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Initiation of human myoblast differentiation via dephosphorylation of Kir2.1 K^+ channels at tyrosine 242

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Myoblast differentiation is essential to skeletal muscle formation and repair. The earliest detectable event leading to human myoblast differentiation is an upregulation of Kir2.1 channel activity, which causes a negative shift (hyperpolarization) of the resting potential of myoblasts. After exploring various mechanisms, we found that this upregulation of Kir2.1 was due to dephosphorylation of the channel itself. Application of genistein, a tyrosine kinase inhibitor, increased Kir2.1 activity and triggered the differentiation process, whereas application of bpV(Phen), a tyrosine phosphatase inhibitor, had the opposite effects. We could show that increased Kir2.1 activity requires dephosphorylation of tyrosine 242; replacing this tyrosine in Kir2.1 by a phenylalanine abolished inhibition by bpV(Phen). Finally, we found that the level of tyrosine phosphorylation in endogenous Kir2.1 channels is considerably reduced during differentiation when compared with proliferation. We propose that Kir2.1 channels are already present at the membrane of proliferating, undifferentiated human myoblasts but in a silent state, and that Kir2.1 tyrosine 242 dephosphorylation triggers differentiation.

KEY WORDS: Human myoblasts, Hyperpolarization, Myoblast differentiation, Potassium channel, Tyrosine phosphorylation

INTRODUCTION

Myoblast differentiation is a multi-step process involving withdrawal from the cell cycle, transcriptional activation of muscle-specific genes, and eventually cell fusion into multinucleated myotubes. Little is known about the signals that control the induction of the differentiation process and our goal was to uncover the molecular mechanism that initiates this induction in human myoblasts.

We have previously shown that human myoblasts must hyperpolarize before they can differentiate (Liu et al., 1998), and that this hyperpolarization occurs via an increased activity of Kir2.1 K^+ channels (Fischer-Lougheed et al., 2001). Hyperpolarization of myoblasts induces a Ca^{2+} influx that is an essential early step of the differentiation process (Bijlenga et al., 2000). We have also established that Kir2.1 channel activation precedes and triggers the expression of myogenin and MEF2, two key transcription factors of the myogenic differentiation program. Kir2.1 channels are upregulated within the first 6 hours of the differentiation process, i.e. several hours before myogenin and MEF2 expression (Konig et al., 2004). To our knowledge, Kir2.1 activation is, so far, the earliest detectable event during human myoblast differentiation.

In the present study, we evaluated on primary cultures of human myoblasts various potential molecular mechanisms that could control Kir2.1 channel expression and/or activity at the onset of the differentiation process. We tested whether Kir2.1 channels were regulated at the level of protein synthesis, vesicular trafficking (from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane), or whether the activity of the channel at the membrane was modulated.

Several types of ionic channels are known to be expressed or repressed during developmental programs, and this is also the case of Kir2.1 channels. For example, several domains in the chicken Kir2.1 promoter regulate the tissue-specific expression of this channel (Mutai et al., 2004). It has also been shown that electrical activity is required for the stability of Kir2.1 mRNA into skeletal muscle cells (Shin et al., 1997). Furthermore, growth factors can regulate the transcription rate of several muscle channels (for example T-type Ca^{2+} channel) (Avila et al., 2006).

With respect to trafficking, Kir2.1 protein contains ER-export motifs that control the targeting of channels to the plasma membrane. These motifs are likely to act as recognition signals for its incorporation into COP-II coated transport vesicles. They were described to be necessary and sufficient for channel export without need for additional interacting partners (Ma et al., 2001; Stockklausner et al., 2001) (but see Grishin et al., 2006). Signaling pathways like the Ras-MAPK pathway also appear to act on Kir2.1 channels trafficking (Giovannardi et al., 2002). In addition, binding sites for anchoring proteins such as the filamin A (Sampson et al., 2003), PSD93δ (Leyland and Dart, 2004) or SAP97 (Leonoudakis et al., 2004) are thought to stabilize Kir2.1 channels at the plasma membrane.

Alternatively, the gating properties of ionic channels at the plasma membrane can be modulated by various intra- and extracellular signaling pathways. Members of the inward rectifying K^+ channel family possess multiple PIP_2-binding sites that are known to be key regulators of their gating properties (Rohacs et al., 2003; Soom et al., 2001). Furthermore, Kir2.1 can be modulated by PKA (Wischmeyer and Karschin, 1996), PKC (Fakler et al., 1994; Jones, 2003) and receptor-activated tyrosine kinases (Hoger et al., 2002; Ruppersberg, 2000; Ruppersberg and Fakler, 1996; Wischmeyer et al., 1998). Recently, it has been suggested that kinases and phosphatases can be intimately associated to channels in a single regulatory protein complex that modulates channel activity (Levitan, 2006).

Using whole-cell patch-clamp recording, we found that Kir2.1 channels activated at the onset of the differentiation process are already present at the plasma membrane during proliferation, but that they are silent. Their activity is induced by dephosphorylation of Kir2.1 tyrosine 242.

MATERIALS AND METHODS

Reagents

Genistein and bpV(Phen) were from Calbiochem (San Diego, California), Brefeldin A (from Penicillium brefeldianum) and cycloheximide from Sigma. Rabbit polyclonal Kir2.1 antibodies were from Alomone Labs

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(Jerusalem, Israel), and chicken polyclonal anti-Kir2.1 IgY were produced by Covalab (Lyon, France). Peroxidase-conjugated donkey anti-chicken IgY were from Jackson Immunoresearch Labs, and mouse monoclonal anti-phosphotyrosine antibody (clone PT-66) from Sigma.

Cell cultures

Clonal cultures of human myoblasts were prepared from single satellite cells and expanded as described by Liu et al. (Liu et al., 1998).

Electrophysiological recordings

Kir2.1 currents were measured in the whole-cell configuration of the patch-clamp technique as in Konig et al. (Konig et al., 2004). During intracellular application of bpV(Phen) through the patch pipette, we decreased the amount of BAPTA in the intracellular solution to 0.2 mM because vanadates are sensitive to Ca^{2+} chelators and Ca^{2+} concentration (Huyer et al., 1997). In these experiments, $CaCl_2$ was added to reach a final concentration of 200 nM, and KCl was increased to maintain osmolarity.

Metabolic labeling

Myoblasts were kept in methionine-free medium for 15 minutes with or without cycloheximide (3 μg/ml). [^{35}S]-methionine (12.5 μCi / well containing 10^5 myoblasts) was then added for 30 minutes. Cells were lysed in triton buffer (PBS, Triton X-100 0.5%, 5 mM EDTA) and proteins were precipitated by TCA before to be mixed with liquid scintillation cocktail (Ready Value). Radioactivity was measured on scintillation counter (Beckman Coulter).

DNA construct and transfection

Mutations of the human pcEGFP-Kir2.1 tyrosines 242, 336 and 366 into phenylalanine residues were carried out with the QuickChange Kit (Stratagene). Mutations were verified by sequencing. Transfections were performed as described by Espinos et al. (Espinos et al., 2001). Proliferating myoblasts were electroporated with pcEGFP-Kir2.1, with the mutants pcEGFP-Kir2.1$_{Y242F}$, pcEGFP-Kir2.1$_{Y336F}$ or pcEGFP-Kir2.1$_{Y366F}$, or, for control experiments, with pcEGFP, and maintained 24 hours in growth medium before electrophysiological recording or other treatments.

