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The calcineurin pathway links hyperpolarization (Kir2.1)-induced Ca^{2+} signals to human myoblast differentiation and fusion

Stéphane Konig[1,*], Anne Béguet[1], Charles R. Bader[2] and Laurent Bernheim[1]

In human myoblasts triggered to differentiate, a hyperpolarization, resulting from K^+ channel (Kir2.1) activation, allows the generation of an intracellular Ca^{2+} signal. This signal induces an increase in expression/activity of two key transcription factors of the differentiation process, myogenin and MEF2. Blocking hyperpolarization inhibits myoblast differentiation. The link between hyperpolarization-induced Ca^{2+} signals and the four main regulatory pathways involved in myoblast differentiation was the object of this study. Of the calcineurin, p38-MAPK, PI3K and CaMK pathways, only the calcineurin pathway was inhibited when Kir2.1-linked hyperpolarization was blocked. The CaMK pathway, although Ca^{2+} dependent, is unaffected by changes in membrane potential or block of Kir2.1 channels. Concerning the p38-MAPK and PI3K pathways, their activity is present already in proliferating myoblasts and they are unaffected by hyperpolarization or Kir2.1 channel block. We conclude that the Kir2.1-induced hyperpolarization triggers human myoblast differentiation via the activation of the calcineurin pathway, which, in turn, induces expression/activity of myogenin and MEF2.

KEY WORDS: Myogenesis, Calcineurin, Hyperpolarization, Human myoblasts

INTRODUCTION

Myoblasts differentiation is a key step for skeletal muscle growth and repair. We have shown previously that one of the earliest events of human myoblast differentiation is a membrane hyperpolarization resulting from to Kir2.1 K^+ channel activity (Fischer-Lougheed et al., 2001; Liu et al., 2003). The goal of the present work was to uncover the molecular link between the Kir2.1-induced hyperpolarization and the expression of myogenin and MEF2, two major transcription factors of the differentiation process.

We are using primary human myoblast cultures derived from single satellite cells. Human myoblasts can proliferate for several months in culture, and terminal differentiation and fusion into myotubes can be induced by serum withdrawal. At the molecular level, the induction of the differentiation process is associated with the expression of an early marker, myogenin. Myogenin belongs to the family of myogenic basic helix-loop-helix (bHLH) transcription factors, which includes MYOD, MYF5 and MRF4 (Braun et al., 1989; Davis et al., 1987; Rhodes and Konieczny, 1989; Wright et al., 1989). These factors are implicated in the specification and in the differentiation of myogenic cells. During myoblast differentiation, activation of muscle-specific genes by myogenic bHLH proteins also requires their interaction with transcription factors of the MEF2 family (Black and Olson, 1998). MEF2 family has four members (MEF2A-D) that bind to a consensus sequence present in several muscle-specific promoters.

Differentiation of human myoblasts requires a hyperpolarization of their membrane resting potential to approximately –70 mV (Fischer-Lougheed et al., 2001; Liu et al., 2003). Preventing this hyperpolarization impedes both expression and activity of myogenin and MEF2, indicating that it is a prerequisite for differentiation (Konig et al., 2004). We proposed that Kir2.1-linked hyperpolarization initiates the differentiation process by increasing cytoplasmic free Ca^{2+} (Arnaudeau et al., 2006; Bijlenga et al., 2000; Liu et al., 2003). The question is what are the signal transduction pathways downstream of this cytoplasmic Ca^{2+} signal that initiate human myoblast differentiation?

In mouse myoblasts, myogenin expression, an early marker for differentiation, has been suggested to be regulated by at least four different pathways: p38 mitogen-activated protein kinase (p38-MAPK), phosphatidyl-inositol 3-kinase (PI3K), Ca^{2+}-calmodulin-dependent kinase (CaMK) and calcineurin (Cuenda and Cohen, 1999; Friday et al., 2003; Xu et al., 2002; Zetser et al., 1999). The p38-MAPK, CaMK and calcineurin pathways appear capable of inducing the transcriptional activity of MEF2 (Tamir and Bengal, 2000). Although it is well known that during myoblast differentiation CaMK (Chin, 2005) and calcineurin (Stiber et al., 2005) activity is strongly controlled by cytoplasmic Ca^{2+}, the role of Ca^{2+} in the activation of p38-MAPK and PI3K is less clear. Activation of p38-MAPK is linked to direct phosphorylations by MKK3 and MKK6 (Derijard et al., 1995; Han et al., 1996), and activation of PI3K is coupled to insulin growth factor (IGF1) tyrosine kinase receptor (Jiang et al., 1998; Kaliman et al., 1996; Kandel and Hay, 1999). The principal downstream target of PI3K is AKT (protein kinase B). Full activation of AKT by insulin or IGF1 requires a phosphorylation at two sites by two separate kinases that both depend on PI3K activity (Alessi et al., 1996; Sarbassov et al., 2005; Stokoe et al., 1997). Whether these four signaling pathways are involved in human myoblast differentiation, and whether they are modulated be the membrane hyperpolarization, however, is not known.

In the present study, we show that the Kir2.1-induced hyperpolarization controls the onset of the differentiation process through the selective activation of the calcineurin pathway, although p38-MAPK, PI3K and CaMK pathways are also required for a full expression of myogenin and MEF2. We find, in addition, that p38-

[1]Department of Basic Neurosciences, University Medical Center, Rue Michel Servet 1, 1211 Geneva 4, Switzerland. [2]Department of Clinical Neurosciences, University Hospital, Geneva, Switzerland.

*Author for correspondence (e-mail: stephane.konig@medecine.unige.ch)

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MAPK and PI3K are already activated during myoblast proliferation, and that CaMK activation can be induced during myoblast proliferation through a Ca^{2+}-dependent mechanism not related to the hyperpolarization. We propose that the differentiation process in human myoblasts is initiated by a membrane hyperpolarization that acts as a molecular switch, forcing differentiation by generating a Ca^{2+} signal responsible for the specific activation of the calcineurin signaling pathway.

