Role of Ca²⁺ signaling in initiation of stretch-induced apoptosis in neonatal heart cells

Xu Dong Liao,[a,b,1] Ai Hui Tang,[c,1] Quan Chen,[b] Hai Jing Jin,[b] Cai Hong Wu,[c] Lan-Ying Chen,[a,*] and Shi Qiang Wang[c,*]

[a] *Cardiovascular Institute and Fuwai Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100037, China*
[b] *State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China*
[c] *State Key Laboratory of Biomembrane and Membrane Biotechnology, College of Life Sciences, Peking University, Beijing 100871, China*

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Abstract

Abnormal mechanical load, as seen in hypertension, is found to induce heart cell apoptosis, yet the signaling link between cell stretch and apoptotic pathways is not known. Using an in vitro stretch model mimicking diastolic pressure stress, here we show that Ca²⁺ signaling participates essentially in the early stage of stretch-induced apoptosis. In neonatal rat cardiomyocytes, the moderate 20% stretch resulted in tonic elevation of intracellular free Ca²⁺ ($[Ca^{2+}]_i$). Buffering $[Ca^{2+}]_i$ by EGTA-AM, suppressing ryanodine-sensitive Ca²⁺ release, and blocking L-type Ca²⁺ channels all prevented the stretch-induced apoptosis as assessed by phosphatidylserine exposure and nuclear fragmentation. Notably, Ca²⁺ suppression also prevented known stretch-activated apoptotic events, including caspase-3/-9 activation, mitochondrial membrane potential corruption, and reactive oxygen species production, suggesting that Ca²⁺ signaling is the upstream of these events. Since $[Ca^{2+}]_i$ did not change without activating mechanosensitive Ca²⁺ entry, we conclude that stretch-induced Ca²⁺ entry, via the Ca²⁺-induced Ca²⁺ release mechanism, plays an important role in initiating apoptotic signaling during mechanical stress.
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Mechanical load is an important biophysical factor influencing cell life. Abnormal mechanical load associated with blood pressure is involved in a number of cardiovascular diseases, such as hypertension [1–3]. Increasing evidence suggests that the chronic mechanical stress causes apoptotic cell death of heart cells, which leads to decreased pumping power and underlies the transition from hypertrophy to heart failure [4,5]. It was suggested that mechanical stretch leads to sustained enhancement of angiotensin II autocrine signaling, which initiates programmed cell death via the p53-dependent Bax gene expression [6,7]. A recent report ascribed both apoptosis and the Bax activation to the production of reactive oxygen species during periodic stretch [8]. It seems that stretch-induced apoptosis is a process involving multiple signaling pathways, but how the mechanical stimulation is translated to biochemical signals that initiate these apoptotic signals is not yet known.

Mechanosensation in many types of cells is often mediated by a class of non-selective cation channels known as stretch-activated channels (SACs) [9]. Activation of SACs can either lead to Ca²⁺ entry directly, or indirectly by activating voltage-sensitive channels [10,11]. Recent studies show that the voltage-sensitive L-type Ca²⁺ channels per se can be activated directly by mechanical stimulation [12]. Ca²⁺ influx through these channels then usually acts as the trigger to mobilize the stored Ca²⁺ in the sarcoplasmic/endoplasmic reticulum via the Ca²⁺-induced Ca²⁺ release mechanism [13–15]. As a result, mechanical stretch usually causes increased intracellular Ca²⁺ activity in cardiac cells [16–19].

Given that excessive elevation of intracellular Ca²⁺ is a major factor leading to apoptosis in many cell types

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[20], we hypothesized that Ca^{2+} signaling may play a role in initiating the stretch-induced apoptosis in cardiomyocytes. Using a sustained mechanical stretch model, here we show that Ca^{2+} is a key mediator of stretch-induced apoptotic signaling in neonatal rat ventricular myocytes; both tonic Ca^{2+} entry and the subsequent Ca^{2+} release from the sarcoplasmic reticulum are required to initiate the apoptotic pathways.

Materials and methods

Cell preparation and culture. Cardiac cells were isolated from the hearts of neonatal Wistar rats (1–3 days after birth). Briefly, the hearts were removed, minced, and trypsinized at 37 °C by gently stirring in Hepes-buffered Saline A (in mmol/L: 137 NaCl, 5.4 KCl, 4.2 NaHCO$_3$, 5.5 glucose, and 1.0 Hepes, pH 7.4) containing 0.1% trypsin (Gibco). Then cells were centrifuged and resuspended in Dulbecco's modified Eagle's medium/F-12 (Gibco) containing 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubation at 37 °C for 90 min, cardiomyocytes and cardiac fibroblasts were prepared from the unattached and attached cells, respectively.

