TRPV4 Channels Mediate Cyclic Strain–Induced Endothelial Cell Reorientation Through Integrin-to-Integrin Signaling

Charles K. Thodeti, Benjamin Matthews, Arvind Ravi, Akiko Mammoto, Kaustabh Ghosh, Abigail L. Bracha, Donald E. Ingber

Abstract—Cyclic mechanical strain produced by pulsatile blood flow regulates the orientation of endothelial cells lining blood vessels and influences critical processes such as angiogenesis. Mechanical stimulation of stretch-activated calcium channels is known to mediate this reorientation response; however, the molecular basis remains unknown. Here, we show that cyclically stretching capillary endothelial cells adherent to flexible extracellular matrix substrates activates mechanosensitive TRPV4 (transient receptor potential vanilloid 4) ion channels that, in turn, stimulate phosphatidylinositol 3-kinase–dependent activation and binding of additional β1 integrin receptors, which promotes cytoskeletal remodeling and cell reorientation. Inhibition of integrin activation using blocking antibodies and knock down of TRPV4 channels using specific small interfering RNA suppress strain-induced capillary cell reorientation. Thus, mechanical forces that physically deform extracellular matrix may guide capillary cell reorientation through a strain-dependent “integrin-to-integrin” signaling mechanism mediated by force-induced activation of mechanically gated TRPV4 ion channels on the cell surface. (Circ Res. 2009;104:1123-1130.)

Key Words: mechanical strain ▶ integrin ▶ TRPV4 ▶ endothelial cell ▶ reorientation ▶ cytoskeleton

Mechanical forces regulate vascular growth and development by influencing endothelial cell growth, survival, differentiation and migration. Local mechanical cues conveyed by extracellular matrix (ECM) attributable to cyclic deformation of blood vessels, hemodynamic forces, or cell-generated traction forces are also potent inducers of directional capillary blood vessel growth and vascular remodeling in vitro and in vivo. For example, the initial step in neovascularization involves reorientation of a subset of capillary endothelial (CE) cells that spread and migrate perpendicular to the main axis of the preexisting vessel toward the angiogenic stimulus; however, the molecular mechanism responsible for this CE cell reorientation response is unknown. Many cell types, including large vessel endothelial cells, realign perpendicular to the direction of the applied force when they experience cyclic stretching (mechanical strain). In the case of macrovascular endothelium, this reorientation response can be prevented by treatment with chemical inhibitors of stretch-activated (SA) ion channels. But neither the identity of these channels nor the mechanism by which they elicit cell reorientation is known.

Endothelial cells express most members of the transient receptor potential (TRP) family of ion channels and TRP vanilloid (TRPV)4 has been reported to mediate flow-induced vasodilation in large vessel endothelium. Here, we show that calcium influx through TRPV4 channels stimulated by mechanically stretching CE cells through their integrin–extracellular matrix (ECM) adhesions promotes cell reorientation by activating phosphatidylinositol 3-kinase (PI3K), thereby stimulating activation of additional β1 integrin receptors. This mechanism is distinct from that used by macrovascular endothelium to sense fluid shear stresses, which is mediated by a mechanosensory complex containing platelet endothelial cell adhesion molecule 1, vascular endothelial growth factor receptor, and VE-cadherin.

Materials and Methods

Cell Culture

CE cells were isolated from bovine adrenal cortex, cloned, and passaged as described previously. Frozen aliquots of these cells (passage, <15), which we have confirmed retain their functionality and differentiation potential, were maintained at 37°C in 10% CO2 on gelatin-coated tissue culture dishes in low glucose DMEM (Invitrogen) supplemented with 10% FCS (HyClone), 10 mmol/L HEPES (JRH-Biosciences), and L-glutamine (292 μg/mL, penicillin (100 U/mL), streptomycin (100 μg/mL) (GPS), as described. Human microvascular endothelial cells from dermis (Cambrex, Walkersville, Md) were cultured in EBM-2 (Cambrex), supplemented with 5% FBS, and growth factors (basic fibroblast growth

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Correspondence to Donald E. Ingber, MD, PhD, Vascular Biology Program, KFRL 11.127.300 Longwood Ave, Children’s Hospital/Harvard Medical School, Boston, MA 02115. E-mail donald.ingber@childrens.harvard.edu

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1123
factor, insulin-like growth factor, vascular endothelial growth factor) according to the instructions of the manufacturer.