Membrane enrichment

Cultured myoblasts were lysed with a hypotonic buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1% NP-40) with protease inhibitors and 1 mM Na_3VO_4. Nuclei were removed and cell extracts were then ultracentrifuged for 1 hour at 100,000 ***g*** at 4°C. Crude membranes pellets were resuspended in hypotonic buffer and used for immunoprecipitation. Protein content was calculated using a Bradford assay (BioRad).

Immunoprecipitation

Crude membrane proteins (10 mg) from non-transfected cells or total lysates (100 μg) from transfected cells were precleared on proteinA-sepharose (Sigma) before incubation with 3.6 μg rabbit anti-Kir2.1 for 2 hours at 4°C. ProteinA-sepharose beads (50 μl) were used to immunoprecipitate Kir2.1 proteins (overnight at 4°C). The beads were pelleted by centrifugation (10,000 ***g*** for 1 minute), and SDS-βmercaptoethanol sample buffer was used to separate immunoprecipitated proteins from the beads (3 minutes at 100°C).

Biotinylation of cell surface proteins

Myoblasts were incubated for 20 minutes with 0.25 mg/ml NHS-Biotin diluted in PBS at 4°C. NHS-Biotin was then quenched for 15 minutes with 40 mM NH_4Cl/PBS at 4°C. For the separation of biotinylated proteins, the same amount of proteins from the different lysates was incubated for 1 hour at 4°C with 50 μl of streptavidin magnetic beads (Roche). Non-biotinylated proteins were separated from the beads with a magnetic particle separator. The beads were then washed three times with hypotonic buffer. Biotinylated proteins were recovered in 50 μl of SDS-βmercaptoethanol sample buffer. After 3 minutes at 100°C, beads were removed with the magnetic separator.

Western blotting

Aliquots were analyzed by western blot as in Konig et al. (Konig et al., 2004). Membranes were incubated with rabbit polyclonal anti-Kir2.1 (1:200), mouse monoclonal anti-phosphotyrosine (clone PT-66, 1:3000) or chicken polyclonal anti-Kir2.1 antibodies (1:250). Primary antibodies were then exposed to horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-chicken antibodies (1:6000).

TIRF measurement

Tracking of membrane-inserted Kir2.1-GFP channels was obtained from total internal reflection fluorescence (TIRF) images as in Marthinet et al. (Marthinet et al., 2005). Image analysis was performed using MetaMorph software.

Statistics

Results are expressed as the mean±s.e.m. Statistical analyses were performed using Student's *t*-test (asterisk in figures indicates $P<0.05$).

RESULTS

Early Kir2.1 channel activity during initial differentiation is neither due to channel synthesis nor to channel incorporation into the plasma membrane

In human myoblasts, Kir2.1 current density reaches its maximum already after an exposure of 6 hours to differentiation medium (Konig et al., 2004). Fig. 1A illustrates a Kir2.1 current elicited at various potentials and its inhibition by 500 μM $BaCl_2$. The current-to-voltage relationships show the typical inward rectification of the Kir2.1 channels (E_K=–84 mV in our recording conditions). In order to determine whether Kir2.1 channel activation at the beginning of the differentiation process was due to new channel synthesis, we inhibited de novo protein synthesis with cycloheximide and evaluated the Kir2.1 channel activity after 6 hours of differentiation (Fig. 1B). Efficiency of the drug was established by measuring [^{35}S]-methionine incorporation. Application of 3 μg/ml cycloheximide for 3 hours blocked 81±6% of total new protein synthesis. Kir2.1 channel activity was measured in myoblasts maintained in differentiation medium supplemented with 3 μg/ml cycloheximide for 6 hours and compared with the Kir2.1 activity measured in myoblasts kept in control differentiating conditions for the same time. The fraction of myoblasts maintained in cycloheximide and expressing a Kir2.1 current was not statistically different from that of myoblasts maintained in control conditions (control=53±9%, n=28, cycloheximide=34±6%, n=46, P=0.117). The Kir2.1 current density of myoblasts kept 6 hours with cycloheximide was, however, smaller than that of myoblasts in control conditions (control=–1.1±0.3 pA/pF, n=28, cycloheximide=–0.4±0.1 pA/pF, n=46, P=0.002, but see below).

In parallel experiments, we assessed Kir2.1 channel activity in the presence of 10 μg/ml Brefeldin A, an inhibitor of endoplasmic reticulum (ER) to Golgi transport (Fig. 1C). This was done to evaluate whether the Kir2.1 channel activation during the 6 first hours of differentiation could be due to a translocation of channels from the ER to the plasma membrane. No statistical difference was observed in the presence of the drug when compared with control. The fraction of myoblasts presenting Kir2.1 current (control=42±6%, n=19, Brefeldin A=40±7%, n=46, P=0.862), as well as the Kir2.1 current density, was similar in both conditions (control=–0.7±0.2 pA/pF, n=19, Brefeldin A=–0.5±0.1 pA/pF, n=46, P=0.558). To verify that Brefeldin A at 10 μg/ml was efficiently inhibiting the ER-to-Golgi transport, myoblasts were transfected with the fusion proteins Kir2.1-GFP, and newly synthesized GFP-channels visualized by confocal microscopy. Unlike what was observed in the control conditions, in the presence of the Brefeldin A most fluorescence was located around the nucleus, demonstrating the efficiency of the drug at blocking the transport from the ER to the plasma membrane.

