The Small Conductance Calcium-activated Potassium Channel Regulates Ion Channel Expression in C3H10T1/2 Cells Ectopically Expressing the Muscle Regulatory Factor MRF4*

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We investigated small conductance (SK) potassium channel-mediated regulation of muscle-specific, ion channel functional expression in the C3H10T1/2-MRF4 cell model system, a stable fibroblast line ectopically overexpressing the myogenic regulatory transcription factor, MRF4. Mitogenic stimulation of C3H10T1/2-MRF4 cells with basic fibroblast growth factor negatively regulates MRF4 transcriptional activity, inhibiting myogenesis. Using patch clamp techniques we found that mitogenic stimulation of C3H10T1/2-MRF4 cells also up-regulated SK. SK is a charybdotoxin-sensitive, apamin-insensitive channel that exerts positive proliferative control in fibroblasts. Mitogen withdrawal, which removes negative regulation of MRF4 thus initiating myogenesis, also eliminated SK channel currents, coincident both with induction of acetylcholine receptor channels, and up-regulation of muscle inward rectifier potassium channels. Addition of the SK channel blocker charybdotoxin to growth factor-containing culture medium overcame basic fibroblast growth factor-induced negative regulation of MRF4, as evidenced by induction of inward rectifier potassium and acetylcholine receptor channel expression identical to that observed in mitogen-withdrawn cells. Thus, the SK channel can govern electrophysiological phenotype in C3H10T1/2-MRF4 cells, consistent with an ability of SK to affect MRF4-dependent transcriptional activity. SK appears to be a pivotal signaling component for growth factor regulation of both cell proliferation and differentiation.

The regulated expression of ion channels is a key component of developmental processes in many cell types. In nonexcitable cells such as lymphocytes and fibroblasts, both potassium and voltage-independent cation channels contribute to proliferative and cell fate control mechanisms (1–6). While in excitable tissues, the expression of both voltage-dependent cation and ligand-gated channels is a culminating event in cell differentiation. The latter scenario is prominent in mammalian skeletal muscle, for which cell maturation is marked by the up-regulation of voltage-dependent channels and conditional expression of different ACh receptor channel subtypes (7). In turn, the progression of muscle fiber differentiation may be affected by the activity of these channels (8–10).

To understand ion channels as both causal and effector agents in cell growth and differentiation, we have looked at the regulation of channel functional expression in the C3H10T1/2-MRF4 myogenic model cell line. C3H10T1/2 (10T1/2) is a multipotent, fibroblast-like cell line that can be manipulated to express a phenotype characteristic of either chondrocytes, adipocytes, or myocytes (11). A muscle phenotype can be selectively produced in 10T1/2 cells via overexpression of the myogenic regulatory transcription factor MRF4. In the presence of bFGF or other mitogenic stimuli, MRF4 is unable to initiate muscle-specific gene expression, even though MRF4 protein levels remain unchanged. Upon bFGF withdrawal, negative regulation of MRF4 is removed, and an MRF4-dependent myogenic program is then induced (12), including both the expression of a number of muscle-specific genes such as those for α-actin and myosin heavy chain, and the fusion of cells to form multinucleate myotubes (13).

We show that bFGF withdrawal and thus initiation of the MRF4-dependent myogenic program in 10T1/2-MRF4 cells stimulates, within 24 h, expression of the muscle-typical ion channels, IRK and ACh receptor. We also extend our earlier work in other fibroblast cell lines including the 10T1/2 parental line, that mitogenic stimulation (of 10T1/2-MRF4) is consistently associated with up-regulation of the SK small-conductance calcium-activated potassium channel, whereas mitogen withdrawal results in SK down-regulation (5, 14–17). Small conductance calcium-activated channels comprise a physiologically defined channel family that vary somewhat in their pharmacology. We and others have shown that the SK channel expressed in fibroblasts is blocked by ChTX, but is insensitive to apamin (5, 14–17). A similar, if not identical, channel has also been found to be important in mitogenic signaling in other cell types (1, 3, 26). In fibroblast cells pharmacological blockade of the SK channel with ChTX inhibits growth factor-stimulated cell proliferation, thus mimicking mitogenic withdrawal (5). The use of ChTX is diagnostic for SK activity in these cells, in that they do not express some of the non-neuronal voltage-gated potassium channels known to be ChTX-sensitive (1, 3, 26). We now show that SK blockade in bFGF-stimulated 10T1/2-MRF4 cells also mimics mitogen withdrawal, causing in these cells the rapid stimulation of MRF4-dependent IRK and ACh receptor expression. These results suggest that SK channel activity is a critical component of the bFGF receptor-initiated signaling events, which ultimately lead to negative regulation of muscle fiber differentiation.

