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Characterization of Caveolae from Rat Heart: Localization of Postreceptor Signal Transduction Molecules and Their Rearrangement After Norepinephrine Stimulation

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Abstract Caveolae are plasma membrane subcompartments that have been implicated in signal transduction. In many cellular systems, caveolae are rich in signal transduction molecules such as G proteins and receptor-associated tyrosine kinases. An important structural component of the caveolae is caveolin. Recent evidence show that among the caveolin gene family, caveolin-3 is expressed in skeletal and cardiac muscle and caveolae are present in cardiac myocyte cells. Both the ANP receptor as well as the muscarinic receptor have been localized to the caveolae of cardiac myocytes in culture. These findings prompted us to conduct a further analysis of cardiac caveolae. In order to improve our understanding of the mechanisms of signal transduction regulation in cardiac myocytes, we isolated cardiac caveolae by discontinuous sucrose density gradient centrifugation from rat ventricles and rat neonatal cardiocytes. Our analysis of caveolar content demonstrates that heterotrimeric G proteins, p21*ras* and receptor-associated tyrosine kinases are concentrated within these structures. We also show that adrenergic stimulation induces an increase in the amount of diverse α- and β-subunits of G proteins, as well as p21*ras,* in both in vivo and in vitro experimental settings. Our data show that cardiac caveolae are an important site of signal transduction regulation. This finding suggests a potential role for these structures in physiological and pathological states. J. Cell. Biochem. 77:529–539, 2000.

Key words: caveolae; caveolin-3; heart; signal transduction; myocytes; adrenergic

Caveolae, also known as plasmalemmal vesicles, are 50–100-nm membrane domains that represent a subcompartment of the plasma membrane. Caveolae are thought to play an important role in most cell types [Anderson et al., 1993]. In capillary endothelial cells, caveolae have been implicated in transcytosis of certain macromolecules, such as albumin and low-density lipoprotein (LDL) [Lisanti et al., 1994] and, more recently, in the functional regulation of NO synthase (eNOS) [Feron et al., 1996; Garcia-Cardena et al., 1997; Ju et al., 1997; Michel et al., 1997]. Caveolar structures in adipocytes are formed during terminal differentiation and are a hallmark of functional adipocytes [Scherer et al., 1994]. Glucose transporter 4 increases in caveolae during terminal differentiation and is compartmentalized together with caveolin 1 [Kandror et al., 1995]. Caveolae are a site of enriched signal transduction molecules in neurons. β-Amyloid precursor also is associated with caveolae-like structures [Bouillot et al., 1996] in neurons.

Abbreviations used: NE, norepinephrine; ANP, atrial natriuretic peptide; eNOS, nitric oxide synthase; M2AchR muscarin 2 acetylcholine receptors; PtdInsP2 phosphatidylinositol bisphosphate, ANF, atrial natriuretic factor.

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The main structural protein of caveolae is caveolin, a protein of approximately 22 kDa [Anderson et al., 1993]. Caveolin exists as a high-molecular-mass homo-oligomer that has the ability to interact with other oligomers and to self-associate into caveolae-like structures in vitro [Sargiacomo et al., 1995]. It has been shown that caveolin can interact with proteins involved in signal transduction, such as G proteins and p21*ras* [Chun et al., 1994; Li et al., 1995, 1996; Mineo et al., 1996; Sargiacomo et al., 1993; Song et al., 1996]. In vitro assays showed that the association between the G protein α-subunits, including Gαs, Gαo, Gαi2, and caveolin can functionally suppress the basal activity of the α-subunits by inhibiting their GDP-GTP-exchange activity [Li et al., 1995]. Both G proteins α and βγ-subunits are concentrated highly in preparations of caveolae membranes purified from diverse sources [Chun et al., 1994; Li et al., 1995; Sargiacomo et al., 1993; Song et al., 1996]. In a similar way, *ras* interacts in a regulated fashion with the portion of the caveolin oriented toward the cytoplasm [Li et al., 1996; Song et al., 1996]. Wild-type *ras*, but not constitutively activated H-*ras*, can interact with caveolin [Song et al., 1996], suggesting a physiological role for *ras*-caveolin association. Similarly, caveolin can associate with *src* and *src*-like tyrosine kinases, negatively affecting their function in vitro [Li et al., 1996].

Three isoforms of caveolin have been cloned and characterized to date. While the expression of caveolin 1 and 2 is broad [Scherer et al., 1997], caveolin 3 is present mainly in skeletal and cardiac muscle cells [Song et al., 1996]. Caveolin 3 levels increase during terminal differentiation in C2C12 skeletal muscle cells [Scherer et al., 1997]. It also has been shown that caveolin 3 is localized with T tubules in skeletal muscle. It has been suggested that caveolin 3 might play a role in the formation of these sarcolemma invaginations during development [Parton et al., 1997].

