Membrane Hyperpolarization Triggers Myogenin and Myocyte Enhancer Factor-2 Expression during Human Myoblast Differentiation*

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It is widely thought that myogenin is one of the earliest detectable markers of skeletal muscle differentiation. Here we show that, during human myoblast differentiation, an inward rectifier K⁺ channel (Kir2.1) and its associated hyperpolarization trigger expression and activity of the myogenic transcription factors, myogenin and myocyte enhancer factor-2 (MEF2). Furthermore, Kir2.1 current precedes and is required for the developmental increase in expression/activity of myogenin and MEF2. Drugs or antisense reducing Kir2.1 current diminished or suppressed fusion as well as expression/activity of myogenin and MEF2. In contrast, LY294002, an inhibitor of phosphatidylinositol 3-kinase (a pathway controlling initiation of the myogenic program) that inhibited both myogenin/MEF2 expression and fusion, did not affect Kir2.1 current. This non-blockade of LY294002 indicates that Kir2.1 acts upstream of myogenin and MEF2. We propose that Kir2.1 channel activation is a required early event that initiates myogenesis by turning on myogenin and MEF2 transcription factors via a hyperpolarization-activated Ca²⁺-dependent pathway.

Skeletal muscle formation arises through the differentiation and fusion of mononucleated myoblasts into multinucleated myotubes. During this process, the resting potential of myoblasts hyperpolarizes from approximately −10 mV to values of around −70 mV (1). We have shown previously that this hyperpolarization to −70 mV is due to expression of the inward rectifier K⁺ channel Kir2.1 (2) and that it precedes fusion (1) and induces an increase in intracellular Ca²⁺ concentration, which is essential for myoblast differentiation and fusion to occur (3, 4).

Myoblast differentiation is a Ca²⁺-dependent multistep process coupled with withdrawal from the cell cycle, repression of genes associated with cell proliferation, and transcriptional activation of muscle-specific genes. Members of the myogenic basic helix-loop-helix (bHLH) protein family (MyoD, Myf5, myogenin, and MRF4) control specification and differentiation of myogenic cells (reviewed in Ref. 5). The myogenic bHLH proteins activate muscle transcription by binding to a consensus sequence referred to as the E box. Myogenesis has been described as beginning with myogenin expression, followed by cell cycle withdrawal and subsequent fusion (6). Activation of muscle gene expression by myogenic bHLH proteins also depends on their interaction with members of the myocyte enhancer factor-2 (MEF2) family of transcription factors (7). The four members of this family (MEF2A–D) bind to a consensus sequence present in several muscle-specific promoters (8). It has been suggested that MEF2 proteins act as integrators of Ca²⁺ signals essentially through the Ca²⁺/calmodulin (CaM)-dependent protein kinase pathway, which activates MEF2 by disrupting the nuclear interactions between MEF2 and histone deacetylase (9), and/or through the calcineurin pathway (10).

In view of the role we proposed for Kir2.1-associated hyperpolarization in the control of Ca²⁺ modulation during myoblast differentiation (3, 4), we decided to examine how Kir2.1 activity, which is required for fusion, may contribute to muscle gene expression in primary cultures of human myoblasts. We found that activation of functional Kir2.1 channels is an early required step of the myogenic program and that the resulting hyperpolarization of the myoblast resting membrane potential triggers the expression and activity of myogenic transcription factors myogenin and MEF2. Thus, an ionic channel is an essential relay in the signaling pathway that leads to expression of key transcription factors.

EXPERIMENTAL PROCEDURES

Cell Cultures—Clonal cultures of human myoblasts were prepared from single satellite cells as described previously (11). Muscle samples were obtained from children during corrective orthopedic surgery according to the guidelines of the local ethical committee. Myoblasts clones were expanded in growth medium (GM) and differentiated into myotubes in serum-free differentiation medium (DM) (12). Mibebradil dihydrochloride was a gift from Hoffmann-La Roche (Basel, Switzerland).

Immunostaining—Cells were fixed with paraformaldehyde, treated with Triton X-100, and incubated in Tween 20 and goat serum in phosphate-buffered saline. Anti-desmin antibody (clone D33, Dako

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The abbreviations used are: bHLH, basic helix-loop-helix; MEF2, myocyte enhancer factor-2; CaM, Ca²⁺/calmodulin; GM, growth medium; DM, differentiation medium; TRITC, tetramethylrhodamine isothiocyanate; DiBAC₄(3), bis(1,3-dibarbituric acid)-trimethine oxonol; pA, picoampere(s); pF, picofarad(s); BAPTA, 1,2-bis(2-aminoethyl)-N,N',N''-tetraacetic acid; MCK, muscle creatine kinase; MAPK, mitogen-activated protein kinase; FITC, fluorescein isothiocyanate; DAPI, 4',6-diamidino-2-phenylindole.
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Cor., was treated alone at 1:50. Anti-myogenin (1:10; clone F5D from Dr. P. L. Purcell) was used together. The secondary antibodies were FITC-labeled anti-mouse IgG (1:100; Dako Corp.) and TRITC-labeled anti-rabbit Ig (1:100; Sigma) together with DAPI (100 ng/ml; Sigma).