**Mechanical Strain Application**

CE cells cultured on fibronectin-coated 6-well Uniflex (Flex Cell International) plates for 24 hours were subjected to uniaxial cyclic stretch (10% elongation; 1 Hz frequency) for 1 to 2 hours using a Flexercell Tension Plus System (Flex Cell International). In some experiments, CE cells were plated on fibronectin-coated 6-well Bioflex (Flex Cell International) for 1 hour and subjected to static stretch (15% elongation) for 1 to 15 minutes. Control cells were maintained under identical conditions in the absence of strain application.

**Measurement of Cell Orientation**

To measure the orientation of cells in cyclic strain experiments, fluorescent images of cells were traced to measure angle with the direction of cyclic strain using ImageJ software (NIH) and reported as percentage cells aligned at 90±30°. For each condition, 5 to 6 fields were evaluated, with approximately 15 to 30 cells per field. Statistical differences between experimental groups were determined using the Student’s t test. All data were obtained from at least 3 separate experiments and are expressed as means±SEM.

**Small Interfering RNA Knock Down of TRPV Channels**

Smart pool small interfering (si)RNAs (10 nmol/L) of TRPV2, TRPV4 (both from Dharmacon), TRP channel (TRPC)1 (Ambion), or control (Qiagen) siRNAs was transfected into CE cells using siLentFect reagent (Bio-Rad) as described. Three days later, cells were used for calcium imaging or reorientation experiments. The knock down of TRPV channel expression was assessed using RT-PCR with species-specific primers and Western blotting.

**Results**

**Capillary Cell Reorientation Induced by Cyclic Strain**

Directional CE cell motility and angiogenesis have been shown to be stimulated by mechanical strain (distortion) in ECM gels and living tissues. To begin to analyze the molecular mechanism by which mechanical strain influences CE cell orientation, we cultured bovine CE cells on flexible fibronectin-coated substrates and subjected them to 10% uniaxial cyclic strain (1 Hz) using a FlexerCell Tension Plus system. Fluorescence microscopic analysis of cells labeled with Alexa 488–phalloidin combined with computerized morphometry revealed that stress fibers thickened in these cells, and most (~80%) realigned perpendicular to the main axis of the applied strain within 2 hours after force application (Figure 1A and 1B). Stress fiber realignment was accompanied by redistribution and reorientation of focal adhesions.
containing vinculin (Figure 1C), focal adhesion kinase, and talin (not shown), which appeared in close association with the ends of newly aligned stress fibers (Figure 1C).

**Strain-Induced Capillary Cell Reorientation Requires β1 Integrin Activation**

The effects of fluid shear on large vessel endothelium and mechanical strain on fibroblasts are mediated by stress-dependent activation of integrin receptors within minutes after force application. When CE cells cultured on flexible fibronectin-coated substrates were exposed to static stretch (15% elongation), β1 integrin activation increased within 1 minute after force application, as indicated by increased phosphorylation of the T788/789 site of the β1 integrin cytoplasmic tail in Western blotting (Figure 2A), which has been shown to correlate with integrin activation. 

Immunofluorescence staining using 12G10 antibodies that only recognize the activated conformation of β1 integrins also showed increased clustering of activated β1 integrins within large streak-like focal adhesions at the cell periphery within 15 minutes after static strain application (Figure 2B). The ability of the 12G10 antibody to detect activated β1 integrins in our CE cells was confirmed using flow cytometry, which demonstrated a significantly increased 12G10 signal after globally activating integrins by treatment with manganese (Figure 1 in the online data supplement, available at http://circres.ahajournals.org). Static strain-induced activation of integrin signaling was confirmed independently by demonstrating increased phosphorylation of mitogen-activated protein kinase (extracellular signal-regulated kinase [ERK]1/2) (Figure 2C) and focal adhesion kinase (Online Figure II) within 5 to 15 minutes after exposure to mechanical strain. Application of uniaxial cyclic strain (10%; 1 Hz) also induced β1 integrin activation within minutes, as measured by enhanced binding to the fibronectin fragment glutathione S-transferase (GST)-FNIII8–11 (Figure 2D and Online Figure III, B) and to the 12G10 antibody, which only ligate the activated form of the β1 integrin receptor (Figure 2E), as well as by increased T788/789 phosphorylation of β1 integrin (Online Figure III, C). Cyclic strain also increased β1 integrin activation in human CE cells, as measured by enhanced binding of GST-FNIII8–11 (Online Figure III, B), and, thus, this appears to be a generalized response in CE cells.

