Published in final edited form as:

J Biomech. 2012 March 15; 45(5): 888–894. doi:10.1016/j.jbiomech.2011.11.031.

Intracellular Ca^{2+} accumulation is strain-dependent and correlates with apoptosis in aortic valve fibroblasts

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Abstract

Aortic valve (AV) disease is often characterized by the formation of calcific nodules within AV leaflets that alter functional biomechanics. *In vitro*, formation of these nodules is associated with osteogenic differentiation and/or increased contraction and apoptosis of AV interstitial cells (AVICs), leading to growth of calcium phosphate crystal structures. In several other cell types, increased intracellular Ca^{2+} has been shown to be an important part in activation of osteogenic differentiability. However, elevated intracellular Ca^{2+} is known to mediate cell contraction, and has also been shown to lead to apoptosis in many cell types. Therefore, a rise in intracellular Ca^{2+} may precede cellular changes that lead to calcification, and fibroblasts similar to AVICs have been shown to exhibit increases in intracellular Ca^{2+} in response to mechanical strain. In this study, we hypothesized that strain induces intracellular Ca^{2+} accumulation through stretch-activated calcium channels. We were also interested in assessing possible correlations between intracellular Ca^{2+} increases and apoptosis in AVICs. To test our hypothesis, cultured porcine AVICs were used to assess correlates between strain, intracellular Ca^{2+} , and apoptosis. Ca^{2+} sensitive fluorescent dyes were utilized to measure real-time intracellular Ca^{2+} changes in strained AVICs. Ca^{2+} changes were then correlated with AVIC apoptosis using flow cytometric Annexin V apoptosis assays. These data indicate that strain-dependent accumulation of intracellular Ca^{2+} is correlated with apoptosis in AVICs. We believe that these findings indicate early mechanotransductive events that may initiate AV calcification pathways.

Introduction

Aortic valve (AV) disease is often characterized by the formation of calcific nodules within thickened AV leaflets, limiting the biomechanical function of the valve. Calcific nodules have long been a major problem in the failure of bioprosthetic replacement heart valves, where the association of extracellular Ca^{2+} with phosphates remaining in cellular debris within the fixed, decellularized scaffolds has been proposed to lead to the nucleation and growth of calcific nodules (Kim, Herrera et al. 1999; Schoen and Levy 2005). In native leaflets, calcification is thought to be an active process involving AV interstitial cells (AVICs). The molecular mechanisms that lead to the formation of these calcific nodules in native leaflets remain unclear; however, AVICs have been shown to form nodule-like structures *in vitro* through differentiation to a phenotype with osteogenic character (Yip, Chen et al. 2009; Balachandran, Sucusky et al. 2010). Nodules have also been characterized by contractile myofibroblastic (i.e., smooth muscle α -actin (α SMA) positive) AVICs and high levels of apoptosis (Benton, Kern et al. 2009; Yip, Chen et al. 2009). Heightened levels of intracellular Ca^{2+} are associated with contraction (Follonier Castella, Gabbiani et al.

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2010), apoptosis (Lemasters, Theruvath et al. 2009), and osteogenic differentiation (You, Reilly et al. 2001; Zayzafoon 2006) in many other cell types as well.

Changes in intracellular Ca^{2+} have been shown to play a significant role in the response of cultured myofibroblasts to mechanical stimuli. Specifically, mechanically-induced changes in intracellular Ca^{2+} lead to short term cellular contraction, allowing the cell to resist externally applied strains (Follonier Castella, Gabbiani et al. 2010). Previously, significant and sustained changes in AV leaflet mechanical properties due to AVIC contraction have been observed due to addition of 90 mM KCl to the media, which results in a rapid Ca^{2+} influx into the cells (Kershaw, Misfeld et al. 2004; Merryman, Huang et al. 2006). *In vivo*, AVICs are continuously subjected to a dynamic strain environment that has been shown to influence AVIC behavior (Huang, Liao et al. 2007; Merryman, Lukoff et al. 2007; Ferdous, Jo et al. 2011); however, to our knowledge, no studies have investigated the role of strain-induced intracellular Ca^{2+} changes in AVICs. We believe that these changes may be important in normal AVIC function as well as disease progression. Correspondingly, mechanical strain has been shown to influence nodule formation in excised AV leaflets (Balachandran, Sucosky et al. 2010). In the current study, we assess the role in intracellular Ca^{2+} accumulation in AVICs and the potential role of these changes in causing AVIC apoptosis. We hypothesize that strain leads to an increase in intracellular Ca^{2+} over time in AVICs. We believe that intracellular Ca^{2+} accumulation is correlated with apoptosis in AVICs and may also play a role in early mechanotransductive events that lead to valve disease.