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1 The abbreviations used are: ACh, acetylcholine; MRF4, muscle regulatory factor 4; bFGF, basic fibroblast growth factor; IRK, inward rectifier potassium channel; SK, small conductance, calcium-activated potassium channel; ChTX, charybdotoxin; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; HS, horse serum; ITS, insulin transferrin sodium selenite; α-BTX, α-bungarotoxin; TEA, tetraethylammonium; EK, potassium equilibrium potential.
loration of MRF4 transcriptional activity. This finding, combined with our previous studies, suggests that the SK channel contributes to the control of cell growth and differentiation via its ability to affect nuclear events including transcriptional activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation—Experiments were performed using both nontransfected 10T1/2 cells (14) and 10T1/2 cells constitutively expressing a rat MRF4 cDNA (10T1/2-MRF4; provided by Dr. S. F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN). Stock 10T1/2-MRF4 cultures were grown on standard tissue culture plastic and maintained as undifferentiated myoblasts by continuous growth in medium consisting of basal medium, Eagle, supplemented with 15% FBS. Cells to be used for patch clamp recording were seeded onto either gelatin or rat tail collagen-coated 35-mm dishes and allowed to grow to confluence in basal medium, Eagle, 15% FBS. These cultures were mitogenically stimulated by either continued maintenance in basal medium, Eagle, 15% FBS, or by switching the medium to low glucose DMEM supplemented with 2% HS and 20 ng/ml bFGF. Cells were induced to differentiate via mitogen withdrawal by one of two methods, either changing the medium to low glucose DMEM supplemented with 2% HS for the entire growth period, or changing to DMEM supplemented with 2% HS and ITS medium supplemented (Sigma) for 24–48 h, followed by growth in DMEM, 2% HS for the balance of the experiment. Although ITS, 2% HS treatment results in a more robust morphological differentiation response compared with 2% HS alone, cells differentiated with ITS-supplemented medium begin to die after about 2–3 days in culture, restricting the time course for looking at channel induction in this system. All media contained 400 μg/ml G418 (Geneticin, Life Technologies, Inc.). All cultures were maintained in a humidified, 5% CO2 atmosphere at 37 °C.

Solutions and Reagents—The standard bath solution used for recording whole-cell SK and IRK currents, as well as single channel ACh receptor currents in outside-out patches contained (in mM): 138 NaCl, 9 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES. The patch pipette solution for these recordings contained (in mM): 150 KCl, 1 MgCl2, 10 HEPES, and 0.1 EGTA. For inside-out patch single channel recordings of calcium-activated potassium channels, the bath and pipette solutions had (in mM): 150 KCl, 1 MgCl2, 10 HEPES, 0.1 EGTA, with free calcium concentration adjusted by addition of CaCl2 (15). A23187 (Sigma), α-BTX (Molecular Probes), TEA, and ChTX (BACHEM Bioscience) were stored as frozen stocks and aliquots of each were diluted to final concentration on the day of use. Application of compounds to the solution changes to excised patches, was accomplished via pressure ejection of solution from blunt-tipped pipettes (5, 15). All solutions were at pH 7.3.

Electrophysiology—Patch clamp apparatus, techniques, and cell preparation for recording were as described previously (5, 15). Single channel amplitudes are means derived from Gaussian fits of amplitude histograms comprised of the number of openings as noted in the figure legends. Whole-cell SK currents were measured during voltage steps to 0 mV (from −70 mV), while intracellular calcium levels were increased either by extracellular application of A23187 (1 μM), or by increasing the free calcium concentration of the patch pipette solution to 10 μM. For inside-out patches SK channels were activated by perfusion of the intracellular patch face with 0.3 μM free calcium. IRK currents were measured during sequential voltage steps from −120 to −10 mV (500-ms duration, 10-mV increments) from a holding potential of −70 mV. Whole-cell ACh receptor currents were recorded at −70 mV in response to 1–100 μM ACh applied extracellularly for the times indicated. ACh receptor channel currents were recorded from outside-out patches at a series of holding potentials from −90 to −30 mV. For testing pharmacological agents the voltage at which current was maximal was used to repetitively evoke currents before, during, and after compound application. All recordings were done at 22–25 °C.

