Calcium influx through a possible coupling of cation channels impacts skeletal muscle satellite cell activation in response to mechanical stretch

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Hara M, Tabata K, Suzuki T, Do MQ, Mizunoya W, Nakamura M, Nishimura S, Tabata S, Ikeuchi Y, Sunagawa K, Anderson JE, Allen RE, Tatsumi R. Calcium influx through a possible coupling of cation channels impacts skeletal muscle satellite cell activation in response to mechanical stretch. Am J Physiol Cell Physiol 302: C1741–C1750, 2012. First published March 28, 2012; doi:10.1152/ajpcell.00068.2012.—When skeletal muscle is stretched or injured, satellite cells, resident myogenic stem cell positioned beneath the basal lamina of mature muscle fibers, are activated to enter the cell cycle. This signaling pathway is a cascade of events including calcium-calmodulin formation, nitric oxide (NO) radical production by NO synthase, matrix metalloproteinase activation, release of hepatocyte growth factor (HGF) from the extracellular matrix, and presentation of HGF to the receptor c-met, as demonstrated by assays of primary cultures and in vivo experiments. Here, we add evidence that two ion channels, the mechanosensitive cation channel (MS channel) and the long-lasting-type voltage-gated calcium ion channel (L-VGC channel), mediate the influx of extracellular calcium ions in response to cyclic stretch in satellite cell cultures. When applied to 1-h stretch cultures with individual inhibitors for MS and L-VGC channels (GsMTx-4 and nifedipine, respectively) or with a less specific inhibitor (gadolinium chloride, Gd), satellite cell activation and upstream HGF release were abolished, as revealed by bromodeoxyuridine-incorporation assays and Western blotting of conditioned media, respectively. The inhibition was dose dependent with a maximum of 0.1 μM (GsMTx-4), 10 μM (nifedipine), or 100 μM (Gd) and canceled by addition of HGF to the culture media; a potent inhibitor for transient-type VGC channels (NNC55–0396, 100 μM) did not show any significant inhibitory effect. The stretch response was also abolished when calcium-chelator EGTA (1.8 mM) was added to the medium, indicating the significance of extracellular free calcium ions in our present activation model. Finally, cation/calcium channel dependencies were further documented by calcium-imaging analyses on stretched cells; results clearly demonstrated that calcium ion influx was abolished by GsMTx-4, nifedipine, and EGTA. Therefore, these results provide an additional insight that calcium ions may flow in through L-VGC channels by possible coupling with adjacent MS channel gating that promotes the local depolarization of cell membranes to initiate the satellite cell activation cascade.

calcium ion influx; mechanosensitive channel; muscle regeneration; satellite cells; stretch-activation; voltage-gated channel

SATELLITE CELLS, a population of resident myogenic stem cells positioned between the basal lamina and the sarcolemma of postnatal skeletal muscle fibers, are normally found in a mitotically and metabolically quiescent (or near-dormant) protracted G1 state (also referred to as G0) in adult muscles. When muscle is injured, overused, or mechanically stretched, these cells are activated to enter the cell cycle, proliferate to produce large numbers of myoblast progeny, differentiate, and fuse with existing muscle fibers or form new fibers. Through this process, many myonuclei are accumulated in the tissue and are responsible for increasing protein synthesis and providing the potential to enhance muscle growth (hypertrophy and hyperplasia) and regeneration. Consequently, satellite cell activation is an initial and crucial step in the process of postnatal myogenesis (reviewed in Refs. 12, 28, 45, 50, 66) and may be triggered by mechanical perturbation of satellite cells and muscle fibers. However, the detailed mechanism is not fully elucidated.

Of all growth factors studied thus far, including fibroblast growth factors (FGFs), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor BB (PDGF-BB), transforming growth factor-βs (TGF-β1 and -2), and epidermal growth factor (EGF), hepatocyte growth factor (HGF) is the only mitogen with an established ability to stimulate quiescent satellite cells to enter the cell cycle early in primary culture assay and in vivo (1, 58; reviewed in Refs. 15, 38, 72), even though Nagata et al. (40) first demonstrated that sphingosine-1-phosphate (SIP), a bioactive sphingolipid metabolite, induces satellite cells to cycle and very recently Sassoli et al. (48) showed that SIP promotes satellite cell renewal and differentiation in damaged muscle. HGF is a heparin-binding protein localized in the extracellular domain of uninjured skeletal muscle fibers by possible association with glycosaminoglycan chains of proteoglycans, and its predominant form is the active disulfide-linked heterodimer of a 60-kDa α-chain and a 30-kDa β-chain (62). The intracellular signaling receptor for HGF is the c-met proto-oncogene; its message and protein have been found in quiescent and activated satellite cells (1, 19, 58). Thus release of HGF from its sequestration in the matrix and subsequent presentation to the receptor c-met may be a critical aspect of the activation of quiescent satellite cells. Recently, Wozniak and Anderson (73) provided a novel insight that HGF released from the matrix may induce c-met RNA expression as an immediate-early gene within 30 min in response to muscle fiber stretch and thus enhance HGF-c-met signaling in the satellite cell activation process.

