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Mechanical Stretch Redefines Membrane Gαq –Calcium Signaling Complexes

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Abstract

Muscle cells are routinely subjected to mechanical stretch but the impact of stretch on the organization of membrane domains is unknown. In this study, we characterize the effect of stretch on GPCR-Gαq protein signaling. Activation of this pathway leads to an increase in intracellular calcium. In muscle cells, GPCR-Gαq signals are enhanced when these proteins are localized in caveolae membrane domains whose curved structure can flatten with stretch. When we statically stretch rat aortic smooth muscle A10 cells by 1–5%, cellular calcium appears unperturbed as indicated by a calcium indicator. However, when we activate the bradykinin type 2 receptor (B2R) / Gαq pathway, we observe a loss in calcium that appears to be mediated through perturbations in calcium-activated stretch receptors. In contrast, if we apply oscillating stretch calcium signals are enhanced. We tested whether the observed changes in B2R-Gαq calcium signals were caused by stretch-induced disruption of caveolae using a combination of silencing RNA technology and growth conditions. We find that stretch changes the ability of monoclonal caveolin antibodies to bind caveolae indicating a change in configuration of the domains. This change is seen by the inability of cells to survive stretch cycles when the level of caveolae is significantly reduced. Our studies show that the effect of calcium signals by mechanical stretch is mediated by the type of stretch and the amount of caveolae.

Summary:

Caveolae are membrane domains that confer mechanical strength to cells. Caveolae also stabilize calcium signals through Gαq activation. This study finds that mechanical stretch inhibits initial calcium release with longer term effects that depend on the nature of the stretch.

Introduction

Smooth muscle cells routinely undergo changes in length as they carry out their biological function. These changes in length result from a number of different factors that cause cells to shift from a relaxed state to a contracted state and are mediated in part by intermediate

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filaments attached to dense band on the sarcolemma. These dense bands contain a large number of proteins including membrane domains called caveolae [1]. Caveolae are protein-rich invaginations on the plasma membrane of many cells and are found at high levels in muscle cells [2,3]. Caveolae are formed from the proteins caveolin1–3 (Cav1–3) and cavins, and in muscle cells caveolin-3 (Cav3) replaces Cav1 [4,5]. The aggregation of ~140 molecules of Cav proteins in the inner leaflet cause the highly curved morphology that distinguishes these domains [6]. Functionally, caveolae provide mechanical strength to cells by reversibly deforming and flattening to provide an extended surface area when cells are mechanically stretched or osmotically swollen (see [7]). In addition to embedding proteins that hold actin filaments to the plasma membrane, caveolae may influence cell signaling by scaffolding related family members of cell signaling pathways (see [8–10]). Our lab has found that caveolae enhance calcium responses generated through the Gαq / phospholipase Cβ (PLCβ) / phosphoinositide (4,5) bisphosphate (PIP_2) signaling pathway by localizing Gαq through specific interactions with caveolin proteins and stabilizing its activated state [11]. The net effect of this interaction is to enhance Gαq responses from external ligands that include angiotensin II, endothelin 1, acetylcholine, serotonin, bradykinin and dopamine.

Calcium levels play a key role in muscle tone and contraction, which are subject to stimuli such as physical stretch, osmotic stress, electrical stimulation, and extracellular agents such as endothelin I. Stretching smooth muscle results in contractions that are mediated by release of calcium from intracellular stores followed by an influx of external calcium [12]. Our lab is interested in understanding how cellular changes brought about by mechanical stretch affect caveolae and Gαq signaling on the single cell level. Cell stretch causes reorganization of actin filaments that underlie the cell substructure to impact caveolae organization [13]. A previous super-resolution study showed that even mild deformation of caveolae caused by a 50% reduction in osmotic strength can provide substantial amount of stabilization energy suggesting that caveolae may play a role in normal responses to stress [14]. The ability of caveolae to adapt and provide stabilizing energy may be related to their role in cardiac and muscle function [5].

The goal of these studies was to test the idea that the pathological conditions related to caveolae may be associated with its effects on calcium signals mediated through GPCR-Gαq. This idea is based on studies showing that Cav3 knock out mice display cardiomyopathy characterized by hypertrophy, dilation and reduced contractility [15] and by noting that Gαq plays a key role in hypertension, vascular remodeling and hypertrophy of vascular smooth muscle cells [16]. We used static and oscillating bidirectional stretch to measure the calcium responses due to Gαq stimulation in rat aortic smooth muscle cells (A10) with normal and reduced caveolae levels. Our studies show that mechanical stress has profound effects on calcium signals and these depend on the nature of the stretch and the abundance of caveolae. These studies highlight the importance of caveolae and the nature of the mechanical stretch in maintaining cell morphology and modulating calcium signaling.

Materials and methods

Cell culture.

Rat aortic smooth muscles A10 cells were purchased from ATCC (Cat# CRL-1476) and maintained in Dulbecco's modified Eagle's medium containing high glucose (GIBCO), 10 % fetal bovine serum (FBS), 1% penicillin streptomycin and 1% of 100mM sodium pyruvate. Cells are split into PDMS (polydimethylsiloxane) stretchable plates or glass-bottom dishes.

Sample preparation.

PDMS stretchable plates were made using the Sylgard ® 184 Silicon Elastomer kit. They were first sterilized by soaking in 70% ethanol, then coated with fibronectin for 30 minutes, washed with HBSS (Hanks' balanced salt solution) and coated again with fibronectin for 30 minutes. This double coating was necessary for the cells to attach and grow.