The analog compensation circuitry of the patch clamp amplifier was used to estimate whole-cell capacitances (expressed in picofarads, pF). Whole-cell currents are normalized to cell capacitance, an indirect measure of membrane surface area and can be expressed as a current density in picofarads/picocoulombs (pA/pF). 10T1/2-MRF4 cells begin to fuse to form myotubes after approximately 40 h growth under mitogen withdrawal. We observed up-regulation of ACh receptor and IRK currents only after 24 h of growth in differentiation medium, that is prior to fusion; so, we restricted our assay for these currents, at all points in the 0–3-day differentiation time course, to cells that appeared uninculturated under phase optics, i.e. cells that had not yet fused. For these experiments, whole-cell capacitance values for mitogen-stimulated (mean = 151 ± 0.6 pF, n = 90 cells) versus mitogen-withdrawn (mean = 13.8 ± 0.4 pF, n = 120) cells were not significantly different, suggesting that we recorded ACh receptor and IRK currents from nonfused cells. Thus the results with these currents reflect physiologic events that occur very early in the differentiation process independent of cell fusion. Mean capacitance values after 3–5 days of growth in differentiation conditions were (for cells with and without currents) 31.7 ± 3.8 (n = 20), 31.3 ± 3.4 (n = 15), and 35.4 ± 6.6 (n = 5) pF.

All statistical results are given as the mean ± S.E. Significant differences in current densities in response to various growth conditions were assessed by a two-tailed, nonpaired Student’s t test at the 0.05 level.

RESULTS

Mitogenic Stimulation with bFGF or High Serum Up-regulates the Calcium-activated Potassium Channel, SK, in 10T1/2-MRF4 Cells—We have previously shown that a ChTX-sensitive, calcium-activated potassium channel, SK, is functionally up-regulated by oncogenic ras or raf transformation of fibroblasts, including 10T1/2 cells (14, 15). The same channel can be induced by stimulation of nontransformed, serum-starved NIH 3T3 cells with either epidermal growth factor or platelet-derived growth factor (5). Fig. 1A shows 1 μM A23187 activation of an outward current recorded at 0 mV in a 10T1/2-MRF4 cell that had been grown under mitogenic stimulation (2% HS + bFGF at 20 ng/ml) for 24 h. We have previously shown in other fibroblast lines that the A23187-activated outward current recorded at 0 mV is exclusively SK, thus the outward current at 0 mV was measured in these cells to give an estimate of SK current density after 24 h (mean ± S.E. = 26.5 ± 4.0 pA/pF, n = 11 cells) and 72 h (20.8 ± 3.0 pA/pF, n = 14) of growth in 2% HS + bFGF. These current densities were clearly greater than the densities in cells grown under mitogen withdrawal (Fig. 1B, 6.8 ± 2.4 pA/pF, n = 12 after 24 h in 2% HS, 0.6 ± 0.6 pA/pF, n = 7 after 72 h in 2% HS). Mitogenic stimulation with high serum (15% FBS) also elevated SK density (13.4 ± 2.6 pA/pF, n = 11). Fig. 1C shows a whole-cell recording from a cell grown in 15% FBS, in which inclusion of 10 μM free calcium in the patch pipette solution activated the outward current at 0 mV. Extracellular application of ChTX blocked >95% of the current, confirming it as SK (experiment replicated for three cells grown in 15% FBS and three grown in bFGF). It should also be noted that in all cases the calcium-sensitive outward current activated immediately upon stepping to 0 mV and showed no voltage or time-dependent inactivation. Thus in all respects, the whole-cell characteristics of the bFGF or high serum up-regulated current in 10T1/2-MRF4 cells are consistent with those of the SK channel current previously described in the 10T1/2 and other fibroblast lines (5, 14, 15). The lack of calcium-sensitive outward current responses in cells grown under differentiating conditions indicates down-regulation of SK (5, 14), and it suggests that these conditions are insufficient for expression of the large conductance calcium-activated potassium channel that is found in mature skeletal muscle.