Western Blotting—Total proteins were separated on an SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Immunoblotting was performed using 1:100 mouse monoclonal antibody against myogenin, 1:100; rabbit polyclonal antibody in Tween/Tris-buffered saline and nonfat milk as follows: mouse monoclonal antibody against myogenin, 1:100; rabbit polyclonal antibody against -tubulin (clone DM1A; Sigma), 1:5000. Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies diluted 1:3000. Antibodies were revealed using ECL reagents and Hyperfilm MP (Amersham Biosciences).

Transfection and Reporter Assay—Transfections were performed by electroporation of 2 × 106 cells with 2 pmol of firefly luciferase plasmid (3MEF2-luc, 4RE-luc, or MCK-luc from Dr. P. L. Puri) together with 1 pmol of the control Renilla plasmid (phRL-TK-luc, Promega) as described previously (13). Cells were maintained under proliferation conditions for 3 days and then split into 24-well plates. When cells reached confluency, GM was replaced with DM for 4 days. (After electroporation, myotube formation is delayed with respect to untreated myoblasts and occurs only after ~3 days in DM (21).) For antisense inhibition of Kir2.1, cells were cotransfected with the 3MEF2-luc plasmid (2 pmol), the phRL-TK-luc plasmid (1 pmol), and either the AS-Kir2.1 plasmid or the empty plasmid pEF-1E (3 pmol). Cells were then processed with the Dual-Luciferase reporter assay kit (Promega). We set to 100% the ratio between the fireplace and Renilla luciferase activities obtained with cells maintained in DM in each set of experiments.

Confocal Monitoring of Membrane Potential—Confocal image acquisition of the fluorescent membrane potential dye bis(1,3-dibarbituric acid)-trimethine oxonol (DiBAC4(3); 1 μm; Molecular Probes, Inc.) was performed using a Nipkow spinning disc confocal microscope. The incident laser (Coherent, Inc.) beam (488 nm) was coupled with a Yokogawa spinning disc confocal scan head (Visithek International QLC100) mounted on an inverted microscope (Zeiss Axiovert 200M). Fluorescence images (520-nm long-pass filter) were captured with a cooled 12-bit TE/CCD interlined Coolscan HQ Photometrics camera (Roper Scientific). Images were acquired with Metafluor Version 5.0r6 software (Universal Imaging Corp.). Cells were plated on glass coverslips and were previously labeled with DiBAC4(3) and imaged with high magnification. Maximum projections were used to evaluate the kinetics and spatial distribution of channel activity.

Electrophysiological Recordings—Kir2.1 currents (I Kir2.1) were measured in the whole-cell configuration of the patch-clamp technique. Signals were recorded at 20–25 °C, low pass-filtered at 1 kHz with an Axopatch 200B amplifier (Axon Instruments, Inc.), and sampled at 2.5 kHz. The pipette resistances were 4–7 MΩ, and the compensation was 70–75%. The cell capacitance was obtained from direct reading of the whole-cell capacitance potentiometer of the Axopatch 200B amplifier. To improve the patching procedure, myoblasts were replated 30–60 min before recordings. An I Kir2.1 of 0 pA/pF was assigned to myoblasts with a whole-cell I Kir2.1 of <5 pA (4).

The extracellular solution contained 100 mM N-methyl-D-glucamine chloride, 5 mM KCl, 2 mM MgCl2, 5 mM Hepes, 50 mM NaOH, 50 mM acetic acid, and 8 mM glucose. The pH was adjusted to 7.4 with N-methyl-D-glucamine. The intracellular (pipette) solution contained 110 mM KCl, 5 mM NaCl, 2 mM MgCl2, 5 mM Hepes, 20 mM BAPTA, 5 mM glucose, and 5 mM MgATP. The pH was adjusted to 7.2 with KOH.

