The LRRC8/VRAC anion channel facilitates myogenic differentiation of murine myoblasts by promoting membrane hyperpolarization

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Skeletal muscle myoblast differentiation involves elaborate signaling networks, including the activity of various ion channels and transporters. Several K⁺ and Ca²⁺ channels have been shown to affect myogenesis, but little is known about roles of Cl⁻ channels in the associated processes. Here, we report that the leucine-rich repeat containing family 8 (LRRC8)/volume-regulated anion channel (VRAC) promotes mouse myoblast differentiation. All LRRC8 subunits of heteromeric VRAC were expressed during myotube formation of murine C2C12 myoblasts. Pharmacological VRAC inhibitors, siRNA-mediated knockdown of the essential VRAC subunit LRRC8A, or VRAC activity-suppressing overexpression of LRRC8A effectively reduced the expression of the myogenic transcription factor myogenin and suppressed myoblast fusion while not affecting myoblast proliferation. We found that inhibiting VRAC impairs plasma membrane hyperpolarization early during differentiation. At later times (more than 6 h after inducing differentiation), VRAC inhibition no longer suppressed myoblast differentiation, suggesting that VRAC acts upstream of K⁺ channel activation. Consequently, VRAC inhibition prevented the increase of intracellular steady-state Ca²⁺ levels that normally occurs during myogenesis. Our results may explain the mechanism for the thinning of skeletal muscle bundles observed in LRRC8A-deficient mice and highlight the importance of the LRRC8/VRAC anion channel in cell differentiation.

Skeletal muscle formation includes the proliferation, differentiation, and fusion of myoblasts into multinucleated myotubes (1, 2). The commitment to terminal differentiation of myoblasts involves a complex system of regulatory signaling pathways (3). This includes the action of ion transport proteins responsible for the hyperpolarization of the plasma membrane (4–8) and an increase of resting cytosolic Ca²⁺ and oscillatory signaling (9–12). The sequential activation of two distinct K⁺ channels, the ether-a-go-go K,10.1 (4) and the inward rectifier Kir2.1 (6), was shown to cause myoblast hyperpolarization from about −10 mV to about −80 mV at the acquisition of fusion-competency. This decrease in turn triggers the activation of T-type voltage-gated Ca²⁺ channels, resulting in an increase in intracellular Ca²⁺ concentration necessary for the differentiation of myoblasts into myotubes (3, 11, 13). Several further K⁺ and Ca²⁺ channels were implicated in myoblast differentiation, including K,7,4 (14, 15), TASK2 and TREK1 (16), IP,3,R1 (17), and store-operated Ca²⁺ channels (18–21). In contrast, little is known about the role played by anion channels in skeletal myogenesis. However, the thinned muscle bundles observed in LRRC8A-deficient (Lrrc8a−/−) mice implicate volume-regulated LRRC8 anion channels in muscle formation (22).

The volume-regulated anion channel (VRAC) is formed by hexameric LRRC8 heteromers (23–25). LRRC8A is the only obligatory subunit and requires conjugation with at least one of the other LRRC8 family members (LRRC8B-E) to form functional plasma membrane channels (24). These channels are ubiquitously expressed in varying subunit configurations in vertebrate cells and mediate the flux of Cl⁻ and organic osmolytes upon activation (25–29). VRAC opens upon osmotic cell swelling by an unknown mechanism. Importantly, it can also be activated under isovolumetric conditions by various signaling pathways (29–32). Besides its role in regulatory volume decrease upon osmotic cell swelling, VRAC has been implicated in various further physiological processes related to cell volume regulation (29). In addition, its impact on membrane potential has been shown to be involved in exocytic insulin release (33, 34) and through its conductance of larger osmolytes such as neurotransmitters it is involved in cell–cell communications such as between astrocytes and neurons (35, 36). The overall physiological importance of LRRC8 channels is demonstrated by the severe phenotypes of Lrrc8a−/− mice deficient in the essential VRAC subunit (22). They exhibit prenatal and postnatal lethality, growth retardation, curly hair, and defective developments in many organs. Notably, mice lacking LRRC8A appear normal at birth but display significantly thinned skeletal muscle bundles at later ages (22); however, the underlying

