Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae

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Running title: Mechanotransduction by RhoA and Rac1
Summary

The Rho family small GTPases play a crucial role in mediating cellular responses to stretch. However it remains unclear how force is transduced to Rho signaling pathways. We investigated the effect of stretch on the activation and caveolar localization of RhoA and Rac1 in neonatal rat cardiomyocytes. In unstretched cardiomyocytes, RhoA and Rac1 were detected in both caveolar and non-caveolar fractions as assessed using detergent-free floatation analysis. Stretching myocytes for 4 minutes activated RhoA and Rac1. By 15 minutes of stretch, RhoA and Rac1 had dissociated from caveolae and there was decreased coprecipitation of RhoA and Rac1 with caveolin-3. To determine whether compartmentation of RhoA and Rac1 within caveolae was necessary for stretch signaling, we disrupted caveolae with methyl β-cyclodextrin (MβCD). Treatment with 5mM MβCD for 1 hour dissociated both RhoA and Rac1 from caveolae. Under this condition, stretch failed to activate RhoA or Rac1. Stretch-induced actin cytoskeletal organization was concomitantly impaired. Interestingly the ability of stretch to activate extracellular signal-regulated kinase (ERK) was unaffected by MβCD treatment, but ERK translocation to the nucleus was impaired. Stretch induced hypertrophy was also inhibited. Actin cytoskeletal disruption with cytochalasin-D also prevented stretch from increasing nuclear ERK, while actin polymerization with jasplakinolide restored nuclear translocation of activated ERK in the presence of MβCD. We suggest that activation of RhoA or Rac1, localized in a caveolar compartment, is essential for sensing externally applied force and transducing this signal to the actin cytoskeleton and ERK translocation.
Introduction

Mechanical stress is recognized as an important extracellular stimulus facilitating cell growth, one example of which is cardiomyocyte hypertrophy. There is a growing list of signaling pathways that are activated by mechanical stress in cardiomyocytes including protein kinase C (PKC), mitogen activated protein kinases (MAPKs), phosphatidylinositol-3 kinase/Akt, Janus kinase/signal transducer and activator of transcription, calcineurin, and nitric oxide synthase (NOS) (1-3). There is also evidence that Rho family small GTPases play a central role in mediating the development of stretch-induced cardiac hypertrophy (4, 5). Rho family small GTPases regulate a variety of cellular functions such as cytoskeletal rearrangement, cell contractility, and cell migration (6). The cytoskeleton communicates with the extracellular matrix through integrins which have been considered as potential mechanosensors (1, 7, 8). There is also evidence that integrins transduce signals via Rho dependent pathways (9). However the molecular mechanisms by which mechanical force is transduced and the steps leading to recruitment of Rho family small GTPases in mechanotransduction in cardiomyocytes are poorly understood.

Caveolae are cholesterol- and sphingolipid-enriched plasma membrane invaginations, which are considered to be important platforms for signal transduction (10-13). There is considerable support for the notion that signaling molecules converge in caveolae (14-16), and that these play an important role in propagating signals in response to agonist treatment in cardiomyocytes. However the possibility that mechanotransduction signals
through caveolar localized molecular pathways in cardiomyocytes has not been previously addressed.

There are two functionally distinct features of caveolae that are critical to their role in signal transduction (17). First, caveolae are considered to function as reservoirs of signaling proteins, with caveolin serving as a negative regulator of these molecules in quiescent cells. Second, caveolae function as clustering sites for ligand-activated G-protein coupled receptors such as the muscarinic cholinergic or sphingosine 1 phosphate receptor (18, 19). In the first regard, several studies indicate that the caveolar scaffolding protein, caveolin-1, can directly interact with signaling molecules (e.g. G-protein subunits, Src family tyrosine kinases, endothelial NOS, PKC, or Ras) within caveolae to negatively regulate their functions (12, 20-23). Accordingly some of these molecules have been shown to be activated and liberated from caveolae upon hormonal stimulation (24-26).