RESULTS

Kinetics of Expression of Myogenic Regulatory Factors in Human Primary Myoblasts—Among the muscle-specific transcription factors that may act in the induction of myoblast fusion, it is generally accepted that myogenin and MEF2 are leading participants (5). Immunocytochemistry was performed on cultured myogenic cells at different times after addition of serum-free DM to observe the kinetics of expression of these transcription factors at the cellular level. A very low level of MEF2 expression was detected in nuclei of proliferating myoblasts (Fig. 1A, GM panels), whereas myogenin was not detected at this stage. After 6 h in DM, expression of myogenin and MEF2 was noticeable in only a few nuclei. Induction of myogenin was apparent in 14% of the nuclei after 12 h in DM and in 42% of the nuclei after 24 h (Fig. 1B). The maximum percentage of myogenin-positive nuclei (57%) was reached after 30 h in DM and remained at this level at 48 h. Significant MEF2 expression, with respect to that noticed in the proliferating stage, was first observed after 24 h in DM (15% of the nuclei) and, as for myogenin, reached a plateau after 30 h in DM (51% of the nuclei). An increase in expression of myogenin and MEF2 was not expected to occur in all myoblasts since only 60–80% of them will eventually fuse (14). It is important to note that human myoblasts began to fuse into myotubes after 24 h in DM (Fig. 1A, arrowheads). The results from examination of protein expression by immunoblotting correlated well with the culture immunostaining results (Fig. 1C). Myogenin protein was detected after 12 h in DM, whereas MEF2 was already present at a low level in myoblasts maintained under proliferation conditions. Basal expression of MEF2 was expected under proliferation conditions, as the antibody we used recognizes several MEF2 isoforms (MEF2A, MEF2C, and MEF2D), and previous studies have shown that some MEF2 isoforms are already present in proliferating myoblasts (15, 16). The increase in MEF2 expression appeared after 24 h under differentiation conditions. Expression of the muscle-specific protein troponin T, which depends upon myogenin bHLH and MEF2 factors, indicates that late steps of differentiation were achieved after 2 days in DM. These results confirm that, in primary human myoblast cultures, myogenin expression is the earliest marker of induction of the myogenic differentiation program and that the developmental increase in MEF2 expression is induced later, yet still before the initiation of cell fusion.

Kir2.1 Channel Activity Precedes Expression of Myogenic Regulatory Factors—We previously demonstrated that, in myoblasts triggered to differentiate, Kir2.1 channel activity induces membrane hyperpolarization to −70 mV, which, in turn, causes intracellular Ca2+ to rise (2, 3). As Ca2+ plays a key role in the activation of the muscle differentiation program (5), we compared the timing of expression of functional Kir2.1 channels with that of the myogenic regulatory factors implicated in the initiation of muscle differentiation.

Previous results have indicated that a low level of mRNA encoding Kir2.1 is present in undifferentiated myoblasts and that increasing levels can be detected throughout differentiation (2). Kir2.1 channel protein was not detectable by Western blotting (presumably due to Kir2.1 low level expression) (data not shown); therefore, we used whole-cell current recordings to evaluate Kir2.1 channel expression and activity in proliferating and differentiating myoblasts (Fig. 2). A very low level of Kir2.1 current density (I Kir2.1) was detected in proliferating myoblasts (I Kir2.1 = 0.16 ± 0.09 pA/pF, n = 35) and in myoblasts after 2 h in fusion-inducing medium (I Kir2.1 = 0.20 ± 0.10 pA/pF, n = 27), and these levels were not statistically different (p = 0.73). In proliferating cells as well as in myoblasts in DM for 2 h, only few cells expressing I Kir2.1 were detected (11% in GM). Interestingly, in these few positive cells, the density of I Kir2.1 was similar to that measured in the positive cells after 6 h in DM. After 6 h in DM, the mean I Kir2.1 increased drastically (I Kir2.1 = −0.79 ± 0.19 pA/pF, n = 44; with 41% of the myoblasts expressing measurable I Kir2.1), reaching a level statistically identical to that measured in cells...
maintained for 15 h in DM ($I_{\text{Kir2.1}} = -0.82 \pm 0.21 \text{ pA/pF, } n = 22, p = 0.91$; with 45% of the myoblasts expressing measurable $I_{\text{Kir2.1}}$) or for 24 h in DM ($I_{\text{Kir2.1}} = -0.81 \pm 0.15 \text{ pA/pF, } n = 55, p = 0.92$; with 51% of the myoblasts expressing measurable $I_{\text{Kir2.1}}$). For direct comparison of the kinetics of expression of Kir2.1 channels with those of myogenin and MEF2 factors, the percentage of myoblasts expressing measurable $I_{\text{Kir2.1}}$ is represented in Fig. 1B.

These results demonstrate that the main pool of Kir2.1 channels is already present and active at the plasma membrane 6 h after the induction of differentiation and suggest that Kir2.1 channel activity precedes myogenin and MEF2 expression. Kir2.1 channel activity in human myoblasts therefore appears to be one of the earliest steps in the establishment of the muscle differentiation program.

Membrane Depolarization through Pharmacological Blockade of Kir2.1 Reduces Myogenin and MEF2 Expression—In previous work, we have shown that Cs$^+$ and mibebradil inhibit Kir2.1 channels in human primary myoblasts (1, 17). To further investigate the role of the Kir2.1-associated hyperpolarization in the initiation of myoblast fusion, we examined the consequences of graded pharmacological blockade of Kir2.1 activity on the differentiation process. In this study, we used 20 mM Cs$^+$ to totally inhibit Kir2.1 channels and 10 mM Cs$^+$ or 5 μM mibebradil to partially suppress Kir2.1 activity. Higher mibebradil concentrations could not be used because of toxic effects on human myoblasts.

We first established a protocol using confocal imaging with the potentiometric fluorescent probe DiBAC$_4$(3) (18) to confirm that the blockade of Kir2.1 channels results in membrane de-
mV. This was used as an internal control for the calibration procedure quality; two procedures (130 mM KCl or gramicidin + 136.3 mM Na\(^+\)) that should theoretically shift the membrane potential to 0 mV actually do so.