Methods

Cell Isolation and Culture

AVICs were harvested from porcine AV leaflets obtained from a local abattoir (Hampton's Meat Processing, Hopkinsville, KY) as described previously (Merryman, Liao et al. 2007). Isolated AVICs were cultured on T-75 tissue culture treated flasks in cell growth media consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic, and 1% amphotericin B antifungal. Cells were grown to 60 – 80% confluence prior to experiments. Previous studies have shown that AVICs retain phenotypic integrity up to 10 passages after initial harvest (Maish, Hoffman-Kim et al. 2003); therefore, we did not exceed this maximum limit. For all strain experiments, serum-free DMEM was used.

Strain System for Real-Time Ca^{2+} Measurements

AVICs were cultured in custom made polydimethylsiloxane (PDMS) wells on flexible membranes and mechanically loaded using a uniaxial stretching apparatus (Fig. 1A). Prior to seeding AVICs, PDMS membranes were functionalized with fibronectin using a modified procedure previously employed to covalently link collagen to PDMS (Fig. 1B) (Wipff, Majd et al. 2009). This functionalization technique was shown to effectively transduce applied strains to cells grown on the membranes (Wipff, Majd et al. 2009). Briefly, the PDMS surface was treated with reactive oxygen groups through exposure to oxygen plasma for 1 min. A 10% solution of 3-aminopropyltriethoxysilane (APTES) in ethanol was immediately added to the membrane to introduce free amines to the surface. After 1 h incubation at 60°C, membranes were washed with PBS and incubated with 3% glutaraldehyde for 20 min. The membranes were washed twice more and incubated with 100 $\mu\text{g/ml}$ fibronectin for 1 h at 37°C. AVICs adhered to the functionalized PDMS surface and exhibited appropriate morphology (Fig. 1C).

Strain System for Flow Cytometric Analyses

For long-term analysis, AVICs were cultured on Pronectin BioFlex® culture plates at 2×10^5 cells per well in cell growth media. These plates were then loaded onto a Flexcell 4000 Tension system and received equibiaxial strains of up to 20% by deforming the membranes over cylindrical loading posts. Using this system, we applied a static strain of 20% to AVICs for 5 min, 15 min, or 3 h. To test more physiologically relevant loading regimens, cyclic strains of 10% or 20% were applied at 1 Hz for 3 h. AVICs were subsequently washed thoroughly in cold PBS to remove excess dye, scraped from the culture plates, and kept on ice prior to flow cytometric analysis.

Real-time Ca^{2+} Analysis

For real-time Ca^{2+} analyses, AVICs were loaded with the Ca^{2+} sensitive dye x-Rhod-1 AM (Invitrogen) for 30 min at 37°C. Following this incubation, AVICs were rinsed twice with indicator free media prior to the addition of strain. To test the hypothesis that intracellular Ca^{2+} accumulates in AVICs as a function of time and strain, AVICs cultured on fibronectin-functionalized PDMS substrates and loaded with the Ca^{2+} -sensitive dye x-Rhod-1 AM were clamped in a custom uniaxial stretch device with a pitch of 0.32 mm/turn (Fig. 1A) and placed above a 20x Zeiss EpiPlan-NeoFluar objective on an inverted microscope (Axiovert 200; Carl Zeiss). Using this system, AVICs were subjected to either 10% or 20% uniaxial static strain. Immediately, following the addition of strain, the objective was readjusted to bring AVICs into focus and the initial fluorescence measurement was taken (corresponding to $t = 0$). Excitation light at 550 ± 15 nm was reflected on the cells by a dichroic mirror (DC590DRLP; Omega Optical). X-Rhod-1 fluorescence emission was collected and recorded at a wavelength of 600 nm (EM 600ALP; Omega Optical) at 20,000 Hz for 5 s in 1 min intervals for a total of 5 min after the addition of strain. In order to compare between samples and correct for inconsistencies in dye loading, each fluorescence reading was normalized to the recorded fluorescence measurement taken immediately after the addition of strain. During post-processing, each 5 s recording was averaged to give a single fluorescence measurement at each recording interval.