To explore whether this mechanical strain–induced wave of β1 integrin activation is required for CE cell reorientation, cells were preincubated with function-blocking anti–β1 integrin (PSD2) antibody for 30 minutes, and then the cells were subjected to uniaxial cyclic strain (10%) for 2 hours. Treatment with this inhibitory antibody, but not isotype-matched control IgG, inhibited strain-induced cell realignment by almost 70% (P<0.001) (Figure 2F), and it prevented reorientation of stress fibers and focal adhesions (Online Figure IV). Before stretching, we did not find any changes in cell morphology or actin staining in antibody-treated cells, confirming that binding of these antibodies did not affect existing adhesions. These results indicate that application of mechanical strain to CE cells through existing integrins that are bound to substrate-immobilized ECM molecules (and hence activated) induces focal adhesion remodeling, stress fiber realignment, and cell reorientation through a mechanism that requires activation of additional β1 integrin receptors.

**PI3K Is Upstream of β1 Integrin Activation in This Mechanical Signaling Cascade**

PI3K has been implicated in the activation of β3 integrins by fluid shear stress in large vessel endothelium; however, it also can act downstream of integrin activation. To explore whether PI3K is involved in early mechanical signaling in microvascular endothelium, CE cells were transfected with green fluorescent protein (GFP) fused to an AKT-PH domain that translocates to the plasma membrane when it binds to the PTK product, phosphatidylinositol 3-phosphate. Bright linear GFP-AKT-PH staining was detected at the peripheral membrane within 1 minute after application of static stretch requirements.
(15%), whereas it remained diffusely distributed throughout the cytoplasm in control (unstrained) CE cells (Figure 3A). Quantification of GFP-AKT-PH translocation by 2 independent parameters (fraction of GFP-AKT-PH in total perimeter of the membrane or GFP-fluorescence intensity ratio between membrane and cytosol) revealed a significant increase in response to mechanical strain that was inhibited by treatment with the PI3K inhibitor LY294002 (Figure 3B). Static stretch also activated PI3K, as determined by enhanced phosphorylation of its downstream target AKT at Ser473 within minutes after force application, as detected in Western blots (Figure 3C). Moreover, stretch-induced translocation of GFP-AKT-PH to the membrane and AKT phosphorylation were both abolished by inhibiting PI3K with LY294002 (Figure 3A and 3D). LY294002 treatment also prevented β1 integrin activation (Figure 3D and 3E) and suppressed focal adhesion kinase activation (Online Figure II). Thus, force application through ECM-integrin adhesions activates additional cell surface β1 integrin receptors by stimulating PI3K.

**Strain-Induced Cell Reorientation Is Mediated by Stress-Activated Ion Channels**

SA ion channels have been implicated in force-dependent alignment of large vessel endothelial cells. Direct force application to cell surface β1 integrins using magnetic tweezers also results in rapid (within 2 to 5 seconds) calcium influx in our bovine CE cells, and this response can be blocked using the general SA channel inhibitor, gadolinium chloride. To confirm that mechanical strain activates SA channels in these CE cells, cells adherent to flexible ECM substrates were loaded with the calcium reporter dye Fluo-4, subjected to static stretch (15% elongation) and calcium influx was measured using microfluorimetry. Functional TRPV4 channels (Online Figure VI). Next, we measured TRPV4 channel activation directly by whole-cell clamp using bovine CE cells transiently transfected with TRPV4-EGFP that gave robust TRPV4 currents in response to 4α-PDD, and we found that substitution of 4α-PDD with N-methyl-d-glucamine for cations in the bathing solution, inhibited activation of inward, but not outward, currents by 4α-PDD, inducing a robust calcium signal in bovine and human CE cells (Figure 4A). RT-PCR analysis also confirmed the presence of TRPV4 mRNA in both bovine and human CE cells (Figure 4B and Online Figure V). We then found that a specific activator of TRPV4 channels, 4-α-phorbol-12,13-didecanoate (4-α-PDD), induced a robust calcium signal in bovine and human CE cells, thus suggesting that both cell types express functional TRPV4 channels (Online Figure VI). Next, we measured TRPV4 channel activation directly by whole-cell clamp using bovine CE cells transiently transfected with TRPV4-EGFP that gave robust TRPV4 currents in response to 4-α-PDD, and we found that substitution of N-methyl-d-glucamine for cations in the bathing solution, inhibited activation of inward, but not outward, currents by 4-α-PDD in these cells (Online Figure VII). We used this approach because TRPV4-like currents in primary endothelial cells are small, transient, and difficult to characterize, as previously described and as we observed as well. Thus, taken together, these findings strongly suggest that CE cells express functional TRPV4 channels, although at a low level.