MATERIALS AND METHODS

Cell cultures and immunocytochemistry

Primary cultures of human myoblasts (progenitors from single satellite cell) were prepared and grown as previously described (Liu et al., 1998). Muscle samples were obtained from children during corrective orthopedic surgery according to the guidelines of the local ethical committee. Single satellite cells obtained after muscle enzymatic dissociation were manually collected under the microscope on a size criteria, transferred into single wells (one cell/well) of a 96 wells container (Becton Dickinson) using a micropipette (clonal culture). Myoblasts were amplified in serum-containing medium (growth medium) and induced to differentiate into myotubes in a serum-free medium (differentiation medium, DM). When indicated, differentiation medium was complemented with 10 μM SB202190 (Calbiochem), 50 μM LY194002 (CellSignaling), 30 μM KN-93 (Calbiochem), 7 μM CsA (Calbiochem) or 5 μM FK-506 (A.G. Scientific).

Immunostaining

Immunostaining was performed as previously described (Konig et al., 2004). Myogenin was revealed using a mouse monoclonal antibody (1/1000, BD Biosciences) and MEF2 using a rabbit polyclonal antibody (1/300, Sc-313 SantaCruz Biotechnology). Immunostaining fluorescence from myoblasts plated on 25 mm coverslips was imaged with a Zeiss Axiovert S100TV microscope using a 40× Fluar 1.3 NA oil-immersion objective (Carl Zeiss AG, Feldbach, Switzerland). DAPI, Alexa 488 and Alexa 546 were respectively excited by the 360±10 nm, 488±10 nm and 546±10 nm line from an Optoscan Monochromator (Cairn Research, Faversham, UK) through a XF2050 dichroic mirror (Omega Optical, Brattleboro, VT) and the fluorescence emission were respectively acquired at 460±30 nm, 520±40 nm and 602±50 nm (XF3063 Omega Optical, Brattleboro, VT) using a cooled, 12 bits TE/CCD interlined Coolsnap HQ camera (Photometrics, Roper Scientific, Trenton, NJ). Image acquisition and analysis was performed with the Metamorph 5.0 software (Universal Imaging, West Chester, PA).

Western blots

Myoblasts were lyzed using PhosphoSafe Extraction Buffer (Novagen). After adding an equivalent volume of Laemmli 2×, extracts were boiled for 3 minutes. Western blots were performed as previously described (Konig et al., 2004), except that saturation with 5% non-fat milk in TTBS was carried out in the presence of phosphatase inhibitors (50 mM NaF and 1 mM sodium orthovanadate). Antibodies were diluted in TTBS with 5% BSA. Antibodies used: mouse monoclonal antibody against myogenin (1/2000, BD Biosciences) and p38-MAPK (1/1000, CellSignaling Technology #9212); rabbit monoclonal antibody against phospho-p38-$MAPK_{Thr180/Tyr182}$ (1/1000, CellSignaling Technology #9215); phopho-AKT_{Ser473} (1/1000, CellSignaling Technology #4058); phopho-AKT_{Thr308} (1/1000, CellSignaling Technology, #4056); and p42/44 $MAPK_{Thr202/Tyr204}$ (1/1000, CellSignaling Technology, #4377).

CaMKII assay

CaMK activity was quantified using the radioactive assay SignaTECT Ca^{2+}/Calmodulin-Dependent Protein Kinase Assay System (Promega). Myoblasts were lyzed using 100 μl PhosphoSafe Extraction Buffer (Novagen) and spun for 5 minutes (13,000 ***g***) at 4°C. Five microlitres of supernatant were collected for each assay and incubated 2 minutes at 30°C in a buffer solution containing the specific peptide for CaMKII. Endogenous activity (which represents the level of CaMKII activity of myoblasts in each conditions of culture) was assessed in a buffer solution containing 5 mM EGTA and 0.5 μCi of γ^{32}P-ATP. Total CaMKII activity (which represents the maximum CaMKII activity of the myoblast sample) was assessed from 1 μl of supernatant (to avoid saturation) in a buffer solution containing non-limiting amounts of Ca^{2+} and calmodulin to allow maximal CaMKII activation in vitro (5 mM $CaCl_2$, 5 μM calmodulin and 0.5 μCi of γ^{32}P-ATP). Specific activation of CaMKII was calculated as the ratio between the endogenous activity in cultured myoblasts and the total activity. Variation of the specific activation of CaMKII throughout the experiments reflects a modification of the CaMKII activity in cultured myoblasts as the total CaMKII remains nearly constant (not shown). For each experiment, the ratio obtained with myoblasts maintained in differentiation medium containing 15 μM BAPTA-AM was set to 1.

p38-MAPK assay

p38-MAPK activity was assessed using the non-radioactive p38-MAPKinase Assay Kit (Cell Signaling Technology, #9820). At the indicated times, cells lysis was carried out with 500 μl of provided lysis buffer. The active form of p38-MAPK was immunoprecipitated (overnight at 4°C) from 200 μl cell extracts with 20 μl phospho-p38-MAPK (Thr180/Tyr182) monoclonal antibody. An in vitro kinase assay was performed directly on the immunoprecipitated phospho-p38-MAPK in presence of 200 μM ATP and using recombinant ATF-2 (recATF-2) as a substrate. Phosphorylated recATF-2 was detected by immunoblotting using a phospho-ATF2 (Thr71)-specific antibody.

Luciferase assay

Using electroporation (Espinos et al., 2001), 2×10^6 human myoblasts were transfected with 2 pmol firefly luciferase encoding plasmid (3MEF2-luc or 9NFAT-luc) together with 1 pmol control plasmid encoding the Renilla luciferase [phRL-TK-luc, Promega (Konig et al., 2004)]. At the indicated times, cells were processed with the Dual-Luciferase reporter assay kit (Promega) as recommended by the manufacturer.

RESULTS

Which regulatory pathways induce human myoblast to differentiate?