After isolation, cells were cultured in a chamber designed for equibiaxial stretch [21]. The culture chamber was bottomed by a silicone elastic membrane (Specialty MFG, MI, USA) fine coated with rat-tail collagen (0.1 mg/mL, in 0.01% acetic acid). Isolated cardiac cells were seeded on the membrane at a density of 5×10^4 cells/cm^2, and cultured in DMEM/F-12 containing 10% fetal bovine serum for 72 h to reach 80% confluence. In myocyte culture, 0.1 mmol/L BrdU (5-Bromo-2′-deoxyuridine, Sigma) was added in the medium in the first 36 h to inhibit proliferation of contaminated non-myocytes. This ensured that >95% cells were myocytes, as identified by anti-α-sarcomeric actin antibody immunostaining. After 24 h serum starvation, the medium was changed to fresh DMEM/F-12 (for apoptosis assay) or Tyrode's buffer (in mmol/L: 137 NaCl, 5.4 KCl, 20 Hepes, 1.2 MgCl$_2$, 1.0 CaCl$_2$, and 10 glucose, pH 7.4) (for measurement of intracellular calcium) before stretch experiments, in which cells were subjected to 20% stretch as described.

Apoptosis assays. We use different assays to identify apoptotic cells after stretch. Phosphatidylserine exposure on cell membrane was detected by Annexin V immunostaining as described [22]. Cells were stained with Annexin V-FITC (25 ng/mL; green fluorescence) simultaneously with dye exclusion (PI, red fluorescence). This assay discriminates between intact (FITC$^-$/PI$^-$), early apoptotic (FITC$^+$/PI$^-$), and late apoptotic cells (FITC$^+$/PI$^+$). Comparative experiments were performed at the same time by bivariate flow cytometry using a FACScan (BD) and analyzed with the CellQuest software on data obtained from the cell population from which debris was gated out.

Nuclear condensation and fragmentation were assessed using DAPI staining [23]. Cells were washed with PBS and stained by 10 ng/mL DAPI (4,6-diamidino-2-phenylindole, Sigma–Aldrich) before visualized using fluorescent microscopy. At least 200 cells were counted in each experiment.

The caspase protease activity was determined using the p-nitroanilide-derived chlorometric substrate (Ac-DEVD-pNA for caspase-3; Ac-LEHD-pNA for caspase-9, Biomol) [24]. Enzyme-catalyzed release of pNA was monitored at 405 nm in a 96-well microtiter plate reader. Caspase activity was expressed as relative absorbance at 405 nm.

Mitochondria membrane potential ($\Delta\Psi_m$) assay. The mitochondria membrane potential was measured by flow cytometry with DiOC6(3) staining [22]. DiOC6(3) (20 nmol/L, Molecular Probes) was loaded into 4×10^5 cells, suspended in 0.5 mL fresh DMEM (pH 7.2), and incubated at 37 °C for 5 min. Each experiment was performed in duplicate. Data were validated by addition of 20 mmol/L CCCP, the mitochondria membrane potential uncoupler, after 5 min of DiOC6(3) loading.

DiOC6(3) fluorescence was examined at 530 ± 30 nm (FL1 detector of BD FACScan). Data were obtained and analyzed with the CellQuest software from a PI negative cell population on a BD FACScan.

ROS flow cytometry assay. Intracellular ROS was examined by flow cytometry with DCF staining. H$_2$DCFDA (5 μmol/L, Molecular Probes) was loaded into 4×10^5 cells, suspended in 0.5 mL fresh DMEM (pH 7.2), and incubated at 37 °C for 15 min. Each experiment was performed in duplicate. DCF fluorescence was examined at 530 ± 30 nm. Data were obtained and analyzed with the CellQuest software from a PI negative cell population on a BD FACScan.

Intracellular calcium measurement. Intracellular calcium level was measured by confocal microscopy [25] in conjunction with the calcium indicator, Fluo-4 (Molecular Probes). In brief, cardiac myocytes were loaded with 5 μmol/L Fluo-4-AM at 37 °C for 20 min in Tyrode's buffer, then gently washed twice, and incubated for another 30 min to ensure complete cleavage of Fluo-4-AM by intracellular ester enzyme to release calcium-sensitive probe (Fluo-4). Fluorescence was visualized by Zeiss LSM510 confocal microscope equipped with an argon laser (488 nm) and 40×, 1.3NA oil immersion objective. The digital images were analyzed with IDL image-processing software (ver5.4). Average fluorescent density of intracellular areas was measured to index calcium level. The data were normalized by the fluorescent density before stretch (control) for statistical analysis.