To confirm that calcium influx through TRPV4 channels mediates the effects of cyclic strain on CE cell orientation, we inhibited PI3K activity, as measured by membrane translocation of GFP-AKT-PH (Online Figure III, D). Finally, the cell and cytoskeletal reorientation normally induced by cyclic strain were greatly suppressed in the presence of this SA ion channel blocker (Online Figure III, E). Thus, mechanical stretch-dependent activation of mechanosensitive calcium channels appears to be required for activation of both PI3K and β1 integrins, as well as subsequent cytoskeletal reorientation in CE cells.

**TRPV4 Channels Mediate Strain-Induced Capillary Cell Reorientation**

We then set out to identify the specific type of mechanosensitive ion channel that mediates the effects of mechanical strain on CE cell orientation. TRPV4 is an interesting potential candidate because it mediates cell sensitivity to osmotic stresses and shear stress–induced vasodilation. To determine whether TRPV4 is the candidate mechanosensitive channel, first we measured its expression in CE cells. Western blot analysis showed a strong band around 85 kDa (and a fainter band at 100 kDa) in both bovine and human CE cells (Figure 4A). RT-PCR analysis also confirmed the presence of TRPV4 mRNA in both bovine and human CE cells (Figure 4B and Online Figure V). We then found that a specific activator of TRPV4 channels, 4-α-phorbol-12,13-didecanoate (4-α-PDD), induced a robust calcium signal in bovine and human CE cells, thus suggesting that both cell types express functional TRPV4 channels (Online Figure VI). Next, we measured TRPV4 channel activation directly by whole-cell clamp using bovine CE cells transiently transfected with TRPV4-EGFP that gave robust TRPV4 currents in response to 4-α-PDD, and we found that substitution of N-methyl-d-glucamine for cations in the bathing solution, inhibited activation of inward, but not outward, currents by 4-α-PDD in these cells (Online Figure VII). We used this approach because TRPV4-like currents in primary endothelial cells are small, transient, and difficult to characterize, as previously described and as we observed as well. Thus, taken together, these findings strongly suggest that CE cells express functional TRPV4 channels, although at a low level.

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knocked down the expression of TRPV4 in bovine and human CE cells using specific siRNA; sham siRNA and siRNA directed against the closely related channel TRPV2 were used as controls. Sequence analysis of smart pool siRNAs confirmed that both siRNA sequences exhibit 80% to 100% homology with bovine and human TRPV4. RT-PCR analysis revealed that TRPV2 and TRPV4 mRNA levels were knocked down by 70% and 90% in bovine and human CE cells, respectively, using this approach, whereas use of a sham control siRNA had no effect (Figure 4B and Online Figure V). We found that TRPV4 protein expression was also knocked down by ~60% and 80% in bovine and human CE cells, respectively (Figure 4C and 4D and Online Figure V).

Importantly, microfluorimetric analysis revealed that application of static stretch (15%) for 4 seconds induced a large wave of calcium influx in bovine CE cells transfected with control siRNAs, whereas this response was significantly inhibited (*P<0.02) in cells treated with TRPV4 siRNA (Figure 4E and 4F). In contrast, use of siRNA directed against the closely related SA channel TRPV2 had no effect (Figure 4E and 4F). siRNA knock down of TRPV4 also inhibited cyclic strain–induced activation of β1 integrins, AKT, and ERK1/2, further confirming that TRPV4 activation is upstream of integrin activation (Figure 5). Pretreatment of CE cells with the general TRPV inhibitor ruthenium red41 or with TRPV4 siRNA also significantly suppressed calcium signaling and cell reorientation induced by application of cyclic strain in CE cells, whereas addition of siRNA against 2 different related SA channels, TRPV2 or TRPC1 (Online Figure V), was ineffective (Figure 6A through 6D). This inhibition was specific for reorientation as transfection of cells with TRPV4 siRNA did not alter the number of viable adherent CE cells when they were cultured on standard tissue culture substrates (Online Figure VIII). Moreover, we found that application of similar cyclic strain, in the presence or absence of ruthenium red, did not effect CE cell proliferation or apoptosis, as measured by Ki 67 staining and poly(ADP-ribosyl) polymerase cleavage (Online Figure IX). Taken together, these results indicate that TRPV4 channels are mechanosensitive calcium channels in CE cells that are...
activated by mechanical strain applied through the integrin-mediated cell–ECM adhesions and that calcium influx through these channels is required for downstream signaling events that drive the cell and cytoskeletal reorientation response triggered by cell stretching.