Membrane hyperpolarization is a crucial early step that allows myoblast to differentiate. Differentiation was induced by replacing growth medium by differentiation medium (see Materials and methods), and myogenin and MEF2 expression were used as differentiation markers. Fig. 1A shows that the percentage of nuclei expressing myogenin and MEF2 is strongly inhibited when differentiation is induced in presence of high extracellular K^+ (116 mM) to prevent membrane hyperpolarization. This figure also illustrates that the inhibition is not due to a toxic effect of high external K^+ as myoblasts are still able to fully differentiate when replaced in a differentiation medium containing 5 mM K^+. From this observation, we hypothesize that an inhibition of the hyperpolarization prevents the activation of one or several regulatory pathways that control myogenin and MEF2 expression.

CaMK, PI3K, calcineurin and p38-MAPK regulatory pathways have been suggested to regulate myogenin and MEF2 expression during murine myoblast differentiation (Xu et al., 2002). We first investigated whether any of these regulatory pathways were involved in human myoblast differentiation (Fig. 1B). Myoblast differentiation was assessed using the percentage of nuclei expressing myogenin or MEF2 over the total number of nuclei. In proliferating myoblasts, very few nuclei were positive for myogenin (6%) or MEF2 (7%). After 3-4 days in differentiation medium, 69% and 71% of the nuclei were positive for myogenin and MEF2, respectively. Inhibition of the hyperpolarization with 10 mM Cs^+ (a Kir2.1 blocker) reduced myogenin and MEF2 expression by 1.9-fold (Konig et al., 2004). Fig. 1B also shows that inhibiting p38-MAPK (with SB202190) and CaMK (with KN-93) reduced myogenin and MEF2 expression to the same level as that obtained by treating cells with 10 mM Cs^+. However, inhibition of PI3K (with LY294002) had a stronger effect on myoblast differentiation,

reducing the percentage of positive nuclei by a 3.8 and 4.1-fold for myogenin and MEF2, respectively. To inhibit the calcineurin pathway, we used cyclosporin A and FK506 mixed together to reduce the toxicity of each drug. Concentrations used were those required to inhibit calcineurin activity fully (see below). Using this strategy, myogenin and MEF2 expression was reduced by 3.7 and 2.6-fold, respectively.

Taken together, these results suggested that p38-MAPK, CaMK, PI3K and calcineurin pathways are all involved in the early steps of human myoblast differentiation. What is less clear is whether one or more of these pathways are controlled by membrane hyperpolarization generated by Kir2.1 channels.

Early activation of calcineurin during human myoblast differentiation requires a membrane hyperpolarization

Our previous work on human myoblasts suggested that membrane hyperpolarization generated by Kir2.1 channels increases intracellular Ca^{2+} and that this step is essential to allow myoblast differentiation to proceed (Arnaudeau et al., 2006; Bijlenga et al., 2000; Liu et al., 2003). As calcineurin is a Ca^{2+}-dependent phosphatase involved in human myoblast differentiation, we tested whether its activity is controlled by a Ca^{2+} signal that could be induced by the hyperpolarization. To assess calcineurin activity we used its ability to dephosphorylate and thereby activate the transcription factor NF-AT. For this purpose, human myoblast were electroporated with a plasmid encoding the luciferase protein under the control of a specific promoter inducible by NF-AT transcription factors (9NF-AT-luc plasmid). The electroporated myoblasts were either maintained in proliferation condition (growth medium) or induced to differentiate in differentiation medium for 1 to 4 days. Kinetics of activation of NF-AT transcription factor in electroporated myoblasts expressing the 9NF-AT-luc plasmid are illustrated in Fig. 2A. It can be seen that NF-AT activity is absent in proliferating myoblasts, is induced during the first 24 hours of differentiation, and then increases during the following days (4.7-fold after 3 days and 10.0-fold after 4 days). Kinetics of activation of NF-AT (calcineurin) was then compared with that of myogenic bHLH and MEF2 (myoblasts were electroporated with a luciferase plasmid controlled by either myogenic bHLH or MEF2 transcription factors). Interestingly, kinetics of activation of NF-AT are similar to that of myogenic bHLH, suggesting that, like myogenic bHLH, calcineurin pathway is activated at the very beginning of the differentiation process. Activation of MEF2 is, however, slower. From these results, we conclude that calcineurin is activated during the very early steps of myoblast differentiation, at the same time as myogenic bHLH and before MEF2.

To evaluate the importance played by the hyperpolarization in the calcineurin activation, myoblasts expressing 9NF-AT-luc plasmid were induced to differentiate either in control differentiation medium, or in differentiation medium supplemented with 10 mM Cs^+ to block Kir2.1 channel activity, or in differentiation medium containing high K^+ (116 mM) to prevent membrane hyperpolarization. Experiments were carried out after 4 days of differentiation to maximize luciferase signals. Fig. 2B shows that both depolarizing conditions strongly inhibited NF-AT activity (by 4.3- and 2.8-fold, respectively), and also that NF-AT activity only depends on calcineurin activity as a combination of cyclosporin A and FK-506, two known calcineurin inhibitors, maintained NF-AT activity to its background level. The strong inhibition of the NF-AT activity obtained in presence of the calcineurin inhibitors clearly confirmed that the activity of the transcription factor NF-AT is due to only the calcineurin activation. A combination of inhibitors was used as a single drug could not fully inhibit calcineurin without inducing a strong toxicity to the human myoblast (data not shown). Finally, as myogenic bHLH and MEF2 activities have been related to calcineurin activity in mouse myoblasts (Friday et al., 2003), we examined whether an inhibition of calcineurin affects these two activities in human myoblasts. Fig. 2C shows that the inhibition of calcineurin by cyclosporin A and FK-506 together reduced myogenic bHLH and MEF2 transcriptional activity by 3.0- and 17.5-fold, respectively.

Together, these results show that suppression of the Kir2.1-induced membrane hyperpolarization inactivates myogenin and MEF2 activities through the inhibition of the calcineurin pathway.

