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A role of BK channel in regulation of Ca^{2+} channel in ventricular myocytes by substrate stiffness

HC Zhao[1][§], Y Yu[1][§], XA Wu[1][§], SS Liu[1], BL Liu[1], J Du[1], B Li[1]*, LH Jiang[2,3]*, XQ Feng[1]*

§Co-first authors

Corresponding Authors:

libome@mail.tsinghua.edu.cn or L.H.Jiang@leeds.ac.uk or Fengxq@mail.tsinghua.edu.cn

1 Institute of Biomechanics and Medical Engineering, Department of Engineering Mechanics, Tsinghua University, 100084, China

2 School of Biomedical Sciences, University of Leeds, United Kingdom

3 Department of Physiology and Neurobiology, School of Basic Medical Sciences, Xinxiang Medical University, China

Abstract

Substrate stiffness is crucial for diverse cell functions, but the mechanisms conferring cells with mechanosensitivity are still elusive. By tailoring substrate stiffness with 10-fold difference, we showed that L-type voltage-gated Ca^{2+} channel current density was greater in chick ventricular myocytes cultured on the stiff substrate than on the soft substrate. Blockage of the BK channel increased the Ca^{2+} current density on the soft substrate and consequently eliminated substrate stiffness regulation of the Ca^{2+} channel. The expression of the BK channel including STREX-containing α subunit that forms stretch-activated BK channel in myocytes, and the BK channel function in myocytes and also in HEK293 cells heterologously expressing STREX-containing α and $β_1$ subunits were reduced in cells cultured on the stiff substrate. Furthermore, in HEK293 cells co-expressing the cardiac $Ca_V1.2$ channel and STREX-containing BK channel, the Ca^{2+} current density was greater in cells on the stiff substrate, which was not observed in cells expressing the $Ca_V1.2$ channel alone or co-expressing with the STREX-deleted BK channel. These results taken together provide strong evidence to show that the stretch-activated BK channel plays a key role in functional regulation of cardiac voltage-gated Ca^{2+} channel by substrate stiffness, revealing a novel mechanosensing mechanism in ventricular myocytes.

Keywords: substrate stiffness, cardiac myocytes, BK channel, voltage-gated Ca^{2+} channel

Introduction

The elasticity or stiffness of extracellular matrix (ECM) often undergoes remarkable changes as a result of physiological and pathological alterations. Matrix stiffness has been increasingly recognized to play a crucial role in a variety of cell functions. For example, the stiffness of cardiac ECM can regulate diverse cell functions such as myocardial cell maturation, morphology, sarcomere organization, electromechanical coupling and gene expression (1-3). Cardiac fibrosis is one of the most important pathophysiological processes involved in cardiac remodeling (4, 5). The elasticity of healthy mature muscles is in the range of 10-150 kPa (6). However, excessive accumulation of collagen components during fibrosis

can increase by several-fold the stiffness of cardiac ECM (6, 7), which stiffens the myocardium, decreases compliance and causes dysfunction of systole and diastole, leading to heart failure and arrhythmia (1, 8). Therefore, a clear understanding of stiffness sensing mechanism is fundamentally essential for gaining insights into cardiac remodeling and disease. However, how ventricular myocytes sense stiffness is still not well-defined. Accumulating evidence supports that cells can detect the mechanical properties of extracellular surroundings by applying traction forces by actomyosin (myosin II) motors via focal adhesions (9,10) and examining the mechanical response (11,12). It is well known that Ca^{2+} is a ubiquitous intracellular signaling molecule (13). Intracellular Ca^{2+} is vital in the reorganization of the actin cytoskeleton through modulation of actin-associated protein activities (13). Increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can enhance stress fiber contractility through phosphorylation of myosin light chain kinase or activation of proteases (13, 14). We thus hypothesize that intracellular Ca^{2+} dynamics has an important role in underpinning the mechanosensitivity of cardiac myocytes.

The large-conductance Ca^{2+}-sensitive K^+ (BK) channel is widely expressed (15), functioning to oppose smooth muscle contraction by hyperpolarizing plasma membrane and reducing voltage-gated Ca^{2+} channel activation and thereby extracellular Ca^{2+} influx (16). The BK channel is expressed at a very low level in cardiac myocytes, leading to the notion that there is no direct link between the BK channel and cardiac function (17). However, the mouse BK channel gene was cloned from cardiac tissues (18) and the functional expression of the BK channel was documented in embryonic chick cardiac myocytes (19). The BK channel in embryonic chick ventricular myocytes is unique in that in addition to activation by membrane potential and intracellular Ca^{2+}, the BK channel can be activated by membrane stretch due to the presence of stress-axis regulated exon (STREX) domain in the C-terminus (20-22). L-type voltage-gated Ca^{2+} channels in cardiac myocytes, particularly the $Ca_V1.2$ channel, serve as the key Ca^{2+} influx pathway to increase the $[Ca^{2+}]_i$ (23) that triggers Ca^{2+} release from sarcoplasmic reticulum and cardiac muscle contraction (24). In this study, we examined the expression of the stretch-activated BK channel and its role in mediating the regulation of L-type Ca^{2+} channel function in chick ventricular myocytes by substrate stiffness.

Polydimethylsiloxane (PDMS) is a widely used elastomeric material to study various cell responses, such as cell spreading and cytoskeletal morphologies in fibroblasts (25), stretch-activated action potential in dorsal root ganglion neurons under mechanical stimulation (26), and neuronal network formation (27). In the present study, we use matrix-coated PDMS to study the effects of substrate stiffness on the BK channel and voltage-gated Ca^{2+} channel. To investigate the mechanism sensing substrate stiffness in ventricular myocytes, the mechanical properties of PDMS substrates were designed to be close to those of cardiac tissues under physiological and fibrosis conditions. Our results provide evidence to show a novel mechanism by which ventricular myocytes senses and respond to substrate stiffness.

Materials and Methods

Preparation of PDMS substrates with different stiffness.

PDMS substrates were prepared by mixing sylgard 184 (Corning, NY, USA) in two different mass ratios of curing agent to base (1:10 and 1:50) as described in our previous study (27). In brief, the mixtures were cast into a 35-mm petri dish and cured at 60°C for 3 h. This was followed by a sterilization step, carried out by exposing them to ultraviolet radiation for 2 h. By using the spherical indentation method (28), the Young's moduli were measured as 457 ± 39 kPa and 46 ± 11 kPa at the 1:10 and 1:50 ratio, respectively, which are referred to as stiff and soft substrates in this study.

Cell culture.

White leghorn chick embryos were obtained from China Agricultural University, Beijing, China. Animal materials used for this study were obtained according to the protocols reviewed and approved by the Institutional Ethical Review Committees of both Tsinghua University and China Agricultural University. All the animal experiments were performed in line with the NIH guidelines (Guide for the care and use of laboratory animals).

Ventricles, dissected from 10-12 day old chick embryos under sterile conditions, were washed 3 times with phosphate-buffered saline (PBS) to avoid red blood cells and, then, cut

into small pieces and exposed to Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (HBSS, Sigma, MO, USA) containing 2 mg/mL collagenase (Sigma) for 10-15 min until they were dissociated. The cell suspension was centrifuged at 1000 rpm for 5 min. After the supernatant was discarded, the cell pellets were resuspended and cultured in high-glucose DMEM culture medium (Corning) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated horse serum (HS). HS-DMEM was selected to inhibit proliferation of low-ratio cardiac fibroblasts coexisting in ventricular myocytes. The cell suspension was plated onto 35 mm-petri dish. After 2 h incubation, non-adherent cells (ventricular myocytes) were collected and transferred to dishes with 10% HS-DMEM. Ventricular myocytes were maintained in an atmosphere of 95% air and 5% CO_2 at 37°C. HEK293 cells were cultured in DMEM medium with 10% fetal bovine serum with 1% penicillin/streptomycin, and grown in 95% air and 5% CO_2 at 37°C.

Cell seeding and sample collection.

Sterilized PDMS substrates were coated with 5 pg/mL poly-D-lysine (MW 220,000: Sigma, MO, USA) at 37°C for 40 min. Isolated ventricular myocytes were seeded onto poly-D-lysine-coated PDMS substrates at a concentration of 1-5 x 10^5 cells per 35-mm petri dish. Ventricular myocytes were allowed 24 h to seed and equilibrate. For mRNA and protein expression assays, after 24 h equilibration in 10% HS culture media, cells were cultured in 2% HS-DMEM for 12 h for starvation and then in 1% fresh HS-DMEM. Cell lysates in 1 mL were collected at 6 h after replacement with 1% fresh HS-DMEM for semi-quantitative RT-PCR, and cell lysates in 300 μL for western blotting. For blebbistatin treatment, blebbistatin (Sigma) was added in culture medium with a final concentration of 5 μM for up to 24 h before cells were used for experiments.

Transfection.