To assess intracellular Ca^{2+} changes in AVICs following the removal of strain. The PDMS substrate was unloaded after 5 min, the 20x objective was refocused, and an initial fluorescence measurement was recorded. As in the strained cases, fluorescence measurements were taken in 1 min intervals for 5 additional min and each measurement was normalized to the first fluorescence recording taken immediately after the release of strain. Percentage Ca^{2+} efflux was calculated from and normalized to the final strained intracellular Ca^{2+} reading.

Ca^{2+} and Apoptosis Flow Cytometry Assays

AVICs were loaded with the Ca^{2+} sensitive dye Fluo-4 AM (Invitrogen) for 30 min at 37°C. After being receiving equibiaxial strain in the Flexcell, Fluo-4 labeled AVICs were washed and scraped in cold PBS, and immediately placed on ice until flow cytometer analysis (within 30 min of strain) with a BD LSR II flow cytometer. For apoptotic analyses, AVICs were resuspended in Annexin V Binding Buffer with Annexin V-PE to assay for apoptosis or propidium iodide to assay for plasma membrane integrity. Annexin V binds to phosphatidylserine that is expressed on the outer surface of plasma membranes in cells undergoing apoptosis. Propidium iodide is impermeant to intact plasma membranes and is used to distinguish apoptotic cells from necrotic cells. Consequently, apoptotic cells stain positive for Annexin V but negative for propidium iodide, whereas necrotic cells stain positive for both markers. To test for general uptake of extracellular molecules during strain, we added 40 μM of the plasma membrane impermeant dye sulforhodamine 101 to AVICs prior to 20% strain. After the removal of strain, AVICs were washed 3 times with

sulforhodamine-free media to remove extracellular dye prior to flow cytometry. For all flow cytometric analyses appropriate compensation controls were utilized to correct for spectral overlap.

Statistics

All data were calculated as the average of at least three independent experimental replications ($n > 3$). All error bars were calculated as standard error of the means. Statistical differences within strain groups were determined by analysis of variance (ANOVA), and pairwise differences were established with post hoc testing using the most stringent pairwise Holm-Sidak test. Repeated measures ANOVA was used to test for differences between samples receiving different strains over a period of time. For all cases, the overall significance level was set at 0.05.

Results

Real-time Intracellular Ca^{2+}

Strain caused significant increase in intracellular Ca^{2+} over 5 min (Fig. 2A $p < 0.005$). Ca^{2+} increases from both strains were well described with least square linear fits to yield a rate of change of 0.0205 for AVICs subjected to 10% strain ($R^2 = 0.99$) and a slope of 0.0295 for AVICs strained to 20% ($R^2 = 0.97$). Using repeated measures ANOVA ($p < 0.05$), the 20% strain led to significantly greater intracellular Ca^{2+} accumulation compared to the 10% strain case. Increases in intracellular Ca^{2+} were visually confirmed by microscopic imaging of Fluo-4 loaded AVICs (Fig. 2C–F)