Inside-out patch single channel recordings from cells grown under bFGF mitogenic stimulation for 24 h were used to confirm the SK identification. In symmetric 150 mM KCl solutions and at a holding potential of −60 mV, increasing the free calcium concentration at the intracellular patch face from <0.1 to 0.3 mM evoked 2-pA inward current single channel openings (Fig. 1D, results are representative of eight patches). In addition, current-voltage plots for this channel type showed mild rectification (figure additional patches), with outward current single channel amplitudes at 60 mV being 1.1 pA, giving conductance values of 33 and 18 pS for −60 and 60 mV, respectively. Further, outward currents were eliminated when the solution at the internal patch face was switched to contain 150 mM NaCl in place of KCl. These results are consistent with
the amplitude and calcium-dependent behavior of the SK channel observed in ras transformed 10T1/2 parental and NIH and balb 3T3 lines (15). Together, the single channel and whole-cell experiments confirm SK as the predominant channel expressed in murine fibroblast cell lines, including 10T1/2-MRF4, and they further establish the close correlation between its up-regulation and mitogenic growth stimulation.

A23187 application (Fig. 1, A and B) or elevated free calcium in the whole-cell pipette solution (Fig. 1 C) also activated an inward current at −70 mV in 10T1/2-MRF4 cells, which we have previously reported for the 10T1/2 line (9). Since $E_{K}$ was set to −70 mV in these experiments (9 mM KCl in the bath and 150 mM in the patch pipette), this current is not likely to be SK. Even if $E_{K}$ was actually more positive due to incomplete equilibration of potassium concentrations between the intracellular compartment and the patch pipette solution, the inward current did not respond to ChTX (Fig. 1 C) (14), further arguing against SK. As for 10T1/2 cells, the inward current in 10T1/2-MRF4 was not sensitive to growth conditions. At present the ionic identity of this current and its mechanism of activation remain unresolved.

Mitogen Withdrawal, Which Removes MRF4 Negative Regulation, Results in Rapid Up-regulation of IRK and Expression of ACh Receptor Channel Currents in 10T1/2-MRF4, but Not 10T1/2 cells—Ectopic overexpression of MRF4 in 10T1/2 cells transforms them into muscle precursors, but the potential to undergo myogenesis via MRF4-dependent transcriptional activation requires differentiating growth conditions, i.e. the withdrawal of mitogen stimulation (13). Thus in the presence of certain mitogens such as bFGF and high serum, there is negative regulation of MRF4-dependent transcriptional activation and thus a lack of muscle specific gene expression (18). Fig. 2A shows that under mitogenic stimulation an IRK channel is expressed at comparably low levels in both 10T1/2-MRF4 (3.6 ± 1.2 pA/pF, n = 9 cells in 15% FBS, 3.9 ± 1.3, n = 10 in 2% HS + bFGF) and 10T1/2 lines (2.9 ± 0.6 pA/pF, n = 28 in 15% FBS). Unlike the SK current, however, the IRK current does not appear to be typical of fibroblast lines, as it is not
present in NIH or \textit{balb} 3T3 fibroblasts grown under mitogenic or nonmitogenic conditions (data not shown). Furthermore, in response to mitogen withdrawal, a condition under which SK is down-regulated and negative regulation of MRF4 transcriptional activity is removed, IRK current density increased significantly in 10T1/2-MRF4 cells (11.4 ± 3.1 pA/pF, \( n = 10 \) in 24-h 2\% HS, 19.9 ± 5.9 pA/pF, \( n = 16 \) in 24-h ITS, 49.8 ± 16.6 pA/pF, \( n = 13 \) in 48-h ITS). Mitogen withdrawal had no effect on IRK current in 10T1/2 cells (1.8 ± 0.4 pA/pF, \( n = 10 \) cells in 24-h ITS) (Fig. 2A). Thus IRK up-regulation in response to mitogen withdrawal occurred only in 10T1/2 cells expressing MRF4.
SK-regulated Ion Channel Expression in 10T1/2-MRF4 Cells