2 The abbreviations used are: VRAC, volume-regulated anion channel; ANOVA, analysis of variance; CBX, carbeneoxolone; DCPB, 4-(2-buty1-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxybutyric acid; DiBAC4(3), bis-(1, 3-dibarbituric acid)-trimethine oxonol; LRRC8, leucine-rich repeat containing 8; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; RFP, red fluorescent protein.
mechanism is unknown. Recently, two groups independently showed that LRRC8A-dependent VRAC is involved in membrane depolarization and activation of voltage-gated Ca\(^{2+}\)/H\(^{+}\) channel–mediated intracellular Ca\(^{2+}\)/H\(^{+}\)signaling in pancreatic β-cells (33, 34), processes which are also known to be important during myoblast differentiation and fusion (11, 37).

In this study, we have investigated the role of VRAC in skeletal myogenesis using mouse C2C12 myoblasts. Our results indicate that VRAC contributes to hyperpolarization and intracellular Ca\(^{2+}\) signals, thereby promoting myoblast differentiation.

**Results**

**Pharmacological inhibition of VRAC impairs C2C12 myoblast differentiation and fusion**

To investigate a putative role of VRAC in myogenesis, we first examined the effects of various pharmacological VRAC inhibitors on myoblast fusion: carbenoxolone (CBX), an inhibitor of VRAC, pannexins, and gap junction–forming connexins (38); 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), a more general Cl\(^{-}\) channel inhibitor (30); and 4-(2-butyl-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxybutyric acid (DCPIB), a VRAC antagonist with high sensitivity at micromolar concentrations (39). As a control, we included niflumic acid (NFA), a potent inhibitor of Ca\(^{2+}\)-activated Cl\(^{-}\) channels with medium to low sensitivity for VRAC, which at the used concentrations may only partly inhibit VRAC (30, 40, 41). We induced C2C12 cells to differentiate by reducing the serum in the growth medium from 10% FBS to 2% horse serum for 4 days in the presence of drugs at various concentrations. The VRAC inhibitors CBX, NPPB, and DCPIB significantly reduced C2C12 myoblast differentiation and fusion in a dose-dependent manner, whereas the Ca\(^{2+}\)-activated Cl\(^{-}\) channel inhibitor NFA did not show any effect (Fig. 1).

**LRRC8A is dispensable for myoblast proliferation but required for normal differentiation**

Because the pharmacological data suggest a role for VRAC in myoblast differentiation/fusion, we next aimed at testing its involvement in this process on a molecular biological level. To this end, we first determined the expression of the VRAC subunits during myotube formation. Western blot analysis revealed that undifferentiated C2C12 myoblasts expressed all five LRRC8 paralogues (Fig. 2, A and B). The amounts of LRRC8A and LRRC8D did not change during the first 4 days of cell differentiation. LRRC8B levels showed the tendency to decrease, whereas LRRC8E levels were strongly reduced already after 2 days of myoblast exposure to differentiation medium (Fig. 2A). LRRC8C protein seemed to decrease as well (Fig. 2B), but because the immunodetection was not unambiguous, we performed quantitative PCR, which revealed a significant decrease in Lrrc8c mRNA levels after 2 days of cell differentiation (Fig. 2C).