In light of the findings cited above we hypothesized that mechanical force might be transduced into cellular signaling pathways in cardiomyocytes via caveolae. In particular, we focused on the localization and activity of Rho family GTPases, RhoA and Rac1, in caveolar microdomains and examined the effect of stretch on their compartmentation and downstream signaling. The data presented here demonstrate that activation of RhoA and Rac1 by stretch requires their proper localization within caveolae and that stretch leads to their dissociation from this compartment. Disruption of caveolae not only prevents RhoA and Rac1 activation but also impairs stretch-induced actin cytoskeletal rearrangement and nuclear localization of ERK.
Experimental Procedures

Materials

Anti-caveolin-3, anti-RhoA, anti-Rac1, anti-Rho-GDP dissociation inhibitor (GDI), and anti-focal adhesion kinase (FAK) monoclonal antibodies were purchased from Transduction Laboratory. Anti-RhoA and anti-Rac1 polyclonal antibodies for immunoprecipitation were purchased from Santa Cruz. Anti-phospho-specific ERK1/2 and anti-ERK1/2 polyclonal antibodies were purchased from Cell Signaling. Protein G-Sepharose was from Amersham Pharmacia Biotech. fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody was from INC. Jasplakinolide and Rhodamine-phalloidin were purchased from Molecular Probes. All other reagents were purchased from Sigma.

Cell Culture

Neonatal rat ventricular myocytes were isolated and cultured as described previously (27, 28). Briefly, hearts were obtained from 1-2-day-old Sprague-Dawley rat pups, digested with collagenase, and myocytes purified by passage through a Percoll gradient. Cells were plated onto silicon membrane, coated with collagen type I (0.5 µg/cm²), that assembled in a biaxial stretch device. Myocytes were maintained overnight in 4:1 Dulbecco’s modified Eagle’s medium/medium 199, supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Cells were then serum starved for 24 hours prior to intervention.
Purification of caveolin-rich membrane fractions

Caveolin-rich microdomains were isolated using the methods of Song et al. with some modifications (20). Briefly, myocytes were washed with ice-cold phosphate buffer saline (PBS) and lysed directly with 600 µl of 0.5M Na₂CO₃ (pH 11). Cells were passed 20 times through a 25-gauge needle and sonicated three 20 sec bursts with minimal output of a Micro Ultrasonic Cell Disrupter (Kontes). A 0.54 ml aliquot of cell lysates was adjusted to 45% sucrose by the addition of 0.54 ml of 90% sucrose prepared in MBS [25 mM 4-morpholineethanesulfonic acid (MES), pH 6.5, 75 mM NaCl] and placed at the bottom of an ultracentrifuge tube. The 45% sucrose-cell lysate mixture was overlaid with 1.08 ml of 35% sucrose and 1.08 ml of 5% sucrose in MBS containing 0.2 M Na₂CO₃. The gradients were centrifuged at 48,000 rpm for 20 hours in an SW50.1 rotor (Beckman Instruments). Twelve 0.27-ml fractions were collected from the top of the gradient. The proteins in each fraction were precipitated with 20% trichloroacetic acid. The precipitated proteins were washed once with 95% ethanol and then resolved with Laemmli sample buffer. The proteins were boiled, separated by 12% SDS-polyacrylamide gel (PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and subjected to immunoblotting. Enhanced chemiluminescence was then performed using SuperSignal chemiluminescent detection system (Pierce).

Preparation of purified GST-fusion protein

The coding sequence for the Rho-binding domain of Rhotekin (RBD) or Rac1-binding domain of p21-activated kinase1 (PBD) cloned into the pGEX vector expression systems were gifts from Dr. Martin Alexander Schwartz (University of Virginia). The
recombinant protein was purified from *Escherichia coli* as described previously (27). Briefly, expression of glutathione S-transferase (GST) fusion proteins were induced with isopropyl β-D-thiogalactopyranoside, and purified by binding to glutathione-agarose beads. The beads were stored at −80°C until use.