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In a series of previous reports, we employed a FlexerCell system (Flexcell International, Hillsborough, NC) to apply cyclic stretch to isolated rat satellite cells and found that mechanical stretch triggers satellite cell activation by rapidly releasing HGF from its tethering in the extracellular matrix and its subsequent presentation to c-met (59, 60, 63, 65; reviewed in Ref. 66). This phenomenon is relevant to satellite cells in living muscle as revealed by an in vivo muscle-stretch model (64). The most important observation from these previous experiments was that the release of HGF was completely dependent on nitric oxide (NO) radical synthesis by NO synthases (NOS) from 1-arginine of the substrate (60, 61). Anderson (4) first pointed to production of NO as a key signal responsible for satellite cell hypertrophy and detachment from the adjacent fiber following crush injury; those results were later verified in in vitro cultures of isolated muscle fibers and their associated satellite cells (5, 6, 71–73; reviewed in Refs. 7, 8). In our subsequent studies, Yamada et al. (75, 76) demonstrated that matrix metalloproteinases (MMPs), a large family of zinc-dependent endopeptidases that collectively degrade one or more extracellular matrix constituents, specifically MMP2, mediate HGF release, possibly by shedding proteoglycan-core proteins in response to the NO radical (reviewed in Refs. 66, 68).

In an effort to understand the events upstream from NO-radical production, recent studies showed that NO-radical/MMP-mediated HGF release and the resulting activation of satellite cells are blocked if calmodulin activity is abolished by the specific inhibitors, calmidazolium, W-13, and W-12; the same report showed that the activation cascade can be turned on when calcium ionophores A23187 or ionomycin are simply added to unstretched control cultures (67). Therefore, the influx of calcium ions and their binding to calmodulin are thought to be involved in the segment of the activation pathway between sensing the mechanical stimulus and synthesis of the NO radicals, consistent with the observation that the calcium-calmodulin complex associates with constitutive NOS (cNOS; i.e., neuronal and endothelial NOS proteins) to activate NOS enzyme activity, as shown in neuronal and endothelial cells (43, 69). However, it is still unclear how quiescent satellite cells sense mechanical stimuli for transduction into an elevation of the intracellular calcium-ion concentration at the most upstream step of the satellite cell activation cascade.

Experiments in this study were designed to test the hypothesis that mechanosensitive cation channels (MS channels) are involved in the mechanotransduction system. MS channels are mechanosensing machinery proteins and are widely distributed from single-celled bacteria to animal and plant cells. A variety of experimental approaches revealed that MS channels respond to a variety of mechanical stimuli, including shear stress, gravity, osmotic pressure, and stretch, by opening nanoscale protein pores that are selectively permeable to cations (mainly calcium and sodium) entering into the cytosol down their steep electrochemical gradients (22, 42; reviewed in Refs. 3, 9, 27, 31, 33). Therefore, MS-channel gating may transduce physical stimuli into electrical signals by generating localized depolarization of the cell membranes (through changes in membrane voltage). This depolarization potentially enables the activation of voltage-gated calcium channels (VGCC channels). Indeed, calcium signaling has a central role in regulating activity of many cell types, including skeletal muscle fibers in which the long-lasting-type of VGCC channels (L-VGCC channels) in transverse tubules act as a voltage sensor in excitation-contraction coupling (44, 56, 57, 70; reviewed by Refs. 14, 21).

In the present paper, we examined the effect of a calcium chelator, EGTA, and selective inhibitors for MS channels (GsMTx-4) and VGC channels (nifedipine and NNC55–0396) with and without cyclic stretch of satellite cell primary cultures on HGF release from the matrix and satellite cell activation. We provide evidence that extracellular calcium-ion influx through a possible coupling of MS and L-VGC channels may be responsible for the mechanosensing machinery of quiescent satellite cells.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM, low-glucose type, 31600–034), normal horse serum (HS, 16050–122), antibiotic-antimycotic (15240–062), and gentamicin (15710–064) were purchased from Invitrogen (Grand Island, NY). Poly-l-lysine (P9155), bovine plasma fibronectin (F1141), protease type XIV (P5147), and 5-bromo-2′-deoxyuridine (BrDU, B5002) were obtained from Sigma (St. Louis, MO). Recombinant mouse HGF (2207-HG) was purchased from R&D Systems (Minnesota, MN). Cation-channel inhibitors, nifedipine [141–05783, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarbonylcacid dimethyl ester], gadolinium chloride (078–02661) and NNC55–0396 (2268, (1S,2S)-2-[3-(1H-benimidazol-2-yl)propyl[methyleniminio][ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-(1-methylethyl)-2-naphthalenyl cyclopropane carboxylate dihydrochloride) were obtained from Wako Pure Chemical Industries (Osaka, Japan), and GsMTx-4 (4393–s, synthetic peptide of the spider venom toxin) was from Peptide Institute (Osaka, Japan).