The IRK current up-regulated in 10T1/2-MRF4 cells in response to differentiating growth conditions (2% HS or ITS) has characteristics of classical muscle-type, strong inward rectifier currents. It displayed rapid voltage-dependent activation at potentials negative to $E_K$, and showed little or no activation at more positive potentials. Time-dependent inactivation of the current became evident at command potentials more negative than $-90 \text{ mV}$, and the rate of inactivation increased from $-90$ to $-120 \text{ mV}$ (Fig. 2A). From a holding potential of $-70 \text{ mV}$, currents evoked by steps to $-120 \text{ mV}$ had a mean inactivation time constant of $2370 \pm 149 \text{ ms}$ (12 cells). In addition to these properties, 10T1/2-MRF4 cell IRK current was pharmacologically similar to muscle IRK. 10 mM TEA blocked the current in both mitogen-stimulated and mitogen-withdrawn 10T1/2-MRF4 cells by similar amounts ($25 \pm 3 \%$ and $33 \pm 3 \%$, $n = 7$ cells each) (Fig. 2B). As for muscle IRK currents, barium was a very effective blocker of IRK current expressed in 10T1/2-MRF4 cells. For mitogenically stimulated and mitogenically withdrawn cells, 100 $\mu M$ barium reduced IRK current measured at $-120 \text{ mV}$ by the same amount, $97 \pm 1 \%$ (6 and 18 cells, respectively). Therefore, IRK currents recorded from 10T1/2 cells were in all ways identical to those in 10T1/2-MRF4 cells, with the exception that mitogen withdrawal did not up-regulate IRK current density in nontransfected 10T1/2 cells.

To directly test for MRF4-dependent up-regulation of nicotinic ACh receptor channels, another measure of muscle-specific gene expression, whole-cell ACh receptor currents were recorded under several growth conditions (Fig. 3). We looked for the presence of ACh receptor channels as a function of MRF4 activation by recording from 10T1/2-MRF4 cells grown under either mitogenic or differentiating conditions. Extracellular application of 100 $\mu M$ ACh revealed low levels of ACh receptor channels in 10T1/2-MRF4 cells grown in 2% HS + bFGF (5.9 $\pm$ 3.6 $\text{pA/pF}$, 3 of 18 cells responding), relative to cells grown in ITS (117.0 $\pm$ 40.0 $\text{pA/pF}$, 14 of 16 cells responding), or 2% HS alone (116.9 $\pm$ 37.4 $\text{pA/pF}$, 8 of 10 cells responding). In the presence of $\alpha$-BTX (5 $\mu M$) ACh current responses from differentiated 10T1/2-MRF4 cells were completely eliminated (eight cells). Therefore, culture conditions which remove negative regulation of MRF4 also cause up-regulation of nicotinic ACh receptor channels. This effect is clearly MRF4-dependent, since 10T1/2 cells grown in ITS were completely unresponsive to ACh application (nine cells). These data are in agreement with previous findings that show muscle regulatory factors are necessary for the activation of the myogenic program and more specifically of muscle specific genes, in this case those for the nicotinic ACh receptor channel.

During development of the mammalian neuromuscular junction there is a switch in ACh receptor expression from embryonic to adult form, based on substitution of $\gamma$ by $\epsilon$ subunits (6, 19). Adult receptors characteristically have larger conductances (60–65 pS) than embryonic receptors (42–45 pS). Fig. 4 shows that 10T1/2-MRF4 cells differentiated with ITS, 2% HS for 48 h exhibit a low conductance type ACh receptor channel (42–44 pS, $n = 4$ patches), characteristic of an embryonic type channel.

The SK Channel Blocker Charybdotoxin Overrides bFGF-induced Inhibition of IRK and ACh Receptor Channel Functional Expression—Mitogen withdrawal from 10T1/2-MRF4 cells decreases SK levels coincident with MRF4-dependent muscle gene activation and myogenic differentiation. If SK activity is a necessary component of mitogenic negative regulation of MRF4, then ChTX should override this negative regulation and initiate myogenic events such as IRK and ACh receptor channel expression. Therefore, we measured whole-cell IRK and ACh receptor current densities from serum-withdrawn cells, chronically treated with bFGF + ChTX, and compared them to the densities in cells treated with bFGF alone.