*Pull-down assay*

Cells were washed with ice-cold PBS, and lysed in 250 μl of cell lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin). Samples were centrifuged for 2 min at 1,000 g to remove nuclei and cell debris. Equal volumes of supernatants were incubated with RBD or PBD beads for 60 minutes at 4°C. After washing the beads for three times with wash buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin), the beads were collected and resolved with 30 μl of Laemmli sample buffer. The precipitated proteins were boiled for 5 min, then separated by 12% SDS-PAGE, transferred to PVDF membranes, and subjected to immunoblotting. Enhanced chemiluminescence was performed using SuperSignal chemiluminescent detection system (Pierce).

*Immunoprecipitation*

Myocytes were washed with ice-cold PBS and lysed with lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1.0% NP-40) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na₃VO₄, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation of RhoA or Rac1 from cell lysates was carried out by incubating 300 μl of total cell lysates
with 1 µg of anti-RhoA or anti-Rac1 polyclonal antibody in the presence of Protein G-Sepharose (10 µl) overnight at 4°C. The precipitated proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane, and probed with anti-RhoA, anti-Rac1, or anti-caveolin-3 antibodies.

**Immunocytochemistry**

Myocytes on stretchers were fixed for 20 minutes in 3.7% paraformaldehyde in PBS, permeabilized for 3 minutes in 0.4% Triton X-100, and blocked in 3% bovine serum albumin for 20 minutes. The silicon membrane was cut and then stained with primary antibodies overnight at 4°C followed by secondary antibody and rhodamine-phalloidin for 1 hour at room temperature. The membrane was mounted on a coverslip and images were captured with a DeltaVision deconvolution microscope system (Applied Precision, Inc., Issaquah, WA.) The system includes a Photometrics CCD mounted on a Nikon TE-200 inverted epi-fluorescence microscope. In general between 30 and 80 optical sections spaced by~ 0.1-0.3 um were taken. Exposure times were set such that the camera response was in the linear range for each fluorophore. The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision, Inc) on a Silicon Graphics Octane workstation. When applicable, image quantitation was performed using the Data Inspector program in SoftWorx.
Results

Characterization of isolated caveolae from cardiomyocytes

Caveolin-3 is a heart and muscle cell predominant isoform of caveolin. We isolated cardiac caveolae from neonatal rat ventricular myocytes (Fig. 1A) using caveolin-3 as a specific marker for cardiac caveolae. Non-caveolar components were defined by the absence of caveolin-3 and by the presence of focal adhesion kinase (FAK) and of Rho-GDI, an endogenous inhibitor of RhoA and Rac1 that sequesters them in the cytoplasm. The caveolin-3 positive bands were concentrated in fractions 5 and 6 in preparations made by the detergent-free purification procedure detailed in Methods. In contrast, FAK and Rho-GDI were detected in fractions 9 to 12 (Fig. 1A). The protein concentration profile obtained using this isolation method was also consistent with that of previous reports (Fig. 1B) (29).

Activation of RhoA and Rac1 in response to stretch

We next determined whether stretch led to the activation of RhoA and Rac1 in cardiomyocytes. Pull-down assays revealed rapid and transient increases in activated (GTP-bound) RhoA and Rac1 in response to 20% equibiaxial stretch (Fig. 2A). The activation of both RhoA and Rac1 peaked at 3 to 5 minutes after the onset of stretch and returned to the basal level by 10 minutes of stretch (Fig. 2B). RhoA and Rac1 activation at 4 minutes of stretch is also shown quantitatively in Figure 5.
Segregation of RhoA and Rac1 from caveolae in response to stretch