Confocal imaging of DiBAC\(_4\)(3) fluorescence was then applied to measure the difference in membrane potential between myoblasts cultured for 20–24 h in control DM and in DM containing different Kir2.1 inhibitors (Fig. 3D). In control DM, the majority of myoblasts (70%) showed a gaussian distribution centered at −70 mV, whereas 30% of the myoblasts were more depolarized, with a gaussian fit centered at −47 mV. Note that the percentage of hyperpolarized cells matches that of the fusion index (60–80%) (14). In the presence of Kir2.1 inhibitors, as expected, the distribution of membrane potentials was shifted to more depolarized levels with respect to control myoblasts. In the perspective of experiments discussed below, it is important to mention that 10 mM Cs\(^+\) and 5 \(\mu\)M mibebradil depolarized the cells to similar levels (−37 ± 1 mV, \(n = 29\); and −36 ± 1 mV, \(n = 34\), respectively), whereas 20 mM Cs\(^+\) produced a stronger effect on membrane depolarization (−17 ± 1 mV, \(n = 34\)).

Myogenin and MEF2 expression was then evaluated in the presence of Kir2.1 channel inhibitors (Fig. 4A), i.e. in depolarized myoblasts. Under control conditions after 24 h in DM, obvious myoblast fusions and a strong induction of myogenin and MEF2 were observed. In the presence of Kir2.1 inhibitors, fusion was markedly impaired, and myogenin and MEF2 expression dropped accordingly. Fig. 4B shows that the percentage of MEF2-positive nuclei was reduced by 10 mM Cs\(^+\) and 5 \(\mu\)M mibebradil and that 20 mM Cs\(^+\) had a robust inhibiting effect. The number of positive nuclei dropped by 82% in presence of 20 mM Cs\(^+\). On the other hand, the proportion of myogenin-positive nuclei seemed to be less affected by Kir2.1 inhibitors. Although myogenin expression was still significantly (\(p < 0.005\)) reduced by 20 mM Cs\(^+\) (−35% fewer positive nuclei), 10 mM Cs\(^+\) or 5 \(\mu\)M mibebradil had no significant effect on myogenin expression (\(p = 0.18\) and 0.06, respectively). The effect of 20 mM Cs\(^+\) was confirmed by immunoblotting. As shown in Fig. 4C, MEF2 expression was characterized by induction of expression of both myogenin and MEF2. Basal expression of MEF2 factors in proliferating myoblasts was confirmed by Western blotting, and a strong induction of MEF2 expression was observed after 48 h in DM. At this time, expression of a late marker of muscle differentiation (troponin T) confirmed the differentiation of myoblasts into myotubes. In the presence of 20 mM Cs\(^+\), the induction of myogenin expression was reduced compared with that under control conditions. Furthermore, in the presence of 20 mM Cs\(^+\), the MEF2 level of expression was maintained at its basal level observed in proliferating myoblasts, and expression of troponin T was not induced. To confirm the importance played by the hyperpolarization in the differentiation process, we maintained the membrane resting potential in the vicinity of 0 mV by raising the extracellular [K\(^+\)] to 130 mM and evaluated myogenin, MEF2, and troponin T expression during the induction of differentiation. As shown in Fig. 4C, treatment with high external [K\(^+\)] totally inhibited the induction of myogenin, MEF2, and troponin T. It is worth mentioning that the effect of both 20 mM Cs\(^+\) and high external [K\(^+\)] was maintained over time in culture (with >95% of the myoblasts still mononucleated under these conditions after 4 days in DM). Fig. 4C also shows that, as already demonstrated in muscle cell lines (19), inhibition of muscle differentiation could be obtained with the phosphatidylinositol 3-kinase inhibitor LY294002 (Cell Signaling Technology). This compound was used in later experiments to evaluate myogenin and MEF2 requirements in the regulation of Kir2.1 channel expression above.
These results show that membrane depolarization resulting from Kir2.1 blockade interferes with expression of myogenin and strongly inhibits later steps controlling the muscle-specific induction of MEF2 and troponin T expression.

Kir2.1 Blockade Strongly Inhibits Activation of the Myogenic Differentiation Program—To confirm that Kir2.1 channel activity and the associated hyperpolarization play a primary role in induction of the myogenic program, we tested Kir2.1 inhibitors on the activity of the muscle creatine kinase (MCK) promoter. Due to its regulation by a synergistic activity of myogenic bHLH and MEF2 transcription factors, the MCK promoter is known to be highly up-regulated during the late stages of muscle differentiation (20). Activation of the MCK promoter was revealed by a luciferase activity assay. Fig. 5 (A) shows that, in human myoblasts undergoing differentiation, the activity of the MCK promoter increased 23-fold. This increased activity of the MCK promoter dropped markedly when myoblasts were induced to differentiate in the presence of Kir2.1 inhibitors. In the presence of 10 mM Cs⁺ or 5 μM mibebradil, MCK activity was reduced to 32 and 40% of the control level, respectively. In the presence of 20 mM Cs⁺, MCK promoter activity was reduced to 10% of its control level and thus was nearly completely inhibited when Kir2.1 activity was blocked. To test whether this inhibition was a consequence of reduced myogenic bHLH and MEF2 activities, we measured the effect of Kir2.1 blockade on myogenic bHLH and MEF2 activities.