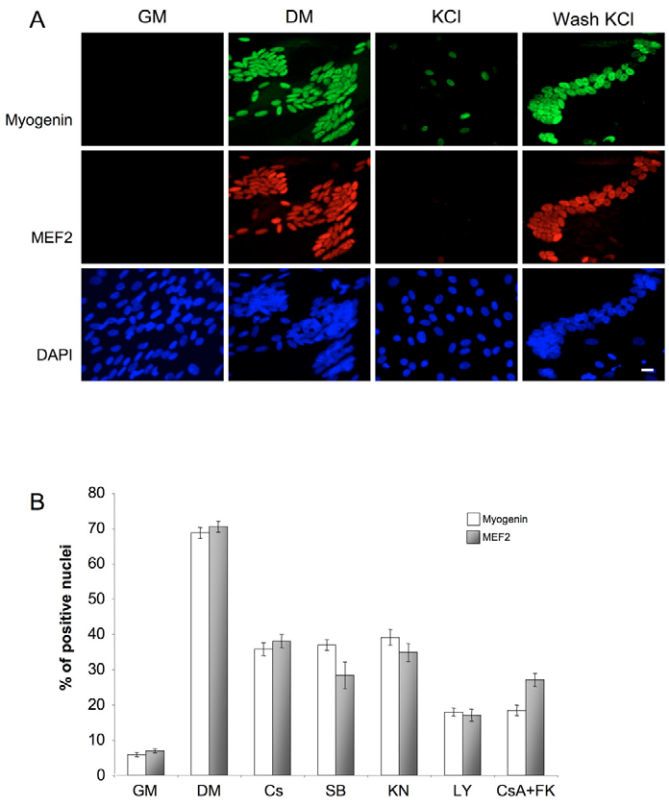

Fig. 1. Calcineurin, CaMK, p38-MAPK and PI3K regulatory pathways are involved in human myoblast differentiation. (**A**) Induction of myogenic differentiation requires a membrane hyperpolarization. Neither myogenin nor MEF2 were detected in myoblast maintained in growing medium (GM). Myoblasts were induced to differentiate for 3 days either in differentiation medium (DM) or in DM in which 116 mM Na^+ was replaced by equivalent concentration of K^+ to prevent the hyperpolarization (KCl). After 3 days in high K^+ differentiation medium, myoblasts were replaced for 3 more days in control differentiation medium (wash KCl) in which they differentiated nicely. Myogenin was revealed with Alexa488, MEF2 with Alexa546, and nuclei with DAPI. Scale bar: 20 μm. (**B**) Differentiation is expressed as a percentage of myogenin- and MEF2-positive nuclei. Myoblast were induced to differentiate for 3-4 days in differentiation medium (DM). Myoblast differentiation is partially inhibited by 10 mM Cs^+ (Cs) to block the hyperpolarization, by 10 μM SB202190 (SB) to block p38-MAPK, by 30 μM KN93 (KN) to block CaMK, by 50 μM LY294002 (LY) to block PI3K, and by a combination of 7 μM cyclosporin A (CsA) and 5 μM FK506 (FK) to block calcineurin. Results are expressed as mean±s.e.m.

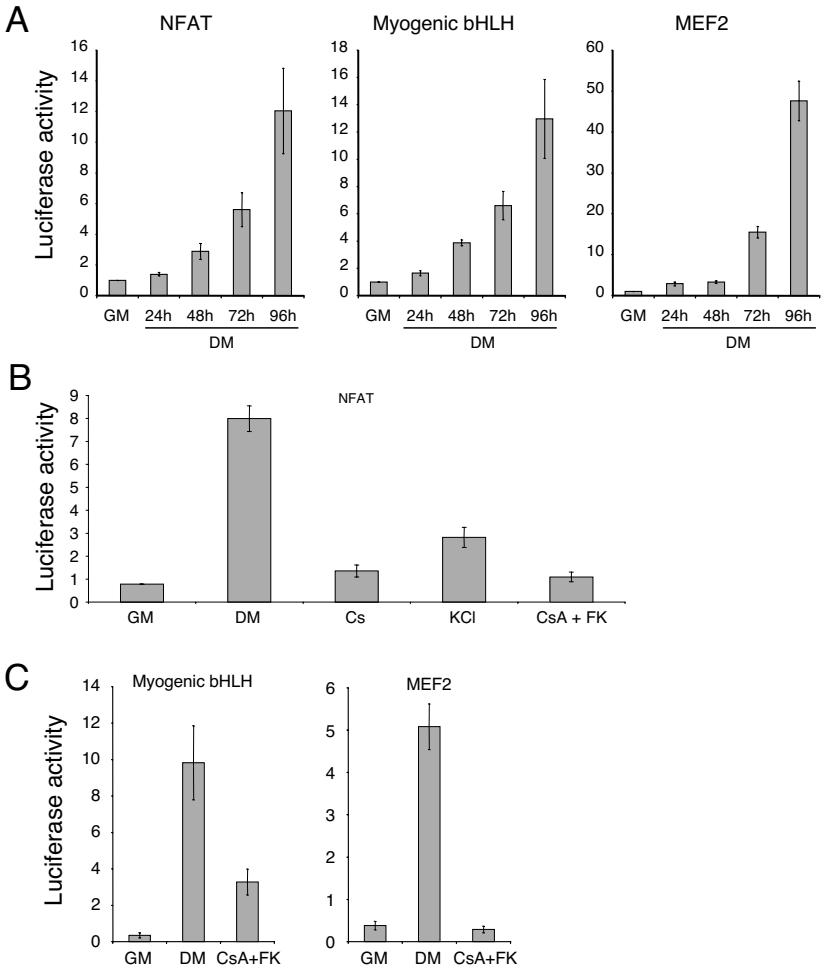

Fig. 2. Hyperpolarization controls the early activation of calcineurin pathway during human myoblast differentiation. Human primary myoblasts were transiently transfected with either 9NFAT-luc or 4RE-luc or 3MEF2-luc plasmids. Luciferase activity is in arbitrary units. Increased level of NFAT transcription factor activity reflects calcineurin activity. (**A**) Kinetics of activation of NFAT, myogenic bHLH and MEF2 transcription factors. Luciferase extracts were prepared from proliferating myoblasts (GM) and from myoblasts maintained for 1 to 4 days in differentiation medium (DM). (**B**) Depolarization inhibits calcineurin activation. Calcineurin activity was assessed using NFAT-luc plasmid. Human myoblasts were induced to differentiate for 4 days either in control conditions (DM) or in presence of 10 mM Cs^+ (Cs), 116 mM high extracellular K^+ (KCl), or a combination of 7 μM cyclosporin A (CsA) and 5 μM FK506 (FK). (**C**) Inhibition of calcineurin pathway strongly decreases myogenic bHLH and MEF2 transcription factors activation. Myogenic bHLH and MEF2 activity was assessed using 4RE-luc and 3MEF2-luc plasmid, respectively. Same conditions as in B. Results are expressed as mean±s.e.m.