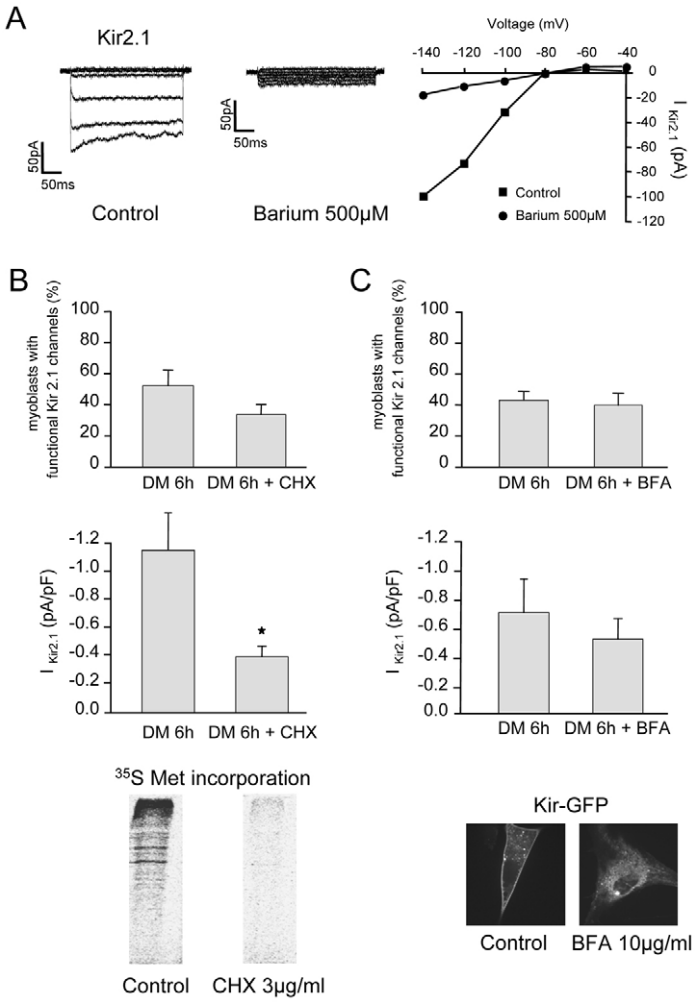

Fig. 1. Kir2.1 channels are neither newly synthesized nor transported to the plasma membrane during the first 6 hours of differentiation. (**A**) Typical Kir2.1 current recorded from one myoblast cultured for 6 hours in differentiation medium (DM). Ba^{2+} (500 μM), as expected, inhibited the current. Voltage-steps were to –40, –60, –80, –100, –120 and –140 mV from a holding potential at –60 mV. Current-to-voltage relationships are shown in control condition and in the presence of 500 μM Ba^{2+}. (**B**) Myoblasts were cultured in DM for 6 hours with or without 3 μg/ml cycloheximide (CHX). Kir2.1 current amplitude was assessed during a 300 ms step to –120 mV from a holding potential at –60 mV. The upper histogram represents the fraction of myoblasts expressing Kir2.1 current (⩾5 pA) and the lower histogram the current density of the total population of myoblasts (including myoblasts with no current, i.e. <5 pA) in control and in cycloheximide conditions. Both histograms are from the same recordings. Leak currents were estimated either by linear extrapolation from the currents recorded at –40 and –60 mV or by addition of 500 μM Ba^{2+} to the external bath solution, and subtracted to the total current. Twenty-eight myoblasts (out of three different clones) with a mean cell capacitances of 32±3 pF were recorded under control conditions, and 46 myoblasts (out of three different clones) with a mean cell capacitances of 27±2 pF in the presence of cycloheximide. Asterisk indicates $P<0.05$ (and in subsequent figures). [^{35}S]-methionine incorporation was performed in the absence or presence of cycloheximide 3 μg/ml during 3 hours to evaluate protein synthesis. (**C**) Myoblasts were cultured in DM for 6 hours with or without 10 μg/ml Brefeldin A (BFA). Histograms represent the fraction of myoblasts with Kir2.1 current and the Kir2.1 current density as in B. Both histograms are from the same recordings. Nineteen myoblasts (out of four different clones) with a mean cell capacitances of 34±3 pF were recorded in control conditions, and 46 myoblasts (out of three different clones) with a mean cell capacitances of 23±2 pF in the presence of Brefeldin A. Representative pictures (confocal microscopy) of myoblasts transfected with Kir2.1-GFP and incubated immediately after transfection in the presence or absence of 10 μg/ml Brefeldin A for 12 hours. In the presence of Brefeldin A, Kir2.1-GFP channels do not reach the plasma membrane.

These data strongly suggest that Kir2.1 channel activation at the onset of differentiation is not due to protein synthesis or trafficking regulations, the two sets of experiments complementing and strengthening each other. Indeed, if a new synthesis of Kir2.1 channels would have taken place during the first 6 hours of differentiation, we should have observed a diminution of the Kir2.1 current after treatment with Brefeldin A. As we did not detect any reduction of the current in the presence of Brefeldin A, we conclude that Kir2.1 activation is not due to a new synthesis followed by transport to the plasma membrane. The diminution of Kir2.1 current density observed in myoblasts kept 6 hours with cycloheximide could be due to either an effect of the drug on channel activity or to an intrinsic instability of the required phosphatase (see below), but not on the inhibition of Kir2.1 protein synthesis.

Kir2.1 channel activation and the fusion process are modulated by a tyrosine phosphorylation

Translational or trafficking regulations being unlikely mechanisms to explain the increase of Kir2.1 channel activity, we then postulated that a pool of Kir2.1 channels could be maintained inactivated at the plasma membrane during proliferation, and that Kir2.1 activation would be the consequence of a functional modulation. It is known that Kir2.1 channels possess a phosphorylation site on the tyrosine 242, and that its phosphorylation inhibits Kir2.1 channel activity (Wischmeyer et al., 1998). To investigate whether such a mechanism could be involved during myoblast differentiation, we tested Kir2.1 current activation in the presence of either bpV(Phen), a tyrosine phosphatase inhibitor, or genistein, a tyrosine kinase inhibitor. As shown in Fig. 2A, the proportion of myoblasts expressing a Kir2.1 current is strongly reduced in the presence of the tyrosine phosphatase inhibitor bpV(Phen). A Kir2.1 current could be recorded in only 10% (2 out of 19) of the myoblasts placed for 6 hours in differentiation medium supplemented with 10 μM bpV(Phen). By contrast, in the control differentiation conditions, 49% of the tested myoblasts had Kir2.1 current (23 out of 47). We should point out that, in the presence of bpV(Phen), the percent of myoblasts with Kir2.1 current is similar to that observed during normal proliferation (Konig et al., 2004). The current density of bpV(Phen)-treated myoblasts was reduced to one tenth of that of non-treated myoblasts ($P=0.005$, Fig. 2A, inset).

We then added genistein, a tyrosine kinase inhibitor, to the differentiation medium; application of 10 μM genistein during 4 hours drastically increased the number of Kir2.1-positive cells. It is important to mention that Kir2.1 channels are not yet activated after 4 hours in differentiation conditions (Konig et al., 2004). Indeed, whereas 45% of the myoblasts (14 out of 31) presented a Kir2.1 current in the presence of the drug, only 14% of the myoblasts (3 out of 22) had measurable Kir2.1 current in the control conditions (Fig. 2B). Consistently, the current density of genistein-treated myoblasts