Upon strain removal (Fig. 2A), intracellular Ca^{2+} decreased over the 5 min measurement period, and 20% strain resulted in significantly more retained intracellular Ca^{2+} compared to 10% strain ($p < 0.001$). To verify that the strain-induced rise in intracellular Ca^{2+} was due to an influx from extracellular Ca^{2+} rather than from a release of intracellular stores, 20% strain was applied to AVICs in Ca^{2+} -free phosphate buffered saline (Fig. 2B). Intracellular Ca^{2+} decreased over the course of 5 min with a significant cumulative decrease observed at 3 min and beyond ($p < 0.005$). These results indicate that the rise in intracellular Ca^{2+} (Fig. 2A) must originate from an influx of extracellular Ca^{2+} . As such, when extracellular Ca^{2+} is removed, the reversed concentration gradient leads to Ca^{2+} efflux from AVICs. To determine whether strain-mediated Ca^{2+} influx was due to non-specific openings in the plasma membrane or to stretch-activated ion channels, 10 μM of the stretch-activated Ca^{2+} channel inhibitor GdCl_3 was added to AVICs immediately prior to the addition of 20% strain. The free Gd^{3+} ions act as a competitive inhibitor to occupy ion channel openings; therefore, the rate of Ca^{2+} accumulation through these channels is slowed when Gd^{3+} is present. The GdCl_3 treatment significantly (repeated measure ANOVA, $p < 0.05$) reduced the rate of Ca^{2+} accumulation after strain (Fig. 2B).

Flow Cytometric Ca^{2+} Analyses

Long-term changes in intracellular Ca^{2+} fluorescence, measured by flow cytometry (Fig. 3A) after static biaxial strain, were in close agreement with real-time measurements for AVICs strained uniaxially to 20% (Fig. 2A). 20% biaxial static strain for 5 min resulted in a significant population shift in measured intracellular Ca^{2+} fluorescence with an $11 \pm 2\%$ mean increase compared to static controls (Fig. 3A, blue line). After 15 min of 20% biaxial static strain, the population mean increased by $41 \pm 3\%$ compared to static controls (Fig. 3A, red line).

We were also interested in assessing the rate by which intracellular Ca^{2+} decreases following the removal of strain in our long-term analyses. To test this, we repeated the

Flexcell experiment whereby Fluo-4 loaded AVICs received 20% static strain for 15 min; however, after release of the strain, one BioFlex® plate was immediately placed on ice for 30 min, and one BioFlex® plate was left at 37°C for 30 min. Samples that were incubated at 37°C after strain did not contain significantly higher levels of intracellular Ca^{2+} than the unstrained controls (Fig. 3B), indicating an active cellular Ca^{2+} removal process that is slowed at lower temperatures. For all of the long-term assays, AVICs were kept on ice prior to flow cytometric analyses; therefore, these results also indicate that the Ca^{2+} signal measured by flow cytometry is an accurate representation of the accumulated Ca^{2+} immediately after the removal of strain.

Finally, to ensure that the measured increases in fluorescence was due to changes in Ca^{2+} levels and not strain-mediated influx of unwashed dyes, we added the plasma membrane impermeant dye sulforhodamine 101 to AVICs and applied 20% equibiaxial static strain for 15 min. Flow cytometric analyses revealed no change in sulforhodamine fluorescence between unstrained controls and strained AVICs (Fig. 3C). These results suggest that strain does not increase membrane permeability to all extracellular substances.

Flow Cytometric Apoptosis Analyses

To examine the relationship between strain, intracellular Ca^{2+} , and AVIC apoptosis, propidium iodide or Annexin V was added to strained AVICs loaded with Fluo-4. Unstrained AVICs display background levels of intracellular Ca^{2+} and retain plasma membrane integrity as indicated by low propidium iodide fluorescence (Fig. 4A). Unstrained AVICs also exhibit little Annexin V fluorescence, indicating that background levels of apoptosis are low (Fig. 4B). In contrast, AVICs equibiaxially strained to 20% for 3 h exhibit increased intracellular Ca^{2+} and intact membranes (Fig. 4C), and the high levels of intracellular Ca^{2+} correlate with high Annexin V fluorescence (Fig. 4D). Therefore, strained AVICs with accumulated intracellular Ca^{2+} have intact plasma membranes (as indicated by a lack of propidium iodide fluorescence in $90 \pm 5\%$ of AVICs with high intracellular Ca^{2+}) and are apoptotic (as indicated by increased Annexin V staining in $76 \pm 0.82\%$ AVICs with high intracellular Ca^{2+}).