Statistical analysis. All results were expressed as means $\pm$ SE of at least three independent experiments unless stated otherwise. Significance was determined with the SPSS10.0 for Student's unpaired t test or one-way ANOVA. Differences between groups were considered significant at a value of $p < 0.05$.

Results

Stretch-induced change of intracellular Ca^{2+} concentration

In the present study, we employed a sustained stretch model to mimic the diastolic volume stress that occurs in many cardiovascular diseases. To investigate the mechanistic link between Ca^{2+} signaling and stretch-induced apoptosis, we first measured the dynamics of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to mechanical stretch in our system. After cultured for 72 h, neonatal rat cardiomyocytes were loaded with the Ca^{2+} indicator, fluo-4, and $[Ca^{2+}]_i$ was visualized by confocal microscopy with 488 nm excitation (Fig. 1A). When the cells were subjected to the 20% sustained stretch, we detected both a phasic and a tonic $[Ca^{2+}]_i$ response in cardiomyocytes. Line-scan imaging revealed that the phasic, high-amplitude $[Ca^{2+}]_i$ change actually consisted of periodic Ca^{2+} transients (Fig. 1B) in most cells. These Ca^{2+} transients diminished quickly, and the number of cells with ongoing Ca^{2+} transients decreased monotonically to the resting level at a time constant of 3.5 min (Fig. 1C).

Although the transient change of $[Ca^{2+}]_i$ is reported by many laboratories [16,19], how the $[Ca^{2+}]_i$ changes during a prolonged stretch is not yet known. The latter is of particular importance for apoptosis, which usually occurs after hours of stretch [26]. We therefore kept monitoring $[Ca^{2+}]_i$ for at least 90 min during stretch. We found that after the phasic $[Ca^{2+}]_i$ response, intracellular
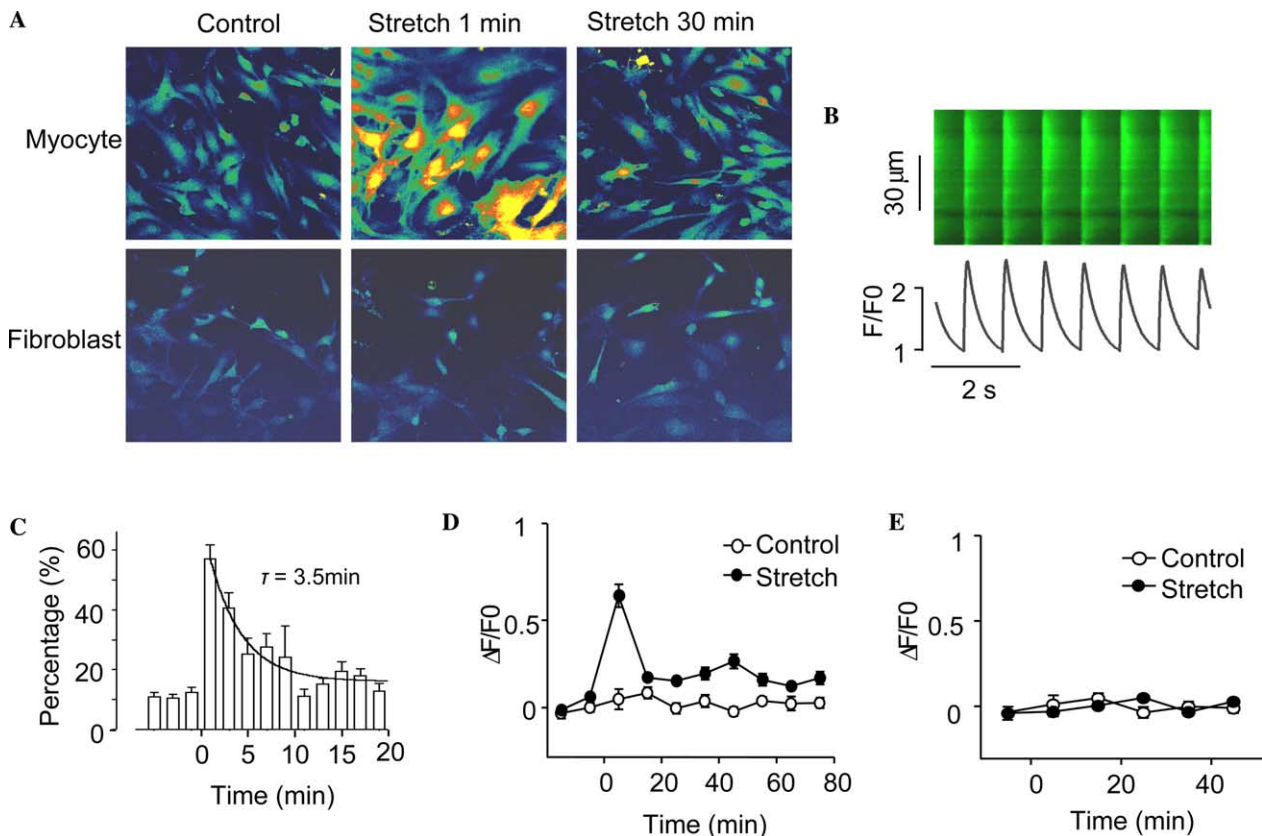
Fig. 1. Mechanical stretch induces both phasic and tonic Ca^{2+} elevation in neonatal rat cardiac myocytes. (A) Intracellular Ca^{2+} was visualized by confocal microscopy using the Ca^{2+} indicator, fluo-4, in both cardiac myocytes and fibroblasts from neonatal rats. Note that stretch induced $[Ca^{2+}]_i$ change in myocytes, but not in fibroblasts. (B) Typical Ca^{2+} transients that occurred in a cardiac myocyte immediately after stretch. The Ca^{2+} transients were recorded with confocal microscopy in line-scan mode. The straight edge of each transient indicates that the transients were activated by action potential, rather than local Ca^{2+} events. (C) The percentage of myocytes with ongoing Ca^{2+} transients decayed monotonically at a time constant of 3.5 min and recovered within ~10 min. (D) Time-dependent change of spatially averaged $[Ca^{2+}]_i$, as indexed by the ratio of flu-4 fluorescence change ($\Delta F/F0$). Note that after 10 min of stretch, when the Ca^{2+} transients diminished, there was a tonic $[Ca^{2+}]_i$ elevation that lasted throughout the whole period of stretch in cardiac myocytes. (E) By contrast, both the phasic and tonic responses were absent in stretched cardiac fibroblasts.