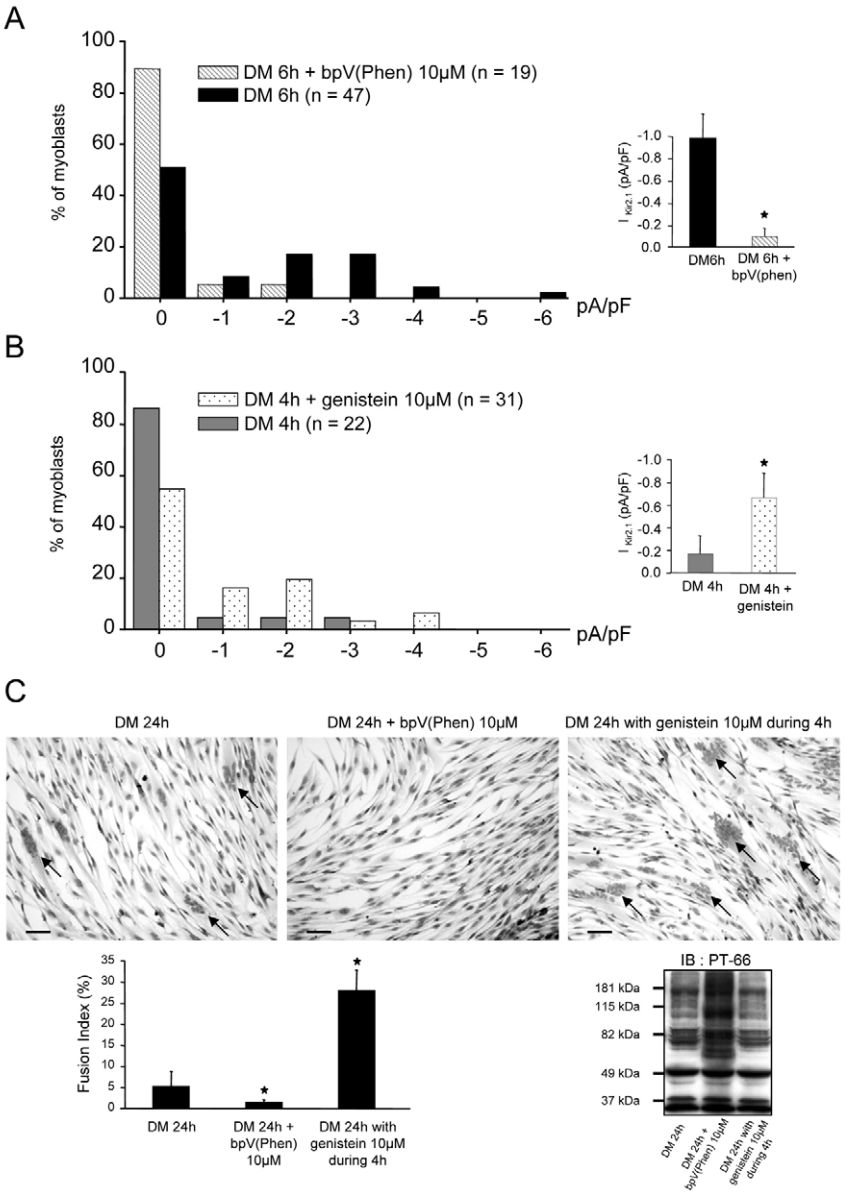

Fig. 2. Kir2.1 channel activation and myoblast differentiation are linked to a tyrosine dephosphorylation. (**A**) Myoblasts were induced to differentiate for 6 hours in the absence or presence of 10 μM bpV(Phen). Kir2.1 current was recorded during a 300 ms step to –120 mV from a holding potential at –60 mV. The histogram represents the fraction of myoblasts with Kir2.1 current density of increasing amplitudes (from 0 pA/pF to –6 pA/pF). Leak currents were estimated as in Fig. 1A. Cell capacitances were 33±3 pF (*n*=47) in DM 6 hours and 20±2 pF (*n*=19) in DM supplemented with bpV(Phen). (Right) Kir2.1 current density in control conditions and when bpV(Phen) is added. This histogram is an alternative representation of the data on the left. (**B**) Myoblasts were induced to differentiate for 4 hours in the absence or presence of 10 μM genistein. Kir2.1 current was measured and results presented as in A. Cell capacitances were 24±3 pF (*n*=22) in DM 4 hours and 24±3 pF (*n*=31) in DM supplemented with genistein. (Right) Kir2.1 current density in control conditions and when genistein is added. This histogram is an alternative representation of the data on the left. (**C**) Fusion index of myoblasts kept for 24 hours in DM (control conditions), or in DM supplemented with 10 μM bpV(Phen) or with 10 μM genistein during the first 4 hours. Fusion index represents the fraction of nuclei within myotubes. As genistein is prepared in DMSO, we verified that the final DMSO concentration (0.1%) had no effect on the fusion index (*n*=6; *P*=0.59, data not shown). Myoblasts were fixed with ice-cold methanol, and stained with Hematoxylin-Eosin. Two different clones were evaluated. Nuclei were counted in three randomly chosen microscope fields for each condition. One microscope field usually contains between 400 and 500 nuclei. A representative picture in each condition is shown. Arrows indicate clusters of nuclei in myotubes. Scale bars: 50 μm. An anti-phosphotyrosine immunoblot on myoblast protein extract shows that 10 μM genistein and 10 μM bpV(Phen) affect overall tyrosine phosphorylation.

was fourfold greater than non-treated myoblasts (*P*=0.036, Fig. 2B, inset). From these observations, we conclude that bpV(Phen) slows down Kir2.1 activation, whereas genistein accelerates it.

As we know that the differentiation process is closely linked to Kir2.1 channel activity (Konig et al., 2006), we examined whether the inhibition of tyrosine kinases and phosphatases also affects the differentiation and fusion process. Experiments were carried out after 24 hours in differentiating conditions, as we wanted to evaluate the beginning of the fusion process. Fig. 2C shows that application of bpV(Phen) slowed down myoblast fusion (down to 2% of fusion, *P*=0.02), whereas genistein accelerated it (up to 28% of fusion, *P*<0.001), in comparison to the control conditions (5% of fusion). The early myotubes (24 hours) that are observed after genistein treatment do not differ morphologically from the early myotubes that appear at a later time (28 hours) in control cultures, when the differentiation is more advanced (data not shown). The immunoblot of Fig. 2C confirms that 10 μM bpV(Phen) enhanced the overall myoblast protein phosphorylation level and that 10 μM genistein reduced it.

Altogether, these results show that activation of Kir2.1 channels and the fusion process are induced by a reduction of tyrosine kinase activity and blocked by an inhibition of tyrosine phosphatase activity.

Kir2.1 current activity is reduced by a tyrosine phosphatase inhibitor

The next step was to determine whether the amplitude of the Kir2.1 current could be modulated by genistein or bpV(Phen) during recording. For that purpose, either bpV(Phen) or genistein was included in the intracellular pipette solution during whole-cell patch-clamp recordings. Fig. 3A (top) illustrates the stability of the Kir2.1 current under control conditions during a 25 minutes recording (left traces are after 1 minute recording and right traces after 25 minutes). When 100 μM bpV(Phen) was added to the pipette solution, Kir2.1 current was reduced by 50% of its initial value after 10 minutes recording, and by 90% after 25 minutes (Fig. 3A, middle traces and Fig. 3B). Finally, co-application of 100 μM bpV(Phen) and 100 μM genistein through the patch pipette (to slow-down a tyrosine re-