Caveolae in cardiac muscle were identified ultrastructurally more than two decades ago [Gabella, 1978; Levin and Page, 1980]. Caveolae were recognized as a site of localization of atrial natriuretic peptide in rat atrial cardiocytes [Page et al., 1994]. Atrial natriuretic receptor and caveolin 3 colocalize in the same membrane invaginations [Doyle et al., 1997]. Other recent evidence points toward a role for caveolae as an active site of signal transduction in heart tissue. eNOS has been shown to coimmunoprecipitate with caveolin 1 and 3 in endothelial and cardiac cells respectively [Feron et al., 1996]. Caveolin 3 negatively regulates the activity of eNOS [Garcia-Cardena et al., 1997; Ju et al., 1997; Michel et al., 1997]. The interaction between eNOS. and caveolin is regulated by Ca^{2+}/calmodulin [Michel et al., 1997]. It has also been shown that muscarine 2 acetylcholine receptors (M2AChR) colocalize with eNOS after agonist stimulation in caveolae, suggesting a potential regulation of eNOS by M2AchR within the caveolar compartment [Feron et al., 1997]. Moreover, levels of caveolin decreased in the myocardium of pressure-overload rats [Oka et al., 1997].

These findings strongly support a role for caveloae in physiological and pathological heart states. In an attempt to clarify this possibility, we identified some components of the caveolae from heart tissue, by isolating caveolin-rich membrane domains from rat ventricles and from primary culture of rat cardiomyocytes. In order to determine whether the caveolar content changes after hormone stimulation and to assess whether cardiac caveolae are a dynamic compartment, we analyzed the content of cardiac caveolae before and after norepinephrine stimulation. Our data suggest that caveolae are an active site of signal transduction regulation in the heart and demonstrate that their molecular components change dramatically after receptor stimulation.

MATERIALS AND METHODS

Myocyte Culture Preparation

Neonatal ventricular myocytes were cultured as previously described [Sean et al., 1988], with some modifications. Hearts from 1- to 3-day-old Wistar rats were removed, and the ventricles were dissected and digested with collagenase type II (108 U/ml, Worthington Biochemical) and with pancreatine (0.6 mg/ml, Gibco-Life Sciences) for 20 min at 37°C. Cells were collected by centrifugation and resuspended in newborn calf serum (Gibco-Life Sciences). These steps were repeated until the ventricles were completely digested. The cells from the digestions were collected and subjected to centrifugation through a discontinuous Percoll gradient (Sigma Chemical Co.). The band containing myocytes was collected and

used as a source of purified cells. These cells were plated on gelatin-coated 100-mm plastic tissue culture plates at a density of $2x10^6$ in culture media consisting of a 4:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium (DMEM) and medium 199 supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS) (all media were from Gibco). These cells were maintained at 37°C in humidified air with 10% CO_2. Cultures were then shifted, 24 h after plating, to a medium containing a mixture 1:1 (vol/vol) of HS and FBS to a final concentration of 10%. To kill fibroblast and endothelial cells, cytosine arabinoside (Ara-C, 10 μM; Sigma) was added. The percentage of myocytes was greater than 90%, as determined by phalloidin staining of actin filaments. All experiments began 48 h after plating. At this time the cells were stimulated with norepinephrine (10 μM; Sigma) for 4 min. At the end of the incubation time, cells were placed on ice, scraped in ice-cold phosphate-buffered saline (PBS) and collected in Eppendorf tubes. After a brief high-speed centrifugation, the cell pellets were stored at −80°C. Many cell preparations were pooled and approximately 5×10^7 cells were used per preparation of caveolae.

Surgical Procedures and Arterial Blood Pressure Measurements

For in vivo experiments, 12-week-old male Wistar rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Polyethylene catheters (PE 50,Clay Adams) were inserted into left femoral vein and left femoral artery for infusions and arterial blood pressure measurements. Arterial blood pressure was measured with a pressure transducer (Statham P23db), and the data were recorded on a Gould Polygraph (Windograf) at a speed of 1mm/sec. After a 15-min stabilization period, a 15-min steady-state arterial blood pressure was recorded, then an intravenous (i.v.) bolus of vehicle (NaCl 0.9%, n = 8) or norepinephrine (1 μg/kg; n = 8) was injected. The animals were killed 3 min after the stimuli with a lethal dose of pentobarbital. Subsequently, the hearts were rapidly excised, weighed, and frozen in liquid N_2. Rats aged approximately 10 weeks were killed after anesthesia; excised hearts were snap-frozen in liquid N_2 and stored at −80°C.

Isolation of Caveolin 3- Rich Microdomains from Hearts and Cardiocytes

Caveolin 3 membrane domains were extracted from rat heart by a modification of a procedure previously carried out with lung tissue, [Lisanti et al. 1994] or from primary cultures [Lisanti et al. 1993]. Frozen rat left ventricles (340–360 mg wet weight) were mechanically ground for 5 min at medium frequency by a metal impactor in Spex/6700 Freezer/Mill apparatus (Glen Creston Ltd., Garden Stanmore, Middle, UK). Typically, 1 ground ventricle was resuspended in 3 ml MES buffered-saline (MBS; 25mM MES pH 6.5, 0.15 M NaCl) containing 1% Triton X-100 or, alternatively, 0.5 M sodium bicarbonate pH 11, using a detergent-free procedure for purification of caveolae. Heart samples were further processed with 10 strokes of a tight-fitting Dounce homogenizer (this was the only grinding treatment for cardiocytes). One-fourth of the tissue homogenate (0.75 ml) was adjusted to 40% sucrose by the addition of equal volume of 80% of sucrose in MBS or sodium bicarbonate and placed at the bottom of an ultracentrifuge tube. An overlay of 1.5 ml each of 5% and 30% sucrose buffered with MES/Na_2CO_3 was added to form a final three-step discontinuous gradient. After equilibrium density centrifugation (14–17 h) at 45,000 rpm in a SW60 rotor (Beckmann Instruments), a sharp band of 30–5% sucrose interface was observed. From the top of the gradient, 0.375 ml /12 fractions were collected and analyzed for an equal amount of protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting

Total protein concentrations in the extracts from both treated and untreated rat ventricles were measured by the Bradford method (Bio-Rad) and separated by electrophoresis in a SDS-polyacrylamide gel with a percentage of acrylamide dependent on the molecular weights of the proteins under investigation. In this study, 20 μl of protein recovered from 12 fractions was denatured by boiling in 2× Laemmli sample buffer and run on polyacrylamide gels. Proteins within the polyacrylamide gel were transferred to a PVDF membrane (Millipore) in CAPS buffer (10 mM CAPS, 20% methanol, pH 11) for 60 min at 70 V. The membrane

was blocked in 5% milk in TBS-T buffer (2 mM Tris, 13.7 mM NaCl, 0.1% Tween-20, pH 7.6) and then washed in TBS-T. Primary antibodies were incubated with the membrane in 3% milk for 1 h and then washed in TBS-T. The membrane then was incubated with antisera coupled with horseradish peroxidase (HRP) (Amersham Life Science) for 1 h and washed in TBS-T. The presence of secondary antibody bound to the membrane was detected using the enhanced chemiluminescence (ECL) system (Amersham-Life Science). The following primary antibodies were used: anti-Gα_s, anti-G α_q, anti-Gα_o, anti-G$_\beta$, *lyn*, and *fgr* purchased from Santa Cruz Biotechnology. Caveolin 1, Caveolin 3, p21*ras*, and *grb2* antibodies were purchased from Transduction Laboratories. Blot bands corresponding with different proteins were quantitated by densitometric scanning (Molecular Dynamics). The intensity of the bands was measured in arbitrary units and was reported on a blot as mean ±SD, taking into account three different experiments.

RESULTS

Isolation of the Caveolae from Rat Ventricles

Caveolae were isolated from rat ventricles as described under Materials and Methods. Sucrose-gradient fractions were analyzed by Coomassie blue staining and by spectophotometric analysis (Fig. 1a,b). An approximately fourfold difference was present in fraction 5, as compared with fraction 11. To assess the distribution of caveolin 3, equal volumes were subjected to Western blotting analysis by probing with an anti-caveolin 3 antibody. Results showed that fraction 5 had the highest caveolin 3 content (Fig. 1c), which corresponded mostly with the sharp band isolated by ultracentrifugation. It was possible to recover caveolae-like structures from fraction number 5, as verified by an electron microscopic analysis (data not shown). Because cardiocytes are not the only cell type of cardiac tissue, it is possible that caveolae extracted with this method could have come from other cells, such as endothelial cells. In order to confirm the source of caveolae, equal volumes of various fractions were subjected to Western blot analysis with an anti-caveolin 1 antibody. No caveolin 1 could be detected by this analysis (not shown), indicating that most of the caveolae recovered with the procedure were of cardiocyte origin. These data demonstrate that (1) cardiac caveolae can

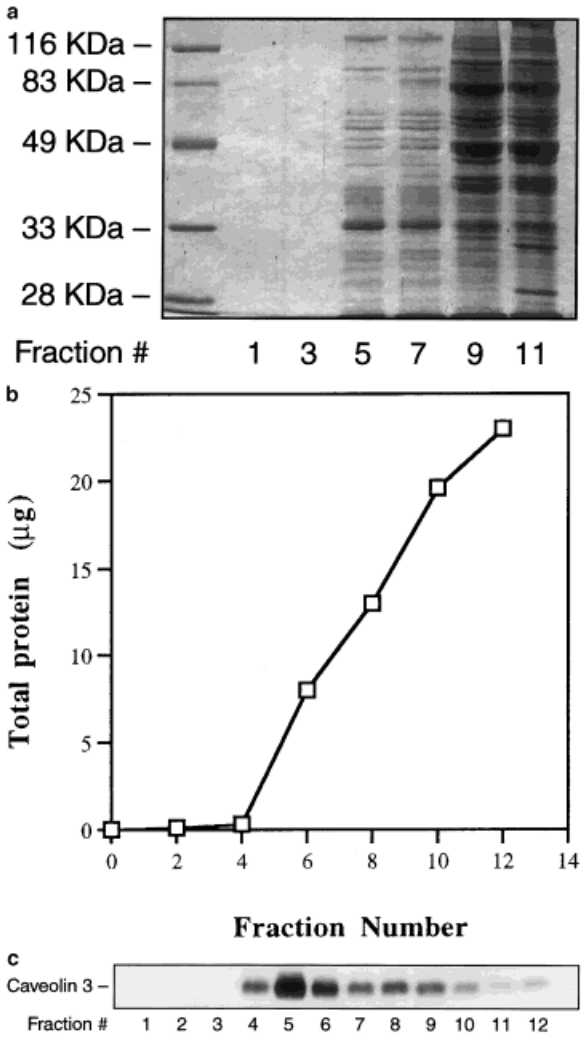


Fig. 1. Total protein and caveolin 3 distribution after caveolar extraction from left ventricular tissue. **a:** Coomassie blue staining of equal volumes (1μl) of the twelve fractions obtained during the caveolar fractionation procedure loaded on a 10% SDS-PAGE. **b:** Diagrammatic representation of the protein concentration throughout the 12 fractions of caveolae extraction. The fraction number is on the ordinate and the concentration, expressed as μg/μl, is on the abscissa. **c:** Western blot analysis of caveolin 3 distribution during caveolae fractionation. Equal volumes (5 μl) of the 12 fractions were loaded onto a 12% polyacrylamide gel, run, transferred onto a membrane, and probed for caveolin 3.

be extracted from whole heart tissue, (2) caveolin 3 is the main caveolin extractable from heart tissue and copurifies mostly with the caveolar fraction, and (3) caveolae extracted by this method are of myocyte origin.