fluorescence pointed to a relatively stable $[Ca^{2+}]_i$ that was still significantly higher than that in cells without stretch (Fig. 1D). With the Ca^{2+} transients already calmed down, the tonic elevation of $[Ca^{2+}]_i$ reflected the change of resting free intracellular Ca^{2+} level. Since the tonic $[Ca^{2+}]_i$ increase, rather than Ca^{2+} transients, is responsible for apoptosis (see Discussion), $[Ca^{2+}]_i$ analysis in the following parts was mainly focused on the tonic phase.

Notably, parallel experiments in cardiac fibroblasts did not show any stretch-related $[Ca^{2+}]_i$ change (Figs. 1A bottom and E), suggesting that stretch-induced Ca^{2+} signal transduction is myocyte-specific.

Ca^{2+}-dependence of the stretch-induced apoptosis

Using the same cell-stretch model as in Ca^{2+} measurements, we investigated apoptosis of neonatal cardiomyocytes in response to stretch. We examined both nuclear condensation, by DAPI staining, and

phosphatidylserine exposure, by Annexin V FITC-based flow cytometry. After 4 h stretch, DAPI staining showed that a significant portion of cells had condensed and fragmented nuclei, which were stained brighter than normal ones (Figs. 2B and C). Flow cytometry data showed that a similar portion of stretched cells is Annexin V-positive (PI-negative) (Figs. 2A and C). These results, together with the observation that caspase-3 is activated during prolonged stretch (Fig. 6C), suggested that the 20% stretch is sufficient to trigger apoptosis in cardiomyocyte. Again, cardiac fibroblasts in parallel experiments did not show any change in Annexin V assay (data not shown), indicating that the stretch-induced apoptosis is also cell type-specific.

