaction with caveolin-3 [6]. We initially used the sodium carbonate/pH 11 technique [7] to prepare lipid rafts and were able to reproduce the findings of Bossuyt *et al.* [6]. That is, NCX1 protein was recovered from nonsolubilized membranes (putative lipid rafts) after sucrose gradient fractionation (Fig. 2). Subsequent experiments found that all integral membrane proteins appeared to be primarily in the lipid raft fraction and we became skeptical of the technique. In fact, the sodium carbonate/pH 11 approach has no rationale and its efficacy has never been verified. There is no precedent describing the successful use of sodium carbonate at pH 11 to solubilize membranes. In fact, alkaline pH has been used for many years to remove peripheral proteins from membranes leaving the membranes otherwise intact [19]. In our hands, the sodium carbonate/pH 11

approach does not work for the preparation of lipid rafts from myocardium. Foster *et al.* [20] also note shortcomings in the sodium carbonate/pH 11 techniques.

We turned to the more conventional use of Triton X-100 to prepare lipid rafts. This preparation relies on the resistance of lipid rafts to solubilization due to their distinct lipid composition and has proven useful in numerous studies. In this case, we find that the $\text{Na}^+\text{-Ca}^{2+}$ exchanger is excluded from lipid raft fractions (Fig. 2). This is true also of two other sarcolemmal ion transporters: the $\text{Na}^+/\text{K}^+\text{-ATPase}$ and the plasma membrane $\text{Ca}^{2+}\text{-ATPase}$. Another study [21] assigned Na^+ pump subunits to lipid rafts based on the sodium carbonate/pH 11 technique but did note that Triton X-100 solubilized the Na^+ pump from sarcolemmal membranes. Although the Na^+ pump subunits, PMCA, and NCX all were in separate fractions than caveolin, it was striking that these proteins did not fractionate in the same way. For example, in Fig. 2, right panel, NCX and the $\alpha 1$ subunits of the $\text{Na}^+/\text{K}^+\text{-ATPase}$ were primarily in fraction 9, whereas the PMCA and the β subunit of the Na^+ pump were in more dense fractions. Perhaps some sarcolemmal components, such as the cytoskeleton, are effecting fractionation even after solubilization.

Immunostaining demonstrates that both NCX and caveolin have a punctate localization pattern throughout the sarcolemma including the transverse tubules (Fig. 5). The amount of overlap in the location of the two proteins, however, is minimal. The lack of colocalization of NCX and caveolin is vividly displayed in Fig. 5c. Discrete “hotspots” of NCX and caveolin alternate in the image. NCX is not necessarily excluded completely from caveolae, however.

Quantitative analysis indicates that a small fraction of NCX may colocalize with caveolin. The validity of the analysis is confirmed by the precipitous decline in the PPI as the plane of one of the images is shifted by a small distance in the x axis (Fig. 5F). A small fraction of NCX in caveolae could explain the co-immunoprecipitation results of Bossuyt *et al.* [6].

In contrast, we find that the DHPR and the adenosine A_1 receptor co-fractionate with caveolin on sucrose gradients after detergent solubilization. This result was expected for the adenosine A_1 receptor as this receptor is a known resident of caveolae in cardiomyocytes [16]. The presence of DHPRs in caveolae has previously been inferred from the effects of methyl- β -cyclodextrin (a reagent that sequesters cholesterol and disrupts lipid rafts) on Ca^{2+} sparks [22]. We now provide direct biochemical data that DHPRs are components of lipid rafts. We found that the adenosine A_1 receptor very efficiently co-immunoprecipitated with caveolin (Fig. 3) consistent with the protein being a constituent of caveolae. The DHPR, however, did not co-immunoprecipitate with caveolin. The implication would be that the DHPR is localized to non-caveolar lipid rafts. This would explain fractionation of the DHPR to lipid rafts and the apparent lack of a physical interaction with caveolae.

The assignment of DHPRs to non-caveolar lipid rafts is complicated, however, by our colocalization analysis (Fig. 7). A significant amount of DHPR colocalized with caveolin-3. One interpretation of this apparent discrepancy is that some of the DHPRs are indeed within caveolae but that this localization is not dependent on a direct interaction with caveolin. Alternatively, the DHPR may bind to caveolin, but the interaction is not sufficiently strong to survive the co-immunoprecipitation process. The overall data do indicate that the cardiac DHPR is a component of lipid rafts, and a significant fraction of the DHPRs may be within caveolae. This is an interesting point as many of the DHPRs are known to localize at junctional sites between the SR and the sarcolemma. Perhaps a substantial fraction of the junctional sarcolemma is lipid rafts; this will require further investigation.

In summary, we find that the majority of $\text{Na}^+\text{-Ca}^{2+}$ exchangers are not in caveolae or lipid rafts. In contrast, the DHPR may have a special lipid environment relevant to EC coupling.

Further investigation is needed to determine if pathological conditions can alter the localization of sarcolemmal proteins with respect to one another.

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Figure 1. Protein and sucrose distribution after gradient fractionation

Rat cardiac tissue was homogenized with either Na₂CO₃ or 1% Triton X-100 and fractionated on a 5-40% sucrose density gradient. (A) Protein concentrations of fractions taken from either the Na₂CO₃ or Triton X-100 gradients after centrifugation. (B) Average sucrose concentrations of the fractions (n = 3) as determined by refractometer. Fraction 1 is from the top of the gradient (the lightest fraction) while fraction 12 is from the bottom.