Characterization of Tissue Cardiac Caveolar Content and Its Modulation After Norepinephrine Stimulation

Caveolae have been shown to be an important site of modulation of signal transduction

for vital cellular functions [Anderson et al., 1993]. It has been demonstrated that *src*-like proteins, G proteins, and p21*ras* are found in caveolae in many experimental systems. Caveolin physically associates with the inactive conformation of these molecules in vivo and in vitro, but not with constitutively active mutants, such as mutants G_s (Q227L), and mutant Ras (G12V) [Li et al., 1995; Mineo et al., 1996; Song et al., 1996]. In addition, caveolin is absent in many tumor cell lines and, when overexpressed, induces cell growth arrest and apoptosis, suggesting an anti-mitogenic role for caveolin [Engelman et al., 1997].

Caveolae in cardiac cells has been shown to be a site of regulation of the muscarinic 2 acetylcholine receptors [M2AChR] [Feron et al., 1997]. After ACh challenge, it is possible to recover M2AChR as well as eNOS from the caveolar compartment. Evidence also indicates that eNOS function is regulated negatively by interaction with caveolin [Feron et al., 1996; Garcia-Cardena et al., 1997; Ju et al., 1997; Michel et al., 1997]. In line with this finding, ANP receptor is found in caveolae and colocalizes with caveolin 3 after the respective agonist stimulation [Doyle et al., 1997; Page et al., 1994]. These findings prompted us to investigate whether molecules involved in the postreceptor step of signal transduction, namely, G protein subunits, tyrosine kinases, and p21*ras*, were present within the cardiac caveolar compartments. We subjected these fractions to Western blot analysis for G protein $\alpha_{s,}$ α_q, and β-subunits, as well as p21*ras* and grb2. The Western blot results show that fraction 5 was a localization site for the above mentioned signal transduction molecules (Fig. 2a). Figure 2b shows a densitometric measurement of the blot reported in Figure 2a.

Because the activity of these molecules is regulated by hormone-receptor interaction, changes in the relative amounts of these molecules were evaluated before and after adrenergic receptor stimulation, which plays a critical role in the control of cardiac function. Rats were injected with a dose of 1 μg/kg of NE. An arterial pressure curve was monitored so that the hearts could be excised after NE began its biological activity on peripheral tissues. Hearts were excised during the descending loop of the pressure curve after NE injection (Fig. 3).

Caveolae were purified from whole hearts both before and 3 min after NE injection. Fractions 5, the richest in caveolin 3, then were subjected to Western blot analysis. Samples were examined from both unstimulated and NE-stimulated heart tissues to detect if any rearrangement occurs in signal transduction molecules after stimulation. Before probing with several antibodies against signal transduction molecules, we measured caveolin expression in these two samples by immunoblot analysis. The same amount of caveolin 3 (Fig. 4a) was observed in both fractions. No caveolin 1 was detected in either sample (data not shown). It can be assumed that any changes of expression of signal transduction molecules detected in the treated samples most likely would reflect rearrangement of the protein storage in the caveolae, rather than reflect to different caveolae concentrations. Fractions 5, which contained the highest percentage of caveolin 3, were subjected to immunoblot analysis for G protein α and β-subunits. $G\alpha_o$, $G\alpha_q$, $G\alpha_s$, and Gβ levels increased in the NE treated hearts (Fig. 4a). In order to determine whether postreceptor molecules of classes other then G protein could be detected in the caveolar fraction and could undergo rearrangement, the p21*ras*, gbr2, *fyn* and *fgr* were tested by immunoblotting (Fig. 4a). While p21*ras* was present in unstimulated cardiac caveolae, its level increased remarkably in caveolae obtained from stimulated heart tissue. This effect was even more marked for gbr2, whose level abruptly changed after NE stimulation. *fyn* and *fgr* were present both in unstimulated and in NE-stimulated caveolae and their levels decreased after NE injection. Figure 4b shows the densitometric measurement of the immunoblots reported in Figure 4a.

These results demonstrate that (1) many types of postreceptor molecules involved in signal transduction are present in cardiac caveolae, and (2) the content and, in some cases, the type, of these molecules change dramatically after agonist-induced receptor stimulation.