Fluo 3-AM [F026, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9- xanthan) phenyl]-2-(2-amino-5-methylphenoxycyethane-N,N,N,N′,N′- tetraacetic acid, pentaaacetoxymethyl ester] was obtained from Dojindo Laboratories (Kumamoto, Japan). EGTA [15224, O, O′-bis(2- aminoethylethylene glycol)-N,N,N′,N′-tetraacetic acid] was obtained from Nacalai Tesque (Kyoto, Japan). Calcium ionophore A23187 (21186) and nomiconic copolymer surfactant Pluronic F-127 (P2443) were purchased from Sigma.

The following materials were obtained for protein expression analysis: D3 mouse monoclonal anti-desmin and G34 mouse monoclonal anti-BruU antibodies from the Developmental Studies Hybridoma Bank (Iowa City, IA); goat polyclonal anti-human HGF antibody (AB-294-NA) and goat polyclonal anti-c-met antibodies (AF527) from R&D Systems; AC-15 mouse monoclonal anti-β-actin antibody (ab6276) from abcom (Cambridge, MA); horseradish peroxidase (HRP)-conjugated AffiniPure donkey Anti-goat IgG (705–036–147) from Jackson ImmunoResearch Laboratories (West Grove, PA); affinity-purified biotinylated horse-anti-mouse IgG (BA-2000) and the HRP-labeled avidin kit (PK-6100) from Vector Laboratories (Burlingame, CA); affinity-purified HRP-conjugated goat anti-mouse IgG (A-4416) and 3,3′-diaminobenzidine (DAB, D5637) from Sigma; HRP-labeled rabbit anti-goat IgG [A41331, Histidine Simple Stain Rat MAX-PO (G)] and normal rabbit serum (426051) from Nichirei BioScience (Tokyo, Japan); Amersham enhanced chemiluminescence (ECL) detection kit (PRN2106), nitrocellulose membrane (Hybond ECL, RPN2020D), and Hyperfilm ECL (28–9068) from GE healthcare (Little Chalfont, UK); MagicMark XP molecular weight standards (LC5602) from Invitrogen; and CanGetSignal immunoreaction solutions (NKB-101) from Toyobo (Osaka, Japan). For the mRNA expression analysis, an RNeasy Micro kit (74004) and QIAshredder homogenizer spin column (79654) from Qiagen (Hilden, Germany), SuperScript III reverse transcriptase (18080–044) from Invitrogen, Oligo(dT) primer (H09876) from Roche (Mannheim, Germany), Ex Taq DNA polymerase (RR001A) from Takara Bio (Osu, Japan), agarose–LE (01157) from Nacalai Tesque (Kyoto, Japan), and Gel Red (41000) from Biotium (Hayward, CA) were additionally used.
Satellite cell isolation and primary culture. Satellite cells were isolated from muscle in the hindlimb and back of 9- to 10-week-old male Sprague-Dawley rats according to Allen et al. (2) with a slight modification (65). Briefly, muscle from the upper hindlimb and back were excised, trimmed of fat and connective tissue, minced with scissors, and digested for 1 h at 37°C with 1.25 mg/ml protease type XIV. Cells were separated from muscle-fiber fragments and tissue debris by differential centrifugation and filtration through nylon cell strainers (100-µm and 40-µm mesh size) prior to a final centrifugation step at 1,500 g for 3 min, and then plated on poly-l-lysine and fibronectin-coated dishes in DMEM containing 10% horse serum (HS), 1% antibiotic-antimycotic mixture, and 0.5% gentamicin (DMEM-10% HS, pH 7.2). Cultures were maintained in a humidified atmosphere of 5% CO2 at 37°C. In addition, companion satellite cell cultures, prepared at the same time, were immunostained for the presence of desmin at 30 h after plating, using a D2 monoclonal anti-desmin antibody, biotinylated anti-mouse IgG antibody, and HRP-labeled avidin, to determine the percentage of myogenic cells present; cultures with less than 95% DAB-positive cells were not used for experiments.