Next, we silenced the expression of the essential VRAC subunit LRRC8A with two individual siRNAs in C2C12 cells, using a scrambled siRNA as control. Western blotting confirmed a robust knockdown of LRRC8A protein, with siRNA2 being more efficient than siRNA1 (Fig. 3A). Notably, siRNA-mediated LRRC8A knockdown did not affect proliferation of C2C12 cells, as shown by the unaffected dehydrogenase activity (Fig. 3B). Hence, we could assess the effect of LRRC8A knockdown on the differentiation and formation of myotubes. Starting 1 day after siRNA transfection, C2C12 cells were exposed to differentiation medium for 3 days. At the start of differentiation induction, the amount of LRRC8A protein was reduced by ~40 and ~70% in cells treated with siRNA1 and siRNA2, respectively, and decreased further during the observed time of differentiation (Fig. 4, A and B). Knockdown of LRRC8A significantly reduced the expression of myogenin, an essential myogenic transcription factor (Fig.
and of myosin, another marker of myoblast terminal differentiation (Fig. 4, A and D and Fig. 5) compared with scrambled siRNA. Furthermore, myoblast fusion was drastically diminished by LRRC8A silencing (Fig. 5). Scrambled control for the siRNA did not affect C2C12 differentiation or fusion (data not shown) and the inhibitory effects of siRNA1 and siRNA2 on myoblast differentiation and fusion correlated with their knockdown efficiencies. Collectively, these results suggest that LRRC8A is dispensable for myoblast proliferation but critically involved in myogenic commitment.

**VRAC activity promotes myoblast differentiation**

To further assess the role of VRAC during myogenesis, we overexpressed LRRC8A in C2C12 cells. Overexpression of LRRC8A alone, without another LRRC8, has previously been shown to suppress endogenous VRAC currents (23, 24). C2C12 myoblasts were transfected with plasmid DNA encoding LRRC8A fused to GFP (LRRC8A-GFP) or GFP alone. The next day, when expression of LRRC8A-GFP was already observable (Fig. 6A), the cells were induced to differentiate by serum withdrawal. 2 days later, more than 20% of GFP-expressing control cells were positive for the differentiation marker myosin, whereas this ratio was significantly lower with only ~7% myosin-positive cells among the LRRC8A-GFP-expressing cells (Fig. 6A). This inhibition of C2C12 myoblast differentiation by LRRC8A overexpression corroborates the notion that the role for LRRC8A indeed lies in its requirement for VRAC activity. Consistently, DCPIB significantly reduced the myogenin mRNA (Myog) expression after 3 days of cell differentiation (Fig. 6B), in agreement with DCPIB impairing myoblast fusion (Fig. 1).
VRAC is required for myoblast hyperpolarization and subsequent increased \([\text{Ca}^{2+}]_i\).

Upon induction of differentiation, myoblasts sequentially hyperpolarize because of the activation of two \(K^+\) channels, \(\text{ether-à-go-go}\) (4) and Kir2.1 (5, 6). This hyperpolarization is completed within the first 6 h of differentiation (7, 42). It induces a small, but sustained, inward \(\text{Ca}^{2+}\) current, sufficient to cause a detectable steady-state increase in intracel-
The thinned skeletal muscle bundles displayed by Lrrc8a−/− mice (22) suggest that lack of LRRCA8 may lead to dysfunction of myoblast proliferation, differentiation, or fusion into multinucleated myotubes. Here, we show that consistent with a previous report (44), inhibition of the LRRCA8-containing anion channel VRAC does not impair the proliferation of C2C12 myoblasts. But we find that indeed VRAC inhibition does impair the differentiation and fusion of C2C12 cells. A role for LRRCA8 in adipocyte differentiation was reported previously (45). In that context, LRRCA8 was proposed to function independent of VRAC activity by regulating insulin-Pi3K-Akt2-GLUT4 signaling through a physical interaction with the Cav1-IRS1-IR inhibitor GRB2 (45). We find that not only knocking down LRRCA8, but also pharmacological VRAC inhibitors and even VRAC current-suppressing overexpression of LRRCA8, impair myoblast differentiation and fusion. This demonstrates that not only the presence of LRRCA8, but also indeed the activity of VRAC plays a crucial role during myogenesis.