Effect of Cyclic Strain on Intracellular Ca^{2+}

We next assayed for intracellular Ca^{2+} accumulation in a more physiologically relevant cyclic strain environment using the Flexcell system. AVICs subjected to 10% equibiaxial cyclic strain at 0.75 Hz for 3 h exhibited a significant ($p < 0.005$) $79 \pm 16\%$ increase in measured intracellular Ca^{2+} compared to unstrained controls. Increasing the applied cyclic strain to 20% resulted in a significant ($p < 0.005$) $98 \pm 22\%$ increase in intracellular Ca^{2+} relative to unstrained controls.

Discussion

Intracellular Ca^{2+} Accumulation in AVICs is Strain-dependent and Correlates with Apoptosis

To our knowledge, this study is the first to demonstrate strain-dependent intracellular Ca^{2+} accumulation in AVICs. The data indicate that extent of strain applied to AVICs is positively correlated with intracellular Ca^{2+} concentration and apoptosis. In our real-time Ca^{2+} measurements, doubling the amount of strain applied to AVICs led to a significant 44% increase in the rate of Ca^{2+} accumulation over 5 min (Fig. 2A). The application of cyclic strain for 3 h also led to a significant increase in AVIC intracellular Ca^{2+} over non-strained AVICs and trended higher with at greater strain levels (Fig. 5). Further, following the removal of strain, significantly more Ca^{2+} was retained in AVICs that were initially subjected to higher strain (Fig. 2B). We believe that these accumulations may be important

in directing AVIC behavior. In glutaraldehyde fixed bioprosthetic valves, Ca^{2+} accumulations in cell remnants is thought to lead to nucleation and growth of nodules (Kim, Herrera et al. 1999; Schoen and Levy 2005). Additionally, a loss in Ca^{2+} homeostasis is known to lead to apoptosis in many cell types (Lemasters, Theruvath et al. 2009), and apoptosis has been shown to play a role in dystrophic calcification processes (Balachandran, Sucusky et al. 2010). Indeed, our data indicate that AVIC apoptosis also increases with strain (Fig. 4). Other studies have identified active osteogenic differentiation of AVICs that leads to ossified nodules (Osman, Yacoub et al. 2006; Yip, Chen et al. 2009). It is interesting to note that moderate increases in intracellular Ca^{2+} have been shown to be required for osteoblast activation in bone remodeling (Zayzafoon 2006), and strain could play a role in inducing this differentiation in AVICs.

Though the real-time data must be extrapolated over several minutes to observe the increased rate of accumulation in our setup, we believe that the linearity of the accumulation suggests that these changes may be relevant on shorter time scales associated with diastolic loading. Additionally, a trending but insignificant increase in intracellular Ca^{2+} is observed when cyclic strain is increased from 10% to 20% for only 3 h (Fig. 5). Further, the data indicate that removal of Ca^{2+} from the cell is a biologically active process that occurs at a constant efflux rate independent of the amount of strain released and that higher strain leads to significantly more retention of intracellular Ca^{2+} over time in AVICs (Fig. 2A). Therefore, we speculate that increased strain could lead to small but gradual accumulations of intracellular Ca^{2+} and pathologic disruption to AVIC homeostasis as discussed in the following model.

Intracellular Ca^{2+} Accumulation is Likely Due to Stretch-Activated Channels

We have demonstrated that intracellular Ca^{2+} accumulation in AVICs is due, at least in part, to a strain-dependent influx of extracellular Ca^{2+} . This influx could be due to either stretch-activated ion channels (Charras, Williams et al. 2004) or non-specific openings in the plasma membrane due to strain (Geddes-Klein, Schiffman et al. 2006). Data from GdCl_3 treated AVICs indicate that strain-induced intracellular Ca^{2+} accumulation occurs through stretch-activated channels in AVICs (Fig. 2B). One potential important characteristic of these channels lies in their increased sensitivity to small changes in strain (Erxleben 1989; Charras, Williams et al. 2004). For the case in which Ca^{2+} diffusion is dictated by the concentration gradient across non-specific pores in the plasma membrane, the rate of accumulating intracellular Ca^{2+} is:

$$\frac{d[\text{Ca}^{2+}]_i}{dt} = A_{\text{pore}} N_{\text{Ca}^{2+}}, \quad (1)$$