Stretch experiments.

A10 cells were grown on the PDMS plates described above and the plates were inserted into stretching device described by Dr. Kristen Billiar and colleagues [17]. The device was then placed on the stage of a Zeiss inverted confocal microscope LSM 510 Meta. Images were taken at room temperature using a 10x or 20x objective. In the experiments described here, the plates were stretched in the x-axis 1%, 2%, 5% and 10%. For static stretch studies, cells were stretched and held for different times, as indicated, before stimulation with 5μM of bradykinin. For oscillating stretch, images were collected immediately before stimulation and then after stimulation at the indicated times.

Calcium imaging.

Cells were labeled with the fluorescent calcium indicator (Calcium Green, Invitrogen) by washing with HBSS and then adding 5 μM of fluorophore diluted in 2ml of HBSS. The cell-dye solution was incubated at 37°C for 45 minutes and then washed twice with HBSS to remove the excess fluorophore. This procedure results in almost complete cell labeling. To determined changes in calcium levels with stimulation and/or stretch, we imaged the calls as described below, and monitored the change in intensity of individual cells as a function of time during the study. The images were transferred to ImageJ to select the region of each cell (i.e. ROI) and the change in intensity over the time series was calculated. The data shown in the figures are compiled over many cells in several independent experiments were the fluorescence intensities were normalized to the initial point, and are presented in arbitrary units. To view cell behavior in the absence of extracellular calcium, A10 cells labeled with Calcium Green (Invitrogen) and HBSS containing 0.63 mM filtered EDTA was added immediately before stretching.

Immunofluorescence measurements.

A10 cells, adhered to glass bottom imaging dishes (from Mat-Tek, MA), were fixed with warm (37° C) 3.7% formaldehyde solution in PBS (1:10 dilute 37% stock with PBS) and incubated for 1 hour. After, the dishes were washed twice with PBS. The medium was then

replaced with 0.2% NP 40 in PBS and incubated for no more than 5 minutes, and replaced with 4% BSA (Bovine Serum Albumin) in 1X TBS and incubated for 30 minutes. The blocking solution was then aspirated and replaced with primary antibody (Caveolin-1 mouse 7C8 from Santa Cruz, TX) diluted in 1% BSA in 1X TBS (1:500). All dishes were incubated at room temperature for 2 hours. The primary antibody was removed and the dishes were washed 3 times with 1X TBS for 3 minutes per wash. The last wash was replaced with appropriate fluorescent secondary antibody 488 from (Invitrogen) diluted in 1%BSA in 1X TBS (1:1000) and incubated for 1.5 hours at room temperature. The secondary antibody was removed from all the dishes and the dishes were washed 3 times with 1X TBS for 3 minutes per wash. The plates were kept away from light at 4°C. Cells were viewed in TBS buffer, on the Zeiss LSM 510 meta microscope using a 40x water objective at room temperature where single pictures and Z-stack pictures were taken. The analysis was performed using ImageJ.

Immunofluorescence measurements with phalloidin.

A10 cells were immunostained with Alexa Fluor 488 phalloidin for Actin (Invitrogen). After cells were split in low and high confluency dishes they were fixed with warm (37° C) 3.7% formaldehyde solution in PBS (1:10 dilute 37% stock with PBS) and incubated for 1 hour. Cells were then washed three times with warm PBS and permeabilized with 0.2% NP 40 in PBS for 5–10 minutes. In the meantime, phalloidin-methanol was diluted in PBS (5μL per 1mL) and after washing the cells with PBS for three times again, cells were stained with the diluted phalloidin for 25 minutes away from light in room temperature. Cells were viewed in PBS buffer, on Zeiss 510 meta microscope at room temperature using a 40x water objective where single pictures of different cells were taken. The analysis was performed using ImageJ.

Data analysis and Statistics.

Data were analyzed using Microsoft Excel and Sigma Plot 13. Statistical analysis included t-tests and ANOVA tests, where appropriate, using Sigma Plot 13. In the figures, the n values represent the number of cells analyzed and these were taken from independent experiments which range in number from 2–6.

Results

Bi-directional static stretch results in calcium efflux.

To determine the effect of mechanic stress on calcium signaling, we grew cells on silicon plates that could be reversibly stretched in the x or y direction using the device described by Billiar and colleagues (17). This apparatus gently stretches silicon plates in the x-y planes and the plate can be placed on the stage of a microscope. Cells can be directly grown on the silicon plates allowing us to continuously observe responses in real time with stretching. We first measured the calcium response of cells grown on the silicon plates with stimulation. These studies were carried out using rat aortic smooth muscle (A10) cells and stimulating calcium responses by the addition of 2μM bradykinin. Once bound to its receptors, bradykinin initiates calcium responses through activation of the Gαq/PLCβ pathway which in these cells, raises the intracellular concentration of calcium from 80 to 280 nM [18]. In Fig. 1 we show the responses of A10 cells to bradykinin where the cells were grown on glass

or silicon plates, and where the calcium response was monitored by the fluorescent calcium indicator, Calcium Green. The calcium response for the cells grown on silicon substrates where slightly reduced compared to those grown on glass due to differences in imaging (i.e. cells on silicon plates were imaged at 10x while those on glass were imaged at 40x). However, the calcium release and recovery behavior was the same for both (i.e. an initial calcium rise over a few seconds after addition of stimulant followed a long, slow recovery).