The plasmids encoding GFP, the full-length BK channel STREX-containing α subunit (GenBank accession number AB072618), STREX-deleted α subunit, or $β_1$ subunit were kindly gifted from Dr. Zhi Qi (Xiamen University, China). The plasmid for L-type Ca^{2+} channel $Ca_V 1.2$ (GenBank accession number NC 000072.6) was kindly gifted Dr. Xiao-dong

Liu (Tsinghua University, China). HEK293 cells were co-transfected with plasmids encoding GFP and STREX-containing BK channel or STREX-deleted BK channel and L-type Ca^{2+} channel, respectively, using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). In brief, for cells with 80% confluence in each 35-mm petri dish, 4 μg plasmid for the BK channel α subunit and 4 μg for the $Ca_V1.2$ channel, together with 1 μg plasmid for GFP were used. In co-transfection with the BK channel $β_1$ subunit, an excessive amount of $β_1$ subunit over the α subunit (a molar ratio of 10:1 mole/mole) was used, and in co-transfection of L-type Ca^{2+} channel with BK channel α subunit and $β_1$ subunit, the expected stoichiometry between them was 1:1. Cells 24 h after transfection were trypsinized and seeded on poly-D-lysine-coated PDMS and incubated for 24-48 h prior to use. The BK channels were expressed at low density in order to record the whole-cell currents without saturating the patch clamp amplifier.

Semi-quantitative reverse transcription polymerase chain reaction.

Total RNA samples were isolated from cultured myocytes using a RNeasy Plus mini Kit (Qiagen, CA, USA), dissolved in RNase-free water, and quantified by measuring the absorbance at 260 nm with a spectrophotometer. The RNA samples were further treated with DNase I (Mbi, Glen Burnie, MD, USA). For each sample, 0.5 μg RNA samples were used to prepare cDNAs using a cDNA synthesis kit (Mbi) in a final volume of 20 μL.

To evaluate the BK channel expression in cultured myocytes, semi-quantitative PCR was performed with a PCR kit (Mbi) using a thermo-cycler (Bio-Rad, Hercules, CA, USA), and the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. PCR primers were designed using Primer 5.0 and the specificity was examined using NCBI's BLAST program. PCR reactions were performed in a total volume of 25 μL containing 1 μL cDNA, following the manufacturer's recommendations. The forward and reverse primer sequences used are: 5'-CCTGAGAAGGGAGTGGGAGACC and 5'-ATGTTGAGTGACGCCAAGATGC for the BK channel α subunit with or without STREX; 5'-AGCCGAGCATGTTGTTTTGAT and 5'-ACGCACACGGCCTGACA for the STREX-containing α subunit; 5'-TGTGCTGTCATCACCTACT and 5'-CATGGCAATAATGAGGAG for the BK channel $β_1$ subunit. PCR consisted of 30

amplification cycles, each with denaturation at 94°C for 30 s, annealing at 58-64°C for 30 s, and elongation at 72°C for 30 s. PCR products were resolved by electrophoresis in 2% agarose gels in Tris-borate/EDTA buffer and visualized by staining with ethidium bromide. The quantification of semi-quantitative PCR was performed by using Quantity One 4.6.3 software (Bio-rad). The mRNA expression of the BK channel subunits was expressed with respect to that of GAPDH in the parallel experiments, and was further normalized to that in cells on the soft substrate, as shown in Fig. 3 *D, E* and *F*.

Western blotting.

Western blotting was conducted at room temperature. Protein samples were prepared by mixing one part of cell lysate sample with one part of Bio-Rad Laemmli Sample Buffer and boiled at 100°C for 3 min. Proteins were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidenedifluoride membrane at 200 mA for 2-3 h. After blocked with 5% non-fat milk suspended in TBST for 2 h, the blots were incubated with the primary antibodies recognizing BK channel α and β_1 subunits both at a dilution of 1:1000 or the primary antibody for $Ca_V1.2$ at 1:500 for 2 h, followed by incubation with HRP-conjugated secondary antibodies at 1:5000 (Santa Cruz) for 2 h. Proteins were visualized via chemiluminescence using a Luminol Reagent Kit (Santa Cruz Biotech) and signals were captured using Kodak X-AR films.

Electrophysiology.

Single-channel inside-out and whole-cell patch clamp recordings were performed, using a MultiClamp 700B amplifier and pClamp 10 software (Molecular Devices) as previously described (21, 22), to measure voltage-gated Ca^{2+} channel currents and BK channel currents from myocytes cultured in vitro for 3 days or GFP-positive HEK293 cells for 24-48 h on the soft and stiff substrates. In single-channel recording, the pipette solution contained following components (in mM): 145 K-gluconate, 1 EGTA, 10 HEPES, 5 glucose, pH 7.4 with NaOH, 310 mOsm. The bath solution contained the same components except for various Ca^{2+} concentrations. The single channel open probability (P_o) in a patch with multiple channels was calculated by using pClamp, based on the equation: $P_o=1-P_c^{1/N}$, where P_c is the

probability when all of the channels are in the closed state and N is the number of channels in the patch.

Whole-cell current recordings were made, using recording pipettes with resistance of 3.0-4.0 MΩ in the extracellular solutions. The cell capacitance and series resistance (< 20 MΩ) were monitored throughout the recording. Signals were filtered at 1 kHz and digitized at 10 kHz. The leak currents were corrected using the online P/6 trace subtraction. The series resistance was compensated by 80%.

The BK channel currents were recorded, using Hanks' balanced salts solution containing (in mM): 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2HPO_4, 10 D-glucose, 4.2 $NaHCO_3$, pH7.4 with NaOH, 310 mOsm. The pipette solution contained (in mM): 1 $CaCl_2$, 145 KCl, 10 EGTA, 10 HEPES, pH 7.3 with KOH and 290 mOsm. Extracellular solutions were applied at 0.5 ml/min. Currents were evoked by applying test pulses from -80 mV to +100 mV with 400-ms duration and an increment of 10 mV at 10-s intervals. The membrane potential was held at -80 mV. Whole-cell Ca^{2+} channel currents were recorded from cultured myocytes and HEK293 cells, mainly using Ba^{2+}-containing bath solutions (in mM): 10 $BaCl_2$, 140 tetraethylammonium (TEA)-Cl, 10 HEPES and 20 glucose, pH 7.3 with TEA-OH and 310 mOsm. Recordings were also made, using Ca^{2+}-containing Tyrode solutions (in mM): 130 NaCl, 2 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 HEPES, 30 glucose, pH 7.3 with NaOH, and 310 mOsm. The pipette solution contained (in mM): 110 CsCl, 10 EGTA, 4 MgATP, 0.3 Na-GTP, 25 HEPES, 10 Tris-phosphocreatine and 20 U/mL creatine phosphokinase, pH 7.3 with CsOH and 290 mOsm. Extracellular solutions were applied at 0.5 ml/min. The membrane potential was held at -50 mV. Currents were evoked by applying test pulses from -80 mV to +60 mV with 40-ms duration and an increment of 10 mV at 10-s intervals. Voltage-dependent channel activation was studied using the tail currents. Amplitudes of the tail currents were normalized to the largest tail current and data were fitted by the Boltzmann function. To study voltage-dependent channel inactivation, patched cells were subjected to 3-s pre-pulse from -100 mV to +50 mV with 10-mV increment, followed by a 20-ms repolarization to -80 mV and then a 40-ms test pulse to +10 mV to elicit Ca^{2+} channel currents through the channels that remained available.

Data analysis.

The data are presented as mean ± SEM, where appropriately. The single channel open probability (P_o)-Ca^{2+} concentration relationship curve was fitted with Hill equation: $P_o = [Ca^{2+}]_i^n/(K_d^n + [Ca^{2+}]_i)$, where n is the Hill coefficient and K_d is the dissociation constant. The P_o-voltage relationship curve was fitted with the Boltzmann equation: $P_o = 1/[1+\exp(V_{1/2}-V)/k]$, where $V_{1/2}$ and k are half-maximal channel activation voltage and slope, respectively. The activation and inactivation curve was fitted to the Boltzmann equation: $I/I_{max} = 1/[1+\exp[(V-V_{1/2})/k])$, where I_{max}, $V_{1/2}$ and k represents the maximal current, half maximal current activation or inactivation voltage and slope, respectively. Statistical analysis was performed using Student's t-test, with $p < 0.05$ to be considered significant.

Results

Stiff substrate upregulates L-type Ca^{2+} channel currents in ventricular myocytes.

Myocytes express L-type voltage-gated $Ca_V1.2$ Ca^{2+} channels that plays an important role in mediating Ca^{2+} influx and excitation-contraction coupling (23, 24). Thus, we set out to examine the effect of substrate stiffness on L-type Ca^{2+} channel currents in cultured chick ventricular myocytes, using Ba^{2+} as the charge carrier in order to avoid complications from Ca^{2+}-dependent effects such as channel inactivation (29). Myocytes cultured on both stiff and soft substrates responded to depolarization pulses with inward Ca^{2+} channel currents (Fig. 1 *A*). The inward currents began to appear at approximately -30 mV, reached the maximum at +20 mV, and reversed at about +60 mV (Fig.1 *B*). The peak current density was significantly higher in myocytes on the stiff substrate compared with that on the soft substrate (Fig. 1 *B*). Similar functional regulation by substrate stiffness were observed when the Ca^{2+} channel currents were recorded using Ca^{2+} as the charge carrier, under which conditions the Ca^{2+} channel exhibited considerable channel inactivation (Fig.1, *C* and *D*). These results consistently indicate that substrate stiffness regulates L-type Ca^{2+} channel in myocytes.