Discussion
In this study, we showed that application of mechanical strain to bound integrins on the CE cell surface stimulates calcium influx through mechanosensitive TRPV4 ion channels, which activates additional β1 integrins and subsequent downstream cytoskeletal reorientation responses. Although cyclic strain induces reorientation of large vessel endothelial cells and this process has been shown to be mediated by activation of SA channels, the present study is the first to analyze this process in microvascular CE cells and to determine the specific molecular identity of these channels. Our work shows the TRPV4 is at least one of the SA channels that is required for activation of β1 integrins and subsequent reorientation of CE cells in response to mechanical strain.

Cell stretching and strain application to integrins have both been implicated as critical regulators of endothelial cell proliferation, migration, and angiogenesis in the past, but how these mechanical signals control vascular development is not known. The present findings provide direct evidence to show that mechanical strain activates β1 integrins in bovine and human CE cells and that this is required for downstream cell and cytoskeletal remodeling events that mediate cell reorientation critical for directional cell motility. Given that we exposed cells to both static and cyclic stretch and similar results were obtained using multiple different assays and probes to assess β1 integrin activation, we believe that these findings unequivocally confirm that mechanical strain activates β1 integrins in CE cells.

The most important finding of this study is the identification of TRPV4 as the SA channel responsible for β1 integrin activation in response to mechanical strain application to microvascular cells. We make this conclusion based on the following observations: (1) bovine and human CE cells functionally express TRPV4 channels that are activated by the selective TRPV4 activator 4α-PDD; (2) the TRPV4 blocker ruthenium red inhibits calcium influx and cell reorientation in response to mechanical strain; and (3) siRNA knock down of TRPV4, but not TRPV2 or TRPC1, inhibits strain-induced calcium influx and capillary cell reorientation. Among all known TRP channels, only TRPV4 has been reported to be mechanosensitive in that it transduces osmotic signals and plays a role in shear stress–induced vasodilation.

TRPV4 is also important for the mechanical behavior of Caenorhabditis elegans, and mice lacking TRPV4 are insensitive to normal levels of noxious mechanical stimuli. Here, we show that activation of TRPV4 by mechanical distortion of cell–ECM adhesions plays a critical role in control of downstream signaling pathways that mediate cell reorientation and vascular development in response to mechanical strain of integrin-mediated cell–ECM adhesions.

Although TRPV2 and TRPC1 channels were shown to mediate stretch-induced calcium signaling when overexpressed in CHO cells and oocytes, we found that knocking down of either TRPV2 and TRPC1 expression using siRNAs did not influence calcium influx or cytoskeletal reorientation in response to mechanical strain, suggesting that these candidate SA channels do not appear to contribute to SA calcium entry in CE cells. TRPV4 channel activation by mechanical strain could be mediated through its interaction with integrins. Other types of TRP channels, such as polycystins and ENaC channels, coimmunoprecipitate with β1 integrins, and TRPV4 has been found to coimmunoprecipitate with α2 integrins, suggesting that it resides in a common mechanosignaling complex with these ECM receptors.

Regardless of the precise mechanism by which TRPV4 channels sense changes in the forces that are balanced across integrins, our findings show that strain-induced calcium influx through these channels activates PI3K. PI3K, in turn, activates additional integrins and related downstream signaling molecules that result in activation of Rho and its target ROCK (Rho-associated kinase), which promote focal adhesion and stress fiber remodeling. The fact that this structural remodeling occurs in a highly oriented manner that is perpendicular to the applied tension field in nonconfluent cells, provides additional evidence to suggest that these events occur locally at the cell surface–ECM interface, where forces are exerted, rather than homogenously throughout the cytoplasm or within lateral membrane junctional complexes that form between cells in a confluent endothelial cell monolayer, as is required for shear stress sensation.

These findings are important because CE cell reorientation plays a crucial role in the directional migration and oriented sprouting that drive angiogenesis. Ion signaling through SA channels has been previously shown to be important for both cell migration and reorientation in response to stress. However, these SA channels were never identified, and the importance of this type of mechanotransduction response for angiogenesis has not been explored previously. The possibility that TRP channels might be involved in vascular development has been raised in the past; however, there has been no evidence to suggest that they play a direct role in endothelial cell reorientation. Importantly, our data show that TRPV4 channels are the SA channels that mediate CE cell responses to mechanical forces and cell–ECM interactions, which are critical for control of cell migration and tube formation during capillary development. Mechanically gated TRPV4 channels therefore appear to mediate a novel stretch-sensitive “integrin-to-integrin” mechanical signaling that is required for CE cell reorientation during angiogenesis, and, thus, these channels may represent new targets for future therapeutic intervention in angiogenesis-dependent diseases, such as cancer, arthritis, and macular degeneration.

Acknowledgments
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Disclosures
None.