Satellite cell cultures on Bioflex-Amino silicone-bottom plates (BF-3001A, Flexcell International) were treated with one of the cation-channel inhibitors (GsMTx-4, nifedipine, gadolinium chloride, or NNC55–0396) or a highly selective calcium chelator EGTA (1.8 mM) for 30 min in DMEM-10% HS. GsMTx-4, nifedipine, and NNC55–0396 are highly selective peptide/chemical inhibitors, and are therefore intensely useful for the study of cellular excitability and intracellular calcium-ion signaling and for characterizing effects of therapeutically beneficial drugs. Although detailed inhibitory mechanisms of these compounds still remain unclear, GsMTx-4 selectively inhibits MS-channel activity when applied to the extracellular face of the cell membrane. It does this by increasing the membrane tension required for activation, suggesting that GsMTx-4 acts as a gating modifier (54).

By comparison, nifedipine binds directly to inactive L-VGC channels, which stabilizes their inactive conformation. NNC55–0396 may bind to transmembrane or intracellular domains of the channels to exert their inhibitory effects specifically on transient-type VGC channels (T-VGC channels) (29; reviewed in Ref. 53). Cells were then subjected to a cyclic-stretch environment [optimized previously at 25% stretch at 12-s intervals, as originally optimized in Tatsumi et al. (59; modified with permission from Elsevier) and shown in A again] in the presence (bar d) or absence (bar e) of 1.8 mM EGTA in DMEM-10% normal horse serum (pH 7.2), followed by an assay to detect activation using bromodeoxyuridine (BrdU) at 48-h postplating. This experiment also included the following control cultures: bar a, unstretched culture; bar h, 2-h stretch culture (equivalent to our regular period of stretch, originally described in Ref. 59); bar b, stretched culture receiving 1.8 mM EGTA for 24–48 h postplating; bar f, positive control culture supplemented with 2.5 ng/ml recombinant mouse hepatocyte growth factor (HGF) for 24–48 h; bar g, HGF culture receiving 1.8 mM EGTA for 24–48 h; bars i and h, control cultures with 1.8 mM EGTA for 24–25 h and 24–48 h, respectively. Bars depict means and SE for 4 cultures per treatment. **Treatment mean was significantly different from the mean of positive control cultures (bar f) at P < 0.01. NS, no significant difference (P > 0.05) in the activation index.

SDS-sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, and 62.5 mM Tris-HCl buffer, pH 6.8) and stored at −80°C until use.

In vitro activation assay. Cultures were pulse-labeled with 10 µM BrdU in DMEM-10% HS for the final 2 h from 46 to 48 h postplating, followed by immunocytochemistry for detection of BrdU using a G3G4 anti-BrdU monoclonal antibody [1:100 dilution in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)] and a HRP-conjugated anti-mouse IgG antibody (1:500 dilution) according to Tatsumi et al. (58). The percentage of BrdU-labeled cells was used as an indicator of activation and entry into the cell cycle.

Immunoblotting and ECL. Mouse recombinant HGF and conditioned media from 1-h stretch cultures with or without a cation-channel inhibitor or EGTA were applied to 10% polyacrylamide gels for electrophoresis under reducing conditions (35) and transferred to nitrocellulose membranes (58). The blots were blocked with 10% powdered milk in 0.1% polyethylene sorbitan monolaurate (Tween 20)-Tris buffered saline (TTBS) prior to incubation with goat polyclonal anti-HGF antibody (1:500 dilution in 1% powdered milk-TTBS additionally containing 0.05% sodium azide) overnight, and subse-
quently treated with HRP-labeled donkey anti-goat IgG antibody at 1:10,000 dilution for 1 h, followed by ECL detection on Amersham Hyperfilm ECL X-ray film. Internal β-actin in cell lysates was visualized with mouse monoclonal anti-β-actin antibody (1:1,000 dilution in CanGetSignal solution 1, overnight) and with biotinylated horse anti-mouse IgG (1:5,000 dilution in CanGetSignal solution 2, for 1 h) and HRP-labeled avidin (1:500 dilution in TTBS, for 30 min).

Calcium imaging. Satellite cell cultures on a silicon membrane (11–004-006, Flexcell International) were loaded with 5 μM fluo 3-AM in 0.2% Pluronic F-127 for 1 h in the dark at 24-h postplating, and then rinsed with PBS and left to equilibrate in DMEM-10% HS (pH 7.2) for 60 min at room temperature. Cultures were then exposed to two cycles of stretch in the presence of 1.8 mM EGTA or a cation-channel inhibitor (0.1 μM GsMTx-4 or 10 μM nifedipine, each added another 30 min prior to the stretch stimulation) under the StageFlexer Jr. system equipped with a 18.5-mm cylindrical loading post; positive-control cells received calcium ionophore A23187 (3 μM) in the media, according to Tatsumi et al. (67). Calcium images were monitored at intervals of 3 s under a Nikon Eclipse 80i fluorescence microscope system (Tokyo, Japan) with an excitation wavelength of 488 nm and a maximum emission wavelength of 526 nm. Fluorescence intensity was assigned from blue to red in color-coded images by the installed Nikon computer software. After imaging, cells were fixed for 10 min in cold methanol containing 0.1% H2O2 and blocked with 5% normal rabbit serum containing 0.6% H2O2 in PBS for 20 min. Blocking was followed by immunostaining for the presence of c-met using R&D Systems goat polyclonal primary antibody (1:100 dilution in PBS containing 5% normal rabbit serum) and Histofine Simple Stain HRP-labeled anti-goat IgG secondary antibody to determine if the calcium-imaged cells were myogenic.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was purified from cultured satellite cells using an RNaseasy Micro kit according to the manufacturer’s recommendation. cDNA was synthesized from total RNA by a reverse-transcriptase SuperScript III kit according to the manufacturer’s recommendation. cDNA was purified from cultured satellite cells using an RNeasy Micro kit (Qiagen, Valencia, CA) and converted into cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The template cDNA was diluted 1:10 and 1:100 in sterile water before use in the PCR reaction. The intron-spanning RT-PCR products were analyzed by agarose gel electrophoresis with 0.01% Gel Red reagent.