The molecular mechanism of VRAC activation is still unknown. Although experimentally this channel is often activated by osmotic cell swelling, various pathways seem to exist that can open VRAC under isotonic conditions (29–32). These include the involvement of signaling by integrins (46) and RhoA (47), which also play important roles in myogenesis (3). The pathways leading to the activation of VRAC during myoblast differentiation remain to be explored.

**VRAC promotes myoblast hyperpolarization**

When human myoblasts are induced to differentiate, the activation of an *ether-a`-go-go* K⁺ channel rapidly hyperpolarizes myoblasts from −8 mV to approximately −35 mV (4, 5), coinciding with a cell-cycle arrest (48). Shortly thereafter, the resting membrane potential of myoblasts drops further to approximately −75 mV, because of the activation of the lular Ca²⁺ ([Ca²⁺]) (11, 13). To investigate whether VRAC influences the membrane potential during myogenic differentiation, we first established a protocol using the potentiometric fluorescent probe DiBAC₄(3) (7, 43) to measure the plasma membrane potential of differentiating myoblasts (Fig. 7A). After 6–8 h of differentiation, C2C12 cells possessed the normal average resting membrane potential of −80 mV in the presence of vehicle (0.1% DMSO) but only −50 mV and −47 mV in the presence of 20 μM DCPiB and 100 μM NPPB, respectively (Fig. 7B). Electrophysiological recordings of C2C12 myoblasts after 20–24 h of differentiation showed a resting membrane potential of −75 mV (Fig. 7C). However, in the presence of 100 μM CBX, the resting membrane potential was found to be −34 mV. These data suggest that VRAC inhibition impaired the normal hyperpolarization.

We next examined whether this impinges on the changes of resting cytosolic Ca²⁺ concentration [Ca²⁺]i, during myoblast differentiation. Using the ratiometric Ca²⁺-sensitive fluorescence dye Fura-2, we observed the expected increase of steady-state [Ca²⁺]i, in C2C12 cells in differentiation medium (Fig. 7D). In the presence of DCPiB, but not of the vehicle DMSO, this increase was abolished (Fig. 7D). When DCPiB was removed after 24 h from the differentiation medium, Ca²⁺ levels increased (Fig. 7D) and myoblast fusion proceeded normally (Fig. 7E). Consistently, VRAC suppression by overexpression of RFP-labeled LRRCA8 (LRRCA8-RFP) prevented the increase of [Ca²⁺]i (Fig. 7F).

Notably, VRAC activity seemed to be required predominantly during the first 6 h of cell differentiation, because the addition of DCPiB after this time did not affect the resting membrane potential of C2C12 myoblasts (Fig. 7B) or their differentiation (Fig. 7G).

In summary, our results suggest that activation of VRAC activity contributes to the hyperpolarization of myoblasts and the rise in intracellular Ca²⁺, thereby supporting myoblast differentiation and fusion.

**Discussion**

**Functional VRAC is required for normal myoblast differentiation**

The thinned skeletal muscle bundles displayed by Lrrc8a−/− mice (22) suggest that lack of LRRCA8 may lead to dysfunction of myoblast proliferation, differentiation, or fusion into multinucleated myotubes. Here, we show that consistent with a previous report (44), inhibition of the LRRCA8-containing anion channel VRAC does not impair the proliferation of C2C12 myoblasts. But we find that indeed VRAC inhibition does impair the differentiation and fusion of C2C12 cells. A role for LRRCA8 in adipocyte differentiation was reported previously (45). In that context, LRRCA8 was proposed to function independent of VRAC activity by regulating insulin-Pi3K-Akt2-GLUT4 signaling through a physical interaction with the Cav1-IRS1-IR inhibitor GRB2 (45). We find that not only knocking down LRRCA8, but also pharmacological VRAC inhibitors and even VRAC current-suppressing overexpression of LRRCA8, impair myoblast differentiation and fusion. This demonstrates that not only the presence of LRRCA8, but also indeed the activity of VRAC plays a crucial role during myogenesis.