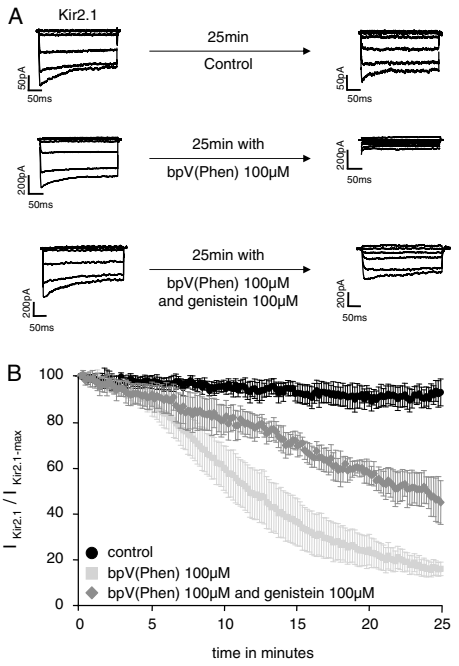

Fig. 3. Kir2.1 current is inhibited by a tyrosine phosphatase inhibitor. Kir2.1 currents were recorded by whole-cell patch-clamp technique in myoblasts induced to differentiate for 24 hours with a patch pipette containing or not (control) 100 μM bpV(Phen). (**A**) Kir2.1 currents recorded from myoblasts cultured for 24 hours in DM, in the absence (top) or in the presence (middle) of 100 μM bpV(Phen), or in the presence of both 100 μM bpV(Phen) and 100 μM genistein (bottom). Left traces represent currents recorded after 1 minute and right traces after 25 minutes. Voltage-steps were to –40, –60, –80, –100, –120 and –140 mV from a holding potential at –60 mV. (**B**) Mean Kir2.1 current amplitude assessed every 10 seconds during a 300 ms step to –120 mV from a holding potential at –60 mV in the absence (n=3) or in the presence (n=5) of 100 μM bpV(Phen), or in the presence of both 100 μM bpV(Phen) and 100 μM genistein (n=3) in the pipette solution. Currents are normalized to the initial current measured 1 minute after breaking the patch. Even though 10 μM genistein was added to the culture medium 2 hours before performing the recordings with both bpV(Phen) and genistein in the pipette, a complete maintenance of channel activity was not achieved.

phosphorylation of the Kir2.1 channels) halved the bpV(Phen)-induced inactivation of the Kir2.1 current (Fig. 3A, bottom traces; Fig. 3B).

These results confirm that Kir2.1 currents recorded in human myoblasts at the onset of differentiation are continuously modulated by tyrosine phosphatase and kinase activities.

Kir2.1 channels are inhibited by a phosphorylation of tyrosine 242

The modulation of Kir2.1 currents by tyrosine phosphatase and kinase activities could be the consequence of the phosphorylation of one of the tyrosines of the Kir2.1 channels itself or it could be linked to a more complex mechanism involving phosphorylation of co-regulators. To evaluate the first possibility, we immunoprecipitated Kir2.1-GFP channels with an anti-Kir2.1 antibody and revealed the tyrosine phosphorylation with an anti-phosphotyrosine antibody (PT-66). Fig. 4A shows that Kir2.1-GFP channels were tyrosine-phosphorylated, and that the channel phosphorylation were halved when tyrosine kinases were blocked by genistein and enhanced fivefold when tyrosine phosphatases were inhibited by bpV(Phen).

We then replaced the tyrosine 242 of the Kir2.1-GFP channel by a phenylalanine (Kir2.1-GFP$_{Y242F}$) and tested the effect of bpV(Phen) on the channel activity. This point mutation was chosen as the tyrosine 242 has been shown to regulate the activity of Kir2.1 channels through its phosphorylation (Wischmeyer et al., 1998). We verified that the Kir2.1-GFP and the Kir2.1-GFP$_{Y242F}$ currents behaved electrophysiologically as the native Kir2.1 current, and that Kir2.1-GFP responded in the same way to bpV(Phen) treatment. Myoblasts transfected with Kir2.1-GFP and Kir2.1-GFP$_{Y242F}$ displayed typical Kir2.1 currents, except that the amplitude of the currents was much larger than the endogenous Kir2.1 current (Kir2.1-GFP=–189±16 pA/pF, n=29; Kir2.1-GFP$_{Y242F}$=–162±16 pA/pF, n=29). Fig. 4B shows that, as the endogenous Kir2.1 current, Kir2.1-GFP current was reduced by at least 90% after 25 minutes when 100 μM bpV(Phen) was added to the intra-pipette solution. However, unlike the Kir2.1 channels, the mutant Kir2.1-GFP$_{Y242F}$ did not inactivate in the presence of 100 μM of bpV(Phen). After 25 minutes bpV(Phen) treatment, the Kir2.1-GFP$_{Y242F}$ current was only slightly decreased (22±8%, n=3), a reduction that is similar to that observed for Kir2.1-GFP currents recorded for 25 minutes without bpV(Phen) (17±6%, n=5). Thus, Kir2.1 channels lacking the tyrosine 242 phosphorylation site are unaffected by the tyrosine phosphatase inhibitor bpV(Phen). However, an experiment similar to that illustrated in Fig. 4A revealed that the Kir2.1-GFP$_{Y242F}$ channel was still tyrosine phosphorylated, suggesting that other tyrosines were available for phosphorylation (data not shown). The Kir2.1 primary sequence contains five to seven tyrosine phosphorylation consensus sites. Therefore, we wondered whether the phosphorylation of one or several other tyrosines would have an effect on Kir2.1 activity. We decided to replace two tyrosine residues, Y336 and Y366, by phenylalanine residues. These tyrosine are the most susceptible to phosphorylation, as predicted by the NetPhos programme (http://www.cbs.dtu.dk/services/NetPhos/); Y242, Y336 and Y366 have phosphorylation scores of 0.976, 0.917 and 0.910, respectively. Using the Prosite program (http://www.expasy.org/tools/scanprosite/), only Y242 and Y366 are potential tyrosine kinase phosphorylation sites. We also verified that these tyrosines are at the surface of the protein, using the available structure of the Kir2.1 cytoplasmic part (Pegan et al., 2005). Kir2.1 current were measured in myoblasts expressing the wild-type Kir2.1-GFP or the mutated channels, Kir2.1-GFP$_{Y242F}$, Kir2.1-GFP$_{Y336F}$ and Kir2.1-GFP$_{Y366F}$, 1 hour after the addition of 200 μM bpV(Phen) to the culture medium. Fig. 4C shows that only the Kir2.1-GFP$_{Y242F}$ current was resistant to bpV(Phen) (P=0.3). By contrast, the wild-type Kir2.1-GFP and the two mutants, Y336F and Y366F, were strongly inhibited by the bpV(Phen) treatment (P<0.001).

From these experiments, we conclude that a tyrosine phosphatase inhibition inactivates Kir2.1-GFP current via the phosphorylation of the tyrosine 242 residue of the Kir2.1-GFP channels.