Statistical analysis. ANOVA procedures were employed to analyze experimental results using the general linear model procedures of SRISTAT2 for Windows software (Social Survey Research Information, Tokyo, Japan). Least-squares means for each treatment were separated, based on least significant differences. Data were represented as means and SE for four cultures per treatment, and statistically significant differences from the mean of control cultures at P < 0.05 and P < 0.01 were indicated. Each experiment was repeated two or three times to verify the reproducibility of results, and, in most cases, one rat was used for each experiment.

RESULTS

It is now clear that, in attempting to construct a cascade pathway that translates mechanical perturbation of quiescent satellite cells or muscle fibers into an activation signal, calcium-calmodulin formation is required for ROS activation that produces the NO radical from l-arginine and which subsequently releases HGF from extracellular tethering (for review see Ref. 66). Therefore, the purpose of this study was to clarify a mechanism that responds to mechanical perturbation of stretch by increasing intracellular calcium-ion concentrations in satellite cell cultures.

The first experiment was designed to examine the significance of extracellular calcium-ion influx in our stretch-activation model of satellite cells in vitro (Fig. 1). Satellite cells were prepared from adult rat skeletal muscles and applied to cyclic-stretch cultures (25% stretch at 12-s intervals as shown in Fig. 1A) for 1 h beginning at 24-h postplating in DMEM-10% HS (pH 7.2) with or without 1.8 mM EGTA (calcium chelator that does not enter into the cytosol), followed by the BrdU-incorporation assay at 48-h postplating (Fig. 1B). This concentration of EGTA decreases free calcium ion concentrations in the culture media (1.8 mM) to about 10 μM and without diminishing cell viability, even through the last 24-h period, as monitored by effects on cell density and BrdU incorporation at the 48-h time point of the assay (bar e and bar i); therefore EGTA was applied to the pilot experiments to determine the dependence of activation on the level of extracellular calcium.

Results clearly showed that EGTA treatment abolished stretch-activation (bar d) down to a level equivalent to that in un-stretched control cultures without (bar a) and with 1.8 mM EGTA (bar h). The 1-h cyclic-stretch cultures (bar c) showed activating activity comparable to cultures receiving 2.5 ng/ml HGF for 24 h from 24-h postplating in the absence or presence of 1.8 mM EGTA (bars f and g, respectively), thereby serving as important controls for other treatment groups in this assay. Together these results provide evidence that extracellular calcium ions are essential in stretch-induced activation of satellite cells in primary culture.