To determine whether mechanical stretch alone affects calcium levels, we carried out studies in which we stretched the cells by 1–10% in length over a 2 min period and held the stretch for 5, 10 or 30 min. This slow, static stretch will allow cells to adapt before any large changes in cell content occur and may be compared to the more physiological oscillating stretch conditions that are seen in many types of smooth muscles. We note that while muscle tissue can accommodate large changes in length, we find that our cell remain healthy (i.e. adherent without changes in morphology) at conditions milder than 10% stretch for 30 min. Fig. 2A shows that stretching the cells by 1% for 5 min results in a slight increase in calcium that returns to basal levels once the stretch is released. Subjecting the cells to a second round of stretching resulted in a similar increase in signal. These results show reversibility in cell calcium levels after the release of mechanical stretch.

While under different levels of stretch, we stimulated the cells with bradykinin and collected images as a function of time. All stretch conditions show the same behavior (Fig. 2B–C). The initial increase in calcium that occurs in the first few minutes under basal conditions is no longer visible, and a large reduction in signal is observed after 5–10 minutes. Interestingly, the drop in calcium was inversely dependent on the amount of stretch where the highest reduction is seen at lower stretch conditions (Table 1). Additionally, we find that calcium levels of these stimulated cells do not recover while under stretch (Fig. 3A–C). We interpreted this calcium behavior to be due to a combination of different mechanisms: an initial intracellular calcium release from calcium stores in the endoplasmic reticulum due to activation of the Gαq/PLCβ pathway, disruption of activating interactions between Gαq and caveolin molecules, and the opening of stretch-activated calcium channels on the plasma membrane that allows extracellular calcium efflux. To test this latter mechanism, we repeated the study under the same conditions but eliminated calcium in the medium by the addition of EDTA (Fig. 4A). These results show that at 1% stretch, the calcium release due to bradykinin stimulation is similar to unstretched cells indicating that the large decrease in calcium levels during stretch is due efflux into the medium. However, at high amounts of stretch, the initial calcium release is reduced (Fig. 4B) suggesting that higher stress is perturbing other calcium mechanisms (see discussion).

Different calcium behavior is observed under oscillating stretch.

Instead of static stretch, we subjected cells to 1% stretch at a frequency of 0.5 Hz (2 s) to better represent physiological stress. In these studies, calcium measurements were taken immediately after release of the oscillating stretch. We find that while calcium responses show an initial increase in level similar to control, they did not recover and continued to increase with time (Fig 5A–B). These results suggest that calcium release through activation of Gαq/PLCβ is intact, but calcium loss through stretch receptors, if operative, either does

not contribute, or is reversible unless the behavior seen for static stretch. The lack of recovery in calcium levels after stimulation suggest that the frequency of the oscillating stretch does not allow for complete calcium recovery after stimulation or alters the gating of calcium channels (see discussion) Repeating these studies in the presence of extracellular EDTA shows eliminates the increase in calcium (Fig 5A).

Mechanical stress impacts caveolae domains.

Caveolin molecules stabilize the activated state of Gαq to enhance calcium signals. We speculated that the reduction in the initial calcium signal under stretch might be caused by a disruption in caveolin-Gαq contacts. Before testing this idea, we first determined how caveolae contributes to calcium responses under stretch. Noting that previous studies have linked the number of caveolae with cell to cell contact [19], we first used cell density to vary caveolae expression, and in Fig. 6A, we show that cells grown at high density have higher levels of Cav1 than those grown at low density. We additionally used siRNA(Cav1) to reduce caveolin expression and lower the number of caveolae (Fig. 6B).

A10 cells grown at high and low densities were treated with siRNA(Cav1) and subjected to oscillating stress. In high density cells, reducing the level of caveolae ~40% as estimated by western blotting had little effect on the calcium response (Fig.7). Low density cells treated with siRNA(Cav1) appeared healthy but subjecting them to stretch and then bradykinin resulted in an initial loss of ~50% of the cell followed continuous calcium efflux.

Super-resolution fluorescence studies indicate that mild osmotic stretch deforms caveolae and reduces calcium signals [14]. To determine whether this is also the case with mechanical stretch, we assessed structural alternations in caveolae by determining the ability of a Cav1 monoclonal antibody to recognize its epitope. Changes in epitope accessibility could be due to disruption in the molecular interactions within the caveolae domain or changes in the molecular caveolin structure. We determined changes in anti-Cav1 immunostaining in A10 cells subjected either static or oscillating stretch. We find a large decrease in the level of immunostaining of Cav1, but a only small increase of Gαi and no change in Gαq (Fig. 8). These results are consistent with a structural deformation of caveolae domains with stretch.

Discussion

In this study, we have determined the effect of increased mechanical stress on caveolae/B2R/Gαq domains that are responsible for calcium signals. This work stems from previous studies where we found a connection between caveolae, membrane tension, and calcium signaling [18]. Caveolae domains are prominent in muscle cells where they have been linked to cardiomyopathies and other muscle pathologies [15]. Caveolae have been shown to provide mechanical strength to cells [20] and our previous work suggests that a single caveola can provide ~180 nN of stabilization in response to stress [14]. Besides this mechanical function, caveolae have been found to enhance calcium signals mediated through the Gαq/PLCβ signaling pathway by stabilizing the activated state of Gαq after GPCR stimulation [18]. Here, our goal was to determine how mechanical stress deforms these membrane domains to impact calcium signals. We used cultured muscle-derived cells where we previously found using osmotic stress that caveolae domains remain intact under mild

deformation [14], and we detected relative changes in calcium using the fluorescence sensor, Calcium Green. Although it is possible that the behavior we see with stretch is an artefact of the dye, we do not believe this is the case since we see systematic and reversible changes in the intensity under various conditions. Also, we used mild bidirectional stretch (1–5%) that is well within normal range of lengthening in these cells and studied the effects on calcium responses upon Gαq activation under static stretch and oscillating stretch to mimic basal tone and contractions and in smooth muscle cells.