Characterization of Caveolae from Primary Cardiocyte Cultures Before and After Ne Stimulation

Artifacts could be generated in the extraction procedure of caveolae from ventricular tissue because noncellular components were present in the homogenate. In addition, the fact that other cellular components of nonmyocyte origin could contribute to the caveolar fraction could not be exclused completely. In order to validate the data obtained from whole rat ventricles, we

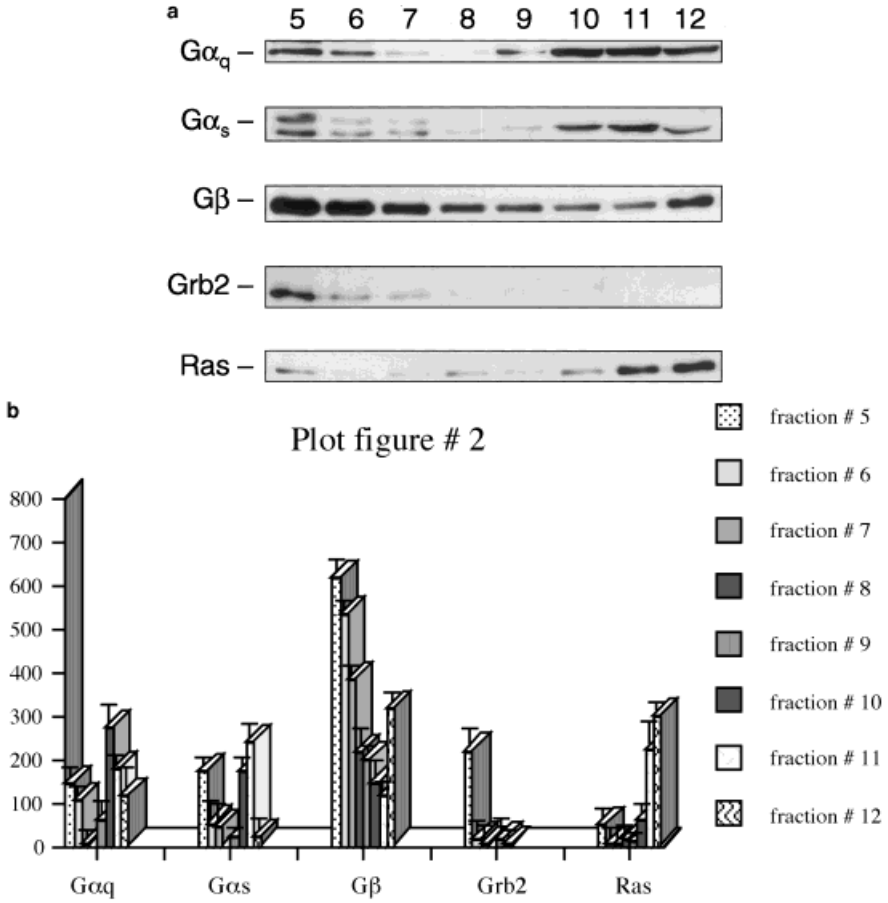


Fig. 2. **a:** Western blot analysis of G proteins, *gbr2*, and *ras* content within fraction 5–12 extracted with a detergent-free procedure from LV tissue. Same amount of proteins (30 μg) from the last 8 fractions were transferred onto a membrane and probed for G proteins, *grb2*, and *ras*. **b:** Densitometric measurement of the blot reported in **a**. Adsorbance is measured as arbitrary units. Mean of three separate experiments ±SD is reported.

extracted caveolae from unstimulated and NE-stimulated (1×10^{-6} M) primary culture cardiomyocytes. Approximately 5×10^7 cells were harvested per preparation. Cells were lysed in detergent containing buffer, ultracentrifuged and caveolae extracted as described. Equal volume fractions were collected and subjected to PAGE and immunoblotting.

Caveolin 3 distribution was similar as compared with that of our in vivo data and was recovered mainly in fraction 5. The protein concentration of fraction 5 was found to be 4 fold lower than that of fraction 12. A similar distribution of caveolin 3 was present in NE-treated fractions. In addition, the pattern of caveolin 3 distribution in NE-stimulated cardiomyocytes was different from the pattern of unstimulated cells. Specifically, it was possible to detect caveolin 3 in fractions 11 and 12 that did not represent the caveolae compartment and that contain predominantly cytoplasmic proteins. This suggests that hormone stimulation induces an internalization of caveolae (Fig. 5a).

Because our in vivo experiments made it clear that NE-caveolae had a high concentration of G protein-subunits, we decided to determine whether NE-caveolae from isolated cardiomyocytes contained a higher concentration of these proteins. Blots were probed for Gα and Gβ proteins (Fig. 5a). Figure 5b shows the densitometric measurement of the blot depicted in Figure 5a.

All the G protein subunits tested displayed increase in fraction 5 of NE-treated cells. In addition, the amount of the same proteins contained in fraction 12 was reduced in NE-treated cells as compared with untreated cells. This finding indicates that the fractions containing Triton-insoluble material, mostly from plasma membrane, were depleted of the G protein-subunits after NE stimulation while, By contrast, the caveolin containing fractions were enriched.