The role of CaMKII in human myoblast differentiation is not linked to the Kir2.1-induced membrane hyperpolarization

As seen in Fig. 1B, CaMK regulatory pathway is a second Ca^{2+}-dependent pathway involved in the control of human myoblast differentiation. We, thus, examined whether this pathway was activated by Ca^{2+} signals induced by the Kir2.1-linked hyperpolarization, in the same way as calcineurin was. Endogenous CaMKII activity was evaluated during myoblast differentiation in control conditions and in conditions that prevent membrane hyperpolarization (10 mM Cs^+ or 116 mM K^+). In order to compare experiments, we normalized the results to the CaMKII activity obtained in presence of 15 μM BAPTA-AM (background). Fig. 3A shows that low levels of CaMKII activity (40% above background) are present in proliferating conditions, and that this activity is increased by 2.3-fold after 24 hours of differentiation. However, this increased activity is insensitive to depolarizing agents (10 mM Cs^+ or 116 mM K^+). This indicates that, unlike calcineurin, CaMKII is not regulated by the Kir2.1-linked hyperpolarization. In agreement with this result, a maximum CaMKII activation could already be detected after a 1-hour exposure to differentiation medium, i.e. before the differentiation-linked hyperpolarization, which occurs after 6 hours of differentiation (Konig et al., 2004).

To investigate the origin of the very early Ca^{2+} signal responsible for CaMKII activation, we examined differentiation at a low extracellular Ca^{2+} concentration (15 μM instead of 1.8 mM), which reduces massively all Ca^{2+} influxes through the plasma membrane of myoblasts [fura-2 Ca^{2+} measurements (Arnaudeau et al., 2006)]. In this low Ca^{2+}-containing differentiation medium, CaMKII activity was reduced to the background level after 1 hour (Fig. 3A). Furthermore, when myoblasts were induced to differentiate in a differentiation medium containing the same Ca^{2+} concentration as in the growth medium (0.7 mM Ca^{2+}), the CaMKII activity was similar to that of proliferating myoblasts kept in growth medium (P=0.78). However, the CaMKII activity in myoblasts proliferating in growth medium containing 1.8 mM Ca^{2+}, was not statistically different to that obtained in control differentiation medium after 1 hour (P=0.14) or after 24 hours (P=0.55), but significantly different to that obtained in control growth medium (P=0.002). Taken together, these results suggest that, in human myoblasts, CaMK activity is directly linked to extracellular Ca^{2+} concentration and that it is unaffected by hyperpolarization.

We then tested the capacity of human myoblast to differentiate (i.e. to express myogenin and MEF2) in differentiation medium containing 0.7 mM Ca^{2+} (CaMKII activity is low) or in growth medium containing 1.8 mM Ca^{2+} (CaMKII activity is increased). As expected, when CaMK is maintained at a low activity in differentiation medium containing 0.7 mM Ca^{2+}, the percentage of positive nuclei for both myogenin and MEF2 was reduced by 2.4 and 1.8-fold respectively (Fig. 3B). This reduction is comparable with that obtained with the CaMK inhibitor KN-93 (Fig. 1A). However, activation of the CaMKII in growth medium containing 1.8 mM Ca^{2+} did not allow any induction of myogenin or MEF2

expression. We also verified that myoblasts maintained in growth medium containing 1.8 mM Ca^{2+} (myoblasts with CaMK activity increased) were still able to proliferate actively. Myoblasts (10^5) were seeded in growth medium containing either 0.7 (control) or 1.8 mM Ca^{2+}, and counted after 48 hours. We found that the proliferation rate was not affected by CaMK activation ($4.7\pm0.3\times10^5$ versus $4.6\pm0.3\times10^5$ myoblasts after 48 hours in 0.7 and 1.8 mM Ca^{2+}, respectively, P=0.74).

In conclusion, these results show that CaMKII activity does not correlate with Kir2.1-linked membrane hyperpolarization but can be modulated by changes in Ca^{2+} fluxes associated with changes in the extracellular Ca^{2+} concentration. Our results also show that an increase of CaMKII activity does not stop or reduce myoblast proliferation and that it is, on its own, not sufficient to induce differentiation.

The observed activities of p38-MAPK and PI3K are neither controlled by membrane hyperpolarization, nor sufficient to induce myoblast differentiation

Given the importance suggested for p38-MAPK in myoblast differentiation (see Fig. 1B), we examined whether this pathway is controlled by the Kir2.1-linked hyperpolarization. To evaluate p38-MAPK activity, we first used antibodies specific for its