It is important to note that the contribution of caveolae in regulating calcium flow in cells subjected to stress is unclear. Besides Gαq, caveolae are thought to localize certain Na^+ channels but not Ca^{2+}-activated Na^+ channels [1]. Previous studies in primary pulmonary endothelin cells whose caveolae has been disrupted show no changes in calcium under basal conditions but calcium levels decrease when the membrane tension increases. [21] However, the opposite pattern occurs in hypoxic cells making the role of caveolae in regulating calcium signals under stress uncertain.

Cells undergo changes in membrane tension routinely when carrying out various functions, such as migrating or engaging in tissue movement. These changes involve spreading of the components in the plasma membrane that destabilizes interactions between integral membrane proteins and lipids. In membranes that contain stretch-activated receptors and/or ion channels, adapt responses to membrane tension can generate ion flow and other changes that allow the cells to to the new environmental conditions. Thus, in the first series of studies, we slowly stretched the cells over a 2 min period to allow for adaption. We find that even small amounts of stretch change the activity of calcium channels to give a large efflux of calcium into the medium. Because this efflux is observed after the initial small rise after stimulation of the stretched cells, it is consistent with activation of calcium-activated channels on the membrane surface. The calcium levels do not recover as long as stretch is applied. By including EDTA in the medium to inhibit these channels, this efflux was no longer observed, and instead, the shape of calcium response curve in cells subjected to 1% stretch is similar to unstretched cells. We also find that the amount of the efflux is reduced as the amount of stretch is higher (Table 1) suggesting that higher stretch affects the integrity of the ion channels by disturbing their membrane configuration. The efflux of calcium may be associated with quenching of the activity of calcium-sensitive proteins and blocking calcium-mediated processes.

Once calcium efflux is eliminated, we find that static stretch above 1% causes a significant reduction in intracellular calcium release. This observation correlates well with the expected deformation of caveolae, as indicated in our immunofluorescence studies, and disruption of Cav1 –Gαq interactions caused by osmotic stress [18]. We note that stretch may also affect other associations involved in this pathway, such as Gαq-PLCβ interactions. However, the reduction in calcium increase with stretch from 1–5% is comparable to the reduction seen when the osmotic strength is reduced from 300 to 150 mOsm [18].

As a more physiologically relevant perturbation, we studied the effect of oscillating stretch on calcium signaling. Here, the cells were stimulated immediately following the stretch release. In sharp contrast to static stretch, these cells show an initial increase upon

stimulation, followed by a steady increase in calcium levels due to calcium influx (Fig. 5). This steady increase in calcium suggests that the rates of calcium recovery are slower than the rate of calcium release so that cells do not fully return to basal calcium during the oscillations. This idea is supported by the data in Fig. 1 showing that recovery after stimulation takes 20–25 s. Since the oscillation frequency is 0.5 Hz, then the recovery is ~10 fold slower indicating that the time for the elevated calcium level to returned to basal level is longer than the time of the oscillations. In tissues and natural systems, we expect that the frequency of oscillating stretch would be optimized to either allow the cell to recover or to allow sustained high calcium and maximize activation of calcium sensitive proteins and enzymes.

Our results clearly show the importance of caveolae in stabilizing the cells under stress. At high cell density, the level of caveolae has little effect on calcium responses. In sharp contrast, lowering the levels of caveolae in low density cells was lethal, and the cells that did survive showed a slow and continuous leakage of calcium. Our results are consist with the idea that muscle tissue, which is enriched in caveolae due to extensive cell-cell contacts, is able to handle stretch through dampening of the forces by caveolae to allow normal Gαq signaling. These data suggest that absent or mutated caveolin proteins may make muscle tissue vulnerable to calcium dysregulation under repeated stress and studies are underway to better understand theses effects.