References
35. Liedtke W, Tobin DM, Bargmann CI, Friedman JM. Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical...
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Materials: Antibodies against activated β1 integrin (clone 12G10) were from Serotec and Chemicon and those directed against T788/789 of β1 integrin cytoplasmic tail, and phospho FAK pY-397 were from Biosource International/ Millipore. Alexa-conjugated phalloidin and secondary antibodies were from Molecular Probes/ Invitrogen. Antibodies against phospho AKT Ser-473, AKT, phospho-ERK1/2, ERK1/2, FAK and PARP were from Cell Signaling, and those against vinculin and actin were from Sigma. The polyclonal antibodies against ki-67 were obtained from Thermo Scientific. Human fibronectin was obtained from BD Biosciences. Gadolinium Chloride, 4-α-PDD and ruthenium red were purchased from Sigma; LY294002 was from Calbiochem. The polyclonal antibodies against TRPV4 were obtained from Affinity BioReagents and MBL International Corporation. The primers used for RT-PCR were: TRPV2 (human: Forward- CAAACCGATTGACCGAGAT; Reverse- GTTCAGCACAGCCTTCATCA and bovine: Forward- CAGCTGGGAGGAAAACTCAG; Reverse- GGGAGGAAGTCCTTTTCCAG), TRPV4 (human: Forward- GACGGGGACCTAGCATCA; Reverse- AACAGGTCCAGGAGGAAGGT and bovine: Forward- GACTACCTGCAGCTGTCG; Reverse- TTCATCCAGGCCAGGAC), TRPC1 (human: Forward- CACTCGTTCGGACCTGCTTT; Reverse- GCAGCTTCGTCAGCACAATCACA; bovine: Forward- CCATTCCGTTGATGCAGCTTT; Reverse- TTATGAAGCATTGCCACCAGCAGC) and GAPDH (Forward- ACCACAGTCATGCACTCAC; Reverse- TCCACCACCCCTGTTGCTGTA)
Morphological and Immunofluorescence Studies: Cells were transfected with GFP-AKT-PH domain (kind gift of Dr. Martin Schwartz) using Effectene (Qiagen, Chatsworth, CA). Cells adherent to flexible ECM substrates and subjected to mechanical stretch were washed in PBS, fixed in 4% paraformaldehyde for 30 min either mounted on glass slides (for visualizing GFP-AKT-PH translocation) or permeabilized with 0.25% Triton-X 100/PBS for 5 min for immunostaining. After blocking with DMEM containing 10% FBS, cells were incubated for 1h with Alexa-phalloidin to visualize stress fibers, washed and mounted on glass slides using fluoromount-G (Southern biotech). For staining focal adhesions, cells were incubated with antibodies against vinculin for 1h followed by rinsing and incubation with Alexa-conjugated secondary antibodies; activated β1 integrins were detected using 12G10 antibody. For measuring proliferation, cells were incubated with antibodies against ki-67 for 1h followed by rinsing and incubation with Alexa-conjugated secondary antibodies. Images were acquired on a Leica Confocal SP2 microscope and processed using Leica software and Adobe Photoshop. GFP-AKT-PH domain translocation to the plasma membrane was quantified measuring either ratio of the perimeter of whole cell membrane and the membrane that contains the GFP-AKT-PH domain or the ratio of GFP fluorescence intensity of translocated GFP-AKT-PH domain and cytosol adjacent to the membrane.

Biochemical Analysis: Our methods for Western blotting have been published previously 1. Membranes containing transferred protein were blocked in 3% BSA/TBST for 1h and incubated overnight with primary antibodies against AKT and phospho Ser-473 AKT (1:1000), phospho-Thr 788/789 of β1 integrin cytoplasmic tail (1: 300-1000), ERK1/2 and phospho ERK1/2 (1:1000), FAK and phospho FAK-pY397 (1:1000), actin (1:1000), PARP (1:1000) and TRPV4 (1:300) at 4°C. The membranes were subsequently washed incubated with HRP-
conjugated secondary antibodies (1:5000) for 1h and washed and incubated with West-Pico ECL reagent from Pierce (USA) and exposed to Kodak X-ray film (Sigma).

**Calcium Imaging:** CE cells adherent to the flexible substrates were loaded with Fluo-4/AM (1-4 µM) for 30 min, washed 3 times in calcium medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.1 mM CaCl2, 1.2 mM KH2PO4, 5 mM NaHCO3, 5.5 mM glucose, and 20 mM Hepes. pH 7.4) and then exposed to static stretch (15% elongation) for 3-4 sec using a ‘Stage Flexer’ (FlexCell International) apparatus that is fixed on a Nikon upright microscope equipped with CCD camera (Spot-RT slider, Diagnostics Corp, USA). Images were acquired for every 4 seconds and analyzed using IP lab software and Microsoft Excel as described². Calcium imaging with TRPV channel activators was performed on cells cultured on MatTek glass bottomed dishes on Leica Confocal Microscope and analyzed using Leica software and Microsoft Excel.