Figure 2. Protein associations with lipid rafts

Proteins involved in ion transport were analyzed by immunoblot to determine localization to lipid rafts after fractionation. The left panel is a representative experiment using Na_2CO_3 , pH 11.0 while the right panel is representative of fractions isolated using 1% Triton X-100. All distributions were determined at least three times.

Figure 3. NCX1 and DHPR are not associated with caveolae as determined by co-immunoprecipitation

Protein A sepharose beads with and without conjugated anti-caveolin antibody were incubated with solubilized sarcolemmal vesicles. After incubation, proteins remaining in the supernatant (Sup) and the proteins bound to the beads (Beads) were collected and analyzed by immunoblot.

Figure 4. Discrete correlation analysis of “colocalization”

(A-D) Single confocal sections after 3D deconvolution of cardiomyocytes immunostained for caveolin-g (A, CAVg, red), caveolin-3 (B, CAV3, green), the overlay of CAV3 and CAVg (C) and after 0.228 μm (6 pixels) x-shift of the CAVg plane (D). Panels a-d are the inset squares in the upper panels at higher magnification showing a $2 \times 2 \mu\text{m}$ grid that was applied to the whole images in panels A and B to measure the correlation coefficient (CC) in each square to construct the CC frequency distribution histograms. Panels E and F illustrate 2D pixel intensity plots for the square marked with an asterisk before (E) and after a 6 pixel shift (F). The grey highlighted area outlines the data points below threshold (0.2 of the square maximum intensity) that are not used for the CC calculation. (G) Histogram showing the frequency distribution of

CC values for CAV3→CAVg for squares selected in the CAV3 image with a pixel value ≥ 0.2 the maximum pixel value of the global image. **(H)** Relationship between the statistical significance ($P_{\text{sign value}}$) and CC of the individual squares in the grid for CAV3→CAVg. Each data point in the plot is the $P_{\text{sign value}}$ and CC of each square. The horizontal red dashed line marks $P = 0.05$. The highlighted area marks the squares with $CC > 0$ and $P < 0.05$. Panels **I** and **J** illustrate the CC histogram and $P_{\text{sign test}}$ -CC plots for CAV3→CAVg after $0.228 \mu\text{m}$ (6 pixels) x-shift of the CAVg plane. **(K)** PPI vs. pixel shift curve showing the PPI reduction as a function of the pixel shift in the x axis of the CAVg plane. Images were acquired at $0.038 \mu\text{m}$ per pixel and every $0.1 \mu\text{m}$ in the z axis.

Figure 5. The Na^+ - Ca^{2+} exchanger (NCX1) does not significantly associate with caveolin-g in rat heart myocytes

(A,a-C,c) Deconvolved single confocal plane immunostained with anti-NCX1 (green) or anti-CAVg (red) antibodies. NCX1 and CAVg labeling show a distinct cluster pattern along the sarcolemma and tubular membranes that do not significantly colocalize as demonstrated in the overlay (C,c). Panels a-c are the regions within the squares in panels A-C at higher amplification; the green and red arrows illustrate the lack of intensity correlation of NCX and CAVg labeling at the sarcolemma. The degree of association was quantified by measuring PPI values in 21 sequential confocal sections acquired every 0.1 μm in the z-axis. (D) CC frequency distribution histogram. (E) Relationship between the statistical significance ($P_{\text{sign value}}$) and CC of the individual squares. (F) Reduction of PPI values as a function of the shift of one plane in the x axis. Similar data were obtained for CAVg vs. NCX. Stacks of single confocal planes of 1024 $\times$ 1024 pixels were acquired every 0.1 μm (z-axis) at 0.058 $\mu\text{m}/\text{pixel}$. Images were 3D deconvolved to reach the diffraction limit of ~ 250 nm. To calculate PPI values a grid of 1.97 $\times$ 1.97 μm per square was used and the CC was calculated after applying a threshold of 0.25 the square maximum pixel intensity.

Figure 6. The adenosine A₁ receptor (AD1R) associates with caveolin-3 (CAV3) in rat heart myocytes

(A,a-C,c) Deconvolved single confocal plane immunostained with anti-AD1R (red) and anti-CAV3 (green) Abs. AD1R and CAV3 labeling shows a similar distinct cluster pattern along the sarcolemma and tubular membranes that significantly colocalizes as demonstrated in the overlay (C,c). Panels a-c are the regions in squares in panels A-C at higher amplification. The degree of association was quantified by measuring PPI values in 21 sequential confocal sections acquired every 0.1 μm in the z-axis where the correlation coefficient (CC) was measured in a grid of 1.97×1.97 μm squares (9232). (D) CC frequency distribution histogram. (E) Relationship between the statistical significance ($P_{\text{sign value}}$) and CC of the individual squares. (F) Reduction of PPI values as a function of the shift of one plane in the x axis validating the association between AD1R and CAV3. Similar data were obtained for CAV3 vs. AD1R. Data acquired and analyzed as described in Fig. 5 and in “Materials and Methods”.

Figure 7. PPI values

The bar graph illustrates lack of significant association between NCX1 and CAVg and a high association level between AD1R and DHPR with CAV3. Values are mean±SE from 8-15 cells for each condition. ** $P < 0.01$ between NCX1→CAVg; * $P < 0.05$ between DHPR→CAV3 and CAV3→CAVg.