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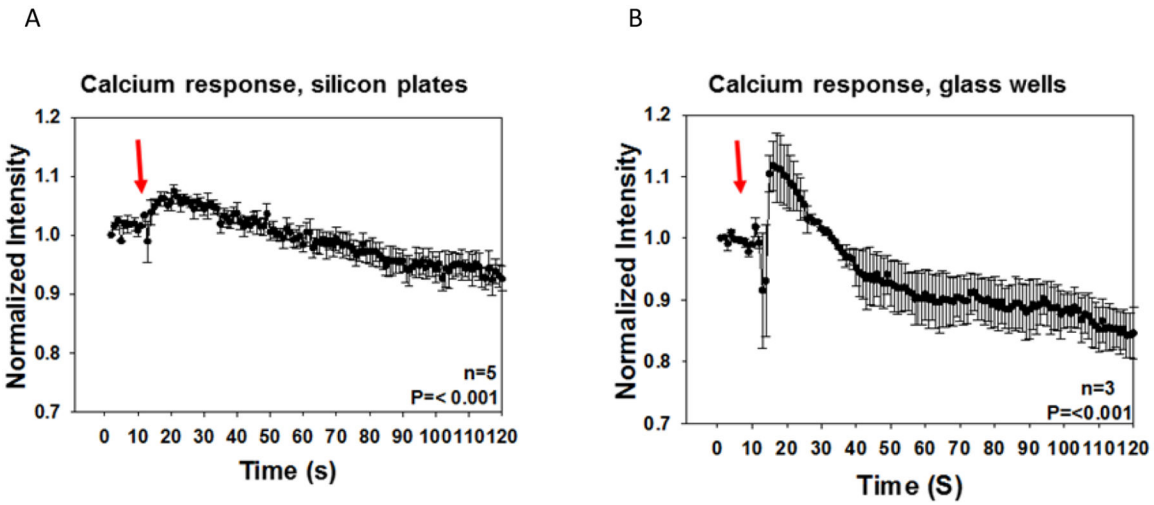
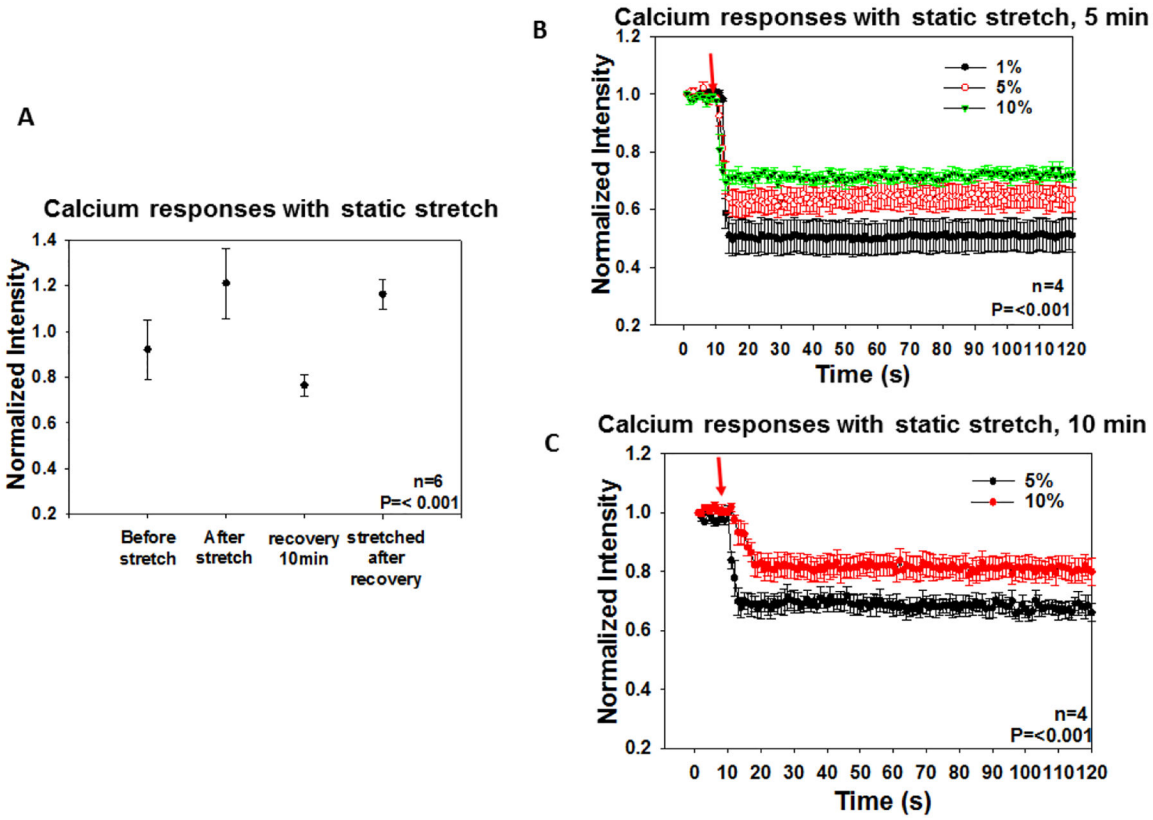


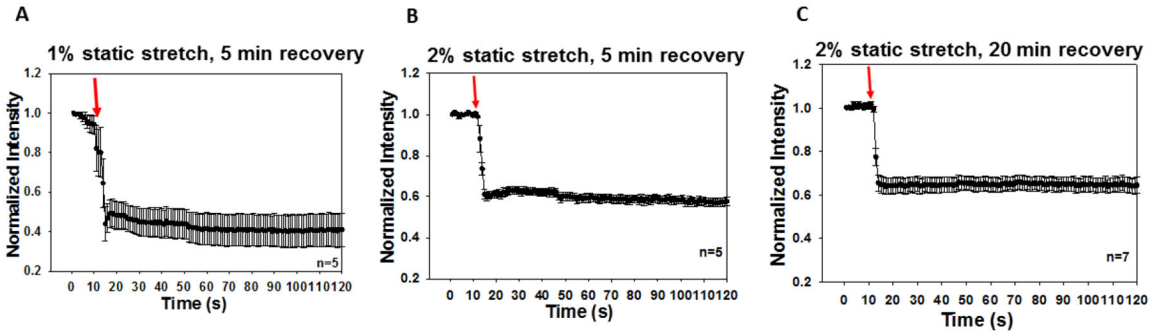
1. Calcium response of A10 cells with the addition of bradykinin.

Normalized response of A10 cells loaded with Calcium Green cultured on PDMS (silicon) plates stimulated by 5 μM bradykinin **(A)** as compared to A10 cells cultured in glass bottom dishes **(B)** where stimulation is indicated by red arrow, and where n refers to the number of independent experiments that sampled 4–6 cells.