Changes in the subcellular localization of RhoA and Rac1 in neonatal rat ventricular myocytes subjected to stretch were assessed using the fractionation procedure described in Figure 1 to purify caveolae. RhoA and Rac1 were distributed in both caveolar and non-caveolar fractions in unstretched cardiomyocytes. Equibiaxial stretch for 4 minutes did not affect the distribution of RhoA or Rac1 in caveolar and non-caveolar fractions (data not shown). However, when equibiaxial stretch was applied to the myocytes for 15 minutes, redistribution of RhoA and Rac1 from the caveolar to a non-caveolar fraction was observed (Fig. 3A). Densitometric analysis of the distribution of caveolin-3, RhoA, and Rac1 in caveolar (fractions 5 and 6) versus non-caveolar (fractions 9 to 12) fractions revealed a 47% reduction in the proportion of RhoA and a 50% reduction in the fraction of Rac1 in caveolae. Concomitant increases in non-caveolar fractions were observed. Importantly, caveolin-3 distribution was not affected by stretch (Fig. 3B).

Effect of methyl β-cyclodextrin (MβCD) on stretch-induced RhoA and Rac1 distribution

The observation that activation of RhoA and Rac1 by stretch appeared to precede their dissociation from caveolae suggested that caveolar compartmentation might be necessary for their initial activation. To address this issue, we used cholesterol depletion with MβCD to disrupt caveolae. Cardiomyocytes were incubated with 5 mM MβCD for 1 hour prior to stretch. The impact of this intervention on the activation and subcellular distribution of RhoA and Rac1 was then examined. Although the destruction
of caveolae was not complete (as evidenced by retention of some caveolin-3 immunoreactivity in fractions 5 and 6), this treatment was sufficient to dissociate both RhoA and Rac1 from caveolae (Fig. 4A). The association of RhoA and Rac1 with caveolin-3 was also examined in immunoprecipitation studies. As expected, caveolin-3 co-precipitated with RhoA and with Rac1 in unstretched myocytes (Fig. 4B). Following fifteen minutes of stretch, the amount of caveolin-3 associated with RhoA and Rac1 was diminished consistent with their dissociation from the caveolar fractions. In addition, the amount of caveolin-3 that co-precipitated with RhoA and Rac1 was decreased by MβCD treatment, confirming dissociation of these small GTPases from caveolar microdomains (Fig. 4B).

**Effect of MβCD on stretch-induced RhoA and Rac1 activation**

The ability of stretch to activate RhoA and Rac1 was then assessed in cells treated with MβCD. As shown in Figure 5, four minutes of stretch activated both RhoA and Rac1 in untreated cells. Pretreatment with MβCD did not significantly affect the basal activity of RhoA and Rac1, however stretch failed to activate either RhoA or Rac1. These observations suggest that the initiation of stretch-induced RhoA and Rac1 activation requires intact caveolar microdomains.

**Effect of MβCD on ERK activity during stretch**

Recent findings from studies of caveolin-3 and -1/3 null mouse demonstrated that chronic loss of caveolae resulted in hyperactivation of ERK in the heart (30, 31). We, therefore, determined whether ERK was activated in myocytes when caveolar structure
was acutely disrupted with MβCD. As shown in Figure 6, MβCD treatment alone did not lead to significant activation of ERK, nor was ERK activation by stretch impaired by MβCD treatment (Fig. 6).

Cytoplasmic retention of active ERK by MβCD in stretched myocytes

When we investigated the subcellular localization of active ERK by immunostaining with a phospho-specific ERK antibody, a different pattern emerged. As shown in Figure 7, in unstretched myocytes, weak phospho-ERK fluorescence (green) and disorganized striated actin filaments (red) were detected in the cytoplasm. One hour of stretch increased the intensity of phospho-ERK staining and nuclear staining was evident in ~90% of the cells. Stretch concomitantly elicited actin fiber alignment. In contrast, in cells treated with MβCD stretch increased phospho-ERK fluorescence but phospho-ERK staining was observed only in the cytoplasm and actin fibers remained disorganized. These data suggest that failure to activate RhoA and Rac1 and to organize the actin cytoskeleton, may prevent nuclear translocation of active ERK.