As aforementioned, chick ventricular myocytes express the BK channel, particularly the stretch-activated BK channel. We hypothesize that under physiological condition, BK channel activation causes membrane hyperpolarization that may reduce voltage-gated Ca^{2+} channel activation and Ca^{2+} influx. Therefore, we next examined the role of the BK channel

in mediating functional regulation of the L-type Ca^{2+} channel by substrate stiffness. Treatment with IBTX, a BK channel blocker, abolished the difference in inward Ca^{2+} current density in myocytes on the soft and stiff substrates without altering in the I-V relationship curve when the Ca^{2+} channel currents were carried by Ba^{2+} (Fig.2, *A* and *B*) or Ca^{2+} (Fig.2, *C* and *D*). These results provide initial evidence to support that the BK channel is crucial in the regulation of the L-type Ca^{2+} channel function by substrate stiffness.

We also evaluated whether substrate stiffness affected voltage-dependent activation and inactivation of L-type Ca^{2+} channel in myocytes. The half-maximal activation voltage ($V_{1/2}$) was -3.6 ± 0.3 mV in cells on the soft substrate, which was significantly more positive than -10.1 ± 0.9 mV on the stiff substrates ($p < 0.05$), but there was no change in the slope (k) (4.8 ± 0.3 and 5.1 ± 0.3 on the soft and stiff substrates, respectively) (Fig.1 *E*). As shown in Fig. 1*F*., stiff substrate caused a positive shift in the voltage-dependent channel inactivation curve. The half inactivation voltage ($V_{1/2}$) was shifted from -55.5 ± 3.2 mV on the soft substrate to -44.3 ± 2.2 mV on the stiff one ($p < 0.05$), while the slope (k) was not significantly different (9.1 ± 0.4 and 8.9 ± 0.8 on the soft and stiff substrates, respectively). These results indicate that substrate stiffness changes L-type Ca^{2+} channel currents by modulating the channel activation and particularly inactivation. Blockage of the BK channel with IBTX reversed these substrate stiffness-induced effects on voltage-dependent channel activation and inactivation (Fig. 2, *E* and *F*). As mentioned above, $Ca_V1.2$ represents the cardiac L-type voltage-gated Ca^{2+} channel (23, 24). Western blotting was performed to analyze the $Ca_V1.2$ protein expression in myocytes on the soft and stiff substrates. There was no significant difference in the protein expression (Fig.1, *G* and *H*). Taken together, these results suggest that substrate stiffness regulates the cardiac L-type Ca^{2+} channel function without effecting on its protein expression and that the BK channel is critical in substrate stiffness regulation of L-type Ca^{2+} channel in myocytes.

Stiff substrate down-regulates the expression of the BK channel α and β₁ subunits.

To further explore the role of the BK channel in substrate stiffness regulation of the cardiac L-type Ca^{2+} channel, western blotting was carried out to examine the BK channel expression. The results show a duplet with 120 kDa and 25 kDa, corresponding to the anticipated size for

the BK channel α and β1 subunits, respectively, as described in previous studies (30). The protein expression levels for both α and β1 subunits in myocytes on the soft substrate were remarkably greater than those on the stiff substrate (Fig. 3, *A* and *B*). To investigate whether such substrate stiffness-dependent changes in the protein expression resulted from their mRNA expression, semi-quantitative RT-PCR assays were performed. The mRNA expression levels for both BK channel α and β1 subunits were elevated in myocytes on the soft substrate compared to those on the stiff substrate (Fig. 3, *C* and *D*). Previous studies showed that that several alternative splicing isoforms of the BK channel α subunit are expressed in chick cardiac myocytes, including STREX-containing α subunit that forms the stretch-activated BK channel (20-22). Hence, semi-quantitative RT-PCR assays were performed to further evaluate expression of the STREX-containing α subunit in myocytes and the effect of substrate stiffness on its expression. The mRNA transcript for STREX-containing α subunit was detected in myocytes on the soft and stiff substrates and, furthermore, the expression level was significantly higher in myocytes on the soft substrate (Fig. 3, *E* and *F*). These results suggest that substrate stiffness regulates the expression of BK channel STREX-containing α and β1 subunits.

Stiff substrate inhibits the BK channel function by reducing channel open probability and sensitivity to Ca^{2+} and voltage.

To characterize the effects of substrate stiffness on the BK channel function, whole-cell recordings were made to measure the BK channel currents in myocytes. There were robust outward K^+ currents, which were strongly suppressed by treatment with 100 nM IBTX, indicating that the outward currents were mainly carried by the BK channel (Fig.4, *A* and *B*). The BK channel current density in myocytes was significantly greater in cells on the soft substrate, for example, the current density at +100 mV was 126 ± 3.6 pA/pF relative to 86.5 ± 3.2 pA/pF on the stiff substrate ($p < 0.05$) (Fig.4, *A* and *B*).

Myocytes express stretch-activated BK channels as well as stretch-insensitive and other types of K^+ channels. In HEK293 cells, a widely used heterologous mammalian cell expression system, there was no detectable expression of the BK channel α and β1 subunits both at mRNA and protein levels (Fig. S1 *A*), and very small K^+ current density that was

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largely insensitive to IBTX (Fig. S1, *B* and *C*), consistently supporting negligible endogenous expression of the BK channel. Thus, to provide direct evidence for substrate stiffness regulation of stretch-activated BK channel, the BK channel STREX-containing α and β_1 subunits cloned from chick ventricular myocytes were transiently co-expressed in HEK293 cells. Fig. 4 *C* shows representative IBTX-sensitive BK channel K^+ currents, which are similar to those reported in previous studies (30). As observed for the native BK channel in myocytes, the current density of recombinant stretch-activated BK channel in HEK293 cells on the soft substrate was considerably higher than that on the stiff substrate, for example, the current density at +100 mV was 134 ± 4.6 pA/pF on the soft substrate, which was significantly greater than 92.5 ± 3.1 pA/pF on the stiff substrate (p < 0.05) (Fig. 4 *D*).

To gain further insights into substrate stiffness regulation of the BK channel, single channel recordings were made to examine the single channel conductance, Ca^{2+} and voltage sensitivity of the BK channel in myocytes. While single channel conductance was not altered by substrate stiffness, the channel open probability was higher on the soft substrate than that on the stiff substrate (Fig. 5 *A*). Previous studies reported that the channel opening probability was strongly dependent of the $[Ca^{2+}]_i$ (21, 22). The increase in the $[Ca^{2+}]_i$ (0.01–1000 μM) greatly enhanced the open probability of the BK channel in myocytes on both soft and stiff substrates (Fig. 5 *B*). The P_o–Ca^{2+} concentration relationship in myocytes on the soft substrates was noticeably left-shifted with respect to that on the stiff substrate; Hill coefficient also significantly increased from 2.2 ± 0.07 on the stiff substrate to 2.8 ± 0.01 on the soft substrate (p < 0.05), suggesting an increase in the Ca^{2+} sensitivity on the soft substrate. The BK channel activation also strongly depends on voltage or membrane potential. Thus, we examined the influence of substrate stiffness on the voltage-dependence of channel activation. The P_o–V relationship was left-shifted in myocytes on the soft substrate (Fig. 5 *C*). $V_{1/2}$ was -28.4 ± 3.0 mV for the stiff substrate, which was much higher than -40.2 ± 0.7 mV for the soft substrate (p < 0.05). Similar effects by substrate stiffness on the open probability (Fig. 5 *D*), and the sensitivity to Ca^{2+} (Fig. 5 *E*) and voltage (Fig. 5 *F*) were observed in HEK293 cells heterologously expressing STREX-containing α and β_1 subunits. However, in HEK293 cells expressing the STREX-deleted BK channel, such substrate stiffness-induced change in whole-cell current density (Fig. S2, *A* and *B*) and single channel open probability

(Fig. 5 *G*) was not observed. There was similar P_o–Ca^{2+} and P_o–voltage relationships on the soft and stiff substrates (Fig. 5, *H* and *I*).

To summarize, the above results provide consistent evidence that substrate stiffness regulates the open probability and the sensitivity to Ca^{2+} and voltage of the BK channel in myocytes and such functional regulation depends on the STREX domain in the BK channel.