Current densities for both IRK and ACh receptor channels were significantly increased when ChTX was present in bFGF-containing growth medium. Extracellular application of 100 $\mu M$ ACh revealed low levels of ACh receptor channels in 10T1/2-MRF4 cells grown in 2% HS + bFGF for 1, 3, or 5 days (mean $\pm$ S.E. $= 5.9 \pm 3.6 \text{ pA/pF}$, $n = 18$; 0.7 $\pm$ 0.7 $\text{pA/pF}$, $n = 7$; 0.5 $\pm$ 0.5 $\text{pA/pF}$, $n = 9$), relative to cells grown in 2% HS + bFGF + ChTX (54.6 $\pm$ 35.3 $\text{pA/pF}$, $n = 9$; 97.7 $\pm$ 54.3 $\text{pA/pF}$, $n = 10$; 97.4 $\pm$ 54.7 $\text{pA/pF}$, $n = 11$) or 2% HS alone as a positive control (116.9 $\pm$ 37.4 $\text{pA/pF}$, $n = 10$; 104.3 $\pm$ 38.7 $\text{pA/pF}$, $n = 10$; 120.0 $\pm$ 41.5 $\text{pA/pF}$, $n = 10$) (Fig. 5A). The ACh activated inward currents observed in cells treated with bFGF + ChTX were blocked by 5 $\mu M$ $\alpha$-BTX (data not shown), confirming them as authentic nicotinic ACh receptor channel responses. Thus ChTX overcame bFGF-induced negative regulation of ACh receptor channel expression.

The results with ChTX up-regulation of ACh receptor channels were paralleled by results for IRK current. For cells grown for 24 h in bFGF and ChTX, peak IRK current amplitudes at $-120 \text{ mV}$ were normalized to cell capacitance (see Fig. 2),
giving a mean current density of 16.7 ± 2.6 pA/pF (n = 11 cells), a significant increase over the density for cells grown in bFGF alone (3.0 ± 0.9 pA/pF, n = 8). After 48-h growth in bFGF and ChTX, mean IRK density was 22.3 ± 4.6 pA/pF, a significant increase compared with cells grown 48 h in bFGF (3.9 ± 1.3 pA/pF). Indeed, the increase in IRK density as a result of chronic ChTX application was comparable to the increase observed when mitogenic stimulation was removed by placing cells in ITS medium for 24 h, a potent differentiation condition. The IRK current present in cells grown with bFGF and ChTX was blocked by 100 μM barium, and it was kinetically identical to the currents recorded from either mitogenically stimulated or withdrawn 10T1/2 and 10T1/2-MRF4 cells.

The ability of ChTX to evoke increases in IRK and ACh receptor channel densities appears to be a function of its blocking action versus SK, combined with the apparent regulatory function of the SK channel for MRF4-dependent transcription. ChTX blocks only potassium channels, and it had no effect on whole-cell IRK currents in bFGF-stimulated 10T1/2-MRF4 cells (data not shown), indicating that SK was the only channel target for ChTX under these conditions. Furthermore, SK channel density remained elevated in the presence of chronic ChTX treatment. After washing off the ChTX-containing growth medium, standard whole-cell recordings and A23187 applications were performed to compare SK current densities in the cells treated as shown in Fig. 5. Mean SK current densities at 1, 3, and 5 days in cells treated with bFGF alone were 26.5 ± 4.0 (n = 11), 20.8 ± 3.0 (n = 14), and 22.1 ± 4.2 pA/pF (n = 18), while in cells treated with bFGF + ChTX the densities were 32.7 ± 10.4 (n = 10), 50.9 ± 11.3 (n = 9), and 13.8 ± 6.0 (n = 10). So SK current densities remained elevated in cells treated with either bFGF or bFGF + ChTX, indicating that the bFGF-activated signaling pathways responsible for maintaining elevated SK levels were unaffected by the toxin. This suggests that signaling from the bFGF receptor, which should negatively regulate MRF4, was also intact; nonetheless ChTX overcame this negative regulation. Further, the presence of MRF4 was obligatory for ChTX to cause up-regulation and induction of IRK and ACh receptor channels, since treatment of 10T1/2 cells with bFGF + ChTX did not change IRK levels, and it did not induce ACh receptor expression. Thus in bFGF-treated 10T1/2-MRF4 cells, ChTX functional blockade of the SK channel was sufficient to allow induction of nicotinic ACh receptor channels, paralleling the ACh receptor induction observed when SK was down-regulated due to bFGF withdrawal.