This issue was further examined by studying the effect of cation-channel inhibitors on stretch-activation of satellite cells (Fig. 2A). In this experiment, stretch was applied to satellite cell cultures for 1 h beginning 24-h postplating in the presence of an increasing concentration of one of the selective inhibitors for MS channels, L-VGC channels and T-type VGC channels (0.01–1 μM GsMTx-4, 0.1–10 μM nifedipine, and 1–100 μM NNC55–0396, respectively) or a less specific classic inhibitor for MS channels (0.1–100 μM gadolinium chloride), and then subsequently assayed for satellite cell activation at 48-h post-plating as described previously. The T-VGC channel inhibitor NNC55–0396 (open square) did not show a significant effect on activation in the range examined here or in another report (29, 55). Exposures to one of the other three cation-channel inhibitors all abolished stretch-stimulated activation in a dose-dependent manner. GsMTx-4 (closed circle) was the most powerful of the three inhibitors, with blocking at an optimal concentration of 0.1 μM vs. 10 μM for nifedipine (closed square) and 100 μM for Gd (open circle). None of these cation-channel inhibitors diminished cell viability, again as
monitored at 48 h by cell density and compared with the BrdU labeling of control cultures receiving cation-channel inhibitors (bars h–j), and by results showing that addition of HGF (2.5 ng/ml) to individual-inhibitor cultures (bars d–g) stimulated satellite cell activation to a level equivalent to HGF (bar c) and stretch (bar b). These observations, therefore, indicated that both MS and L-VGC channels are essential in the stretch activation of satellite cells and may be mediating the influx of extracellular calcium ions in response to mechanical stretch. Also, as shown in Fig. 2B, the implication of MS and L-VGC channels in the activation cascade was further emphasized by Western blot detection of the 60-kDa α-chain of HGF in conditioned media (serum-free DMEM, pH 7.2); media were collected from the 1-h stretch cultures that had received the optimal dose of GsMTx-4 (0.1 μM), nifedipine (10 μM), or EGTA (1.8 mM) and assayed for HGF. Results qualitatively demonstrated that the cyclic stretch culture stimulates HGF release (consistent with our original results) (lane m), and even in the presence of stretch, GsMTx-4 (lane n), nifedipine (lane o), and EGTA (lane p) diminish release of HGF with an apparently similar potency. This indicates that HGF release from extracellular matrix depends on the upstream MS- and L-VGC-channel activities. In fact, the messages of α1S and α1C subunits of L-VGC channels were detected by RT-PCR analysis for satellite cells at 24-h postplating, which is the time at which the stretch treatment was applied in the present and our previous experiments (65, 75–77) (Fig. 3A). Because L-VGC-channel proteins are now classified into CaV1.1, CaV1.2, CaV1.3, and CaV1.4 according to their corresponding α1-subunits, α1S, α1C, α1D, and δIF (34, 36), our PCR results indicate that CaV1.1 and CaV1.2 may exist in quiescent satellite cells and be responsible for calcium ion influx by coupling with MS-channel gating that senses mechanical perturbation of satellite cells.

Finally in a series of extracellular calcium ion dependency experiments, calcium ion influx was documented by analysis of calcium imaging studies for two-cycle stretched satellite cells at 24-h postplating in the presence or absence of cation-channel inhibitors (Fig. 4). Calcium ionophore treatment (3 μM A23187, without stretch or treatment with channel inhibitors), stimulated increase in intracellular calcium ion concentrations, consistent with our previous result (67) and therefore served as a positive control within this assay (row A). Cyclic stretch also
mechanical stretch in skeletal muscle satellite cells in culture. In mediating the extracellular calcium ion influx in response to direct evidence that both MS and L-VGC channels are essential for a considerable time. It is well documented that one of the skeletal muscle repair and hypertrophy has been appreciated for a considerable time. It is well documented that one of the earliest events, triggering the activation of quiescent satellite cells is initiated by mechanical or chemical stimuli and enables satellite cells to migrate and enter the cell proliferation cycle. Bischoff (11) was among the first to explore the bio-

discussion

The importance of satellite cell proliferation activity in skeletal muscle repair and hypertrophy has been appreciated for a considerable time. It is well documented that one of the earliest events, triggering the activation of quiescent satellite cells, is initiated by mechanical or chemical stimuli and enables satellite cells to migrate and enter the cell proliferation cycle. Bischoff (11) was among the first to explore the bio-

chemical link between satellite cell activation and mechanical perturbation of muscle tissue. His classical experiments used a crushed-muscle extract, which he found could stimulate satellite cells associated with isolated fibers to synthesize DNA and divide. Johnson and Allen (30) later reported that the same crushed-muscle extract could stimulate adult rat satellite cells in culture to enter the cell cycle earlier than adult rat cultures in control medium. Shortening of the lag phase observed with freshly isolated adult satellite cells has since been used as an assay for satellite cell activation (1, 2, 58), and this assay was employed in experiments that first demonstrated that HGF was unique in its ability to activate satellite cell division in vitro (1). In subsequent experiments, the active agent in crushed-muscle extract was shown to be HGF, which was present in uninjured muscle and located primarily in the extracellular matrix (58, 62). In further experiments, Tatsumi et al. (59 – 61, 64) showed that satellite cells were stimulated to enter the cell cycle when subjected to mechanical stretch in primary culture and living muscle. We also demonstrated that satellite cell activation was due to rapid release of HGF from its extracellular tethering and the subsequent presentation to the receptor c-met, and later verified that HGF release is dependent on calcium-calmodulin formation and the downstream NO radical-mediated activation of matrix metalloproteinases (MMPs) (47, 67, 75, 76).