Substrate stiffness regulation of L-type Ca^{2+} channel depends on stretch-activated BK channel

To further confirm the importance of the BK channel, particularly the STREX-containing stretch-activated channel, in substrate stiffness regulation of cardiac L-type Ca^{2+} channel, whole-cell Ca^{2+} channel currents were determined in HEK293 cells that expressed the $Ca_V1.2$ channel alone, or co-expressing with STREX-containing BK or STREX-deleted BK channel and then cultured on substrates with different stiffness. In cells only expressing the $Ca_V1.2$ channel, there was no significant difference in the Ca^{2+} channel current density in cells cultured on the soft and stiff substrates (Fig. 6, *A* and *B*). Substrate stiffness also had no effect on voltage-dependent channel activation or inactivation (Fig. 6, *C* and *D*). In striking contrast, in cells co-expressing the $Ca_V1.2$ Ca^{2+} channel and STREX-containing BK channel, the Ca^{2+} current density at voltages from 0 to 20 mV in cells on the stiff substrate was significantly higher than that on the soft substrate (Fig.6, *E* and *F*). Stiff substrates also caused a negative shift in the voltage-dependent activation curve with the $V_{1/2}$ value changing from -2.5 ± 0.4 mV on the soft substrate to -7.6 ± 0.8 mV on the stiff substrate ($p < 0.05$) without change in the slope value (4.3 ± 0.3 and 4.5 ± 0.5 on the soft and stiff substrates, respectively). Moreover, stiff substrate induced a positive shift in the voltage-dependent inactivation curve with the $V_{1/2}$ changing from -53.2 ± 3.1 mV on the soft substrate to -41± 3.6 mV on the stiff substrate ($p < 0.05$) and the k value changing from 9.2 ± 0.8 on the soft substrate to 8.8 ± 1.2 on the stiff substrate (Fig.6, *G* and *H*).   However, in cells co-expressing $Ca_V1.2$ channel and STREX-deleted BK channel, the Ca^{2+} current density exhibited no significant difference in cells on the soft and stiff substrates (Fig. 6, *I* and *J*). The Ca^{2+} current density in cells co-expressing the STREX-deleted BK channel was slightly lower than, but not significantly different from, that in cells expressing the Ca^{2+} channel alone. Voltage-dependent channel

activation and inactivation were not significantly altered by substrate stiffness (Fig. 6, *K* and *L*).

Myosin-mediated traction forces are critical in substrate stiffness regulation of the BK and L-type Ca²⁺ channels.

Cells can detect the mechanical properties of ECM by the mechanical response to the traction forces generated by myosin II in stress fibers (9, 10). Traction forces are greater in cells on the stiff substrate (31). Blebbistatin is known to specifically inhibit myosin and thereby the traction forces (31). Therefore, we finally examined whether myosin-mediated traction forces are critically involved in sensing the substrate stiffness by examining the effects of treatment with blebbistatin at a low dose on the BK channel and L-type Ca²⁺ channel in myocytes on the stiff substrate. Treatment with 5 μM blebbistatin resulted in relatively higher BK channel currents (Fig. 7, *A* and *B*) and lower Ca²⁺ channel currents (Fig. 7, *C* and *D*), which were similar in amplitude to those on the soft substrate (Fig.7, *B* and *D*). These results suggest that myosin-mediated traction forces are critical in sensing substrate stiffness and regulating the BK and L-type Ca²⁺ channels in myocytes.

Discussion

In the present study, we have shown that substrate stiffness regulates the BK and L-type voltage-gated Ca²⁺ channels in ventricular myocytes and that the stretch-activated BK channel is critical in substrate stiffness regulation of cardiac L-type Ca²⁺ channel. These findings reveal a novel mechanosensing mechanism in cardiac myocytes.

The L-type Ca²⁺ channel plays a critical role in mediating Ca²⁺ influx as a signal in the excitation-contraction coupling in cardiac myocytes. It has been documented that the stiffness of cardiac ECM undergoes remarkable increase during cardiac fibrosis (32). In this work, we showed that the change in substrate stiffness significantly altered cardiac L-type Ca²⁺ channel function in chick ventricular myocytes (Fig. 1, *A-D*), suggesting that cardiac L-type Ca²⁺ channel may function significantly differently under physiological and disease conditions. We further demonstrated that such substrate stiffness regulation mainly results from change in voltage-dependent channel activation and inactivation (Fig. 1, *E* and *F*) but not protein

expression (Fig. 1 *H*).　We also provided evidence to show that substrate stiff regulates the BK channel in chick ventricular myocytes. Stiff substrate downregulated mRNA and protein expression of both BK channel α and β_1 subunits (Fig. 3, *A-D*) and reduced the BK channel currents (Fig. 4, *A-D*). Further analysis of single channel recordings revealed that stiff substrate reduced the channel open probability (Fig. 5, *A* and *D*) and its sensitivity to voltage and Ca^{2+} (Fig. 5 *B-F*). The BK channel is long known to be expressed in cardiac myocytes but its role in cardiac function is less well-established (17). Here, we showed that inhibition of the BK channel abolished substrate stiffness regulation of cardiac L-type Ca^{2+} channel (Fig. 2, *A-D*), supporting the idea that the BK channel plays a critical role in substrate stiffness regulation of L-type voltage-gated Ca^{2+} channel in chick cardiac myocytes.

Previous studies demonstrated that the STREX-containing α subunit forms stretch-activated BK channel in embryonic chick cardiac myocytes and in recombinant systems and the sensitivity of the BK channel to membrane stretch is determined by the STREX domain located in the intracellular C-terminus (21, 22). Here we have confirmed the expression of STREX-containing α subunit in chick ventricular myocytes and further demonstrated that its expression level was down-regulated on the stiff substrate (Fig. 3, *E* and *F*). In HEK293 cells heterologously co-expressing the cardiac Cav1.2 channel and STREX-containing BK channel, the Ca^{2+} channel current (Fig. 6, *E* and *F*) and voltage-dependent channel activation and inactivation (Fig.6, *G* and *H*) were significantly altered by substrate stiffness in a similar manner to that observed in ventricular myocytes (Fig.1, *E* and *F*).　However, these effects of substrate stiffness on L-type Ca^{2+} channel were largely lost in HEK293 cells expressing the Ca$_V$1.2 channel alone (Fig. 6, *A-D*) or co-expressing with STREX-deleted BK channel (Fig. 6, *I-L*). These results, together with the aforementioned observation that pharmacological inhibition of the BK channel prevented substrate stiffness regulation of cardiac L-type Ca^{2+} channel, provide consistent evidence to indicate that the STREX-containing BK channel plays an essential role in the process during which substrate stiffness regulates L-type Ca^{2+} channel in myocytes. However, despite that this intracellularly located STREX domain is firmly established as the sensor for membrane stretch, it is unlikely to directly detect the stiffness of extracellular matrix and substrates. Instead, the STREX domain is more likely to serve as a transducer in the mechanism by

which cells sensing substrate stiffness. It is known that cells detect ECM by application of actomyosin-mediated traction forces (13, 31). We showed that treatment of myocytes on the stiff substrate with low-dose blebbistatin significantly increased the BK channel current (Fig. 7, *A* and *B*) and at the same time lowered the L-type Ca^{2+} channel current (Fig. 7, *C* and *D*), with the amplitudes of maximal K^+ and Ca^{2+} channel currents being similar to those in cells on the soft substrate (Fig.7, *B* and *D*). These results suggest that myosin-mediated traction forces are critically engaged in substrate stiffness sensing and regulation of the BK channel and L-type Ca^{2+} channel in myocytes.

While our results provide strong evidence to support a critical role of the STREX-containing BK channel in functional regulation of the cardiac L-type Ca^{2+} channel by substrate stiffness, it remains unclear how the BK channel regulates the Ca^{2+} channel. Increased BK channel activity can induce membrane hyperpolarization that inhibits Ca^{2+} channel activation. This mechanism may occur under physiological conditions but it is highly unlikely in our experimental conditions where the membrane potential was clamped. It is tempting to hypothesize a physical coupling between the BK channel and L-type Ca^{2+} channel via which activation of the BK channel evokes conformational changes in the L-type Ca^{2+} channel. This hypothesis is consistent with the changes in voltage-dependent activation and inactivation. There is accumulating evidence to show that change in substrate stiffness can cause the membrane protein and cytoskeleton remodeling (33, 34), and previous studies reported that the BK channel and voltage-gated Ca^{2+} channel form protein complexes in neurons and other types of cells, where Ca^{2+} influx through the voltage-gated Ca^{2+} channel facilitates BK channel activation (35, 36). In cardiac myocytes, L-type Ca^{2+} channel serves as the main Ca^{2+} influx pathway to stimulate ryanodine receptor-mediated Ca^{2+}-induced Ca^{2+} release to further increase the $[Ca^{2+}]_i$ and thereby activate the Ca^{2+}-activated K^+ channels including BK channel (23, 24). Therefore, there are two possible mechanisms by which Ca^{2+} induces BK channel activation in cardiac myocytes, namely, Ca^{2+} release from sarcoplasmic reticulum through ryanodine receptor and directly Ca^{2+} influx mediated by the L-type Ca^{2+} channel. Another interesting observation was that expression of both STREX-containing α and $β_1$ subunits was significantly regulated by substrate stiffness (Fig.7 *B* and *D*). It is known that the transcriptional co-activators, Yap and Taz (also known as WWTR1), identified as a

part of the Hippo pathway, are located in the nucleus in epithelial and mesenchymal stem cells cultured on the stiff substrates but translocate to the cytoplasm in cells on the soft substrates (37). It remains to be investigated in future studies whether such transcriptional mechanisms contribute to substrate stiffness regulation of the BK channel expression.