**Integrin Activation Assay:** β1 integrin activation was measured using a glutathione S-transferase (GST) fusion protein consisting of of FN III repeat 8-11 domains or 12G10 antibodies³. Briefly, cells subjected to mechanical stretch were incubated with either 5 µg/ml of GST-FN III 8-11 protein or 12G10 antibodies in PBS containing Ca²⁺ and Mg²⁺ for 30 min at 37°C, washed and lysed in SDS-sample buffer. The samples were separated on SDS-PAGE and the bound reagents were assessed on Western blots using anti GST-antibodies and HRP-conjugated secondary antibodies.

**FACS analysis:** Activated β1 integrin expression was measured with specific antibodies (12G10) using flow cytometry⁴. Briefly, cells were incubated with 12G10 antibody in FACS buffer (PBS containing 1% bovine serum albumin) for 20 min on ice and fixed in 4% paraformaldehyde for 15 min. After fixation, the cells were washed twice with FACS buffer and
incubated with PE-conjugated secondary antibodies (Vector Laboratories, USA) for 20 min on ice. The cells were then washed twice and analyzed on Guava Personal Cytometer (Guava Technologies). Isotype-matched IgG and secondary antibodies alone were used as controls.

Whole-Cell Patch Clamp Experiments: One day after bovine CE cells were transfected with TRPV4-EGFP, they were plated on gelatin-coated glass coverslips and allowed to grow for ~24 h prior to recording. Cells were recorded in the whole-cell mode using borosilicate glass pipettes (1-3 MΩ) containing (in mM): 120 CsMeSO₃, 10 EGTA, 2 MgCl₂, 10 HEPES; pH and osmolarity were adjusted with CsOH or HMeSO₃, as needed, to 7.2 (23°C) and ~300 mOsm, respectively. Cells were bathed in a solution containing (in mM): 138 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES; pH and osmolarity were adjusted with NaOH or HCl, as needed, to 7.4 (23°C) and ~310 mOsm, respectively. Cells were held at -40 mV for 3-5 min to allow for intracellular dialysis. Bath superfusion was stopped prior to initiating the recording of currents resulting from the indicated voltage protocol applied every 5 s. Immediately before use, a 4-α-PDD stock solution (2 mM in EtOH, on ice) was diluted 1:50 in bath solution under vortex and 50 µl was subsequently added to a still bath (0.5 ml) by pipette (final concentrations of 4 µM 4-α-PDD and 0.2% EtOH) and mixed by 3 gentle up/down pipette strokes. Once a clearly discernable 4-α-PDD-induced current was observed (typically after a 10-30 sec delay), the bath solution was changed to a Na⁺- and Ca²⁺-free solution (in mM: 145 N-methyl-D-glucamine-Cl, 10 HEPES, adjusted to pH 7.4 and ~310 mOsm with HCl) by restarting the superfusion. In some cases, the original bath solution was later re-introduced as a wash.
Supplementary References


Supplementary Figure Legends

**Online Figure I. 12G10 antibody detects β1 integrin activation by manganese on bovine CE cells.** Flow cytometric analysis of activated β1 integrin expression on bovine CE cells detected using the 12G10 antibody in the absence and presence of manganese (Mn^{2+}). Note that the expression of activated β1 integrin is
increased following treatment with manganese. The isotype-matched control IgG is shown as a red peak.

**Online Figure II. Mechanical strain-induced FAK activation is dependent on PI3 kinase activity.** Representative Western blot showing static mechanical strain (15%, 15 min) dependent tyrosine phosphorylation of FAK (FAK-pY397 antibody) in bovine CE cells in the absence and presence of the PI3 kinase inhibitor, LY 294002 (LY, 40 µM).