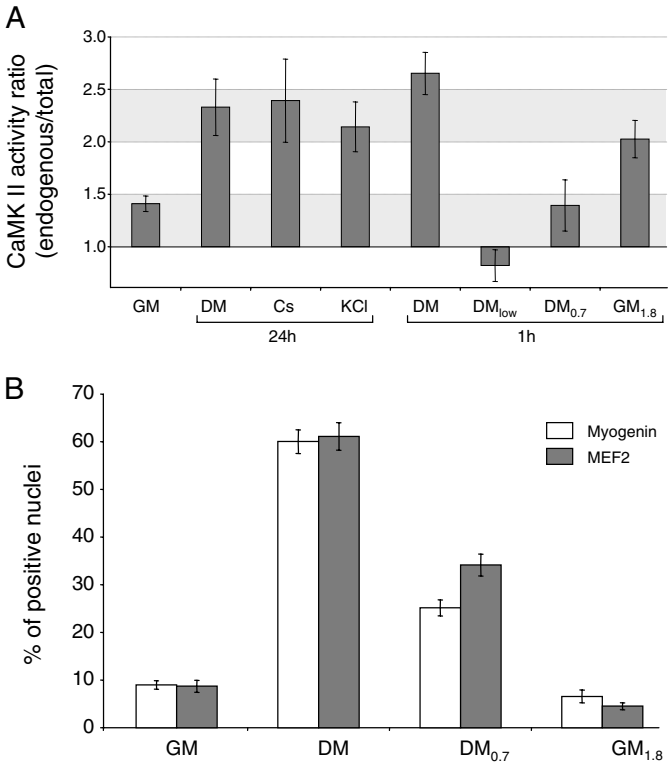

Fig. 3. CaMKII activation is unrelated to the membrane hyperpolarization. (**A**) CaMKII activity was assessed by measuring the ability of myoblast extracts to phosphorylate a specific peptide substrate in presence of γ32P-ATP. Total cell extracts containing phosphatase inhibitors were prepared from proliferating myoblast (GM) or proliferating myoblast with 1.8 mM Ca^{2+} ($GM_{1.8}$), and from myoblast induced to differentiate for 1 or 24 hours in differentiation medium (DM), or in DM containing 10 mM Cs^+ (Cs), 116 mM KCl (KCl), 15 μM mM Ca^{2+} (DM_{low}) or 0.7 mM Ca^{2+} ($DM_{0.7}$). Endogenous CaMKII activity (activity in cultured) was assessed in absence of added Ca^{2+} or calmodulin; total CaMKII activity (maximum activity of the myoblast sample) was assessed after addition of 5 mM Ca^{2+} and 5 μM calmodulin. Specific activation of CaMKII was calculated as the ratio between the endogenous and the total activity. For each experiment, the ratio obtained with myoblasts maintained in differentiation medium containing 15 μM BAPTA-AM was set to 1. Results are expressed as mean±s.e.m. (**B**) CaMKII activation in high Ca^{2+} proliferating medium does not induce myoblast differentiation. Differentiation is expressed as a percentage of myogenin- and MEF2-positive nuclei. Results are expressed as mean±s.e.m.

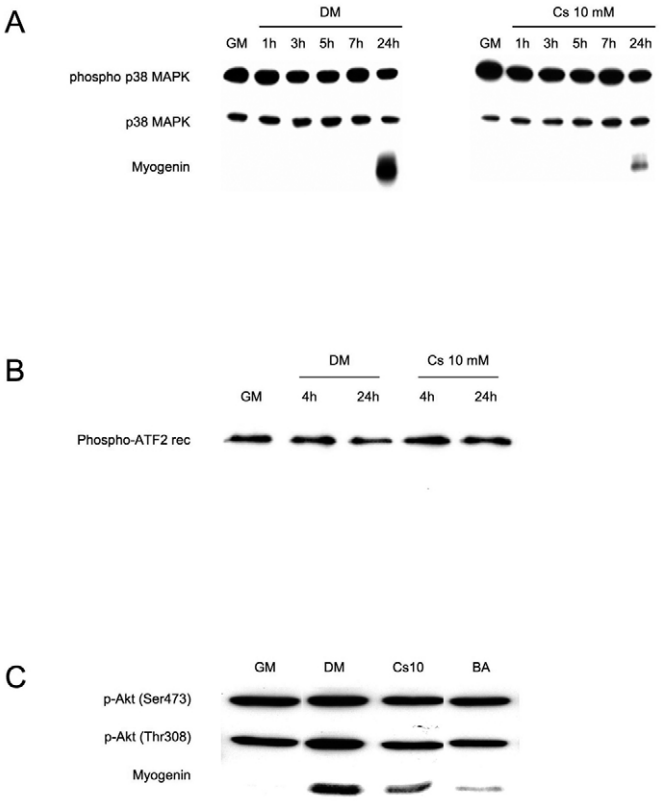

Fig. 4. p38-MAPK and PI3K are activated in proliferation and are not controlled by the differentiation-linked hyperpolarization. (**A**) Activation of p38-MAPK pathway was detected by western blot using an antibody directed against phospho-p38-MAPK (Thr180/Tyr182). Phospho-p38-MAPK is present in proliferating myoblasts (GM), is maintained in differentiating myoblasts (DM) and depolarization induced by 10 mM Cs^+ (Cs10 mM) does not affect the level of phosphorylation. By contrast, myogenin expression associated with differentiation is reduced in presence of Cs^+. Protein extracts were prepared at the indicated time. (**B**) Phosphorylation of recombinant ATF2 by immunoprecipitated phospho-p38-MAPK was used to assess p38-MAPK activity. ATF2 phosphorylation has been detected with an antibody specific for phospho-ATF2 (Thr71). Phospho-p38-MAPK was immunoprecipitated from proliferating myoblasts (GM), and from myoblasts differentiated for 4 and 24 hours in presence or absence of 10 mM Cs^+. (**C**) Detection of phospho-AKT by western blot. Myoblasts were grown in media without exogenous insulin. Phospho-AKT (Ser473/Thr308) is present in proliferating myoblasts (GM) and myoblasts differentiated for 24 hours (DM). Phospho-AKT expression is not affected by 10 mM Cs^+ (Cs10) or 15 μM BAPTA-AM (BA). Myogenic differentiation is confirmed by myogenin expression.

phosphorylated (activated) form. We found that p38-MAPK was already phosphorylated in proliferating myoblasts (Fig. 4A) and that it remained phosphorylated during the first 24 hours of the differentiation process. The presence of phosphorylated p38-MAPK during proliferation made it unlikely that this pathway would be controlled by the differentiation-linked hyperpolarization. This was confirmed by showing that phosphorylation of p38-MAPK is not sensitive to Cs^+ (Fig. 4A). When myoblasts were induced to differentiate in the presence of 10 mM Cs^+ to block the hyperpolarization, the expression of phospho-p38-MAPK was not affected. The efficiency of the Cs^+ treatment in this experiment is illustrated by the marked downregulation of myogenin expression in presence of 10 mM Cs^+. These results demonstrate that the activation and the maintained activity of the p38-MAPK regulatory pathway are not correlated with the Kir2.1-induced hyperpolarization during human myoblast differentiation.