The results presented in Fig. 5 (B and C) reveal a very low activity of both bHLH and MEF2 families in undifferentiated myoblasts and a strong activation of these transcription factor activities upon myotube formation (16- and 43-fold, respectively). Exposure to either Cs⁺ or mibebradil under differen-
ation conditions decreased both myogenic bHLH and MEF2 activities. Treatment with 10 mM Cs⁺ (Cs 10), 20 mM Cs⁺ (Cs 20), and 5 μM mibebradil (Mib 5) reduced myogenic bHLH activity to 46 and 44% of its control activity, respectively, and 20 mM Cs⁺ reduced the activity by 76% (Fig. 5B). The activity associated with MEF2 dropped by 84 and 95% compared with the control cells in the presence of 10 and 20 mM Cs⁺, respectively, and 5 μM mibebradil reduced the activity by 83% (Fig. 5C). We thus observed a decrease in myogenic bHLH activity that was less important than that observed for MEF2 activity in the presence of Cs⁺ (Kir2.1 inhibitor) or mibebradil. This is consistent with the well described Ca²⁺-dependent activity of the MEF2 transcription factor and our previous observation that hyperpolarization of myoblasts allows [Ca²⁺]i to increase (3, 4).

To confirm these pharmacological results, we evaluated the effect on MEF2 activity of an antisense against the human Kir2.1 mRNA (AS-Kir2.1). We used a bicistronic vector allowing enhanced green fluorescent protein expression together with expression of the full-length cDNA encoding Kir2.1 in an antisense orientation. This plasmid was previously described to be able to reduce both Kir2.1 current and myoblast fusion (2). Myoblasts were transfected with the AS-Kir2.1 vector, and MEF2 activity was assessed. Transfection with the bicistronic vector without the first cistron (expressing enhanced green fluorescent protein alone) did not modify the MEF2 activity and was used as control. Fig. 5D shows that the specific blockade of Kir2.1 channel expression decreased MEF2 activity by 50%. It was not surprising that the antisense inhibition was not as impressive as that shown for 20 mM Cs⁺, as about one-third of the Kir2.1 current is still present after antisense

**Fig. 4. Blockade of Kir2.1 channel activity inhibits myoblast differentiation.** Shown are the results from immunocytochemical analysis of cells maintained in GM, in DM for 26, or in DM 26 h with the indicated drugs: 10 mM Cs⁺ (Cs 10), 20 mM Cs⁺ (Cs 20), and 5 μM mibebradil (Mib 5). A, cells stained for myogenin (FITC) and MEF2 (TRITC) and with DAPI to visualize the nuclei. Scale bars = 20 μm. B, decrease in the number of myogenin- and MEF2-positive nuclei by inhibitors of Kir2.1. Five randomly selected fields were analyzed in two independent experiments. Error bars correspond to S.E. C, Western blot of equal amounts of total proteins from cells maintained in DM in the absence or presence of either 20 mM Cs⁺ (Kir2.1 inhibitor) or 50 μM LY294002 (LY, phosphatidylinositol 3-kinase inhibitor). After 24 or 48 h, cells were lysed, and protein expression was analyzed for myogenin, MEF2, and troponin T (a marker of late differentiation). An antibody against α-tubulin was used to show the amount of protein applied to gel. The last three lanes represent an experiment with high external [K⁺]. Myoblasts treated with 130 mM KCl (and control (Cont) myoblasts in 5 mM external K⁺) were induced to differentiate for 48 h. To maintain the osmolarity constant, 125 mM NaCl usually present in DM was replaced with 125 mM KCl.
treatment (2), whereas 20 mM Cs\textsuperscript{+} reduces Kir2.1 current by 95% (1). The results obtained using AS-Kir2.1 are thus consistent with those obtained with pharmacological inhibition of Kir2.1 activity and confirm that Kir2.1 channels contribute to the control of transcription factor activity.

Increase in Kir2.1 Channel Activity during Myoblast Differentiation Is Independent of Myogenin and MEF2 Activities—