Jasplakinolide restores nuclear translocation of active ERK

To further examine the involvement of the actin cytoskeleton in nuclear translocation of ERK we tested the effect of, an actin polymerizer jasplakinolide, on the translocation of active ERK by stretch. Actin fibers could not be detected with phalloidin staining in these studies because jasplakinolide competes with phalloidin for binding to actin fibers. We, therefore stained for α-actinin, an actin-binding protein. Following jasplakinolide treatment, the actin cytoskeleton was organized and stretch led to the nuclear
translocation of phospho-ERK in a significant fraction (~40%) of the MβCD treated cells (Fig. 7). Furthermore we determined that treatment of myocytes with cytochalasin-D, an actin depolymerizer, did not affect ERK activation but prevented stretch induced organization of the actin cytoskeleton and led to retention of active ERK in the cytoplasm of most cells (Fig. 8). This is consistent with a previous study with cytochalasin-D which suggested that nuclear translocation of active ERK is facilitated by an organized actin cytoskeleton (32).

The precise molecular relationship between RhoA and Rac1 activation, actin cytoskeletal reorganization, and ERK nuclear translocation remains to be explored. However, ERK signaling in cardiomyocytes has been associated with the development of hypertrophy, characterized by increases in cell size, actin myofilament organization and ANF expression. Cardiomyocytes stretched continuously for 24 hours show clear increases in each of these parameters (Fig. 9). Concomitant treatment with MβCD prevents these changes. While long term MβCD treatment could disrupt myriad signaling pathways that regulate hypertrophy, the data are consistent with a requirement for caveolae in transduction of stretch to hypertrophic growth, possibly mediated via RhoA, Rac1 and ERK signaling pathways.
Discussion

The findings presented here demonstrate by direct biochemical analysis that stretch increases the amount of activated RhoA and Rac1 in cardiomyocytes. This observation extends published evidence demonstrating more indirectly that Rho is activated by stretch in vascular smooth muscle and cardiac muscle cells (4, 33).

The precise mechanism by which mechanical force is transduced into activation of downstream signaling cascades remains unknown. The data presented here suggest that the compartmentation of RhoA and Rac1 in caveolae play a critical role in mechanotransduction in cardiomyocytes.

Using a detergent-free purification of caveolae we determined that a significant fraction of RhoA and Rac1 are localized in caveolar microdomains of cardiomyocytes. Furthermore we demonstrate that mechanical stretch results in the dissociation of RhoA and Rac1 from the caveolar fraction and decreases the amount of caveolin-3 associated with RhoA or Rac1 in immunoprecipitates. We also provide evidence that short term MβCD treatment leads to complete loss of compartmentation of RhoA and Rac1 within caveolae and that this manipulation prevents stretch induced RhoA and Rac1 activation. These data implicate caveolae as the sites at which RhoA and Rac1 are activated by stretch. From a cellular perspective, electron microscopic studies suggest that caveolar structures open and become flat when cells are stretched beyond 7% of their resting length (34). Therefore, it might be hypothesized that stretch, by altering caveolar morphology, renders caveolar localized RhoA and Rac1 more accessible to guanine
nucleotide exchange factors which catalyze the release of GDP and its replacement by GTP (8).

In addition to affecting RhoA and Rac1 activation, MβCD treatment prevents stretch induced actin cytoskeletal organization. The role of RhoA and Rac1 in activation of molecules responsible for actin cytoskeletal reorganization (e.g. Rho kinase, LIM kinase, p21-activated kinase 1 (PAK), cofilin) is well documented (35, 36). It has also been demonstrated that filamin, a protein involved in actin cytoskeletal assembly through PAK, is a caveolin binding protein (37, 38). The ability of caveolar disruption to prevent stretch mediated actin cytoskeletal organization could therefore result either from failure to activate RhoA and Rac1 or from impaired binding of cytoskeletal regulatory molecules to caveolin/caveolae.