These observations suggest that the stretch-activation of satellite cells is a cascade of sequential events including (in order) an influx of calcium ions and their binding to calmodulin, NO radical production by cNOS, MMP activation, HGF release from the matrix, and presentation of HGF to the signaling receptor c-met (see a review of Ref. 66). In this model, a complex of HGF and proteoglycan extracellular domain would be shed from the matrix and presented to the receptor c-met. In fact, HGF associated with heparan sulfate moieties has a greater affinity for c-met relative to HGF alone, and therefore, has enhanced HGF signaling activity (20). Such a complex has been detected in PBS extracts from crushed muscle and intact muscle incubated in the NO donor, sodium nitroprusside (SNP) (Allen et al., unpublished data), supporting the above insight. In related experiments by Wozniak and Anderson (73), released HGF may induce c-met expression as an immediate-early gene, and thus also enhance HGF-c-met signaling in the satellite cell activation process. They additionally observed that the level of satellite cell activation was increased in unstretched fibers from mdx and nNOS-knockout mice and in unstretched normal fibers by treatment with a competitive NOS-inhibitor, \( \text{N}^2 \)-nitro-l-arginine methyl ester (l-NAME), suggesting that a NO-independent HGF/c-met signaling pathway, and possibly other signaling pathways, control activation in muscle deficient in dystrophin and nNOS (73).

It has not been determined how quiescent satellite cells sense mechanical stimuli to generate biochemical signals that elevate intracellular calcium ion concentrations; therefore, in the present work, the involvement of MS and VGC channels in the mechanotransduction system was examined. When satellite cell cultures were incubated with the MS-channel inhibitor GsMTx-4, the L-VGC channel inhibitor nifedipine, or a less specific inhibitor, gadolinium chloride, stretch-induced HGF release from the matrix and the resulting cell activation were abolished completely in a dose-dependent manner. This was revealed by Western blotting of conditioned media and a BrdU-incorporation assay (Fig. 2). By contrast, the T-VGC-
channel inhibitor NNC55–0396 did not have inhibitory effects on the activation index. Addition of the calcium-chelator EGTA to culture media also inhibited the stretch-activation response and HGF release, indicating the significance of extracellular calcium ions in the activation cascade (Figs. 1 and 2B). This means that free calcium ions stored in mitochondria may now be excluded from a role in this activation pathway. Calcium-imaging analyses verified these results, and therefore provide direct evidence that both MS and L-VGC channels are essential in calcium ion influx in response to mechanical stretch and that calcium ions may flow in through L-VGC channels (Fig. 4). A possible explanation for these observations can be addressed by a possible model of MS/L-VGC-channel coupling, in which the MS channel responds to mechanical stimuli by gating cations; this would result in local depolarization of the plasma membrane, a signal that is then quickly sensed by L-VGC channels (Ca_{v1.1}, Ca_{v1.2}) that allow a significant amount of calcium ions to permeate the cell and lead to calcium-calmodulin formation (see Fig. 5B for details). In the nifedipine treatment, in which MS channels are expected to retain biological activity to gate-selective cations, significant calcium influx was not observed, suggesting that MS channels concerned in the activation pathway allow entry of cations other than calcium ions and/or gate an undetectable level of calcium ions that is not sufficient to activate calmodulin-NOS signaling directly but is enough to depolarize the cell membrane.

Considering the possible implication of MS channels in the mechanosensing mechanism of satellite cell activation, it is worth noting the valuable study by Formigli et al. (25), which provided the first experimental evidence that the transient receptor potential canonical channel 1 (TRPC1) represents an essential component of stretch-activated cation channel signaling in the mouse C2C12 myoblast cell line, as assayed by whole cell patch-clamp and atomic force microscopic pulling. That study indicated that TRPC1 plays a crucial role in mechanotransduction during regulation of the early phases of myoblast differentiation. TRPCs (TRPC1–7) are a family of cation channels that assemble as homo- or heterotetramers to form voltage-independent, nonselective cation- (Na^{+} and Ca^{2+}) permeable pores (16, 46). Although numerous studies have shown

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**Fig. 4.** Effect of cation-channel inhibitors and EGTA on the increase in intracellular calcium ion concentration in response to stretch. Calcium-imaging analysis was conducted under the StageFlexer Jr. system using fluo 3-loaded satellite cells that were treated with individual cation-channel inhibitors (0.1 μM GsMTx-4, 10 μM nifedipine) for 30 min just prior to adding 2 cycles of stretch at 24-h postplating. Row + EGTA, cells were incubated with 1.8 mM EGTA in DMEM-10% HS just prior to the addition of the stretch. Companion cells were treated with 3 μM A23187 (a calcium ionophore) and served as a positive control (row A23187) (67). Fluorescence intensity was assigned from blue to red in color-coded images; the normal resting free calcium-ion level was represented by blue, and gradual increases were demonstrated by a change to yellow and then red. After calcium imaging, cells were visualized by immunostaining to confirm the presence of c-met, a marker molecule for myogenic cells in our primary cell cultures (DAB-stained images not shown).