Given our hypothesis that Kir2.1 channel activity is a crucial step acting upstream of myogenin and MEF2 in triggering myoblast differentiation, we would expect expression of Kir2.1 to occur independently of myogenin and MEF2 expression and activity, even when expression and activity of these factors are blocked. In other words, we expect Kir2.1 channel proteins to be expressed in the presence of two muscle differentiation inhibitors, Cs\textsuperscript{+} and the phosphatidylinositol 3-kinase inhibitor LY294002 (Fig. 4C). Fig. 6 illustrates the presence of functional Kir2.1 channels in myoblasts 24 h after transfer of the cells into DM (i) under control conditions, (ii) when myogenin and MEF2 activities were blocked by a 24-h exposure to 20 mM Cs\textsuperscript{+}, and (iii) when myogenin and MEF2 muscle-specific expression was blocked by 50 μM LY294002. To measure Kir2.1 channel activity after the Cs\textsuperscript{+} treatment, cells were washed with DM for 15 min prior to recording. We found that \( I_{\text{Kir2.1}} \) density in myoblasts treated either with 20 mM Cs\textsuperscript{+} (\( I_{\text{Kir2.1}} = -0.78 \pm 0.21 \) pA/pF, \( p = 0.90 \)) or with 50 μM LY294002 (\( I_{\text{Kir2.1}} = -0.83 \pm 0.27 \) pA/pF, \( p = 0.95 \)) was not different from the density measured in control myoblasts treated with 5 μM mibebradil, \( p < 0.0001 \); and 10 mM Cs\textsuperscript{+} compared 20 mM Cs\textsuperscript{+}, \( p < 0.005 \). Antisense inhibition of Kir2.1 channel expression decreased MEF2 activity (D). Results represent the mean of at least five independent experiments. Error bars correspond to S.E.
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Fig. 7. Whole-cell properties of the T-current in fusion-competent myoblasts (from Ref. 3). A, activation (●) and inactivation (□) conductance curves. Left inset, current traces recorded during steady-state inactivation voltage protocol; right inset, current traces recorded during depolarizing steps (activation protocol). B, computed T-channel window current from the curves shown in A. In the window domain, T-channels cycle permanently between the closed, open, and inactivated states; and thus, statistically, a tiny fraction of the T-channels is always in the open state.

This work has demonstrated that Kir2.1 channel activity and its associated hyperpolarization (i) modulate the bHLH and MEF2 myogenic transcription factor families, two of the most important protagonists in the control of the myogenic differentiation program (7), and (ii) do not depend upon the activity of these transcription factor families. This suggests that, in human myoblasts, Kir2.1 channel activation is an early trigger event in the differentiation process. We have explained the position of Kir2.1 upstream of transcription factors by the fact that it creates the permissive conditions allowing the Ca\(^{2+}\) influx known to be critical for myoblast differentiation and fusion.

Our current understanding of a major mechanism controlling this Ca\(^{2+}\) influx is as follows. Human myoblasts must hyperpolarize to approximately −70 mV (i.e. their membrane potential must become more negative inside the cell) before they can proceed through the differentiation process (2). Hyperpolarization occurs through expression of Kir2.1 K\(^+\) currents. (Under physiological conditions, open K\(^+\) channels allow an efflux of positively charged potassium ions when the potential of the cell is more positive than \(E_K\); this efflux of K\(^+\) leaves negative charges inside the cell.) We propose that the purpose of the hyperpolarization is to increase the driving force for Ca\(^{2+}\) and to set the myoblast resting potential in a voltage range where the necessary inward Ca\(^{2+}\) flux can take place.

It may seem surprising that hyperpolarization increases Ca\(^{2+}\) influx, as in many familiar situations (such as neurotransmitter release), Ca\(^{2+}\) influx is associated with a depolarization that opens voltage-gated calcium channels. In our model, the Ca\(^{2+}\) influx is generated by a “window current” through T-type Ca\(^{2+}\) channels (T-channels) (3), which are expressed at about the same time as Kir2.1. The mechanism underlying the window current is explained in Fig. 7. T-channels are known to activate rapidly from a closed to an open state upon depolarization (Fig. 7A, traces d and e during increasing step depolarizations from a holding voltage of −100 mV). However, T-channels rapidly switch off and remain in an inactivated state as depolarization persists. (See the drop in current size at a fixed depolarized voltage to −30 mV following previous voltage holdings at progressively more depolarized levels; traces a–c in Fig. 7A were recorded after holding the myoblast for 10 s at −100, −60, and −35 mV, respectively.) To move T-channels out of the inactivated state, the cell membrane has to rehyperpolarize, which resets the T-channels to the closed state. How then would T-channels be activated by hyperpolarization? A careful analysis of the T-channel characteristics of myoblasts showed that a steady-state Ca\(^{2+}\) current through T-channels exists in the voltage domain where the activation and inactivation current-voltage relationships overlap. In this domain, the equilibrium between the closed, open, and inactivated states is such that T-channels cycle continuously between the three states and that a tiny fraction of the T-channels is always open (Fig. 7B). The magnitude of the T-channel window current (\(I_{\text{window}}\)) at various membrane potentials can be computed from the product of the two curves in Fig. 7A multiplied by the driving force on K\(^+\) (\(V_{\text{m}} - E_K\)) at each membrane potential \(V_{\text{m}}\). The amplitude of the window current in myoblasts is small (<0.6 pA), but is sufficient to induce a measurable change in [Ca\(^{2+}\)]\(_i\), over time (3).