Unexpectedly, our data also suggest that stretch induced actin cytoskeletal rearrangement plays a role in insuring the proper localization of active ERK. Disruption of caveolae with MβCD did not prevent ERK activation by stretch. Indeed the finding that ERK is activated in the presence of MβCD suggests that some stretch-induced signaling pathways remained intact. Possibly integrin signaling pathways are engaged and lead to ERK activation in the absence of intact caveolae (see Fig. 10). Most notably, however, MβCD treatment led to cytoplasmic retention of activated ERK in response to stretch. The possibility that the impaired ERK localization is related to the failed cytoskeletal organization is consistent with another recent report demonstrating that cytochalasin-D, an actin cytoskeleton disrupting agent, impairs integrin stimulated
nuclear translocation of active ERK in NIH 3T3 cells (32). Experiments presented here indicate that this also occurs in cardiomyocytes and in response to stretch. Stretch-induced ERK activation and nuclear translocation in mesangial cells has also been reported to be disrupted via destabilization of the actin cytoskeleton resulting from elevating NO or treatment with 8-bromo-cGMP (39). Jasplakinolide reverses the destabilization and allows proper ERK localization in mesangial cells (39). Similarly, in cardiomyocytes, jasplakinolide restores nuclear translocation of active ERK induced by stretch, even in the presence of MβCD.

The mechanism by which an intact actin cytoskeleton participates in the transport of active ERK into the nucleus remains unknown. Nonetheless our data suggest that organization of the actin cytoskeleton is required for mechanotransduction in cardiomyocytes. Mechanotransduction and G-protein coupled agonists are known to utilize ERK signaling pathways to induce cardiomyocyte hypertrophy (1, 40, 41). We demonstrate here that MβCD treatment prevents stretch induced hypertrophic increases in ANF gene expression and myocyte cell size. While there are many potential molecular events inhibitable by MβCD our findings suggest that the ability of MβCD treatment to block RhoA or Rac1 activation, cytoskeletal organization and ERK translocation affects nuclear responses.

In summary, our findings demonstrate that stretch activates and subsequently dissociates RhoA and Rac1 from a caveolar compartment in rat cardiomyocytes. Mechanotransduction via this pathway appears to require organization of the actin cytoskeleton, a response that contributes to the nuclear localization of ERK. We
suggest that proper cellular compartmentation of RhoA and Rac1 within caveolae is necessary for transducing mechanical force into spatially regulated ERK signals.

Footnotes

The abbreviations used in this paper are: MβCD, methyl β-cyclodextrin; ERK, extracellular signal-regulated kinase; NOS, nitric oxide synthase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; GDI, GDP dissociation inhibitor; FAK, focal adhesion kinase; FITC, Fluorescein isothiocyanate; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; MBS, MES-buffered saline; PAGE, polyacrylamide gel; PVDF, polyvinylidene difluoride; GST, glutathione S-transferase; RBD, Rho-binding domain of rhotekin; PBD, Rac1-binding domain of p21-activated kinase1; jaspla, jasplakinolide; Cyto-D, cytochalasin-D.
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Figure Legends

Figure 1.

Characterization of purified caveolae. (A) Detergent-free isolation of caveolae from unstretched neonatal rat ventricular myocytes. Non-linear sucrose gradient centrifugation (SW50.1) at 48,000 rpm for 20 hours yielded 12 fractions designated as 1 to 12 from the top. Proteins were separated by the 12% SDS-PAGE and immunoblotting was performed with antibodies indicated. Western blotting with anti-caveolin-3 antibody identified fractions 5 and 6 as caveolaer fractions, while fractions 9-12 were estimated as non-caveolaer fractions assessed by the presence of FAK or Rho-GDI. (B) Protein concentration profile of the fractions collected from sucrose gradient ultracentrifugation was determined by Bradford analysis using known concentrations of BSA as standards.

Figure 2.

Time course of activation of RhoA and Rac1 by stretch. (A) Neonatal rat ventricular myocytes plated on silastic membranes coated with collagen type I were subjected to 20% static equibiaxial stretch for the time indicated. Pull-down assays with Rho-RBD or Rac-PBD were performed to determine the activity of RhoA (upper panel) or Rac1 (bottom panel) in response to stretch. RhoA or Rac1 activity is indicated by the amount of RBD- or PBD-bound RhoA or Rac1, normalized to total RhoA or Rac1, respectively. (B) Quantitation of relative RhoA and Rac1 activity. Values are means ± SEM from two independent experiments.
Figure 3.