Kir2.1-GFP channels are not internalized during bpV(Phen)-treatment

Phosphorylation of the tyrosine 242 of Kir2.1-GFP channels reduces the whole-cell Kir2.1-GFP current. This could be the consequence either of a change in gating properties of the Kir2.1 channel itself (reduction of the probability of opening) (Wischmeyer et al., 1998) or of an internalization of the Kir2.1 channels (Tong et al., 2001). We evaluated a possible internalization of the channels by bpV(Phen) using the technique of cell-surface protein biotinylation at 4°C, followed by a separation of the biotinylated proteins from the non-biotinylated with streptavidin magnetic beads. The biotinylated

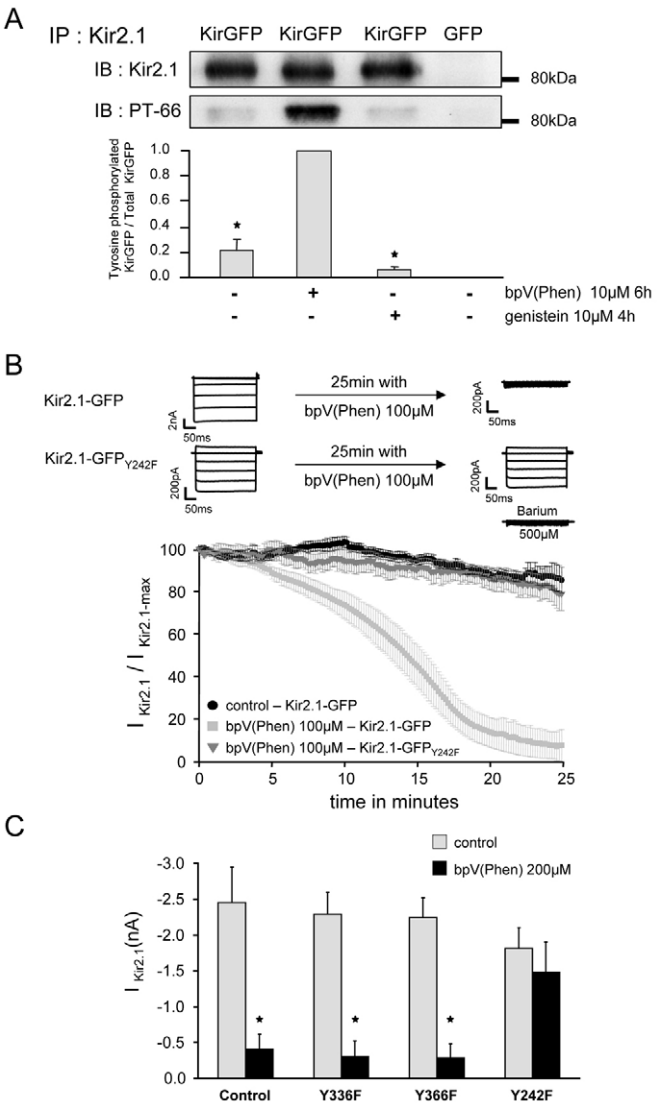

Fig. 4. Kir2.1 channels are modulated through the phosphorylation of the tyrosine 242. (**A**) Myoblasts expressing Kir2.1-GFP were incubated either with 10 μM bpV(Phen) for 6 hours or with 10 μM genistein for 4 hours. Myoblasts transfected with a vector containing only GFP was included in these experiments as a control. In all conditions, myoblasts were treated for 30 minutes before lysis with 10 μM bpV(Phen) to avoid unspecific dephosphorylation (this does not affect Kir2.1 current, data not shown). Kir2.1-GFP channels were immunoprecipitated from cell lysates with an anti-Kir2.1 antibody. Immunoprecipitated proteins were separated on SDS-PAGE, and revealed first with an anti-phosphotyrosine antibody (lower lane) and then reblotted with an anti-Kir2.1 antibody (upper lane). IP is for immunoprecipitation and IB for immunoblot. Bands were quantified by Optiquant software and represented as a histogram. The fraction of Kir2.1-GFP channels that are tyrosine-phosphorylated was normalized to the maximum tyrosine-phosphorylated Kir2.1-GFP channels obtained in the presence of 10 μM bpV(Phen). Results were obtained from three independent experiments. (**B**) Kir2.1-GFP and Kir2.1-GFP$_{Y242F}$ currents were recorded in transfected proliferating myoblasts with a patch pipette containing or not (control) 100 μM bpV(Phen). Top: examples of Kir2.1-GFP and Kir2.1-GFP$_{Y242F}$ currents recorded in the presence of 100 μM bpV(Phen) during voltage-steps as in Fig. 3A. Addition of Ba^{2+} (500 μM) blocked the Kir2.1-GFP$_{Y242F}$ current at the end of the 25 minute recording. Bottom: Kir2.1 current amplitude was assessed as in Fig. 3B. The graph represents the mean Kir2.1 current of Kir2.1-GFP transfected cells for 25 minutes recording in the absence (n=5) or in the presence (n=3) of 100 μM of bpV(Phen), and of Kir2.1-GFP$_{Y242F}$ transfected cells recording in the presence of 100 μM of bpV(Phen) (n=3). (**C**) Myoblasts expressing Kir2.1-GFP (control), Kir2.1-GFP$_{Y336F}$ (Y336F), Kir2.1-GFP$_{Y366F}$ (Y366F) and Kir2.1-GFP$_{Y242F}$ (Y242F) channels were incubated with 200 μM bpV(Phen) for 1 hour. The Kir2.1 current density was evaluated before (gray) and after bpV(Phen) treatment by whole-cell patch-clamp. Nine to 16 cells were recorded in each condition; capacitances of each group of cells vary between 10±2 and 14±2 pF (not significantly different).

fraction of Kir2.1-GFP channels was evaluated before and after bpV(Phen) treatment (10 μM for 6 hours since this protocol massively reduced Kir2.1 current; Fig. 2A, inset). Fig. 5A shows that the fraction of Kir2.1-GFP at the plasma membrane was not significantly different after the 6 hours treatment by bpV(Phen) (P=0.91, n=4). Controls were carried out with non-biotinylated myoblasts and with GFP-transfected myoblasts.

To confirm this result, we addressed the same issue using TIRF microscopy (total internal reflection fluorescence microscopy). This method is a powerful optical technique that allows the study of cellular membrane activities, such as secretion or internalization of fluorescent molecules. As TIRF measurements could not exceed 1 or 2 hours because of cell migration, we applied 200 μM bpV(Phen) to have a rapid inhibition of the Kir2.1-GFP current. Fig. 5B showed that Kir2.1 current is largely inhibited by a 60 minutes bath application of 200 μM bpV(Phen). Kir2.1 current density was –188 pA/pF and –35 pA/pF before and after 60 minutes bpV(Phen) treatment (n=3 and n=5 respectively; P=0.02). The variation of fluorescence intensity due to Kir2.1-GFP channels at the plasma membrane in the presence of 200 μM of bpV(Phen) was followed by TIRF microscopy in sister cultures. If internalization of Kir2.1-GFP channels occurs, the fluorescence intensity seen by TIRF microscopy should decrease. It can be seen in Fig. 5B that a 60-minute treatment with 200 μM bpV(Phen) did not affect the fluorescence intensity (n=4; P=0.59). As a control, we verified that, in the absence of bpV(Phen), the fluorescence of Kir2.1-GFP channels was constant during the 60-minute recording (n=3, data not shown).

Altogether, these results strongly suggest that Kir2.1-GFP current inactivation is not due to an endocytosis of the channels but that it is linked to a direct tyrosine phosphorylation of the Kir2.1 channels.