In order to determine whether Ca^{2+} signaling plays a role in the stretch-induced apoptosis in cardiomyocytes, we sought to chelate and stabilize the $[Ca^{2+}]_i$ by introducing into the cell EGTA, a Ca^{2+} buffer with a K_d of 150 nmol/L. When the myocytes were treated with the cell-permeable EGTA-AM (2 μmol/L), the number of
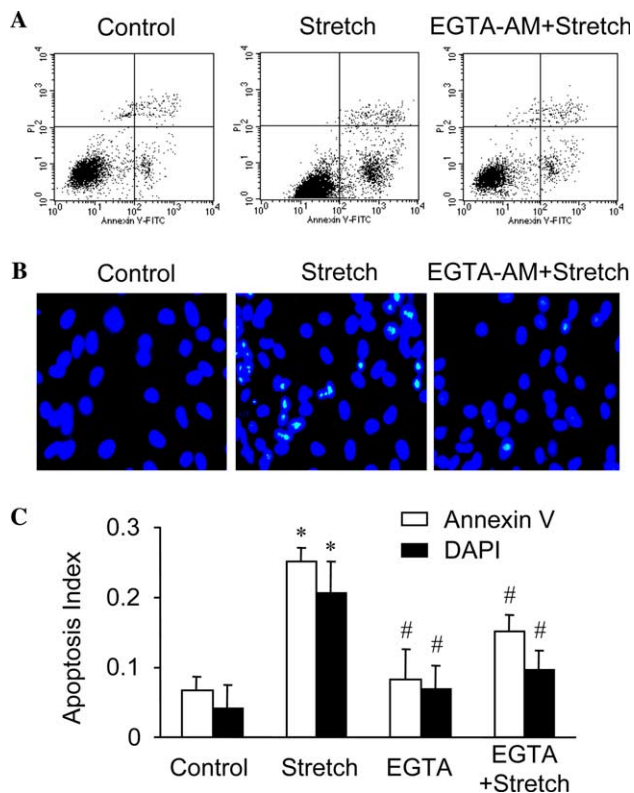
Fig. 2. Stretch-induced apoptosis in cardiac myocytes is Ca^{2+}-dependent. The apoptosis in cardiac myocytes was assayed both by Annexin-V-based flow cytometry (A) and DAPI-based fluorescent microscopy (B). Buffering intracellular calcium by pretreatment of EGTA-AM (2 μmol/L) 30 min before stretch markedly suppressed stretch-induced apoptosis. (C) Statistic data of myocyte apoptosis in response to stretch and/or EGTA-AM treatment. $*p < 0.05$ vs. control; $\#p < 0.05$ vs. stretch.

apoptotic cells after 4 h stretch, as indexed by Annexin V flow cytometry (Fig. 2A) and DAPI staining (Fig. 2B), was decreased by 57% (from $25.2 \pm 1.8\%$ to $15.0 \pm 2.5\%$) and 66% (from $20.7 \pm 4.5\%$ to $9.7 \pm 2.7\%$), respectively (Fig. 2C). Since EGTA per se did not interfere with apoptosis (Fig. 2C), and all factors that blocked $[Ca^{2+}]_i$ change also inhibited apoptosis (see below), Ca^{2+} signaling plays an essential role in the stretch-induced apoptosis in neonatal cardiac myocytes.

Mechanism of the apoptotic Ca^{2+} signal

We next sought to investigate how the apoptotic Ca^{2+} signal is initiated by stretch. It has been suggested that the SACs in the sarcolemma mediate a stretch-sensitive non-specific cation current [11,27], which may either contribute directly to the Ca^{2+} entry or activate voltage-gated Ca^{2+} channels by depolarizing the cell membrane. We therefore tested in our experimental system whether Gd^{3+}, a SAC blocker [28], could interfere with the $[Ca^{2+}]_i$ change. We found that $GdCl_3$ (100 μmol/L) only partially inhibits the stretch-induced $[Ca^{2+}]_i$ elevation

(Fig. 3A) and could not prevent stretched cells from apoptosis (Fig. 5), suggesting that another mechano-sensation mechanism may exist.

Recent work has suggested that the classic voltage-gated L-type Ca^{2+} channel (LCC) can be activated directly by mechanical stimulation [12] and may serve as a potential mechanosensor. It was also shown that blockage of LCCs can suppress the Ca^{2+} transients initiated by stretch [29]. We therefore tested whether the tonic elevation of $[Ca^{2+}]_i$ is also LCC-related. We found that a specific LCC antagonist, nifedipine (Nif) (2 μmol/L), almost completely blocked the tonic change of $[Ca^{2+}]_i$ (Fig. 3B). Since the $[Ca^{2+}]_i$ increase is also absent without extracellular Ca^{2+} (unpublished observation), these data suggest that the stretch-induced $[Ca^{2+}]_i$ elevation is triggered by Ca^{2+} influx through LCCs. These results also imply that the role of SACs in stretch-induced Ca^{2+} entry, if any, is indirect, i.e., to activate LCCs by depolarizing the membrane potential.

In cardiac cells, the direct contribution of LCC Ca^{2+} influx to the intracellular Ca^{2+} change is usually small; its major role is to trigger Ca^{2+} release from ryanodine
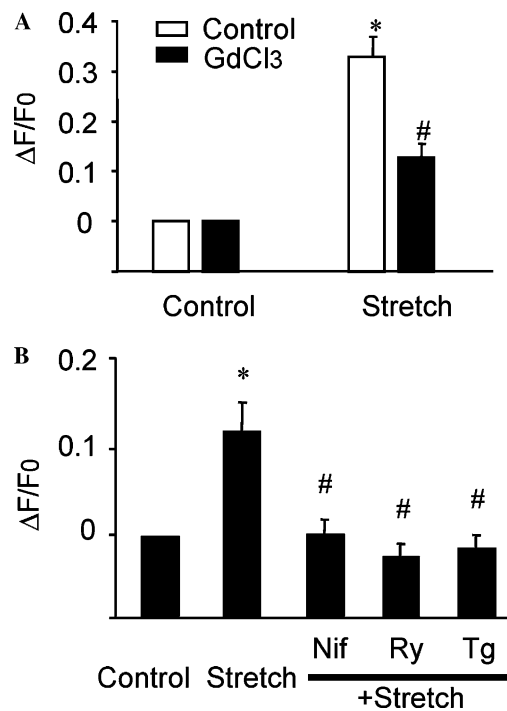