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**Fig. 5.** A possible mechanism of satellite cell activation in response to mechanical stimuli. A: Ca-CaM, calcium-calmodulin complex; L-Arg, L-arginine; NOS, NO synthase; MMP2, matrix metalloproteinase 2. [Reproduced from Tatsumi and Allen (66) with some modifications with permission from John Wiley and Sons. Copyright 2008.] B: Calcium ion influx mechanism was examined in the present study and the model was schematically presented. Steps 1 and 2: cations stream into the cell through mechanosensitive cation channels (MS channel) to induce local depolarization of the cell membrane (changes in membrane potential); step 3: depolarization promotes gating of adjacent L-type voltage-gated calcium-ion channels (L-VGC channel), and the result is calcium ion influx that promotes formation of the Ca-CaM complex that initiates NOS activity.
that TRPC1 may be store-operated calcium-ion entry channels (SOCs) in various cell types (23, 74), there is also evidence that this channel is able to translate membrane stretch into cation currents across the plasma membrane (17, 37, 52). TRPC5 and -6 were also shown to be stretch-gated and GsMTx-4-sensitive cation channels and TRPC6 plays an essential role of mechanosensing in smooth muscle cells (26, 51; reviewed in Ref. 13); therefore, at least at this time, TRPC1, -5, and -6 may be reasonable candidates for the MS channel in the satellite cell activation pathway concerned here. Our rat satellite cell cultures expressed TRPC1 message, as revealed by RT-PCR, while TRPC6 was not detected at 24-h postplating (Fig. 3B), which is the time point at which the stretch treatment was applied in the present and our previous experiments (65, 75–77); this provides a supportive background for the implication of TRPC1 in the above model of stretch-activation. TRPA1 (18), TRPV4 (10), and TRPM7 (41) are also reported to be stretch-sensitive channels and therefore cannot be excluded from the list of candidate MS-channels at the present time, and the field awaits a study to examine their GsMTx 4 sensitivity and expression in quiescent satellite cells.

Another important observation by Formigli et al. (25) is that TRPC1 activity and stretch-induced cation influx in C2C12 myoblasts were modulated by sphingosine-1-phosphate (S1P), an important bioactive sphingolipid-metabolite that mainly acts through G protein-coupled receptors present on mammalian cells, and thereby regulates numerous cell functions including cell proliferation, differentiation, and apoptosis (78). In line with this, TRPC1 expression is reported to be significantly upregulated during myogenesis, especially in the presence of S1P, supporting the physiological relevance of the sphingosine-S1P-TRPC1 axis in regulating C2C12 cell growth and differentiation (24, 25, 39). Notably, Nagata et al. (40) clearly showed that S1P can induce satellite cells to enter the cell cycle, whereas inhibiting the sphingolipid signaling cascade (which generates S1P mainly from sphingomyelin and sphingosine by N-SMase, ceramidase and sphingosine kinase) reduces the number of satellite cells able to divide in response to mitogen stimulation. A similar observation was reported by Sassoli et al. (48); S1P promotes satellite cell renewal and differentiation in damaged muscle. Although the S1P-mediated activation pathway is not well elucidated at the present time, considering the regulatory role of S1P in TRPC1 gating in C2C12 myoblasts (25), one possible hypothesis may be that S1P associates with TRPC1 (and/or TRPC5, 6) directly or indirectly and can trigger satellite cell activation even without stretch stimuli. Alternatively, S1P could drastically decrease the threshold intensity of stretch that is required for TRPC gating; this would be followed by L-VGC-channel activation and lead to influx of extracellular calcium ions and their subsequent presentation to calmodulin to form calcium-calmodulin complexes as the cNOS activator. In line with this, it is worth noting the excellent study by Sbrana et al. (49), which showed that S1P activates the cytoskeleton contraction that in turn stretches the cell membrane and increases its stiffness; therefore such changes may improve the mechanosensitivity of unstretched C2C12 myoblasts. This S1P-TRPC scenario was recently addressed by some focused studies on TRPC functions related to sphingolipid biology and therefore does not exclude other possibilities including that the S1P pathway is not dependent on either TRPC signaling or the activation cascade concerned here.

In summary, the present experiments bridge the gap between mechanical stretch stimuli and the increase in intracellular calcium-ion concentration by demonstrating that inhibition of MS channels and L-VGC channels in vitro, prior to mechanical stretch, prevents the influx of extracellular calcium ions, HGF release from the matrix, and reentry of adult satellite cells into the cell cycle. The physiological significance of the MS channel gating-initiated pathway described using cultured satellite cells remains to be verified in muscle fibers, as does the crucial role of calcium-calmodulin formation in the NO-radical production by cNOS that was described recently in isolated satellite cells in culture (67). Nonetheless, the current report demonstrated that in isolated satellite cells, a functional coupling of MS-channel and L-VGC-channel gating plays the central role in mechanosensing machinery that instigates the activation of satellite cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


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