Previous studies reported that Ba^{2+} can block (38, 39) or activate the BK channel via the Ca^{2+}-bowl site (40) or be ineffective in activating the BK channel (41). The present study used Ba^{2+} as the charge carrier to measure the L-type Ca^{2+} channel currents to rule out Ca^{2+}-dependent mechanisms such as channel inactivation (Fig. 1, C). Here, we showed that substrate stiffness regulation of L-type voltage Ca^{2+} channels was observed regardless of Ca^{2+} or Ba^{2+} as the charge carrier (Fig. 1, A-D). Furthermore, IBTX was effective in inhibiting the BK channel currents (Fig. 2, B and D). The L-type Ca^{2+} channel current carried by Ba^{2+} was significantly regulated by substrate stiffness in HEK293 cells co-expressing STREX-containing BK channel and $Ca_V1.2$ channel (Fig.6 F). All these results suggest that Ba^{2+} at the concentration used in this study was ineffective in inhibiting the cardiac BK channel endogenously expressed in myocytes and heterologously expressed in HEK293 cells.

In conclusion, this study manifests a key role for the STREX-containing BK channel in substrate stiffness regulation of L-type Ca^{2+} channel function in cardiac myocytes. This finding helps to understand the influence of mechanical properties of ECM or biomaterials on cardiac function and remodeling under physiological and pathophysiological conditions, but also provides useful information for tissue engineering approaches to repair and regenerate cardiac tissues.

Author Contributions

X.Q.F, H.C.Z, L.H.J, B.L, conceived the research, designed the experiments, prepared and revised the manuscript. H.C.Z, Y. Y., X.A.W. S.S. L, B.L. L and J.D performed the experiments and analyzed the data. All authors participated in discussion and commented on the manuscript.

Acknowledgement

Conflict of Interest

None declared.

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Figure legends

FIGURE 1 Stiff substrate enhances L-type Ca^{2+} channel currents in ventricular myocytes.

(*A*) Representative whole-cell recordings of Ba^{2+} currents from ventricular myocytes cultured on the soft and stiff substrates. (*B*) Mean I-V relationship from 10 cells in three preparations for each case. (*C*) Representative whole-cell recordings of Ca^{2+} currents from cells cultured on the soft and stiff substrates. (*D*) Mean I-V relationships from 9 cells in three preparations for each case. (*E*) Voltage dependence of channel activation determined with the tail currents from 10 cells. (*F*) Voltage dependence of channel inactivation from 12 cells in three preparations cultured on the soft and stiff substrates. (*G*) Representative western blots showing the protein expression of Ca^{2+} channel α subunits in cells on the soft and stiff substrates. (*H*) Summary of the mean data from 4 independent experiments. * $p < 0.05$, comparison the current density at the same voltage. NS, no significant difference.

Figure 2 BK channel is critical in substrate stiffness regulation of L-type Ca^{2+} channel in ventricular myocytes.

(*A*) Representative whole-cell recordings of Ba^{2+} currents from cells cultured on the soft and stiff substrates after treatment with 100 nM IBTX for 10 min. (*B*) Mean I-V relationships from 12 cells in three preparations for each case. (*C*) Representative whole-cell recordings of Ca^{2+} currents from cells cultured on the soft and stiff substrates after treatment with 100 nM IBTX for 10 min. (*D*) Mean I-V relationships from 10 cells in three preparations for each case. (*E*) Voltage dependence of L-type channel activation determined with tail currents from 12 cells cultured on the soft and stiff substrates after treatment with 100 nM IBTX for 10 min. (*F*) Voltage dependence of L-type channel inactivation from 12 cells in three preparations on the soft and stiff substrates after treatment with 100 nM IBTX for 10 min.

FIGURE 3 Substrate stiffness regulates protein and mRNA expression of the BK channel α and β1 subunits.

(*A*) Representative western blots showing BK channel α and β1 subunit proteins in ventricular myocytes cultured on the soft and stiff substrates. (*B*) Summary of the mean protein expression from 4 independent experiments, normalized to that in cells on the soft and stiff substrate. (*C*) RT-PCR analysis of the mRNA expression of α and β1 subunits in cells on the soft and stiff substrates. (*D*) Summary of the mean mRNA expression from 4 independent experiments, normalized to that in cells on the soft and stiff substrate. (*E*) RT-PCR analysis of the BK STREX-containing α subunit in cells on the soft and stiff substrates. (*F*) Summary of the mRNA expression for the BK STREX-containing α subunit from 4 independent experiments, normalized to that in cells on the soft and stiff substrate. * $p < 0.05$.

FIGURE 4 Soft substrate increases the BK channel currents in ventricular myocytes and HEK293 heterologously expressing the stretch-activated BK channel.

(*A*) Representative whole-cell recordings of outward K^+ currents from cells on the soft and stiff substrates before (left panels) and 10 min after application of 100 nM IBTX (middle panels) and IBTX sensitive currents (right panels). (*B*) Mean I-V relationships of the IBTX-sensitive currents from 10 cells in three preparations for each case. (*C*) Representative whole-cell recordings of outward K^+ channel currents from HEK293 cells heterologously expressing the BK channel STREX-containing α and β1 subunits and cultured on the soft and stiff substrates before (left panels) and 10 min after application of 100 nM IBTX (middle panels) and IBTX sensitive current (right panels). (*D*) Mean I-V relationships of the IBTX-sensitive current in 11 cells from three preparations for each case. * $p < 0.05$, comparison the current density at the same voltage.

FIGURE 5 Substrate stiffness regulates the single channel open probability, Ca^{2+} and voltage sensitivity of the BK channel.

(*A*) Representative inside-out recordings of single-channel currents in ventricular myocytes on soft and stiff substrate at a membrane voltage of +40 mV with 0 μM Ca^{2+} (left panel), and summary of P_o from 10 cells in three preparations for each case (right panel). (*B*) The P_o-Ca^{2+} relationships of the BK channel currents from 7 cells in three preparations. The membrane potential was held at +40 mV. (*C*) The P_o-voltage relationships of the BK channel currents from 8 cells in three preparations for each case. 3 μM Ca^{2+} was used. (*D*) Representative

inside-out recording of single-channel currents in HEK293 cells heterologously expressing the BK channel STREX-containing α and β1 subunits at a membrane potential of +40 mV with 0 μM Ca^{2+} (left panel), and summary of P_o from 11 cells for each case (right panel). (*E*) The P_o-Ca^{2+} relationships of the BK channel currents in HEK293 cells heterologously expressing the BK channel STREX-containing α and β1 subunits on the soft and stiff substrate from 7 cells in three preparations for each case. The membrane potential was held at +40 mV. (*F*) The P_o-voltage relationship curves of the BK channel currents in HEK293 cells heterologously expressing the BK STREX-containing channel α and β1 subunits on the soft and stiff substrate from 7 cells in three preparations for each case. 3 μM Ca^{2+} was used. (*G*) Representative inside-out recording of single-channel currents in HEK293 cells heterologously expressing BK channel STREX-deleted α and β1 subunits at a membrane voltage of +40 mV with 0 μM Ca^{2+} (left panel), and summary of P_o from 11 cells for each case (right panel). (*H*) The P_o-Ca^{2+} relationships of the BK channel currents in HEK293 cells heterologously expressing the BK STREX-deleted channel α and β1 subunits on the soft and stiff substrate from 7 cells in three preparations for each case. The membrane potential was held at +40 mV. (*I*) The P_o-voltage relationships of the BK channel currents in HEK293 cells heterologously expressing the BK channel STREX-deleted α and β1 subunits on the soft and stiff substrate from 7 cells in three preparations for each case. 3 μM Ca^{2+} was used. *, $p < 0.05$. NS, no significant difference.

FIGURE 6 **Regulation of L-type $Ca_V1.2$ Ca^{2+} channel function by substrate stiffness requires STREX-containing BK channel.**

(*A*) Representative whole-cell recordings of Ba^{2+} currents from HEK293 cells expressing the $Ca_V1.2$ channel on the soft and stiff substrates. (*B*) Summary of the mean I-V relationships from 10 cells in three preparations for each case. (*C*) Voltage dependence of the $Ca_V1.2$ channel activation determined with tail currents from 10 cells for each case. (*D*) Voltage dependence of the $Ca_V1.2$ channel inactivation from recordings in 10 HEK293 cells expressing the $Ca_V1.2$ channel in three preparations cultured on the soft and stiff substrates. (*E*) Representative whole-cell recordings of Ba^{2+} currents from HEK293 cells co-expressing the STREX-containing BK channel and $Ca_V1.2$ Ca^{2+} channel on the soft and stiff substrates.