Differentiation of human myoblasts is linked to a tyrosine dephosphorylation of endogenous Kir2.1 channels

A key experiment of this study was to evaluate endogenous Kir2.1 channels tyrosine phosphorylation, and also to assess whether this tyrosine phosphorylation was modulated during differentiation. This experiment was difficult to perform, as there are only a few hundreds Kir2.1 channels at the plasma membrane of each myoblasts. Therefore, 300×10^6 proliferating myoblasts and the same number of myoblast kept for 6 hours in differentiation medium were harvested. Total lysates were ultracentrifuged to enrich the membrane fraction. Kir2.1 channels of the enriched membrane fraction of the two populations were immunoprecipitated using a rabbit IgG anti-Kir2.1 antibody and revealed on SDS-PAGE with a

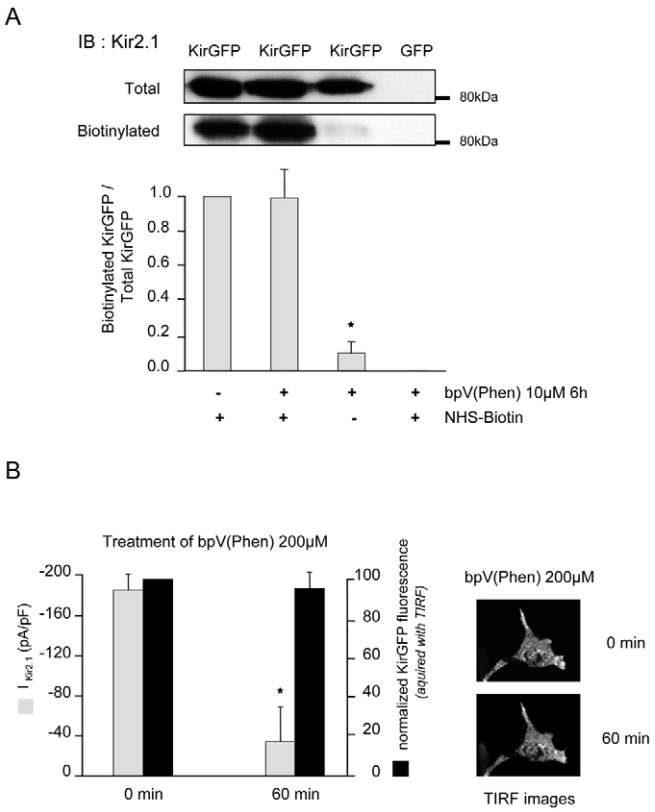

Fig. 5. Kir2.1-GFP channels are not internalized by the bpV(Phen)-treatment. (**A**) Myoblasts expressing Kir2.1-GFP channels were incubated with or without 10 μM bpV(Phen) for 6 hours. After cell surface protein biotinylation, myoblasts were lysed and an equal amount of proteins from each condition was precipitated using streptavidin magnetic particles. Myoblasts expressing Kir2.1-GFP channels on which no biotinylation was performed and myoblasts transfected with a vector containing only GFP were included as controls. Total proteins and biotinylated proteins were separated on SDS-PAGE and revealed with an anti-Kir2.1 antibody. Bands were quantified by Optiquant software. The histogram represents the fraction of biotinylated Kir2.1-GFP channels normalized to the fraction of biotinylated Kir2.1-GFP channels observed in the absence of bpV(Phen). Results were obtained from four independent experiments. (**B**) Myoblasts expressing Kir2.1-GFP channels were incubated with 200 μM bpV(Phen) for 1 hour. The Kir2.1-GFP current density (gray) was evaluated before (*n*=3) and after (*n*=5) bpV(Phen) treatment by whole-cell patch-clamp technique. In parallel experiments, Kir2.1-GFP channels fluorescence (black) was followed by TIRF microscopy for the 1 hour bpV(Phen) treatment. The histogram represents Kir2.1-GFP current density before and after bpV(Phen) treatment and the average fluorescence from four myoblasts (mean of 7-8 regions per cell) measured during the first 3 and the final 3 minutes of the 1 hour bpV(Phen) treatment. The fluorescence was normalized to the fluorescence measured during the first 3 minutes of the bpV(Phen) treatment. TIRF pictures of a myoblast at the beginning and at the end of the 1 hour bpV(Phen) treatment are shown on the right.

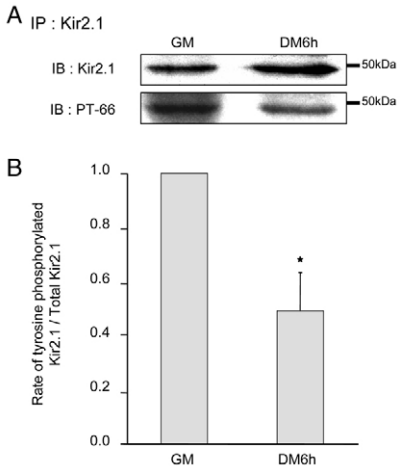

Fig. 6. Tyrosine dephosphorylation of endogenous Kir2.1 channels at the onset of differentiation. (**A**) Myoblasts (300×10^6) were cultured in growth medium (GM) or in differentiation medium for 6 hours (DM 6h). Both populations of myoblasts were treated for 30 minutes preceding lysis with 10 μM bpV(Phen) to avoid unspecific dephosphorylation. An equal amount of proteins (10 mg) from each population was immunoprecipitated with a rabbit anti-Kir2.1 antibody. Immunoprecipitated proteins were separated on SDS-PAGE, and revealed with chicken anti-Kir2.1 antibody (upper lane) and then reblotted with an anti-phosphotyrosine antibody (PT-66, lower lane). Bands were quantified by Optiquant software. (**B**) The histogram represents the fraction of endogenous Kir2.1 channels that are tyrosine-phosphorylated normalized to the tyrosine-phosphorylated Kir2.1 channels in proliferating conditions. Results were obtained from three independent experiments.

chicken IgY anti-Kir2.1 antibody. We employed a chicken IgY antibody because the heavy-chain of the rabbit IgG has the same molecular weight as Kir2.1 channels. Fig. 6A (upper lane) shows that endogenous Kir2.1 channels were detectable, and equally present in proliferation and differentiation conditions. We then reblotted the same membrane with an antibody against phosphotyrosine residue, which showed that endogenous Kir2.1 channels of proliferating myoblasts were tyrosine-phosphorylated, and that this tyrosine phosphorylation was reduced by 50% after 6 hours in differentiation condition (Fig. 6B).

These final results confirm that endogenous Kir2.1 channels of proliferating myoblasts are tyrosine phosphorylated, and that the induction of differentiation is linked to a tyrosine dephosphorylation of the endogenous Kir2.1 channels.

DISCUSSION

Our study demonstrates that the increase of Kir2.1 channel activity, which is tightly linked to the induction of human myoblast differentiation, is due to the dephosphorylation of tyrosine 242 of the channel itself. By activating Kir2.1 channels, this dephosphorylation is responsible for the early hyperpolarization that, via a Ca^{2+} signal, turns on the calcineurin pathway and triggers the differentiation process (Arnaudeau et al., 2006; Konig et al., 2006; Konig et al., 2004). To our knowledge, the Kir2.1 tyrosine 242 dephosphorylation is thus the earliest molecular mechanism described yet in the cascade of events that leads to the irreversible induction of myoblast differentiation and fusion.