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**LRRC8/VRAC in myoblast differentiation**

![LRRC8/VRAC in myoblast differentiation](image-url)
inward-rectifying K⁺ channel Kir2.1 (5, 6, 37). Hyperpolarization to similar values was shown for C2C12 myoblasts (49), as we also observed here. It was reported that the Kir2.1 channel is active at the plasma membrane already after 6 h of cell differentiation (7, 42). We found that myoblasts did not fully hyperpolarize, but remained at an intermediate resting potential when VRAC was inhibited, consistent with a function of VRAC before that of Kir2.1. In agreement with a role of VRAC in the early phase of myoblast differentiation, volume-activated Cl⁻ currents drastically decrease during differentiation of C2C12 cells (50). We also observed significantly decreased expression of LRRC8C and LRRC8E after 2 days of cell differentiation. However, it is questionable if this underlies the reduction of VRAC currents, as the protein levels of the other paralogues, especially of LRRC8A, remained unchanged.

It may seem surprising that the anion channel VRAC contributes to membrane hyperpolarization, because in other situations it is associated with depolarization, such as during pancreatic insulin release where its activity triggers the early opening of voltage-gated Ca²⁺ channels (33, 34). Before the
induction of differentiation, C2C12 myoblasts were shown to express very low level of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter NKCC1, which is then up-regulated during differentiation (51). Thus, undifferentiated C2C12 possibly has a low intracellular Cl\(^{-}\) concentration. This would lead to an influx of chloride and hence membrane hyperpolarization upon VRAC activation within the first several hours of myoblast differentiation. Alternatively, VRAC could indirectly affect the membrane potential by modulating the activity of Kir2.1 during myoblast differentiation. It may influence regulatory pathways, such as those leading to the dephosphorylation of Kir2.1 at tyrosine-242 (42) or Cdo signaling (52).

In conclusion, we show that the volume-regulated anion channel VRAC, by controlling hyperpolarization and cytosolic Ca\(^{2+}\) signals, plays a critical role in myoblast differentiation. Our findings highlight the hitherto unknown importance of Cl\(^{-}\) channels in cell differentiation. Given that hyperpolarization is generally necessary for stem cell differentiation (8, 53), VRAC may have roles in other differentiation processes, such as osteoblastogenesis (54, 55).

**Experimental procedures**

**Cell culture and drugs**

C2C12 mouse skeletal muscle myoblasts (American Type Culture Collection, CRL-1772; kindly provided by P. Knaus, Freie Universität, Berlin, Germany) were maintained in growth medium (DMEM supplemented with 10% FBS, 100 units ml\(^{-1}\) penicillin, and 100 µg ml\(^{-1}\) streptomycin) at 5% CO\(_2\) at 37 °C. To induce myogenic differentiation, cells at 90–100% confluency were rinsed with Dulbecco’s PBS and then switched to differentiation medium (DMEM supplemented with 2% horse serum, 100 units ml\(^{-1}\) penicillin, and 100 µg ml\(^{-1}\) streptomycin). Upon induction of differentiation, medium containing the following drugs or vehicle if applicable (DMSO from PAN-Biotec) was replaced daily: CBX (Sigma-Aldrich, C4790), NFA (Sigma-Aldrich, N0630); NPPB (Tocris Bioscience, 0593), and DCPIB (Tocris Bioscience, 1540). For stock solutions, NFA, NPPB, and DCPIB were dissolved in DMSO, CBX in water.