**REFERENCES**

This study has identified several changes in the electrophysiological phenotype of 10T1/2 cells that occur prior to and during myogenic initiation under the control of the ectopically expressed muscle regulatory factor, MRF4. The major current found in mitogenically stimulated 10T1/2-MRF4 myoblasts, in which MRF4 activity is negatively regulated and myogenesis is suppressed, is due to an SK channel. This channel appears identical to the SK channel previously associated with positive control of mitogenesis in other fibroblasts (14–17). Calcium-activated potassium channels, specifically SK, have been correlated with proliferative control in nonexcitable cells (1, 3), including murine fibroblasts similar to the 10T1/2 parental line (5). We have shown in NIH 3T3 cells that the partial mitogen epidermal growth factor produces only transient SK up-regulation, while the full mitogen platelet-derived growth factor produces persistent SK up-regulation. Like platelet-derived growth factor in NIH 3T3 cells, bFGF produces persistent SK up-regulation in 10T1/2-MRF4 cells, consistent with maintenance of a proliferative versus a differentiated cell state.

In addition to down-regulation of SK, removal of MRF4 negative regulation via mitogen withdrawal causes rapid up-regulation of IRK and induction of ACh receptor channels in 10T1/2-MRF4 cells. Up-regulation of IRK is characteristic of mammalian muscle cell differentiation (20–23), and characterization of the current present in 10T1/2-MRF4 cells shows it to be of the classical muscle type. It displays rapid activation, progressively faster time-dependent inactivation as a function of increasing membrane potentials beyond −90 mV, and block by external TEA and barium. Kubo (20) also observed IRK current in the 10T1/2 line and found it to be up-regulated in 10T1/2 cells expressing a muscle phenotype. These cells were selectively subcloned based on their fusogenic potential provoked by 5-aza-2′-deoxycytidine-induced DNA hypomethylation, and resultant transcriptional activation of muscle specific genes. As a result the cells in which IRK up-regulation was observed had likely been expressing muscle genes for weeks, as dictated by the subcloning procedure. Our results suggest that MRF4 activation per se has a very immediate and dramatic effect on IRK channel expression, indicating that IRK up-regulation is likely to occur very early on in muscle cell differentiation. In addition, they suggest that MRF4 may transcriptionally activate the muscle IRK channel gene. The induction of nicotinic ACh receptor channels coincident with MRF4 activation is consistent with the finding that MRF4 efficiently trans-activates an ACh receptor reporter gene construct in 10T1/2-MRF4 cells (24). However, the present study is the first to show MRF4-dependent functional expression of the endogenous channel gene. The single channel data show that mitogen-deprived 10T1/2-MRF4 cells express ACh-activated channels of relatively low conductance, similar to embryonic channels in authentic muscle. Although the number of recordings was limited, this result provides preliminary evidence that MRF4 induces the embryonic ACh receptor subtype that is contingent on expression of the γ subunit (versus the adult ε subunit) (6, 19).
Mitogen withdrawal removes MRF4 negative regulation resulting in IRK and ACh receptor expression and ultimately complete myogenic differentiation; however, the signaling pathways underlying mitogen control of MRF4 have yet to be identified. Two findings now suggest that the SK channel may be an important component of this signaling. First, mitogen withdrawal results in down-regulation of SK coincident with initiation of the myogenic program. Second, we find that ChTX functional block of the SK channel in bFGF-stimulated 10T1/2-MRF4 cells up-regulates IRK and induces ACh receptor channel expression, indicating that SK contributes to the negative regulation of MRF4 imposed by the mitogen. Inclusion of ChTX in the bFGF-containing growth medium up-regulated IRK and induced ACh receptor channel expression as early as 24 h following the start of toxin treatment. Furthermore, ACh receptor currents could be recorded in cell cultures maintained in bFGF + ChTX for as long as 5 days. Thus in terms of IRK and ACh receptor expression, SK channel functional blockade precisely mimics the removal of MRF4 negative regulation seen with mitogen withdrawal, suggesting that the SK channel contributes to mitogen regulation of transcriptional control via MRF4 and possibly other muscle regulatory factors. Mitogenic stimulation of human muscle satellite cells in vitro also has been shown to up-regulate a calcium activated potassium conductance and prevent myogenic progression (25). Like SK, this current is blocked by ChTX and TEA, and it is insensitive to apamin (although it displays voltage dependence more typical of large conductance, calcium-activated potassium channels). The coincidence of calcium-activated potassium conductance up-regulation with mitogenic stimulation in satellite cells, fibroblasts, and T lymphocytes (26) suggests conservation of a central role for calcium-activated potassium channels in mitogenic signaling events in mesoderm-derived cells. The results of the present study with the 10T1/2-MRF4 myogenic model provide the first suggestive evidence linking SK channel activity to control of a defined transcriptional regulatory complex. In this way, they begin to establish a possible mechanism by which SK could affect the fundamental cellular events of proliferation and myogenic differentiation. Coupling between SK activity and transcriptional control could rely on the channel’s ability to set the membrane potential and indirectly influence voltage-independent calcium influx, an important element in mitogenic stimulation.