Detection of Functional Kir2.1 Channels and Myogenin Expression—To evaluate the position of Kir2.1 channels in the hierarchy of the myoblast differentiation program, we compared the kinetics of expression of myogenin with those of Kir2.1 current density. Myogenin expression is believed to be one of the earliest steps in the process of differentiation, occurring even before expression of p21 and cell cycle arrest (6). In human myoblasts, myogenin proteins were detected after 12 h in DM and, as expected, preceded the developmental boost in expression of MEF2. We have shown that functional Kir2.1 channels could be detected several hours before expression of myogenin and MEF2. This result suggests that the activation of Kir2.1 channels not only is independent of myogenin expression, but also could act as a key event in the initiation of the myoblast differentiation process.

Mechanisms of Modulation of MEF2 and Myogenic bHLH by Kir2.1 Channels—The fine control of myoblast differentiation is associated with a precise control of the activities of transcription factors. In this work, we have shown that Kir2.1-associated hyperpolarization plays a key role in the activation of the MEF2 and myogenic bHLH families, as Kir2.1 inhibitors reduce the activities of both families of transcription factors. There is increasing evidence demonstrating that Ca\(^{2+}\) regulates MEF2 activation mainly through activation of CaM-dependent protein kinase and by calcineurin (9, 10, 22–25). As expected, MEF2 activity could be reduced by Kir2.1 inhibitors to levels close to those observed in undifferentiated myoblasts. MEF2 activity has been shown to be turned on when class II histone deacetylases are translocated from the nucleus to the cytoplasm after phosphorylation by CaM-dependent protein kinase (9). The high sensitivity of MEF2 activity to Kir2.1 inhibition is consistent with our model linking the hyperpolarization induced by Kir2.1 channel activation to intracellular Ca\(^{2+}\) regulation, as it would predict a reduction in CaM-dependent protein kinase activity. In fact, we have shown recently that an experimental setting of the membrane potential that accelerates myoblast differentiation increases [Ca\(^{2+}\)]\(_i\) (4). In this work, we have demonstrated that inhibition of Kir2.1 currents during myoblast differentiation results in a strong inhibition not only of MEF2 activity, but also of MEF2 expression.

MEF2 transcription factors have already been detected at...
low levels in human proliferating myoblasts. Previous studies have shown that MEF2 factors pre-exist in myoblasts (16) and that the MEF2D isoform is present in proliferating myoblasts (15). During differentiation in vitro, the proportion of cells that show an induction of MEF2 expression increases to reach 55%. When Kir2.1 channels are blocked, this number is strongly reduced. This result suggests that functional Kir2.1 channels control, in some way, MEF2 expression. The skeletal MEF2C promoter contains binding sites for MyoD and MEF2 factors. Expression of MEF2 is thus strongly regulated by synergistic action of myogenic bHLH and MEF2 transcription factors (26). The reduced expression of MEF2 in the presence of inhibitors of Kir2.1 channels could thus reflect the decrease in the activities of both myogenic bHLH and MEF2 transcription factors. Expression of the myogenin protein preceded the increase in MEF2 expression and was found to be less sensitive to Kir2.1 current blockade. This result confirms that myogenin and MEF2 expression is not regulated in the same way throughout the process of myoblast differentiation. Expression of myogenin has been recently described to be under the combined influences of p38 MAPK and the Ca^{2+}-dependent regulating pathways associated with calcineurin and CaM-dependent protein kinase (22, 27). The differential sensitivity of myogenin and MEF2 expression to Kir2.1 inhibition suggests that the p38 MAPK pathway may be sufficient to induce myogenin expression in human myoblasts. It is interesting to note that inhibition of Kir2.1 led to a stronger decrease in expression of a late marker of muscle differentiation (troponin T) compared with expression of myogenin, which is considered to be an early marker of muscle differentiation. This shows that troponin T expression depends on both myogenin and MEF2 factors. The MCK promoter/enhancer is primarily regulated during muscle differentiation by two E boxes and two MEF2-binding sites (8, 28, 29). Reduced MCK promoter activity by Kir2.1 blockade could thus reflect the loss of activity of either the MEF2 or myogenic bHLH family or both. It is important to note that, for each promoter tested, there was a perfect correlation between the activity of the promoter and the resting membrane potential. According to our model, the hyperpolarization induced by Kir2.1 activation, at the onset of myoblast differentiation, will lead to an increase in the intracellular Ca^{2+} concentration. This suggests that the Kir2.1-induced hyperpolarization and subsequent changes in membrane potential modulate the Ca^{2+}-dependent mechanism involving the CaM-dependent protein kinase/class II histone deacetylase pathway.

Relationship between Membrane Depolarization and Differentiation—The use of the potentiometric fluorescent probe DiBAC_4(3) coupled with spinning disc confocal microscopy allowed us to get a precise measurement of the membrane potential of myoblast populations over long periods. Using this noninvasive technique, we were able to evaluate the membrane potential of myoblasts submitted to chronic exposure to Kir2.1 inhibitors. We found that a large proportion of cells induced to differentiate for 20–24 h in control DM hyperpolarized to approximately −70 mV, a result that is comparable with that obtained with patch-clamp recordings (4). This population of myoblasts with a membrane potential at −70 mV (70% of the cells) is in relative agreement with patch-clamp measurements done at the same time and showing that 50% of the tested myoblasts expressed Kir2.1 channels. The difference in the percentage of myoblasts expressing Kir2.1 channels compared with those that were hyperpolarized to −70 mV can be explained by the experimental procedure used in our patch-clamp experiments.