**Immunofluorescence staining**

After the indicated time in differentiation medium, C2C12 cells growing on coverslips were rinsed with PBS and fixed in 4% paraformaldehyde/PBS for 15 min at room temperature. Cells were subsequently permeabilized in 0.2% Triton X-100/PBS for 20 min and blocked in 3% BSA/PBS for 1 h. Then, cells were incubated overnight at 4 °C with a monoclonal anti-myogenin antibody (clone MF20, 0.28 g per lane) was separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane (Macherey & Nagel). Blotted membranes were subsequently blocked in 5% skim milk in TBS-T (20 mM Tris, pH 7.6, 150 mM NaCl, and 0.02% Tween-20) for 1 h at room temperature, incubated with primary antibodies overnight at 4 °C, and stained with HRP-conjugated secondary antibodies for 40 min at room temperature. Primary antibodies were rabbit anti-LRRC8A-E subunits (1 µg ml\(^{-1}\); kindly provided by T. J. Jentsch, FMP and MDC Research, Berlin, Germany) (24, 56), mouse anti-myogenin (clone F5D, 1 µg ml\(^{-1}\); Developmental Studies Hybridoma Bank), anti-myosin (clone MF20, 0.28 µg ml\(^{-1}\); Developmental Studies Hybridoma Bank), rabbit anti-GAPDH (1:4100, 1:2500; Cell Signaling Technology). Secondary antibodies were goat anti-mouse and goat anti-rabbit (1:5000; Jackson ImmunoResearch Laboratories). Signals were detected using an enhanced chemiluminescence reagent (HRP juice; PJK GmbH) and a ChemiSmart5000 digital imaging system (Vilber-Lourmat). Densitometric quantification was performed with the Fiji software (57).

**Cell transfection**

For siRNA experiments, C2C12 myoblasts were transfected with 15 nm siRNA (scrambled siRNA: a nontargeting negative control siRNA; Lrcc8a siRNA1: sense, CCU UGU AAG UGG GUC ACC ATT; Lrcc8a siRNA2: sense, GAU CGA CAC CAG UAC AAC UTT; Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. For overexpression, 2 µg ml\(^{-1}\) of plasmid DNA of pEGFP-N1-LRRC8A, pmRFP-N1-LRRC8A (kindly provided by T. J. Jentsch) (24) or expression vectors pEGFP-N1, pmRFP-N1 (Clontech) was transfected into cells using FuGENE 6 (Promega) according to the manufacturer’s instructions. Cells were induced to differentiate 1 day after transfection.

**Cell proliferation assay**

C2C12 myoblasts were seeded at 5000 cells per well in 96-well plates and transfected with siRNA or plasmids the next day. Cell viability was evaluated with the Cell Counting Kit-8 (Sigma-Aldrich) at 24 and 48 h post transfection. The absorbance of the water-soluble formazan dye produced from tetrazolium salt WST-8 by cellular dehydrogenase activity was measured at 450 nm using a microplate reader (Biochrom).

**Quantitative real-time PCR**

Total RNA was isolated from C2C12 cells with a NucleoSpin RNA Kit (Macherey & Nagel). SuperScript II Reverse Transcriptase (Invitrogen), Oligo (dT)\(_{20}\) Primer (Invitrogen), and 1 µg of total RNA as template were used for cDNA synthesis. To assess gene expression, standard quantitative PCR was conducted with Power SYBR Green PCR Master Mix (Applied Biosystems) on StepOnePlus Real-Time PCR System (Applied Biosystems). Results were analyzed with the comparative cycle threshold C\(_T\) (ΔΔC\(_T\)) method by using Gapdh as the reference expression.
gene. Primers included LrreC-F, 5’-TCC TTT TCT GCG GAT ACC CT-3’; LrreC-R, 5’-AAC TCG GTC ACC GGA ATC AT-3’; Myog-F, 5’-CCA AGG TCT CCT GTG CTG ATG-3’; Myog-R, 5’-TTG GCA AAA CCA CAC AAT GC-3’; Gapdh-F, 5’-TGC GAC TTC AAC AGC AAC TC-3’; Gapdh-R, 5’-GCC TCT CTT GCT CAG TGT CC-3’.