(*F*) Summary of the mean I-V relationships from 12 cells in three preparations for each case. (*G*) Voltage dependence of the $Ca_V1.2$ channel activation determined with tail currents from 12 cells for each case. (*H*) Voltage dependence of the $Ca_V1.2$ channel inactivation from 11 cells in three preparations cultured on the soft and stiff substrates. (*I*) Repres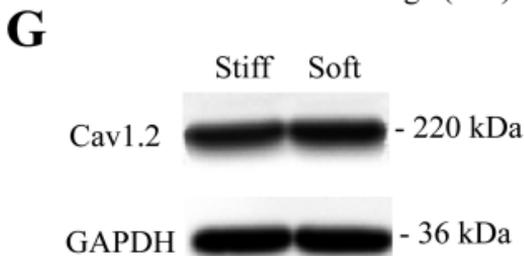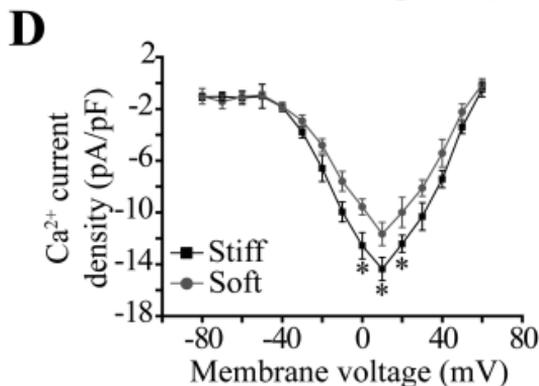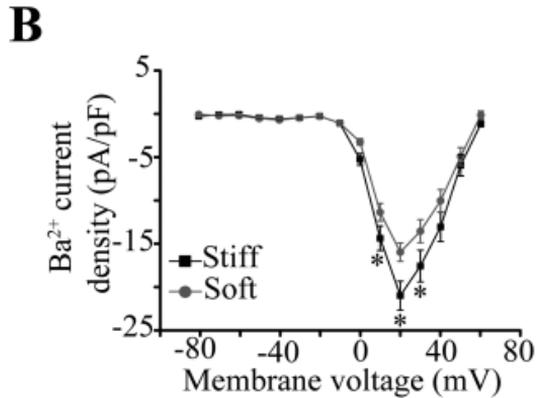entative whole-cell recordings of Ba^{2+} currents from HEK293 cells co-expressing the STREX-deleted BK channel and $Ca_V1.2$ channel on the soft and stiff substrates. (*J*) Summary of the mean I-V relationships from 12 cells in three preparations for each case. (*K*) Voltage dependence of the $Ca_V1.2$ channel activation determined with tail currents from 12 cells for each case. (*L*) Voltage dependence of the $Ca_V1.2$ channel inactivation from 11 cells in three preparations cultured on the soft and stiff substrates.   *, $p < 0.05$, comparison of the current density at the same voltage.

FIGURE 7 **Increase BK channel current and decrease L-type Ca^{2+} channel current in ventricular myocytes on stiff substrate after treatment with low-dose blebbistatin.**   (*A*) Representative whole-cell recordings of outward K^+ currents from cells after treatment with 5 μM blebbistatin on the stiff substrate and cells on the soft substrate before (upper panels) and 10 min after application of 100 nM IBTX (middle panels) and IBTX sensitive currents (bottom panels). (*B*) Mean I-V relationships from 10 cells in three preparations for each case. (*C*) Representative whole-cell recordings of Ba^{2+} currents from cells on the stiff substrates after treatment with low-dose blebbistatin and cells on the soft substrate without blebbistatin treatment. (*B*) Mean I-V relationships from 11 cells in three preparations for each case.
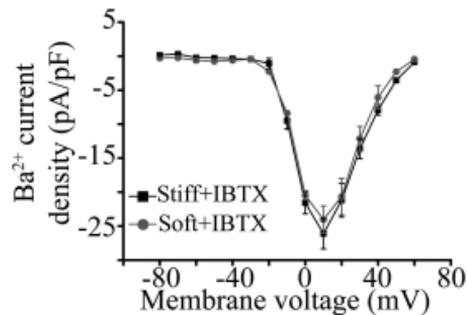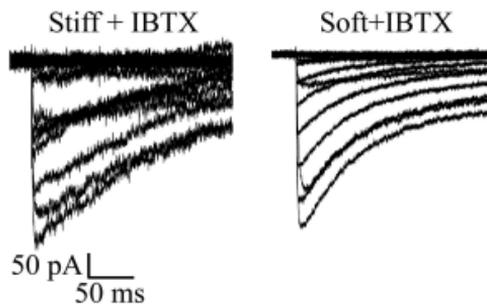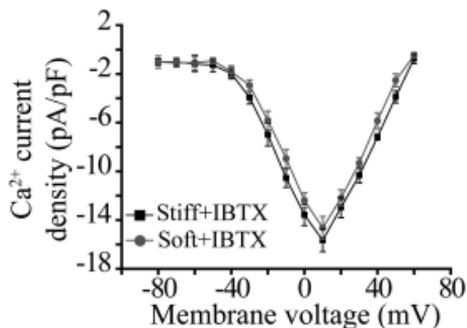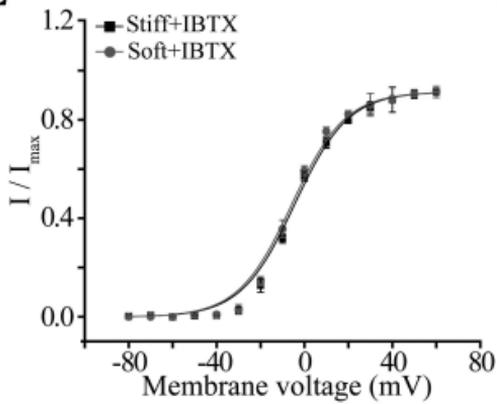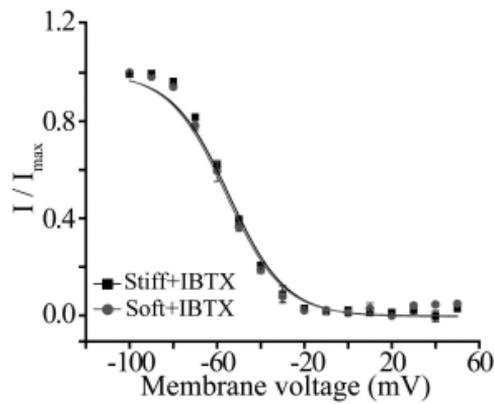
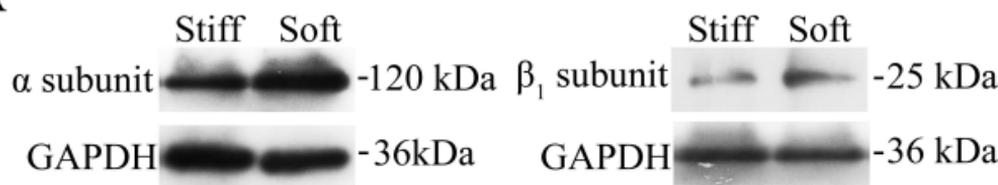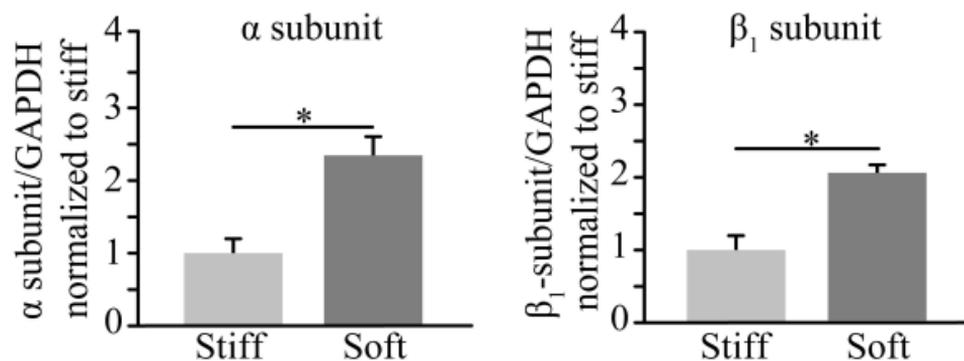**Fig. S1 Endogenous expression of BK channel α and β1 subunits and K^+ currents in HEK293**
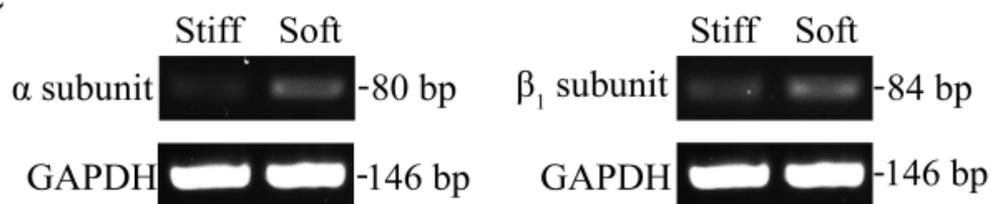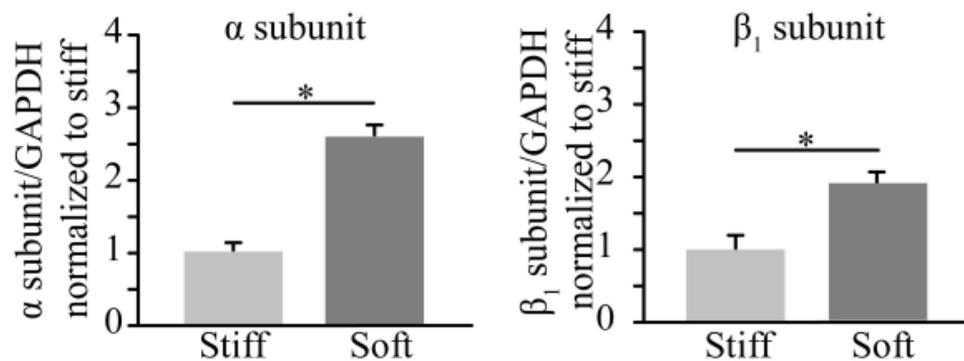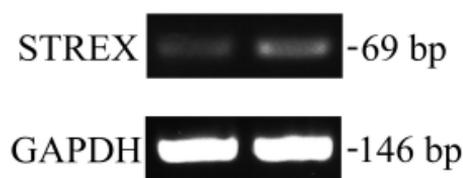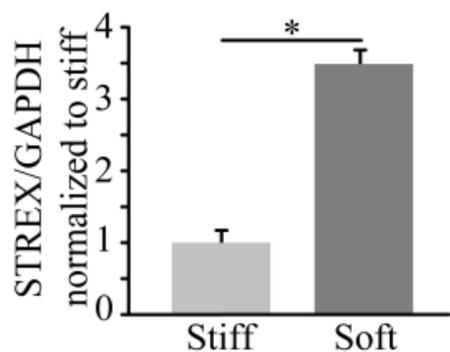
(A) Representative western blots showing lack of protein (upper panels) and mRNA expression (lower panels) of the BK channel α and $β_1$ subunits in HEK293 cells on the soft and stiff substrates. (*B*) Representative whole-cell recordings of K^+ channel currents from HEK293 cells on the soft and stiff substrates (upper panels) and 10 min after application of 100 nM IBTX (lower panels). (*C*) Summary of the mean I-V relationship curves of the K^+ channel currents from 11 cells in three preparations.