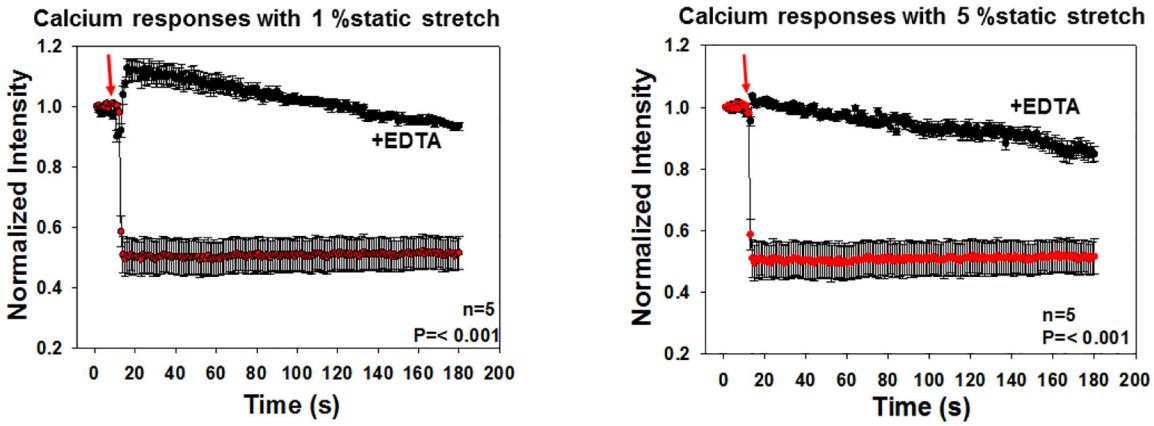
2. Static stretch results in calcium efflux.

(**A**) Normalized changes in Calcium Green intensity in unstimulated A10 cells where the signal was monitored before and after 1% static stretch for 5 minutes. Cells were allowed to recover for 10 minutes, re-stretched and recorded. (**B**) A similar study were 1%, 5% and 10% static stretch was applied for 5 minutes, and (**C**) where 5% and 10% static stretch was applied for 10 minutes. The time of stimulation is indicated by red arrow, and where n refers to the number of independent experiments that sampled 4–6 cells. ANOVA testing comparing the mean time point in throughout each graph shows statistically significant differences $p<0.001$.



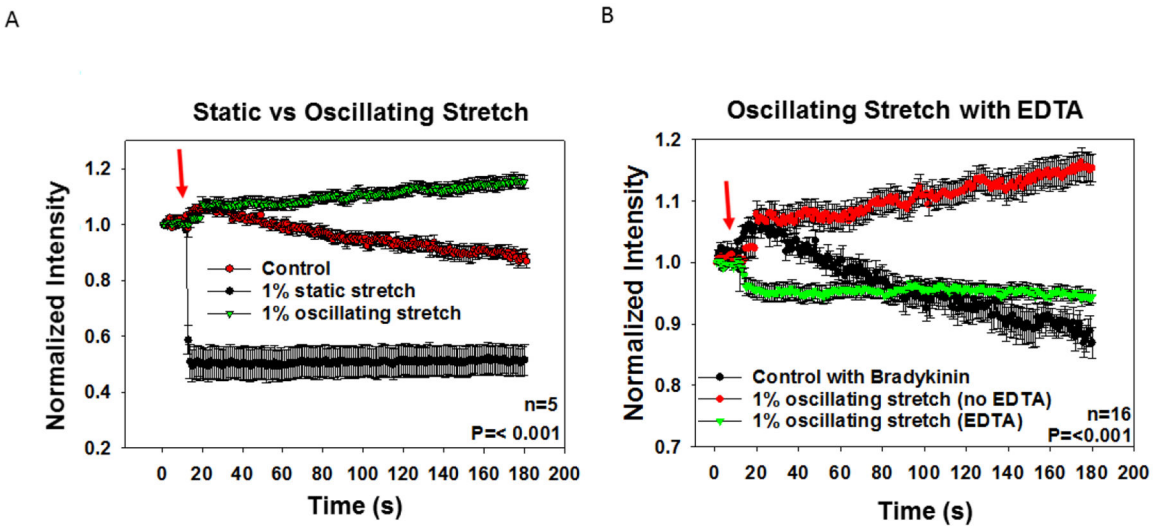
3. Static stretch irreversibly effects calcium responses.

Normalized changes in Calcium Green intensity in A10 cells in which 1% static stretch was applied for 5 minutes and allowed to recover for 2 minutes (**A**) or 5 minutes (**B**) before stimulating the cells with bradykinin. (**C**) A similar study where 2% static stretch was applied for 5 mins and allowed to recover for 20 minutes before stimulation. The time of stimulation is indicated by red arrow, and where n refers to the number of independent experiments that sampled 4–6 cells. ANOVA testing to compare the mean time point in throughout each graph shows statistically significant differences $p<0.001$.