Krause et al. (9) observed a large up-regulation of ACh receptor density in cultured human myoblasts when they were switched from proliferative to differentiating growth conditions, but before fusion to myotubes. Our results are comparable to theirs in that we recorded large ACh receptor currents from nonfused uninucleate cells which had been growing in conditions that induce differentiation, again indicating that ACh receptor induction, like IRK up-regulation, is well underway prior to fusion. Indeed, it has been suggested that ACh receptor expression and subsequent stimulation accelerate the fusion process in cultured human myoblasts. The finding that MRF4-dependent IRK and ACh receptor expression is subject to control by the SK channel further suggests that ion channel activity serves to regulates myogenic differentiation as well as to mark its progression. The 10T1/2 and 10T1/2-MRF4 cell lines provide a compelling model system in which to explore the mechanisms by which SK and other channel types perform this regulatory function.
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REFERENCES
1. Leonard, R. J., Garcia, M. L., Slaughter, B. S., and Reuben, J. P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10094–10098
2. Day, M. L., Pickering, S. J., Johnson, M. H., and Cook, D. I. (1993) Nature 365, 560–562
3. Dubois, J. M., and Rouzaire-Dubois, B. (1993) Prog. Biophys. Mol. Biol. 59, 1–21
4. Wang, Z., Estacion, M., and Mordan, L. J. (1993) Am. J. Physiol. 265, C1239–C1246
5. Huang, Y., and Rane, S. G. (1994) J. Biol. Chem. 269, 31183–31189
6. Kopta, C., and Steinbach, J. H. (1994) J. Neurosci. 14, 3922–3933
7. Duclert, A., and Changeux, J. P. (1995) Physiol. Rev. 75, 339–368
8. Hu [Missing reference]
9. Krause, R. M., Hamann, M., Bader, C. R., Liu, J.-H., Baroffio, A., and Ber [Missing reference]
10. Su, C. T., Huang, C. F., and Schmidt, J. (1995) FEBS Lett. 366, 131–136
11. Reznikoff, C. A., Brankow D. W., and Heidelberger, C. (1973) Cancer Res. 33, 3231–3238
12. Olson, E. N. (1992) Dev. Biol. 154, 261–272
13. Rhodes, S. J., and Konieczny, S. F. (1989) Genes Dev. 3, 2050–2061
14. Rane, S. G. (1991) Am. J. Physiol. 260, C104–C112
15. Huang, Y., and Rane, S. G. (1993) J. Physiol. 461, 601–618
16. Repp, H., Draheim, H., Ruland, J., Seidel, G., Beise, J., Fresek, P., and Dreyer, F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3403–3407
17. Draheim, H. J., Repp, H., and Dreyer, F. (1995) Biochim. Biophys. Acta 1269, 57–63
18. Hardy, S., Kong, Y., and Konieczny, S. F. (1993) Mol. Cell. Biol. 13, 5943–5956
19. Camacho, P., Liu Y., Mandel G., and Breym, P. (1993) J. Neurosci. 13, 605–613
20. Kubo, Y. (1991) J. Physiol. 442, 711–741
21. Trautmann, A., Delaporte, C., and Marty, A. (1986) Pflugers Arch. 406, 163–172
22. Genoi, T., and Hasegawa, S. (1991) Pflugers Arch. 419, 657–661
23. Wieland, S. J., and Gong, Q.-H. (1995) Am. J. Physiol. 268, C490–C495
24. Yutzey, K. E., Rhodes, S. J., and Konieczny, S. F. (1990) Mol. Cell. Biol. 10, 3934–3944
25. Hamann, M., Widmer, H., Baroffio, A., Aubry, J.-P., Krause, R. M., Kaelin, A., and Bader, C. R. (1994) J. Physiol. 475, 305–317
26. Lewis, R. S., and Calahan, M. D. (1995) Annu. Rev. Immunol. 13, 623–653