Fig S2 Effect of substrate stiffness on STREX-deleted BK channel current.

(A) Representative whole-cell recordings of Ba^{2+} currents from HEK cells heterologously expressing the BK channel STREX-deleted α and $β_1$ subunits cultured on the soft and stiff substrates. (*B*) Mean I-V relationship curves from 9 cells in three preparations.
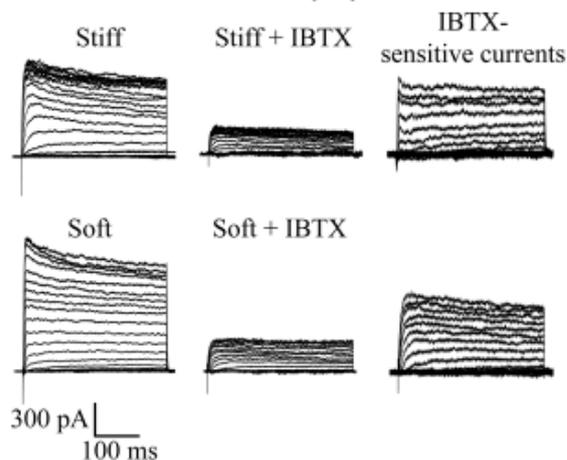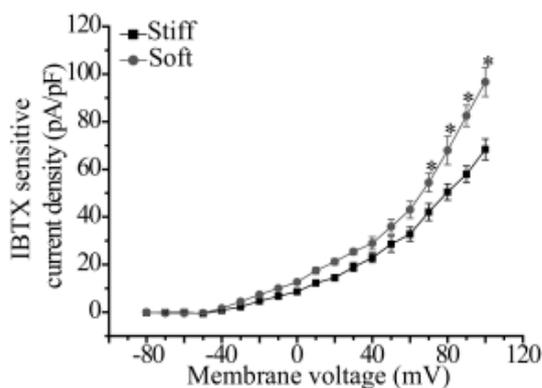
A.

Stiff + IBTX   Soft+IBTX

200 pA |
      |__
      10 ms

B.

C.

Stiff + IBTX   Soft+IBTX

50 pA |
      |___
      50 ms

D.

E.

F.

A

Ventricular myocytes

Stiff   Stiff + IBTX   IBTX-
sensitive currents

Soft   Soft + IBTX

300 pA |
100 ms

B

Ventricular myocytes

■ Stiff
● Soft

IBTX sensitive
current density (pA/pF)

Membrane voltage (mV)

C

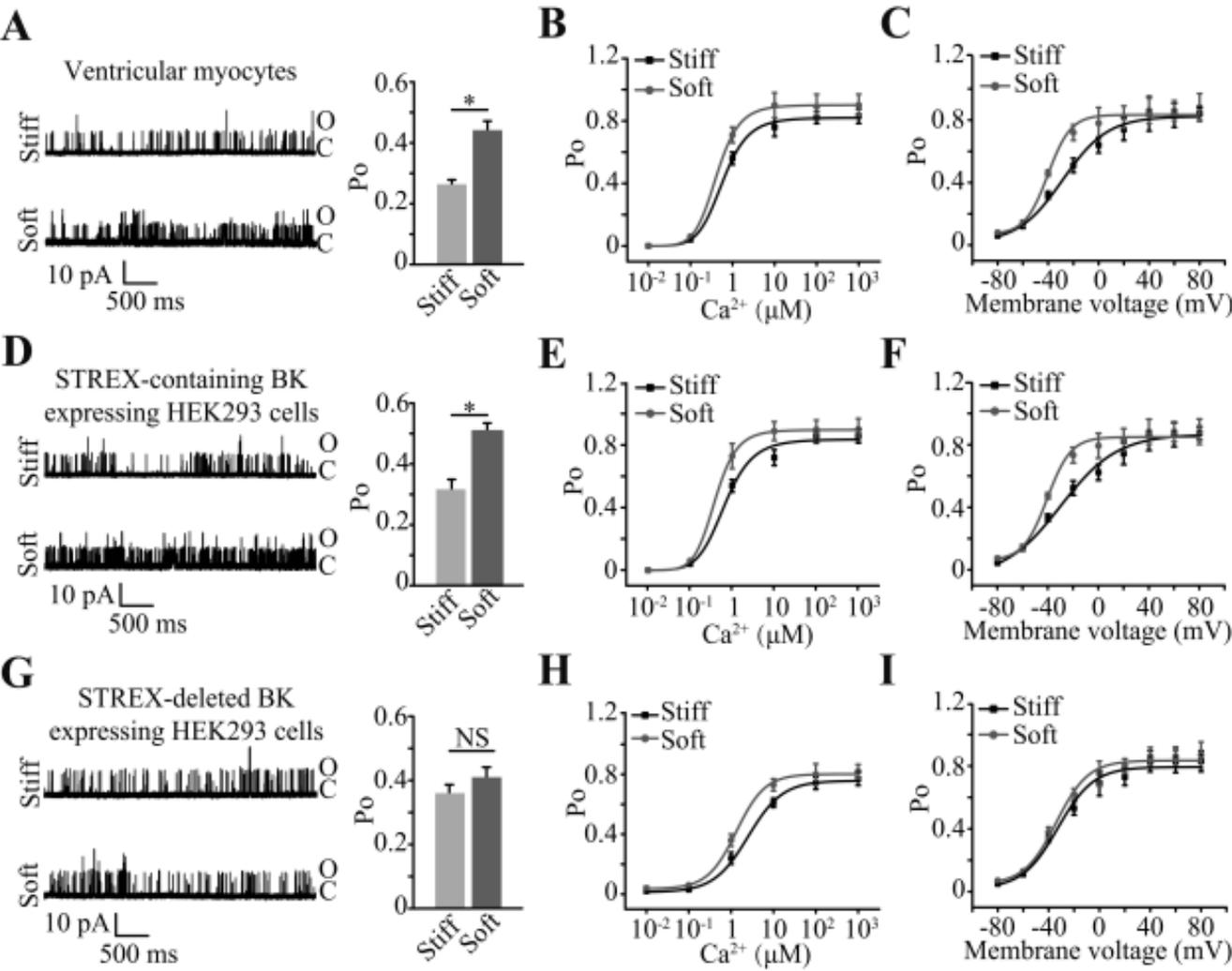BK expressing HEK 293 cells

Stiff   Stiff + IBTX   IBTX-
sensitive currents

Soft   Soft + IBTX

300 pA |
100 ms

D

BK expressing HEK 293 cells

■ Stiff
● Soft

IBTX sensitive
current density (pA/pF)