where $[\text{Ca}^{2+}]_i$ is the concentration of intracellular Ca^{2+} , A_{pore} is the total area of all open pores, and $N_{\text{Ca}^{2+}}$ is the molar flux of Ca^{2+} through the open pores. A_{pore} cannot be determined directly; however, if a constant flux is assumed for any given strain, the ratio of Ca^{2+} accumulation rates between two different strains should be proportional to the ratio of pore area for each strain. If an elliptical pore is assumed,

$$A_{\text{pore}} = \frac{\pi d_1 d_2}{4}, \quad (2)$$

where d_1 and d_2 are the diameters of the elliptical axes. Therefore, the ratio of two pore areas is equivalent to the ratio of the products of the diameters of each ellipse, or

$$\frac{(A_{pore})_{\varepsilon=20\%}}{(A_{pore})_{\varepsilon=10\%}} = \frac{(d_1 d_2)_{\varepsilon=20}}{(d_1 d_2)_{\varepsilon=10}}, \quad (3)$$

where the subscripts outside the parentheses denote the strain magnitudes. A change in uniaxial strain from 10% to 20% and a plasma membrane Poisson ratio of 0.49 (Charras, Williams et al. 2004), results in an off axis strain of -4.9% . The strains in each axis can then be used to relate change in the length of each axis for an applied uniaxial strain from 10% to 20%. Substituting these relations into Equation 3 yields a 4.6% increase in total pore area, which would correspond to a 4.6% increase in the rate of intracellular Ca^{2+} accumulation. However, the ratio of slopes from Fig. 2A gives a 44% increase in the rate of intracellular Ca^{2+} accumulation from 10% to 20% strain. This indicates an increased sensitivity to intracellular Ca^{2+} accumulation at higher strains previously reported for mechanosensitive ion channels (Erxleben 1989; Charras, Williams et al. 2004). The probability of opening mechanosensitive channels has been observed to have a sigmoidal dependence on strain, whereby a relatively small change in strain can lead to a higher number of open channels and a large Ca^{2+} influx (Charras, Williams et al. 2004). This sensitivity could have pathological consequences whereby small increases in AV strain lead to significant increases in intracellular Ca^{2+} . A graphic illustration of a proposed Ca^{2+} accumulation mechanism in AVICs is shown in Fig. 6. In this model, heightened strain leads to increased Ca^{2+} influx through an increased number of opened channels. This Ca^{2+} then accumulates over time as the rate of efflux from AVICs is constant and cannot counteract the increased load, resulting in a loss of AVIC homeostasis.

Limitations

Herein, we were concerned with the rate of intracellular Ca^{2+} accumulation as a function of strain and how increased strains may lead to residual Ca^{2+} build up in the cytoplasm. We believe these data give an indication of changes in accumulation rates with strain; however, further work is needed to address possible accumulations on shorter timescales associated with diastolic loading and the potential for significant accumulations to lead to changes in AVIC behavior.

Conclusion

The results of this study provide insight into AVIC responses to mechanical strain and early mechanotransductive events that may contribute to AV calcification. Studies are needed to clarify the molecular mechanisms associated with both the strain-mediated intracellular Ca^{2+} increase and the active cellular-mediated decrease. Finally, identification of important pathways and downstream consequences of increased intracellular Ca^{2+} may reveal therapeutic targets for AV disease.

Acknowledgments

We would like to acknowledge M.K. Sewell-Loftin and Stephanie C. Preston for assistance in creating PDMS structures for the real-time fluorescence measurements. We would also like to thank Hampton's Meat Processing for donation of porcine AV tissue. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). This work was supported by the AHA (09GRNT2010125) and NIH (HL094707), both to WDM. JDH was supported by an AHA Pre-doctoral Fellowship (10PRE4290020).

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Fig. 1.

Strain apparatus for real-time microscopy. (A) AVICs were cultured on molded PDMS gels that were functionalized with fibronectin. The gels were held between two clamped ends of the strain apparatus, and strain was applied by turning a screw with a pitch of 0.32 mm/turn to increase the distance between the two clamped ends. (B) APTES was used to silanize PDMS that had been activated via O₂ plasma treatment. The PDMS substrate was functionalized using glutaraldehyde to crosslink primary amines on fibronectin to primary amines on the APTES chain. (C) Using these methods, AVICs adhered to the PDMS surface and displayed appropriate morphology.