Using confocal imaging with the fluorescent probe DiBAC_4(3), we also found that populations of differentiating myoblasts treated with 10 mM Cs⁺ or 5 μM mibefradil were depolarized by −30 mV to −37 ± 1 and −36 ± 1 mV, respectively. The similarity of the final potentials reached under these two conditions indicates that, although mibefradil at 5 μM can inhibit many channels (T-type Ca^{2+} channels, L-type Ca^{2+} channels, delayed rectifier K⁺ channels, and ether-a-go-go K⁺ channels) (17), its main effect on the membrane potential of differentiating myoblasts is best explained by inhibition of Kir2.1 channels (also described in Ref. 17).

The effects of 10 mM Cs⁺ and 5 μM mibefradil on membrane potential showed the crucial contribution of Kir2.1 channels to myoblast hyperpolarization. However, we also found that Cs⁺ at a higher concentration (20 mM), which is more effective in inhibiting myoblast differentiation and fusion, had a more pronounced effect on membrane depolarization than expected (−17 ± 1 mV, n = 34). If the potential reached with 10 mM Cs⁺ was very close to the value obtained by current-clamp experiments during acute exposure to this concentration (−46 ± 4 mV) (1), the even more depolarized potential reached with 20 mM Cs⁺ contrasts with our previous data of patch-clamp recording, in which acute exposures to 30 mM Cs⁺ depolarized the cells to −40 ± 1 mV (1). We attribute the additional depolarization observed here to the effect of long-term exposure to Cs⁺. Indeed, as myoblasts depolarize near −40 mV, the human ether-a-go-go channels present in myoblasts are activated (21), and this could lead to an intracellular accumulation of Cs⁺, as this ion can permeate ether-a-go-go channels (30). There is also a possibility that Cs⁺ could enter the cells via nicotinic acetylcholine receptors (12, 31). This chronic accumulation of Cs⁺ could affect the resting potential of myoblasts by blocking pumps or others K⁺ channels, including the human ether-a-go-go channel. These mechanisms could explain the additional depolarization to −17 mV during chronic exposure to Cs⁺.

It is important to mention that the inhibition by 20 mM Cs⁺ of the fusion process, of activation of the MCK promoter, and of the activities of the myogenic bHLH and MEF2 factors was consistently more efficient than the blockade by 10 mM Cs⁺. This indicates that mechanisms other than T-channels must contribute to Ca^{2+} entry in differentiating myoblasts when the membrane potential of myoblasts is outside the T-channel window domain (more depolarized than −30 mV) (3).

Hierarchy in the Differentiation Program—We have shown that the presence of functional Kir2.1 channels precedes the activation of the myogenic program of differentiation. This early expression of Kir2.1 at the onset of myoblast differentiation is in agreement with the key role we proposed for Kir2.1-induced hyperpolarization in this developmental process. In this context, it was important to verify that activation of Kir2.1 channels did not require the induction of myogenin and MEF2 expression. We showed that the specific inhibition of phosphatidylinositol 3-kinase by LY294002 totally blocked the expression of myogenin and MEF2 that is normally observed upon induction of myoblast differentiation. We measured Kir2.1 currents in myoblasts induced to differentiate for 24 h in the presence of LY294003 and found that Kir2.1 activity was not modified under these conditions. This result clearly indicates that activation of Kir2.1 current is independent of the activation of MEF2 and myogenic bHLH transcription factors and that it must precede activation of the differentiation program leading to fusion. Furthermore, the observation that the density of I_{K\text{Kir2.1}} in myoblasts induced to differentiate remained nearly constant over a period characterized by changes in transcription factor expression and activity confirms that these myogenic regulating pathways do not affect the activation of Kir2.1 channels. Also, exposure to 20 mM Cs⁺, which blocked differentiation and fusion, did not prevent Kir2.1 protein ex-
pression. Together, these observations point to Kir2.1 activation as being an early and Cs\(^+\)-insensitive step in the process of differentiation leading to myoblast fusion.

The mechanism controlling the activation of Kir2.1 current during myoblast differentiation is still an open question. Since Kir2.1 mRNA is detected in proliferating myoblasts (2), a regulatory post-transcriptional mechanism is most likely involved. In neuronal cells, export of K\(^+\) channels from the endoplasmic reticulum to the plasma membrane has been proposed to control the surface density of K\(^+\) channels (reviewed in Ref. 32). On the other hand, the activation of Kir channels at the membrane may be modulated. Kir2.1 channels, in particular, have been reported to be regulated by tyrosine kinases (33) and, interestingly, by phospholipids, especially phosphatidylinositol 4,5-diphosphate (34). The precise pathways implicated in the regulation of Kir2.1 channel activation at the onset of myoblast differentiation remain to be explored.

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