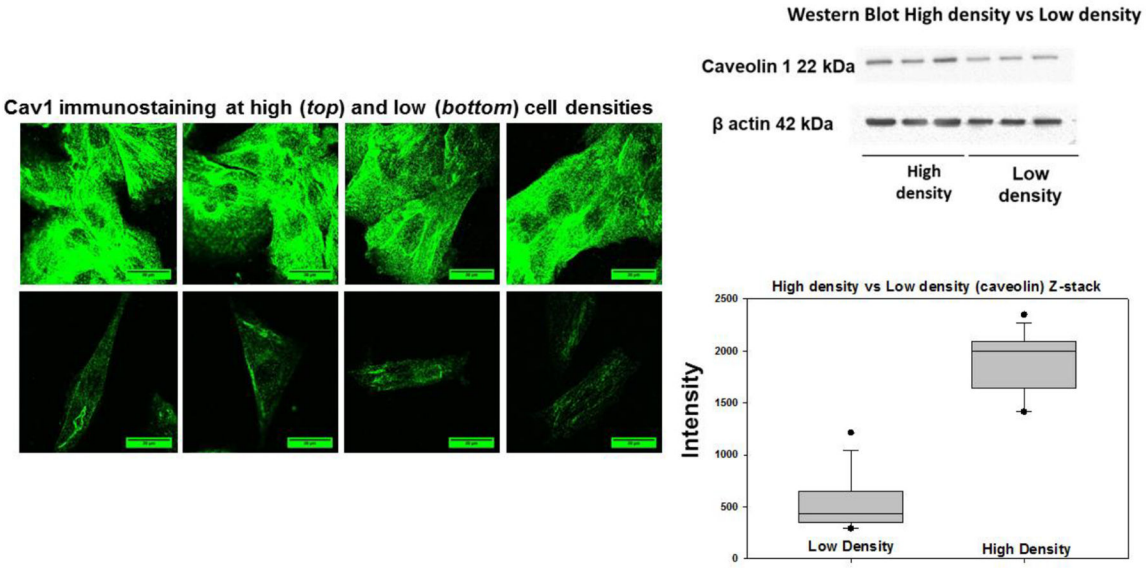
4. Removing extracellular calcium gives unpertured calcium responses.

(Left) Normalized change in Calcium Green intensity in A10 cells in which 1% static stretch was applied for 5 minutes before the exposure to 0.63mM EDTA and subsequent stimulation with bradykinin. *(Right)* an identical study in which cells were stretched at 5%. The time of stimulation is indicated by red arrow, and where n refers to the number of independent experiments that sampled 4–6 cells. ANOVA testing to compare the mean time point in throughout each graph shows statistically significant differences $p<0.001$.



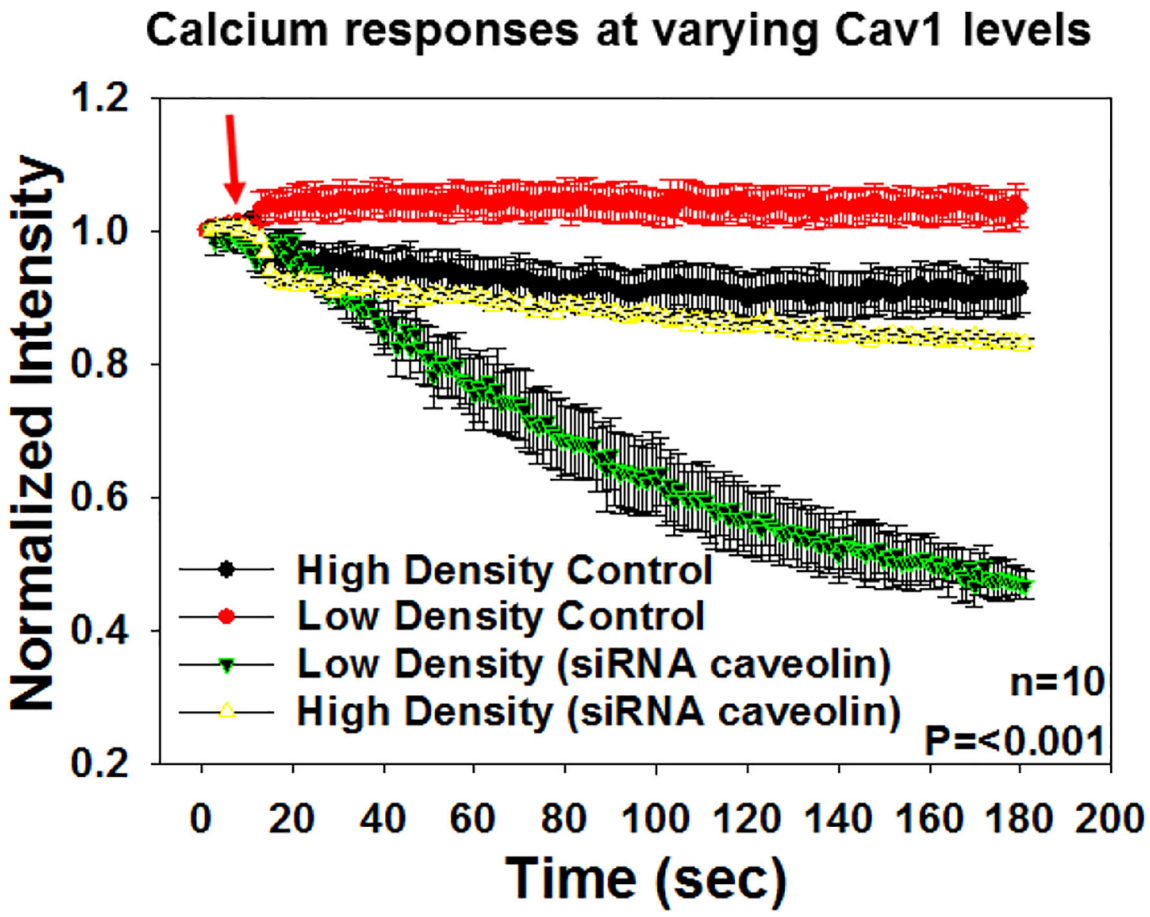
5. Calcium behavior of static and oscillating stretch.