**Effect of stretch on the distribution of Cav-3, RhoA, and Rac1.** (A) Fractionation of Cav-3, RhoA, and Rac1. Western blotting with antibodies against caveolin-3, RhoA, and Rac1 was used to compare control myocytes (plated on membranes but not stretched) with myocytes exposed to 20% stretch for 15 minutes. Each fraction was obtained by detergent-free fractionation using sucrose gradient ultracentrifugation and western blotting was performed as described for figure 1. (B) Densitometric analysis of the distribution of Cav-3, RhoA and Rac1 was carried out on the immunoblots using ChemiImager 4400 (Alpha Innotech Corp.). The percent of the total protein in caveolar fractions (Cav; fractions 5 and 6) or non-caveolar fractions (Non-cav; fractions 9 to 12) was determined by dividing these values by the total density of fractions 1 through 12. The experiments were repeated at least three times to give the averaged value ± SEM as shown. *: p<0.05 compared with unstretched.

Figure 4.

**Effects of MβCD on the distribution of Cav-3, RhoA, and Rac1.** (A) Fractionation of Cav-3, RhoA, and Rac1. Cardiomyocytes were treated with or without 5 mM of MβCD for 1 hour followed by detergent-free purification of caveolae using sucrose gradient ultracentrifugation. Proteins were separated by the 12% SDS-PAGE and immunoblottings were performed with antibodies against Cav-3, RhoA, and Rac1. (B) Immunoprecipitation. Cardiomyocytes subjected to 20% stretch for 15 minutes or treated with 5 mM of MβCD for 1 hour were immunoprecipitated with anti-RhoA or anti-
Rac1 monoclonal antibody and then probed with antibodies against caveolin-3, RhoA, or Rac1. The experiments were repeated three times with similar results.

*Figure 5.*

**Effects of stretch with or without MβCD on the activation of RhoA and Rac1.**

Cardiomyocytes were plated on silastic membranes but not stretched, or were stretched 20% for 4 minutes, with or without prior treatment with 5 mM MβCD for 1 hour. Pull-down assays were performed and then blots were probed with antibody against RhoA (left) or Rac1 (right). Bar graphs show the relative bands intensities of active RhoA or Rac1 divided by total RhoA or Rac1. The experiments were repeated four times and averages ± SEM are shown. *: p<0.05 compared with unstimulated cells.

*Figure 6.*

**Effects of stretch with or without MβCD on the activation of ERK1/2.**

Cardiomyocytes were treated as described in Fig 5. Cell lysates were prepared and proteins separated by 8% SDS-PAGE, followed by immunoblotting with phospho-specific ERK or total ERK antibody. The experiments were repeated three times and averages ± SEM are shown. *: p<0.05 compared with unstimulated cells.
**Figure 7.**

**MβCD impairs nuclear translocation of active ERK via disassembly of actin cytoskeleton.** Cardiomyocytes were pretreated with or without 5 mM MβCD or with combination of 5 mM of MβCD and 50 nM of jasplakinolide for 1 hour prior to stretch. Localization of activated ERK was examined using phospho-specific ERK antibody. Actin fibers were visualized with rhodamine-conjugated phalloidin except in experiments with jasplakinolide where α-actinin, was stained. Scale bar: 10 µm.

**Figure 8.**

**Cytochalasin-D prevents nuclear translocation but not activation of ERK by stretch.** Cardiomyocytes were pretreated with 2 µM cytochalasin-D for 1 hour prior to stretch. Localization of activated ERK was visualized using phospho-specific ERK antibody and subsequent staining with FITC-conjugated goat anti-rabbit antibody Actin fibers were visualized with rhodamine-conjugated phalloidin. Scale bar: 10 µm.