Fig. 2.

Real-time measurements of Ca^{2+} in uniaxially strained AVICs. (A) AVICs subjected to 10% uniaxial strain exhibit a linear increase in intracellular Ca^{2+} over the course of 5 min (closed circles). AVICs subjected to 20% uniaxial strain also exhibit a linear increase in intracellular Ca^{2+} (open circles). The removal of strain after 5 min leads to a similar rate of decrease in both strain cases. (B) AVICs strained to 20% in the absence of extracellular Ca^{2+} exhibit a slight decrease in intracellular Ca^{2+} . AVICs treated with 10 μM prior to the addition of 20% strain show a significant decrease in the rate of intracellular Ca^{2+} accumulation. (C) Microscopic image of show low levels of intracellular Ca^{2+} in unstrained AVICs. (D–F) Representative microscopic images show intracellular Ca^{2+} increases in AVICs strained to 20% over time.

Fig. 3.

Flow cytometry analyses of Ca^{2+} in strained AVICs. (A) AVICs subjected to 20% equibiaxial static strain exhibit significant ($p < 0.05$) population shifts in intracellular Ca^{2+} accumulation at 5 min ($11 \pm 2\%$ increase in population mean, blue line) and 15 min ($41 \pm 3\%$ increase in population mean, red line) compared to unstrained AVICs (black line). (B) Significant accumulated intracellular Ca^{2+} is retained in AVICs kept on ice for 30 min after receiving 20% equibiaxial strain for 15 min ($p < 0.005$, black bars); however, when kept at 37°C , intracellular Ca^{2+} returns to control levels (gray bar). (C) Intracellular levels of the plasma membrane impermeant dye, sulforhodamine does not change in AVICs exposed to 20% strain for 15 min (red line) compared to unstrained AVICs (black line).

Fig. 4.

Flow cytometry analysis of Ca^{2+} and viability in strained AVICs. (A) Unstrained AVICs exhibit low levels of intracellular Ca^{2+} and have intact plasma membranes as indicated by propidium iodide staining. (B) Few unstrained AVICs are apoptotic as shown by Annexin V staining. (C) AVICs subjected to 20% static strain for 3h have increased intracellular Ca^{2+} , but retain plasma membrane integrity. (D) Apoptosis is correlated with accumulated Ca^{2+} as $76 \pm 0.82\%$ of Annexin V-positive AVICs have increased intracellular Ca^{2+} .

Fig. 5. Intracellular Ca²⁺ in AVICs exposed to cyclic strain. A flow cytometric histogram indicates strain-dependent calcium accumulation in AVICs experiencing either 10% (blue line) or 20% (red line) cyclic strain for 3h (Fig. 5A). AVICs exposed to 10% cyclic strain for 3h exhibit a $79 \pm 16\%$ increase in Fluo-4 fluorescence. When the cyclic strain is increased to 20%, AVICs exhibit a $98 \pm 22\%$ increase in Fluo-4 fluorescence (Fig. 5B).

Fig. 6.

Proposed mechanism of Ca^{2+} handling in AVICs. (A) The probability of opening (P_{open}) a stretch activated channel is a sigmoidal function of strain. When no strain is applied (case a, x-axis), no channels are open. When low strain is applied (case b), the P_{open} increases and some channels open. When a larger strain is applied (case c), P_{open} increases dramatically and a large number of channels open. (B) Physiologic strain during diastole therefore may open some channels, causing a small influx of Ca^{2+} into AVICs. This Ca^{2+} is pumped back out of AVICs during systole (cycle 1). Increased diastolic loading under pathologic conditions may lead to greater strain on AVICs, more open channels, and a greater influx of Ca^{2+} (cycle 2). Assuming a constant efflux independent of strain, AVICs are not able to sufficiently remove the accumulated intracellular Ca^{2+} , which leads to apoptosis after multiple cycles.