DISCUSSION

This report characterizes the caveolar compartment from rat heart and from isolated rat neonatal cardiocytes. Specifically, we investigated whether cardiac caveolae contain postreceptor molecules of the heterodimeric G protein

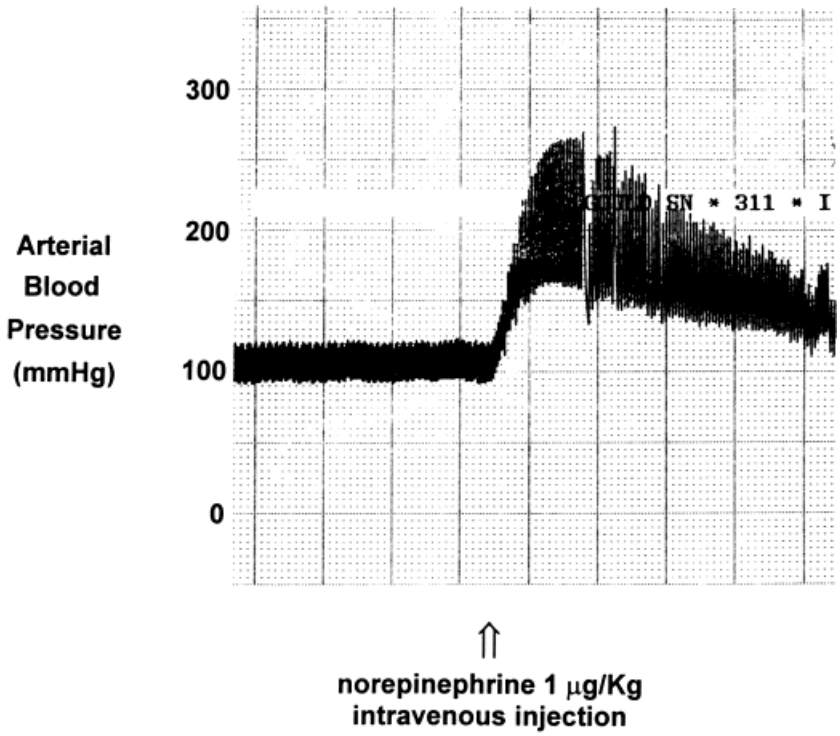


Fig. 3. Diagrammatic representation showing the monitoring of arterial pressure curve after injection of 1 μg/kg of NE. Hearts were excised in the descending limb of the curve.

family, as well as *ras*- and *src*-related tyrosine kinases. Previous reports suggested the existence of the caveolar compartment in cardiac cells. In fact, cardiac caveolae were shown to contain type B ANF receptor, ANF itself, and caveolin 3 in atrial myocyte cells. Moreover, studies have demonstrated the presence of muscarinic receptor type 2 in the caveolar compartment, and the physical association between caveolin 3 and M2AChR after ACh stimulation of adult rat cardiac ventricular myocytes [Feron et al., 1997]. PtdInsP2 was recently found in the caveolar compartment [Pike and Miller, 1998].

G proteins including p21*ras* play a critical role in the heart in mediating physiological and pathological stimuli from outside. A deregulated and constant activation of these molecules causes cardiac hypertrophy in transgenic mice by their overexpression.

Deregulated expression of $G\alpha_s$ or $G\alpha_q$, two G proteins involved in the β and α adrenergic receptor signaling, promotes heart failure [D'Angelo et al., 1997; Iwase et al., 1996]. Similarly, *ras* overexpression induces cardiac hypertrophy. This prompted us to ask whether cardiac caveolae contained p21*ras*, heterodimeric G proteins and tyrosine kinases of the *src* family. We set up the conditions for caveolar extraction from rat left ventricles to establish the feasibility and specificity of the technique to permit the characterization of this compartment from ventricles in different pathological states of human and animal models. This separation technique, previously used to obtain caveolae from lung tissue, was modified to extract caveolae from heart tissue. Our results indicate that caveolin 3 is readily detectable with the caveolar extraction methodology employed, while caveolin 1, which is particularly rich in endothelial cells is not detectable. This finding strongly suggests that the extracted caveolae were primarily, if not exclusively, of cardiac origin. In order to validate the in vivo data, we extracted caveolae from a minimum of 5×10^7 rat neonatal cardiocytes. We were not able to extract caveolae from 1×10^7 cells, which indicates that a minimal bulk number of cells are required for the technique to be successful.

The adrenergic system plays a fundamental role in regulating both cardiac inotropism (a function mainly ascribed to the β-adrenergic receptor) and the cardiac hypertrophic response to stress (mainly through the α-adrenergic receptor). We decided to investigate whether indiscriminate stimulation of both the αand βtypes of receptors present on the myocyte membrane would induce a rearrangement of the caveolar content of the pro-