We also verified directly phospho-p38-MAPK activity by evaluating the ability of immunoprecipitated phospho-p38-MAPK from proliferating and differentiating human myoblasts to phosphorylate recombinant ATF2. Fig. 4B shows that recombinant ATF2 is phosphorylated by phospho-p38-MAPK immunoprecipitated from proliferating myoblasts, as well as by myoblasts induced to differentiate for 4 and 24 hours. The same kinase activity was observed in presence of 10 mM Cs^+, confirming by a direct assessment of phospho-p38-MAPK activity that the Kir2.1-linked hyperpolarization is not involved in the activation or maintained activity of the p38-MAPK regulatory pathway during human myoblast differentiation.

Activation of the PI3K regulatory pathway depends upon insulin/IGF1 signaling (Jiang et al., 1998; Kaliman et al., 1996; Kandel and Hay, 1999). In order to avoid unwanted stimulation of PI3K, insulin was omitted from both growth and differentiation media in the experiments involving this pathway.

A key downstream molecule activated by PI3K is AKT (protein kinase B). Thus, as readout for PI3K activity, we used AKT phosphorylation. When the PI3K pathway is activated, AKT is phosphorylated at two sites, Ser473 and Thr308. Fig. 4C shows that AKT is already phosphorylated at the two sites in proliferating myoblasts, and that the phosphorylation level is comparable with that of myoblasts induced to differentiate for 24 hours. This result shows that, like p38-MAPK, PI3K is already fully active in proliferating human myoblast. Treating myoblast with 10 mM Cs^+ to inhibit Kir2.1-induced hyperpolarization had no effect on AKT phosphorylation (Fig. 4C), indicating that the hyperpolarization is not modulating the PI3K regulatory pathway. In the same experiment, a downregulation of myogenin expression indicated that Cs^+ was efficient at inhibiting differentiation. Fig. 4C also shows that the AKT pathway is not regulated by Ca^{2+}. In the presence of 15 μM BAPTA-AM, AKT phosphorylation was not affected although myogenin expression was markedly inhibited.

Taken together, these results show that PI3K and p38-MAPK pathways are activated during myoblast proliferation. They are not controlled by the Kir2.1-linked hyperpolarization, but they are required for a full myoblast differentiation to take place.

DISCUSSION

This work performed on primary cultures of human myoblasts demonstrates that the Kir2.1-linked hyperpolarization specifically activates the Ca^{2+}-dependent calcineurin pathway, and that this pathway is tightly linked to the induction of the differentiation process. This work also shows that activity of the CaMK, p38-MAPK and PI3K pathways, although required for a full differentiation to take place, does not interfere with myoblast proliferation, and is not affected by the differentiation-induced hyperpolarization.

Our model is that the Kir2.1-linked hyperpolarization and the resulting calcineurin activation constitute the molecular switch that forces myoblast to differentiate and fuse to form myotubes (Fig. 5).

Calcineurin signaling pathway

In skeletal muscle, the calcineurin pathway has been first implicated in transcriptional regulation of slow fiber genes (Chin et al., 1998). Calcineurin is a serine/threonine phosphatase that is activated during sustained elevations of intracellular Ca^{2+} (Klee et al., 1998), as occur

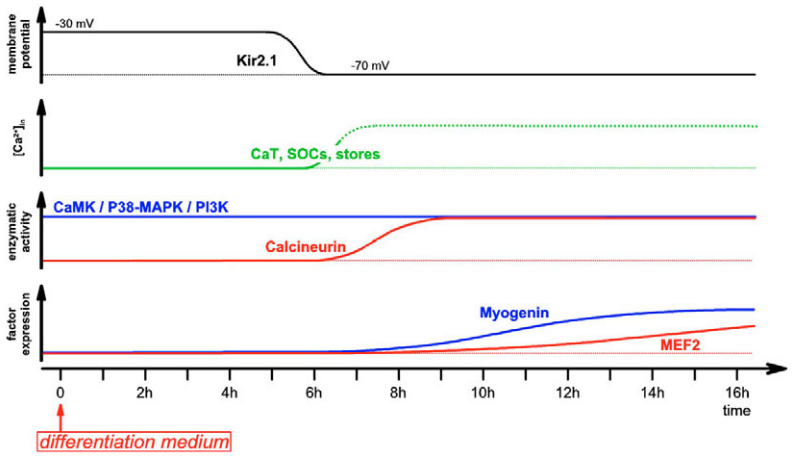

Fig. 5. Schematic representation of the role of membrane hyperpolarization during human myoblast differentiation. Chronological illustration of the mechanisms of myoblast differentiation, that begins with calcineurin activation as a consequence of Kir2.1-induced membrane hyperpolarization. Calcineurin activation is strictly associated with Kir2.1 activity and the onset of the differentiation process. Activation of Ca^{2+} influx through T-type Ca^{2+} channels (CaT) or store-operated channels (SOCs), or release of Ca^{2+} for endoplasmic reticulum (stores), could provide the increase of intracellular Ca^{2+} concentration responsible for myoblast differentiation and calcineurin activation (Arnaudeau et al., 2006). However, p38-MAPK, PI3K and CaMK are (or can be) activated during myoblast proliferation without inducing myoblast differentiation. CaMK can be activated by an increase in extracellular Ca^{2+} concentration not linked to the differentiation process. Myogenin and MEF2 expression is observed after calcineurin activation.

in muscle fibers by slow type motoneuron firing (Schiaffino and Serrano, 2002). In vivo, the downstream substrates for calcineurin implicated in the fast-to-slow fiber switch include the transcription factors NFAT and MEF2 (Chin et al., 1998; Wu et al., 2000a). A role for calcineurin at early steps of differentiation in mouse myoblasts has also been suggested (Friday et al., 2000; Xu et al., 2002). However, the trigger of a change in calcineurin activity at the beginning of the differentiation process, i.e. prior to innervation, remained to be explained.