**Online Figure III. Stretch-activated (SA) calcium channels are upstream of mechanical strain-induced β1 integrin phosphorylation.** A) Relative change in cytosolic calcium in Fluo-4 loaded CE cells in response to applied static strain (15%, 3 sec, arrow) in the absence (♦) and presence (○) of gadolinium chloride (25 µM;Gd) (F/Fo = ratio of normalized Fluo-4 fluorescence intensity relative to time 0). B-C) Representative Western blots showing cyclic strain dependent binding of GST-FNIII8-11 fragment in bovine and human (HCE) CE cells (B), and β1-integrin phosphorylation in CE cells (C) in the absence and presence of gadolinium. D) Percentages of CE cells displaying GFP-AKT-PH domain translocation to the plasma membrane when subjected to 0 or 15% static stretch in the absence and presence of gadolinium chloride (p<0.022). E) Percentage of CE cells oriented 90 ± 30° degrees (aligned) relative to the direction of applied cyclic strain in the absence and presence of gadolinium chloride (p<0.0002).

**Online Figure IV. β1 integrin activation is required for cyclic strain-induced CE cell reorientation.** Immunofluorescence photomicrographs of CE cells exposed to 0 or 10% uniaxial cyclic strain (arrow indicates the direction of applied strain) in the
absence and presence of the function-blocking anti-β1 integrin antibody P5D2 and stained for vinculin (green) and stress fibers (magenta). Colocalization of vinculin and stress fibers is shown in white. Scale bar: 25 µm.

Online Figure V. siRNA knock down of TRP channels in human CE cells. A) Representative RT-PCR results confirming knockdown of TRPV4, TRPV2 and TRPC1 mRNA levels in human CE cells using specific siRNAs. B) Western blotting analysis showing that the same TRPV4 siRNA produced comparable suppression of protein expression.

Online Figure VI. TRPV4 channels are functionally expressed in CE cells. Relative changes in cytosolic calcium measured in Fluo-4 loaded bovine (a) or human (b) CE cells in response to the specific TRPV4 activator 4-α-PDD (2 µM) or the TRPV4 blocker ruthenium red (RR, 2 µM) in the absence or presence of extracellular calcium. Arrows denote the time drugs were added to cells.

Online Figure VII. Activation of TRPV4 currents by 4-α-PDD in bovine CE cells transiently transfected with TRPV4-EGFP. Conventional whole-cell patch clamp recording shows activation of an outwardly rectifying current by 4-α-PDD (4 µM) in bovine CE cells expressing TRPV4-EGFP. Traces shown were recorded 10 s (control, black line), 20 s (4-α-PDD, blue line), 50 s (NMDG, red line) or 135 s (wash, cyan line) after 4-α-PDD addition. Note that when NMDG was substituted for cations in the bathing solution, it inhibited activation of inward, but not outward, currents induced by 4-α-PDD.
Online Figure VIII. TRP siRNA treatment does not affect human CE cell morphology or viability. Phase contrast photomicrographs of human CE cells transfected with indicated siRNA shown in cell culture. Scale bar: 100 µm.

Online Figure IX. Cyclic strain did not affect CE cell proliferation or apoptosis. CE cells were exposed to cyclic strain as mentioned in materials and methods and either fixed and stained using ki67 antibody and positive cells were quantified (A) or cells were lysed and subjected to SDS-PAGE and Western blot analysis for PARP (poly (ADP-ribose) polymerase) cleavage to assess apoptosis (B).

Online Figure X. Representative full Western blots for TRPV4 expression in bovine (A) and human (B) CE cells, which correspond to the blots shown in Fig. 4C (bovine) and Online Fig.V (human), respectively.

Online Figure XI. Representative full Western blots for β1 integrin activation and actin levels corresponding to the blots shown in Fig. 2A (β1 integrin phosphorylation), Online Fig.IIIB (GST-FN-binding) and Fig.5A (binding of 12G10 antibody).

Online Figure XII. Representative full Western blots for ERK activation (phospho and total ERK1/2) in bovine CE cells, which correspond to the blots shown in Fig. 2C.

Online Figure XIII. Representative full Western blots for AKT phosphorylation (phospho and total AKT) in bovine CE cells, which correspond to the blots shown in Fig. 3D.
**Online Figure XIV.** Representative full Western blots for FAK phosphorylation (phospho and total FAK) in bovine CE cells, which correspond to the blots shown in Online Fig. II.
Online Figure I

Log fluorescence

Cell number

100

Log fluorescence

10

100

1000

10000

10

1

10

100

0

0

0

0

IgG

-Mn^{2+}

+Mn^{2+}
Online Figure II

Con Str+LY Str

FAK-pY397

FAK
Online Figure IV

Control \hspace{1.0in} \text{Strain} \hspace{1.0in} + \text{P5D2}

P5D2
A

% positive nuclei

B

proform

Cleaved fragment

Con  Str  Str+RR
Online Figure X
Online Figure XI
ERK1/2-phospho  ERK1/2- Total

Con  Str  Con  Str

42 kDa