Fig. 3. Mechanistic analysis of stretch-induced $[Ca^{2+}]_i$ elevation in cardiac myocytes. (A) Inhibition of stretch-activated channel (SAC) with $GdCl_3$ reduced stretch-induced calcium elevation in cardiac myocytes. Cells were pre-incubated and kept in Tyrode's buffer containing 100 μmol/L $GdCl_3$. Data shown are fluo-4 fluorescence ratios before and 10 min after stretching cells. (B) The effect of nifedipine (Nif, 2 μmol/L), ryanodine (Ry, 10 μmol/L) or thapsigargin (Tg, 10 μmol/L) on stretch-induced $[Ca^{2+}]_i$ elevation. Data in stretch groups were from myocytes after 45 min of stretch. Control cells were monitored for 45 min without stretch. $*p < 0.05$ vs. control; $\#p < 0.05$ vs. stretch.

receptors (RyRs) in the sarcoplasmic reticulum (SR) [14], a process known as Ca^{2+}-induced Ca^{2+} release (CICR) [13]. To assess the role of CICR in the stretch-induced $[Ca^{2+}]_i$ elevation, we inhibited SR Ca^{2+} release either by ryanodine (Ry), a specific antagonist of RyRs, and by thapsigargin (Tg), which blocks SR Ca^{2+}-AT-Pase and empties the SR Ca^{2+} content. Both ryanodine (10 µmol/L) and thapsigargin (10 µmol/L), added 30 min before stretch, eliminated the $[Ca^{2+}]_i$ change during stretch (Fig. 3B), indicating that the RyR Ca^{2+} release plays an important role in the stretch-induced increase of $[Ca^{2+}]_i$.

Altogether, the above results suggest that the stretch-induced tonic increase of $[Ca^{2+}]_i$ has mainly resulted from the Ca^{2+}-induced Ca^{2+} release from SR, in which the triggering Ca^{2+} signal from LCCs is activated either directly by stetch or indirectly as the result of SAC activation.

The role of Ca^{2+} signaling in initiation of apoptotic cascades

Since stretch-induced apoptosis is Ca^{2+}-dependent, any factor that prevents the $[Ca^{2+}]_i$ elevation would be expected to suppress apoptosis. Indeed, when the Ca^{2+} entry from LCCs was blocked by nifedipine (2 µmol/L), the sustained stretch could no longer induce detectable change in nuclear morphology visualized by DAPI staining nor could it increase Annexin V-positive cells (Fig. 4). Similarly, inhibition of SR Ca^{2+} release by Ry (10 µmol/L) also prevented stretch-induced apoptosis (Fig. 5). These results indicate that both Ca^{2+} entry and subsequent SR Ca^{2+} release are engaged in the stretch-induced apoptosis.

In several reports addressing the stretch-induced apoptosis, the activation of apoptotic cascades was attributed to signaling events other than Ca^{2+} elevation, such as the increase of reactive oxygen species (ROS) or angiotensin II [6,8]. To determine the relationship between Ca^{2+} and the major apoptotic pathways, we investigated the effects of Ca^{2+} buffering by EGTA-AM
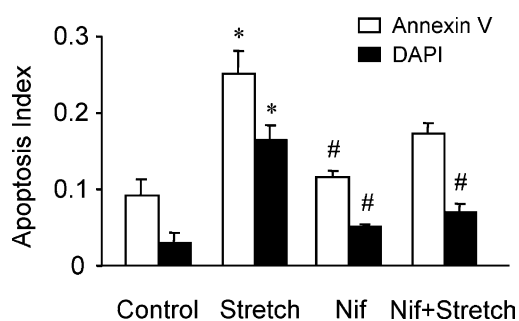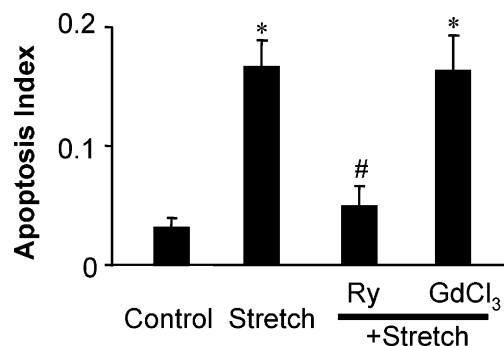