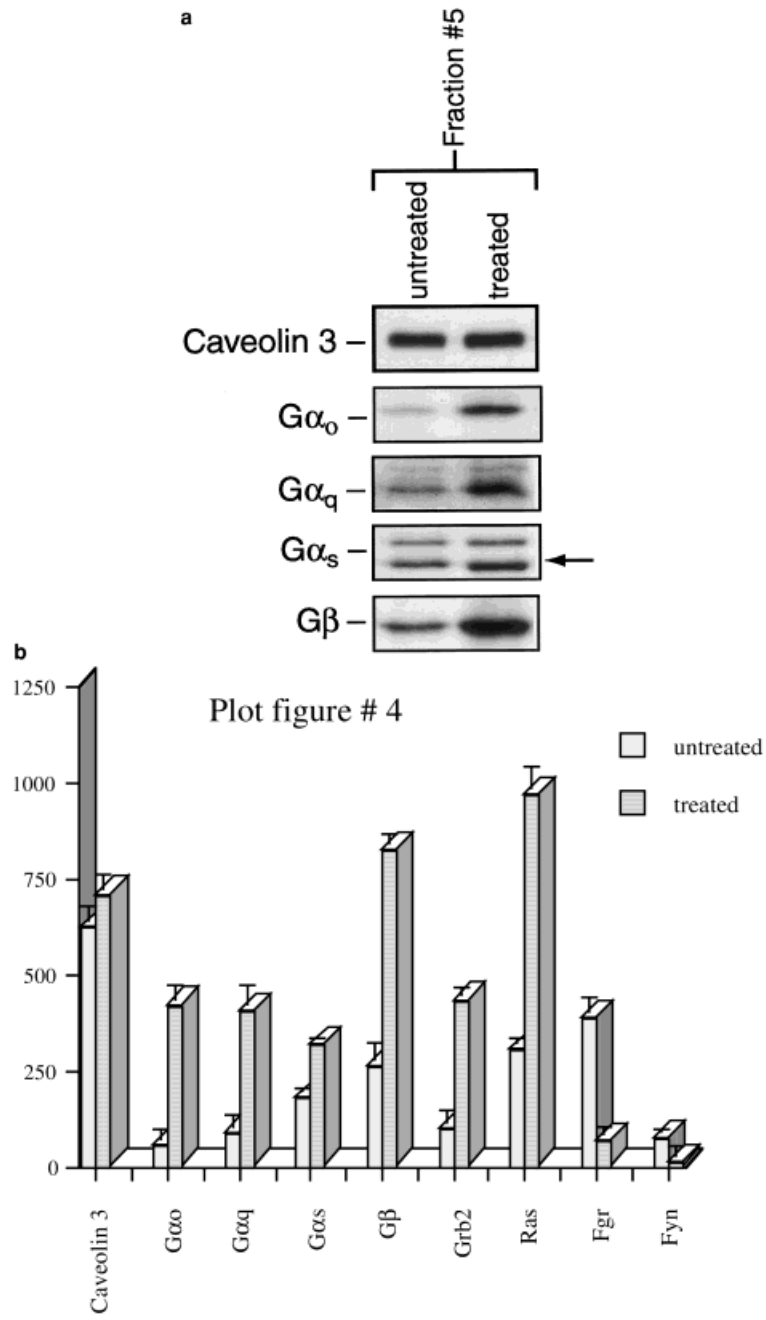
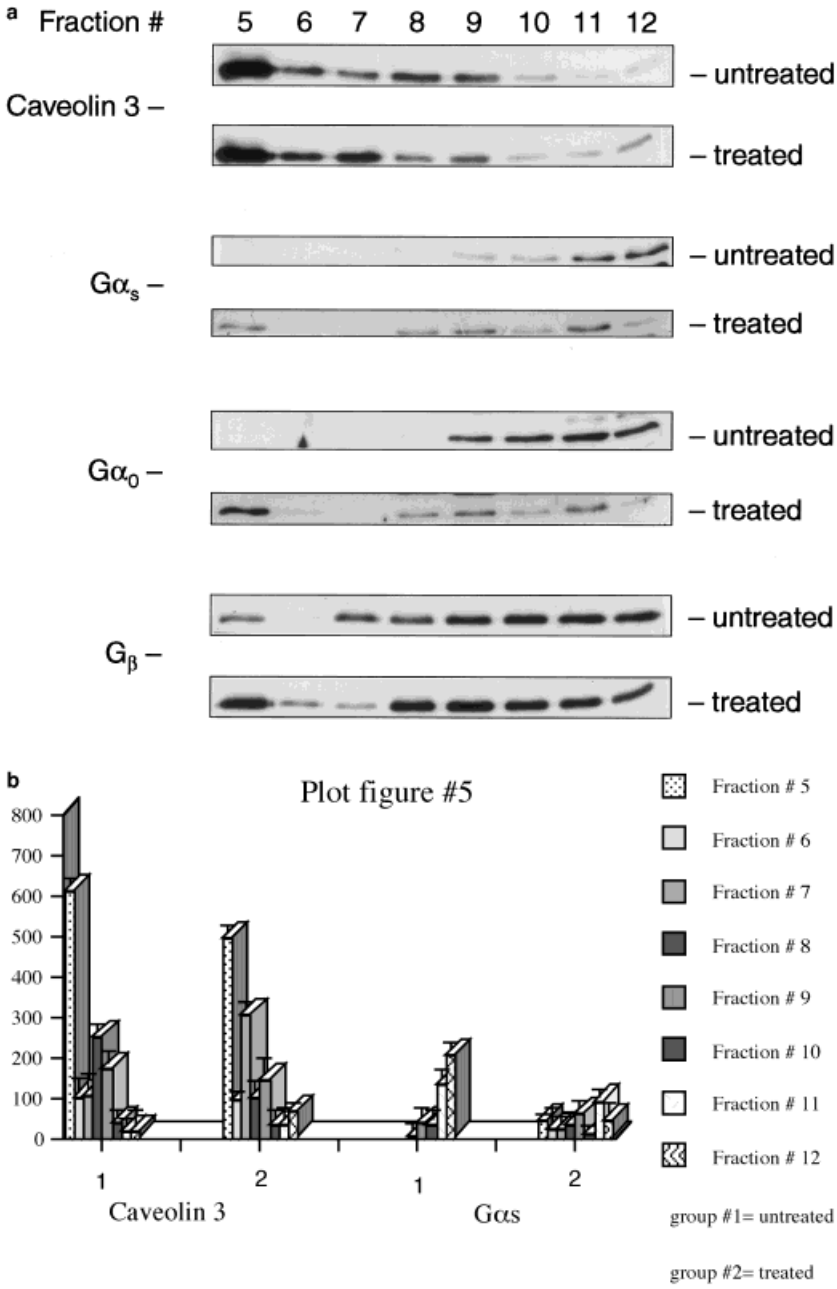