We have previously shown that Kir2.1 mRNA was present in proliferating myoblasts (Fischer-Lougheed et al., 2001). We could exclude in the present work that the induction of Kir2.1 channel activity is due to new channel synthesis or transport of Kir2.1 channels from the endoplasmic reticulum to the plasma membrane. We propose that, in human proliferating myoblasts, Kir2.1 channels are kept silent at the plasma membrane by phosphorylation of tyrosine 242, and that these channels are ready to turn on the differentiation process in response to extracellular stimuli that induce their dephosphorylation.

Silent Kir2.1 channels are already present at the plasma membrane of proliferating myoblasts

Our results show that upregulation of Kir2.1 channels is not the consequence of gene expression or trafficking regulation. Neither cycloheximide nor Brefeldin A had major effects on the activation of Kir2.1 channels during the first 6 hours of differentiation. In addition, biotinylation and TIRF experiments showed that Kir2.1-GFP channels are still present at the plasma membrane even after the complete inhibition of Kir2.1 current by bpV(Phen). Together, these observations suggest that Kir2.1 channels are localized at the cell surface of proliferating myoblasts but in a silent state.

In agreement with this hypothesis, a recent study reported that the active Kir2.1 channels could represent only a small fraction of the total channels present at the plasma membrane. Using the FRAC technique (function recovery after chemobleaching), Sun et al. (Sun et al., 2004) showed that many 'Kir2.1 sleeping molecules' were present on plasma membrane of HEK293 cells stably overexpressing Kir2.1 channels, and concluded that only a small fraction of channels at the plasma membrane contributed to the overall channel activity.

Myoblast differentiation is modulated by a tyrosine phosphorylation

Kir2.1 channel increased activity and the fusion process are induced by a tyrosine kinase inhibitor, genistein, and reduced by a tyrosine phosphatase inhibitor, bpV(Phen). The interpretation of these pharmacological studies is, however, complicated by the fact that such inhibitors will affect all intracellular pathways in which a tyrosine phosphorylation is involved. For example, genistein inhibits cell proliferation in prostate and breast cancer cells (Kousidou et al., 2006; Ouchi et al., 2005). We did not specifically look at the effect of genistein or bpV(Phen) on human myoblast proliferation as the drugs were added to cells only under differentiation conditions. Indeed, our goal was to analyze the early effect of tyrosine kinase and phosphatase inhibitors on Kir2.1 channel activity and on the induction of the differentiation process. Although genistein and bpV(Phen) probably affect many proteins of human myoblasts, these inhibitors directly target Kir2.1 channels, and modulate the differentiation and fusion process in a way closely related to the level of Kir2.1 channel phosphorylation and activity. From these results, we conclude (1) that during proliferation, a tyrosine kinase activity phosphorylates Kir2.1 channels and blocks the entry of myoblasts in the differentiation process and (2) that, although the kinase is still active in the early phase of differentiation, an increase in tyrosine phosphatase activity targeting Kir2.1 channels is required to trigger differentiation.

Kir2.1 current is directly modulated by the tyrosine 242 phosphorylation

Evidences for a possible regulation of ionic channels by a direct tyrosine phosphorylation and dephosphorylation has substantially increased over the last years (Davis et al., 2001). Concerning Kir2.1 channels, Wischmeyer et al. (Wischmeyer et al., 1998) showed that Kir2.1 channels overexpressed in HEK293 cells were suppressed by perorthovanadate, and that this inhibition was reversed by genistein. Our observations in primary cultures of human myoblasts are in good agreement with these results, the only difference is that the Kir2.1 inhibition by bpV(Phen) in human myoblasts requires a longer application of the drug to be completed. Kir2.1 current is inhibited by bpV(Phen) within 10 minutes in HEK293 cells overexpressing Kir2.1 channels (we confirmed these results ourselves, data not shown), whereas it required about 25 minutes in human myoblasts. This difference could be explained either by a lower level of tyrosine kinase activity in human myoblasts or by a localization of the enzyme that makes it less accessible to the drug.

The effect of genistein on Kir2.1 current is less striking than that of bpV(Phen). Application of genistein alone did not result in Kir2.1 currents activation in proliferating myoblasts. A possible explanation is that the tyrosine phosphatase that dephosphorylates Kir2.1 channels is lacking or not activated in proliferating myoblasts. However, co-application of genistein with bpV(Phen) in differentiated myoblasts expressing Kir2.1 channels reduces the bpV(Phen)-induced Kir2.1 inhibition. Recently, it has been shown that a genistein pretreatment blocks the EGF-induced inhibition of Kir2.1 channels in urothelial cells (Sun et al., 2007). This observation supports the hypothesis that Kir2.1 channels activity can be directly modulated by a tyrosine phosphorylation. Finally, we showed that bpV(Phen) did not inactivate the current through Kir2.1 channels when these channels were mutated on the tyrosine 242. The mutant mimics a dephosphorylated channel that is constitutively active. This experiment strongly suggests that the modulation of Kir2.1 channel activity occurs via the phosphorylation and dephosphorylation of its tyrosine 242.

Differentiation is triggered by the dephosphorylation of endogenous Kir2.1 channels

A major finding of this work is that endogenous Kir2.1 channels are tyrosine phosphorylated in proliferating myoblasts, and that phosphorylation is reduced at the onset of differentiation. We therefore propose that Kir2.1 channels are kept silent during proliferation by a tyrosine phosphorylation due to activated kinase receptors. Several ionic channels have been described to be inhibited by such mechanism. For example, when Kv1.3 is co-expressed in HEK293 cells with EGFR, the channel becomes tyrosine-phosphorylated and the current inhibited, mimicking the regulation of Kv1.3 by Fas in T lymphocytes (Bowlby et al., 1997). Similarly, when Kir2.1 channel is co-expressed with EGFR and NGFR in oocytes, its activity decreases after EGF and NGF exposure (Wischmeyer et al., 1998). Recently, it has been shown in bladder urothelial cells that Kir2.1 channel activity is strongly inhibited by EGF (Sun et al., 2007). In our human myoblast cultures, one of the main differences between proliferating and differentiating conditions is a reduction of growth factors in the differentiation medium. This difference could, on its own, explain a reduction of tyrosine kinase activity in differentiating conditions. However, the efficiency of the Kir2.1 channel dephosphorylation at the onset of differentiation may also depend on the induction of a tyrosine phosphatase activity. Indeed, recent literature underscores the major role of tyrosine phosphatases in signal transduction pathways (Tiganis and Bennett, 2007). The control of the Kir2.1 channel activation throughout differentiation is thus likely to be dependent on the fine tuning of both kinase and phosphatase activities. A possible candidate for this dephosphorylation event could be SHP-2, a tyrosine phosphatase required for myogenesis (Kontaridis et al., 2004). SHP-2 has also been proposed to promote slow skeletal muscle fiber growth by coordinating signals from the extracellular matrix to the NFAT pathway (Fornaro et al., 2006).

In conclusion, we propose that during proliferation, endogenous Kir2.1 channels are maintained silent at the plasma membrane by a tyrosine phosphorylation. Then, during the first hours of differentiation, a fine balance between tyrosine kinase and tyrosine phosphatase activities dephosphorylates Kir2.1 channels at tyrosine 242, and thereby triggers the activation of the channels. These

results provide the basis for further investigation into the link between extracellular signaling and the induction of a differentiation process through the modulation of ionic channels.

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