Fig. 5. The effect of ryanodine (Ry, 10 µmol/L) and GdCl₃ (100 µmol/L) on stretch-induced apoptosis. Apoptosis was accessed by DAPI assay. *$p < 0.05$ vs. control data; #$p < 0.05$ vs. stretch.

on various intermediate apoptotic events. As shown in Fig. 6, mechanical stretch led to increase of intracellular ROS (Fig. 6A), disruption of mitochondria membrane potential (Fig. 6B), and activation of both caspase-9 and caspase-3 (Fig. 6C), all of which are among the key steps toward apoptosis during stretch. Notably, buffering intracellular Ca^{2+} by EGTA-AM prevented all of these stretch-induced events (Fig. 6), suggesting that without elevation of $[Ca^{2+}]_i$, stretch-induced apoptotic signaling is blocked at a very early stage. In other words, the stretch-induced elevation of $[Ca^{2+}]_i$ is among the initial signaling events and presumably acts as a transducer between mechanical stimulation and downstream signaling cascades in triggering apoptosis of cardiomyocytes.

Discussion

An intriguing question the present study aimed to address is how stretch can activate apoptotic signaling. Our working hypothesis is that Ca^{2+} is the essential mediator that links the mechanical stimulation to intracellular targets. We have the following major findings: (1) the stretch-induced apoptosis is largely Ca^{2+} dependent; (2) the apoptotic Ca^{2+} signal, in the form of $[Ca^{2+}]_i$ elevation, is initiated via the Ca^{2+}-induced Ca^{2+} release from the SR, in which the stretch-induced Ca^{2+} entry through LCCs and SACs provides the trigger signal; and (3) the elevation of $[Ca^{2+}]_i$ is a prerequisite for activation of apoptotic cascades associated with stretch.

Since the classic finding by Moss et al. [18], many studies have demonstrated the stretch-activated Ca^{2+} response in cardiac myocytes. Most studies, however, were focused on the transient response during myocyte length change and suggested that mechanical stretch, within the physiological range, alters only the amplitude of periodic Ca^{2+} transients without changing the resting $[Ca^{2+}]_i$ level [16,19]. An increase of resting $[Ca^{2+}]_i$ can be

Fig. 4. The effect of nifedipine (Nif) on stretch-induced apoptosis in cardiac myocytes, as assessed by Annexin V and DAPI assays. *$p < 0.05$ vs. control; #$p < 0.05$ vs. stretch.