Fig. 4. **a:** Changes of caveolin 3, G proteins, *grb2, ras, frg,* and *fyn* in caveolae (fraction 5) extracted from rat left ventricle, before and after NE injection (1 μg/kg). Same amounts of proteins (30 μg) from fraction 5 of treated and untreated (NE, 1 μg/kg) animals were transferred onto a membrane and probed for caveolin 3, G protein α- and β-subunits, *grb2, ras, frg, fyn.* A representative result from a single treated and untreated animal is shown. **b:** Densitometric measurement of the blot reported in **a**. Adsorbance is measured as arbitrary units. Mean of three separate experiments ±SD is reported.

teins belonging to the p21*ras*, *src*, and G protein receptor family. The results show that the categories of proteins, localized in other cellular systems in the caveolae after hormone stimulation, undergo similar changes in plasma-membrane compartmentalization after NE stimulation both in vitro and in vivo. Our model system does not distinguish between α- and β-receptor pathways because rats were injected with NE, which is a nonspecific agonist of both receptor types. The animal heart tissue data appear to contrast with those obtained from isolated cardiocytes. However, there always is a degree of adrenergic activity in the living animal, while the cell culture system permits starvation from NE. Therefore, it is reasonable that caveolae extracted from beating hearts might contain various amounts of G proteins. It was also possible to detect an increase in the amount of caveolin 3 proteins in



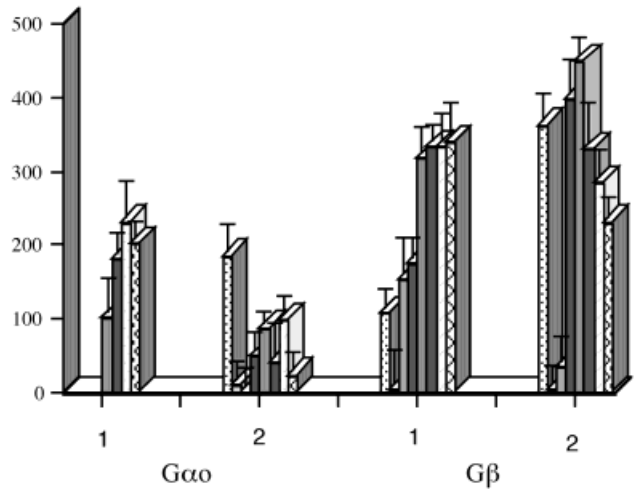


Fig. 5. **a:** Western blot analysis of caveolae content after fractionation of primary cardiomyocytes. 5×10^7 cells, untreated or pretreated with 1×10^{-6} M NE, were subjected to detergent-free caveolar extraction. Fraction content was then analyzed. Same amount of protein/fraction (30 μg) was transferred onto a nylon membrane and probed for caveolin 3, G protein α, and β-subunits. **b:** Densitometric measurement of the blot reported in **a**. Adsorbance is measured as arbitrary units. Mean of three separate experiments ±SD is reported.

fractions 11 and 12, after NE stimulation of primary cardiocytes corresponding with noncaveolar, cytoplasmic flowthrough. This suggests, but does not prove, that the whole caveolar compartment is recycled after hormone stimulation. The chain of events suggested by our results consists of hormone-receptor interaction, activation of postreceptor signal transduction molecules, localization of these molecules in the caveolar compartment and their inactivation by interaction with caveolin, and eventually internalization of the caveolar compartment. It is interesting to note that $G\alpha_q$ is present in the caveolar compartment and its concentration is modified by NE, probably after α-adrenergic challenge. $G\alpha_o$ seems to be more diffusely distributed in sucrose fractions. It is consistently present in the caveolar compartment and is not significantly modified after NE challenge. The β-subunit of the G protein also is present in abundance in caveolae and increases both in tissue extracts and in cells after NE stimulation, suggesting again that the G proteins are in a biologically inactive state when recruited to caveolae.

It will be of interest to ascertain whether G protein receptor kinases (GRKs) also localize to caveolae after hormone stimulation in future studies. We demonstrate here also that p21*ras* is present in cardiac caveolae and increases after NE stimulation. We did not investigate whether other proteins of the *ras* signal transduction follow a pathway similar to that of p21*ras*, although this is certainly a possibility. Because constitutively active H-*ras* is unable to bind caveolin 1 and bears defects in membrane localization, it is possible that caveolae regulate the level of activity of p21*ras*.

A similar phenomenon was described in smooth muscle cells after stimulation with bradykinin. Bradykinin increased the amount of active G protein and p21*ras* in the caveolar compartment [de Weerd and Leeb-Lundberg, 1997].

In summary, we provide evidence that caveolae are a critical component of the signal transduction in cardiac cells. We have demonstrated that cardiac caveolae content undergoes a profound rearrangement after NE stimulation, both in vitro and in vivo. This study raises the possibility of characterizing the cardiac caveolar content in different physiological as well as in pathological conditions in animal and human models of heart disease more definitively.

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