**Figure 9.**

**Stretch-induced cardiac hypertrophy is blunted by MβCD.** Cardiac hypertrophy was assessed by ANF expression and myofibril organization. Unstimulated or stretched myocytes treated with or without MβCD were fixed, permeabilized, and incubated with anti-ANF polyclonal antibody. Myocytes were double stained with FITC-conjugated goat anti-rabbit IgG for ANF or Rhodamine-phalloidin for F-actin.
Figure 10.

Model depicting the role of caveolar RhoA and Rac1 in regulating actin cytoskeleton organization and ERK nuclear translocation in cardiomyocytes. Caveolar localized RhoA and Rac1 are specifically activated by stretch and lead to reorganization of actin cytoskeleton. ERK activation may occur via an integrin dependent pathway. Activation of ERK and reorganization of the actin cytoskeleton promoting translocation of active ERK and to the nucleus in response to stretch.
Figure 1

A

5% 45%
sucrose

1 2 3 4 5 6 7 8 9 10 11 12
fractions

Cav-3
FAK
Rho-GDI

Caveolae  Non-caveolae

B

Protein concentration (mg/ml)

0.0 0.5 1.0 1.5

0 1 2 3 4 5 6 7 8 9 10 11 12
fractions
Figure 2

A

![Western blots showing Active RhoA, Total RhoA, Active Rac1, and Total Rac1.](image)

B

![Graph showing RhoA and Rac1 activity over time of stretch.](image)
Figure 3

A

Cav-3 | RhoA | Rac1
---|---|---
**Unstretched**
[Image]
[Image]
[Image]
**Stretch (15min)**
[Image]
[Image]
[Image]

B

| Cav-3 | RhoA | Rac1 |
|---|---|---|
| **Unstretched** | **Stretch (15min)** | **Unstretched** |
| [Graph] * | [Graph] * | [Graph] * |

* indicates statistically significant differences.
Figure 4

A

| Cav-3 | RhoA | Rac1 |
|-------|------|------|
| Unstretched | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| Unstretched + M CD | ![Image](image4) | ![Image](image5) | ![Image](image6) |

B

| Stretch | M CD | IP: RhoA | IP: Rac1 |
|---------|------|----------|----------|
| -       | -    | ![Image](image7) | ![Image](image8) |
| +       | -    | ![Image](image9) | ![Image](image10) |
| -       | +    | ![Image](image11) | ![Image](image12) |
Figure 5

Active RhoA
Total RhoA

Active Rac1
Total Rac1

Active/Total RhoA (relative rate)
Active/Total Rac1 (relative rate)

Stretch
M CD
- + + - - - + + - - + + + +
Figure 6

Figure showing a bar graph comparing P-ERK/Total ERK (relative rate) under different conditions. The graph includes bars for Stretch and MCD treatments, with and without p-ERK1 and p-ERK2 markers.

- Stretch: - , +, +, -
- MCD: - , - , +, +

The graph indicates increased P-ERK/Total ERK with positive markers, particularly under the + conditions for p-ERK1 and p-ERK2.
Figure 7

Stretch (-)  
Control

Stretch (+)  
M CD (+)

M CD  
+ jasplakinolide
Figure 8

A

Stretch (-) Stretch (+) Stretch (-) Stretch (+)

Cytochalasin-D - - + + Cytochalasin-D - - + +

B

Stretch (-) Stretch (+)

Cytochalasin-D

p-ERK1 p-ERK2

ERK1 ERK2

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Figure 9

ANF

Myofibril Organization

Unstretched

Stretched (24 hr)

Unstretched + M CD

Stretched (24 hr) + M CD
Figure 10

Hypertrophic responses

stretch

RhoA  Rac1

caveolae

integrin

activation

ERK

translocation

nucleus

Hypertrophic responses

actin cytoskeletal rearrangement

cytochalasin-D

jasplakinolide

M CD
Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae
Shuji Kawamura, Shigeki Miyamoto and Joan Heller Brown

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