(**A**) Normalized Calcium Green intensities in A10 cells with stimulation of control cells (no stretch) as compared to cells that underwent 1% static stretch for 5 minutes and 1% oscillating stretch for 5 minutes. (**B**) A similar study as in (**A**) except that cells were subjected to 1% oscillating stretch for 30 minutes in the presence and absence of EDTA. The time of stimulation is indicated by red arrow, and where n refers to the number of independent experiments that sampled 4–6 cells. ANOVA testing to compare the mean time point in throughout each graph shows statistically significant differences $p<0.001$.



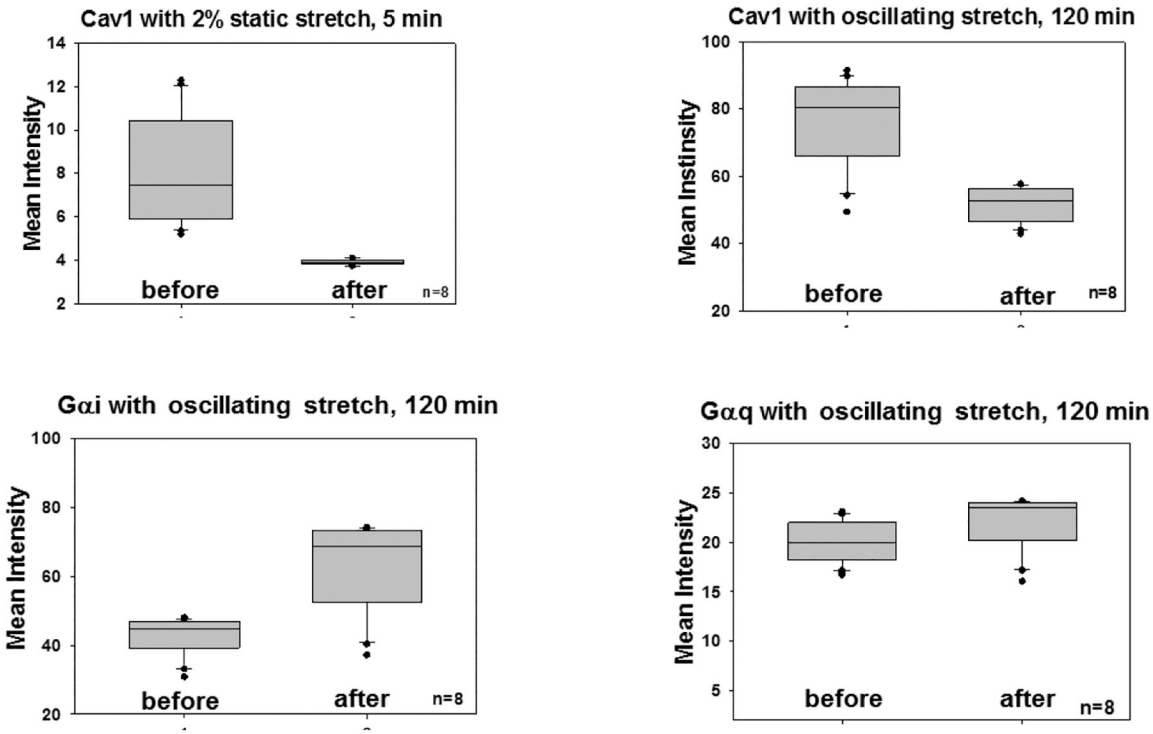
6. Caveolin expression increases with cell density.

(Left) Cells were grown at high density (*top panels*) or low density (*bottom panels*) and immunostained with Anti-Cav1. (*Middle top*) A sample western blot of Cav1 from high and low density cells and (*middle bottom*) a compilation of band intensities from western blots from anti-Cav1 compared to anti-actin where the data are significantly different as determined using a Student t-test, $p<0.001$. (*Right*) A sample western blot showing the inverse correlation between actin and Cav1 levels where LD=low density and HD=high density.



7. The effect of oscillating stretch on calcium behavior varies with the level of caveolae.

Normalized calcium responses in A10, grown at high density and showing high levels of caveolae, or grown at low density showing low levels of caveolae, were monitored after treatment of 1% oscillating stretch for 30 min. Normalized calcium responses were also monitored in cells treated with siRNA(Cav1). The time of stimulation is indicated by red arrow, and where n refers to the number of independent experiments that sampled 4–6 cells. ANOVA testing to compare the mean time point in throughout each graph shows statistically significant differences $p<0.001$.



8. Caveolae reorganizes with stretch.

A10 cells were treated with 2% static bi-directional mechanical stretch for 5 minutes (*top left*), or 1% oscillating stretch for 2 hour (*top right*), fixed and immunostained with Anti-Cav1 and viewed. The mean intensities were normalized and averaged after subtracting for background for the number of cells analyzed in each figure. Changes in immunostaining were statistically significant for Cav1 ($p=<0.001$, Student t-test) (*upper panels*) but not for Gαi or Gαq ($p=0.040$, Student t-test and $p=0.070$, Signed Rank test respectively) (*bottom panels*).

TABLE 1

Net decrease in Calcium Green signal under different static stretch conditions

STRETCH APPLIED		% Reduction in Intensity
1%	5 minutes	58%
2%	5 minutes	50%
5%	5 minutes	42%
10%	5 minutes	32%
5%	10 minutes	36%
5%	30 minutes	38%
10%	10 minutes	22%

The % reduction in Calcium Green signal in cells that were stretched and then stimulated for n=3–12 samples and where SD ranges from 2–6%.