Membrane voltage (mV)

A Ventricular myocytes

B

C

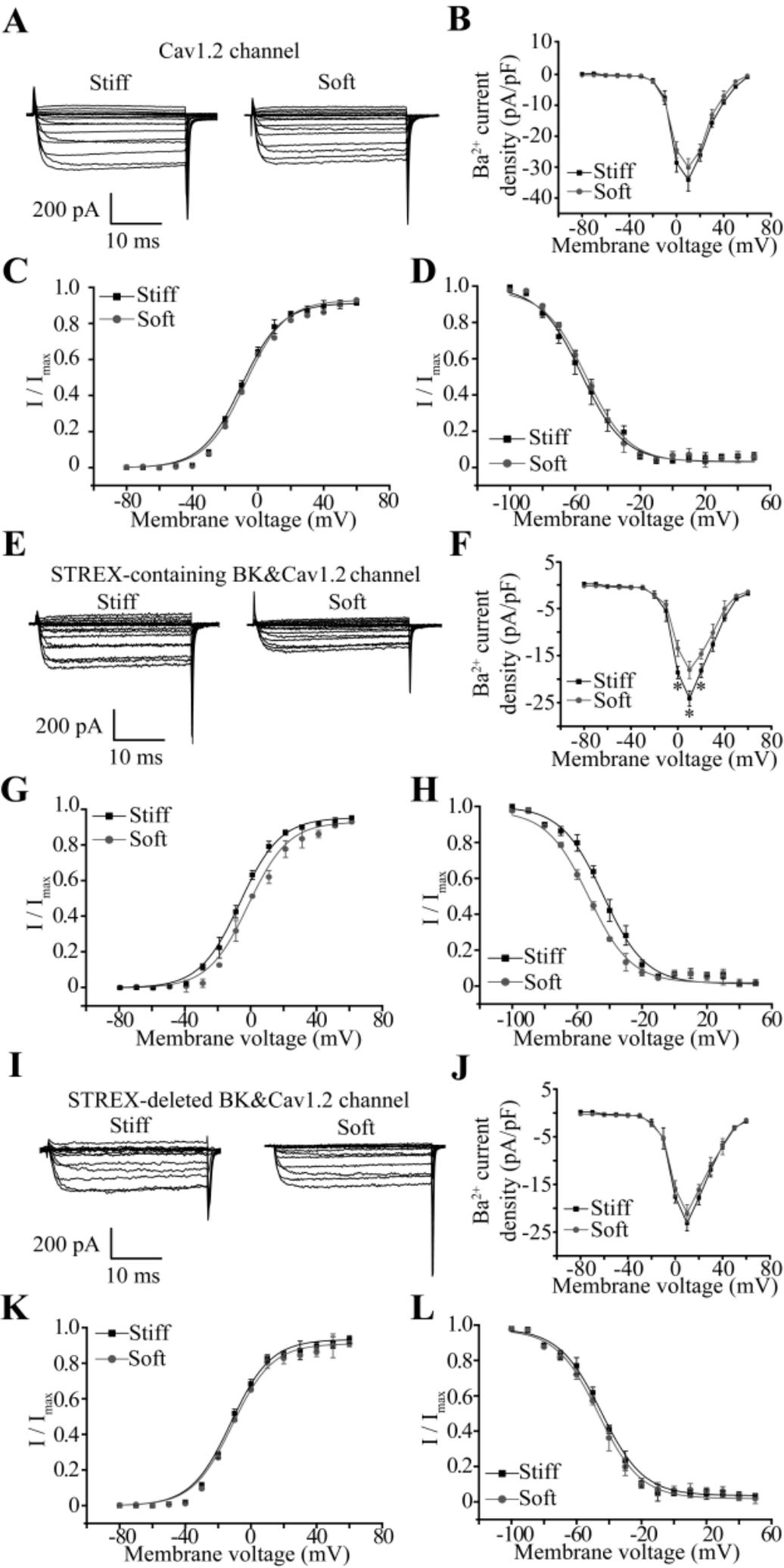**D** STREX-containing BK expressing HEK293 cells

E

F

G STREX-deleted BK expressing HEK293 cells

H

I

A Cav1.2 channel
Stiff   Soft
200 pA
10 ms

B [Ba²⁺ current density vs Membrane voltage graph — Stiff, Soft]

C [I/Imax vs Membrane voltage graph — Stiff, Soft]

D [I/Imax vs Membrane voltage graph — Stiff, Soft]

E STREX-containing BK&Cav1.2 channel
Stiff   Soft
200 pA
10 ms

F [Ba²⁺ current density vs Membrane voltage graph — Stiff, Soft]

G [I/Imax vs Membrane voltage graph — Stiff, Soft]

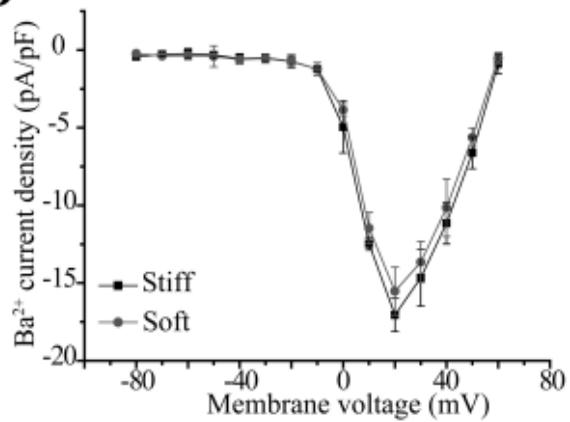**H** [I/Imax vs Membrane voltage graph — Stiff, Soft]

I STREX-deleted BK&Cav1.2 channel
Stiff   Soft
200 pA
10 ms

J [Ba²⁺ current density vs Membrane voltage graph — Stiff, Soft]

K [I/Imax vs Membrane voltage graph — Stiff, Soft]

L [I/Imax vs Membrane voltage graph — Stiff, Soft]

A

Stiff + Bleb   Soft

IBTX−

IBTX+

IBTX-sensitive
current

200 pA |
100 ms

B

IBTX-sensitive current density (pA/pF) vs Membrane voltage (mV)

- Stiff+Bleb
- Soft

C

Stiff + Bleb   Soft

200 pA |
10 ms

D

Ba²⁺ current density (pA/pF) vs Membrane voltage (mV)

- Stiff
- Soft

A HEK293 cells

GAPDH — 36 kDa ____ 36 kDa

α subunit — 120 kDa β$_1$ subunit — 25 kDa

GAPDH — 233 bp ____ 233 bp

α subunit — 80 bp β$_1$ subunit — 84 bp

B

Stiff   Soft

Stiff + IBTX   Soft + IBTX

50 pA

100 ms

C

- Stiff
- Stiff + IBTX
- Soft
- Soft + IBTX

K$^+$ current density (pA/pF)

Membrane voltage (mV)

A
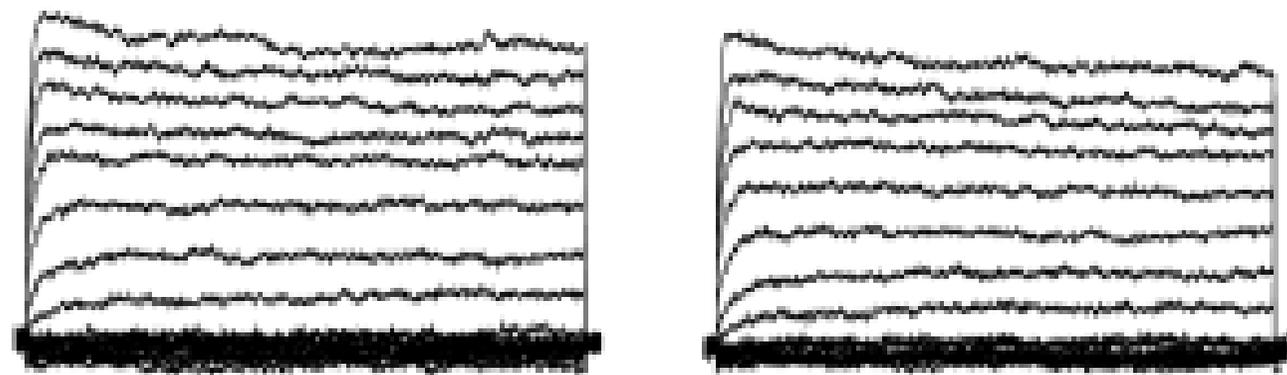
HEK293 cells

Stiff   Soft

500 pA|__
100 ms

B

■ Stiff
● Soft