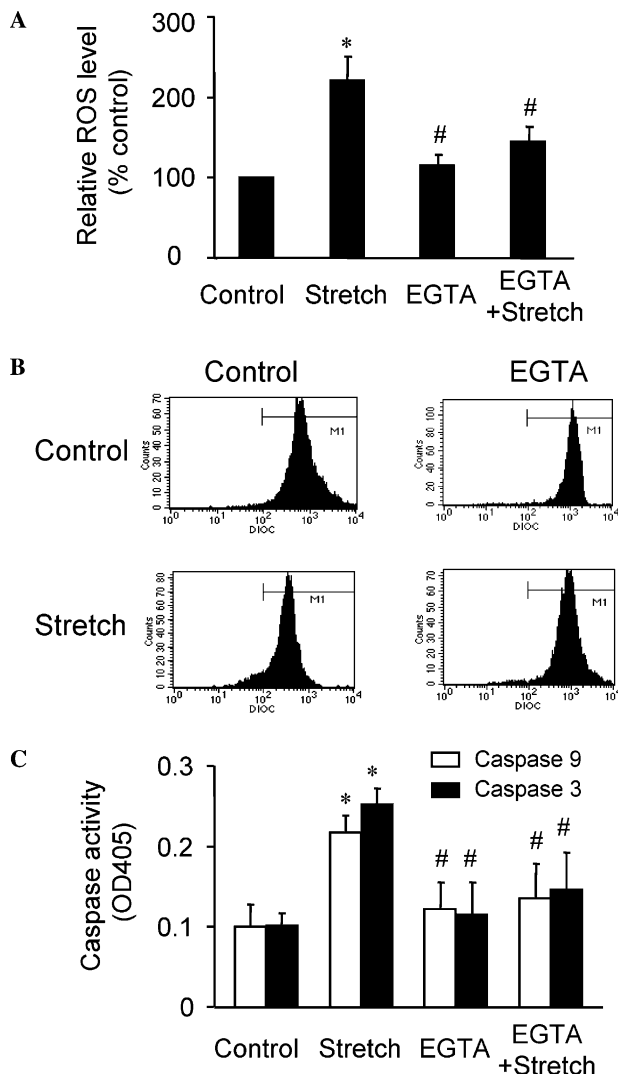**A**

B

C

Fig. 6. The effect of $[Ca^{2+}]_i$ buffering on intracellular events associated with stretch-induced apoptosis in cardiac myocytes. EGTA-AM (2 μmol/L) was applied 30 min before stretch. (A) ROS concentration was evaluated by DCF-based flow cytometry. (B) Mitochondrial membrane potential was detected by DIOC6(3)-based flow cytometry. (C) Caspase-3 and caspase-9 activity was determined by pNA-based assay. *$p < 0.05$ vs. control; #$p < 0.05$ vs. stretch.

detected only when the cells are stretched over 15% [29]. Despite these information about the immediate Ca^{2+} response after stretch, how the $[Ca^{2+}]_i$ changes during sustained stretch (beyond 10 min) is not clear. In order to correlate the Ca^{2+} events with apoptotic process, which usually takes place after hours of stretch, we measured the $[Ca^{2+}]_i$ change over a long duration for at least 90 min when the cells were stretched by 20%. We found that sustained stretch causes both transient and tonic response of $[Ca^{2+}]_i$. While the transient response recovers automatically in minutes, the tonic $[Ca^{2+}]_i$ increase lasts for hours throughout the stretch period.

Then, the question arises whether the transient or the tonic Ca^{2+} signal is responsible for apoptosis. Intui-

tively, transient Ca^{2+} elevation should not be apoptotic, because the heart in any healthy individual keeps beating (each with a Ca^{2+} transient) for decades without significant loss of cells. This notion is recently confirmed by our observation that removing the Ca^{2+} overload-inducing stimulation in 10 min prevented apoptosis that would otherwise occur (unpublished observations). Recent study shows that periodic elongation of rat ventricular myocytes at a frequency of 1 Hz, although reaching 20% in stretch amplitude, failed to induce apoptosis [30]. This contrasts with our present results and also with other reports [6], in which 20% sustained stretch is sufficient to initiate apoptotic signaling. Interestingly, in another study, 25% (but not 5%) periodic stretching at 1 Hz did result in apoptosis in neonatal rat cardiomyocytes [8]. Based on our present Ca^{2+} study, the above differences can be explained by different patterns of intracellular Ca^{2+} response: periodic stretch tends to result in a series of Ca^{2+} transients, rather than the tonic elevation of $[Ca^{2+}]_i$ seen in sustained stretch situation; the resting $[Ca^{2+}]_i$ change in periodic stretch model needs larger stretch amplitude. This principle apparently has clinical relevance, because it suggests that high diastolic pressure is of more significance than systolic pressure in the pathogenesis of apoptosis-related heart diseases.

Despite the wealth of information on the relationship between Ca^{2+} signaling and apoptosis (for review see [20,31]), the role of Ca^{2+} signal in mechanical load-induced heart cell apoptosis has not yet been characterized. Recent studies have revealed that the stretch-induced apoptosis is mediated by ROS [8] or angiotensin II signaling [6], but how these signaling pathways are activated by mechanical stimulation is still obscure. In the present study, we provide several lines of evidence suggesting that Ca^{2+} signal is among the earliest apoptotic events evoked by stretch: (1) Stretch activates Ca^{2+} entry through LCCs and SACs, both of which are themselves mechanosensitive. (2) SR Ca^{2+} release from RyRs, a major contributor of the stretch-induced $[Ca^{2+}]_i$ increase, is triggered by the LCC Ca^{2+} entry via CICR mechanism, a direct process not involving any intermediate signaling pathways [15]. (3) Stretch-induced apoptosis does not occur when the $[Ca^{2+}]_i$ increase is blocked at different signaling stages, e.g., by extracellular Ca^{2+} free solution, EGTA permeation, and ryanodine and nifedipine perfusion. (4) Known apoptotic pathways activated by stretch, such as ROS generation [8], mitochondria membrane potential corruption, and caspase-9/-3 activation, cannot be initiated without $[Ca^{2+}]_i$ elevation. Although we have not yet tested the signaling crosstalk between Ca^{2+} and angiotensin II, it is generally known that the exocytosis of many hormones is Ca^{2+}-dependent [32]. Based on these facts, we propose that Ca^{2+} signaling, besides its known roles in general apoptotic signaling [20], provides the

necessary link between the mechanical sensation and apoptotic pathways in heart cells suffering mechanical stress.

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