**Fluorescence measurement of plasma membrane potential**

Membrane potential measurements using the fluorescent bis-oxonol type plasma membrane potential indicator DiBAC4(3) were performed as described previously (7, 43). C2C12 myoblasts growing in 8-well chambers (Sarstedt) were washed twice and then incubated at 37 °C in imaging buffer containing (in mM): 144 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, pH 7.4) with DiBAC4(3) (1 μM; Molecular Probes) for 30 min. After incubation, measurements were performed at room temperature on a DMi8 fluorescence microscope (Leica Microsystems) equipped with a 63×/1.40 NA oil-immersion objective. DiBAC4(3) fluorescence images were acquired at 16-bit, 4× binning, and 50-msec exposure with a FITC filter set (Ex: 480/40, Dc: 410, Em: 510/84; AHF analysentechnik) with the Winfluor software. At the end of each experiment, R\text{\text{\text{\text{\text{\text{max}}}}} was achieved by the addition of 1 μM ionomycin. No significant variation of R\text{\text{\text{\text{\text{max}}} was observed between experiments.

**Electrophysiological measurement of plasma membrane potential**

Whole-cell patch clamp recordings of the resting membrane potential were performed at room temperature with a Multi-Clamp 700B (Axon Instruments, Molecular Devices) and electrodes with an average resistance of 4 MOhm (range: 3.3–4.8 MOhm) in a submerged chamber containing the external solution containing (in mM): 144 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, pH 7.4, with NaOH, 329 mOsm. C2C12 myoblasts were visualized using IR differential contrast optics and an IR video camera (PIKE F-145B, Allied Vision). Recordings were filtered at 10 kHz and sampled at 20 kHz with a Digidata 1550A (Axon Instruments, Molecular Devices). The internal solution consisted of (in mM): 125 potassium gluconate, 5 KCl, 1 EGTA, 2 Na2ATP, 2 MgATP, 0.3 Na2GTP, 10 sodium phosphocreatine, 10 HEPES, pH 7.25, with KOH, 280 mOsm. Recorded traces were corrected for liquid junction potential. The resting membrane potential was determined from the first 15 s of each recorded trace to avoid membrane potential changes because of cytosol washout.

**Cytosolic Ca\text{\text{\text{\text{\text{2\text{+}}}}} imaging**

C2C12 cells growing on glass-bottom dishes (MatTek) were loaded with mix of Fura-2 AM (Invitrogen) and Pluronic F-127 (0.02%; Invitrogen) in culture medium at 37ºC for 30 min and then washed with imaging buffer containing (in mM): 145 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, pH 7.4. Imaging was performed at room temperature on a DMi8 microscope (Leica Microsystems) equipped with a 63×/1.40 NA oil-immersion objective and an OcraFlash 4.0 camera (Hamamatsu). Samples were excited with an Optoscan monochromator (Cairn Research) at 340 or 380 nm; emission was recorded using a Fura-2 filter set (Dc: 410, Em: 510/84; AHF analysentechnik) with the Winfluor software. The membrane potential or 340/380 ratio (Ca\text{\text{\text{\text{\text{2\text{+}}}}} imaging) from each field per sample was compiled and depicted as mean ± S.D.

**Statistical analysis**

All data are presented as mean ± S.D.; p values between two groups were determined by a two-tailed unpaired Student’s t test. For three or more groups, a one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test was performed. p values are indicated according to convention: *, p < 0.05; **, p < 0.01, and ***, p < 0.001, n.s. = not significant.

**Author contributions**—L. C. and T. M. B. formal analysis; L. C. and T. M. B. investigation; L. C., T. M. B., U. K., and T. S. methodology; L. C. and T. S. writing-original draft; L. C. and T. S. writing-review and editing; L. C. and T. S. conceptualization; U. K. and T. S. resources; U. K. and T. S. supervision; T. S. funding acquisition; T. S. project administration.

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