In human myoblasts induced to differentiate, we showed that calcineurin activation is linked to the Kir2.1 linked membrane hyperpolarization. In presence of either Cs^+ or high K^+, calcineurin activity is reduced to a level close to the basal level measured in proliferating myoblast. In a previous work, we showed that blockade of Kir2.1 channels with 10 mM Cs^+ depolarizes myoblast induced to differentiate to approximately –35 mV (Konig et al., 2004), while high external K^+ (116 mM) clamps the resting membrane potential in the vicinity of 0 mV. The remaining potential of about –35 mV in presence of 10 mM Cs^+ is probably due to activity of ether-à-gogo and/or ether-à-gogo-related gene K^+channels (Bijlenga et al., 1998; Liu et al., 2003). Here, we show that calcineurin activity is already fully inactivated by 10 mM Cs^+, which suggests that, during the early stages of human myoblast differentiation, calcineurin activation is strictly controlled by the activity of Kir2.1 channels and the signals they induce.

There are evidences that L-type Ca^{2+} channels are essential to initiate calcineurin activation in neurons and in rat ventricular myocytes (Graef et al., 1999; Perrier et al., 2004). In addition, in differentiating C2C12 myoblasts, it was clearly shown that calcineurin is a downstream mediator of IGF-1-induced signaling through L-type Ca^{2+} channels (Spangenburg et al., 2004). In human myoblasts, however, inhibition of L-type Ca^{2+} channels has no effect on the early steps of the differentiation process, whereas T-type Ca^{2+} channels activity induced by the Kir2.1 linked hyperpolarization plays an important role (Bijlenga et al., 2000). Specifically, we could show that activation of the T-type Ca^{2+} channels at hyperpolarized potentials allows a sustained low amplitude increase of intracellular Ca^{2+} (Bijlenga et al., 2000) that is compatible with the Ca^{2+} signals that activate calcineurin (Klee et al., 1998). However, we recently showed that all clones of human myoblasts do not exclusively use T-type Ca^{2+} channels to differentiate (Arnaudeau et al., 2006). We found that, as they differentiate, different clones of human myoblasts can use, in addition to or as substitutes for T-type Ca^{2+} channels, others sources of Ca^{2+} such as an influx through store operated channels or a release of Ca^{2+} from the endoplasmic reticulum via IP3 receptors. However, all these Ca^{2+} signals are suppressed when the hyperpolarization is blocked.

Thus, we propose that, in differentiating human myoblasts and prior to the innervation of myotubes, there is already a first activation of calcineurin that links the early activation of the Kir2.1-induced membrane hyperpolarization and its associated Ca^{2+} signals to the expression/activity of myogenin and MEF2 transcription factors.

CaMK regulatory pathway

CaMK is another Ca^{2+}-dependent pathway involved in human myoblast differentiation. CaMKII activation is, however, not linked to Kir2.1-induced hyperpolarization. Indeed, CaMKII activities measured at 1 hour in differentiation medium (before activation of Kir2.1 channels) or at 24 hours (long after membrane hyperpolarization took place) are statistically identical. Furthermore, blockade of Kir2.1 channels with 10 mM Cs^+ or inhibition of the hyperpolarization with 116 mM K^+ have no effect on the CaMKII activity. These results suggest that the Ca^{2+} signals that are linked to the hyperpolarization do not control the CaMKII activity, and that at least two different Ca^{2+} signals must exist to activate sequentially CaMK and calcineurin. This is in agreement with previous work suggesting that these two enzymes are activated by different Ca^{2+} signals, i.e. transient high amplitude Ca^{2+} spikes preferentially activate CaMKII, whereas sustained low-amplitude Ca^{2+} increases rather induce calcineurin activity (McKinsey et al., 2002).

Our results also show that CaMKII activation depends on extracellular Ca^{2+} concentration (influx). We found that the CaMK activity measured in myoblasts kept for 1 hour in differentiation medium containing only 15 μM Ca^{2+} is massively reduced, and that CaMK activity is similar in myoblasts kept in either growth or differentiation medium containing the same Ca^{2+} concentration (this was tested for 0.7 and 1.8 mM Ca^{2+}). CaMKII activation can thus be induced either in proliferation or in differentiation conditions. It seems to depend only on extracellular Ca^{2+} concentration, and its activation does not affect the rate of myoblast proliferation. In vivo, it is possible that CaMKII is activated in proliferating myoblasts according to extracellular Ca^{2+} concentration. Indeed, we expect that during a massive myofiber degeneration or after a muscle injury, Ca^{2+} concentration could be locally increased. It is worth recalling that CaMK activation on its own does not induce myoblast differentiation but is required for an optimal differentiation to take place. It is possible that a graded CaMK activation, depending on extracellular Ca^{2+} concentration, could optimize myoblast differentiation and muscle regeneration.

p38-MAPK and PI3K regulatory pathways

Several authors have proposed that p38-MAPK is activated after the induction of myoblast differentiation and that this activation promotes myoblast differentiation (Cuenda and Cohen, 1999; Wu et al., 2000b; Zetser et al., 1999). Inhibition of p38-MAPK in human primary myoblasts decreases myoblast differentiation, which confirms an implication of this pathway in human cells. However, using two different approaches, we found that p38-MAPK is already activated in proliferating myoblasts, and that this pathway is not regulated during the early steps of the differentiation process. Our results are in agreement with a recent work, suggesting that p38-MAPK plays a major role in triggering differentiation of quiescent satellite cells into proliferating myoblasts rather than myoblasts into myotubes (Jones et al., 2005). However, as p38-MAPK activity is required for a full myoblast differentiation to proceed, it was important to show that this pathway was not affected (downregulated) by the differentiation-linked hyperpolarization. We found, indeed, that p38-MAPK activity is Ca^{2+}-independent and insensitive to the change in potential generated by Kir2.1 channel activation.

Kinetics of activation of PI3K during human myoblast differentiation is similar to that of p38-MAPK. We found that PI3K is active in proliferating myoblasts and that its activity is neither Ca^{2+}-dependent nor hyperpolarization sensitive. It should be noted, however, that, both in proliferating and differentiating myoblasts, PI3K is activated in the absence of added insulin in the culture media. As differentiation medium does not contain any serum and as our cultures are purely composed of myoblasts (see Materials and methods), we hypothesize that either human myoblasts are able to secrete IGF1, or that PI3K is activated via an IGF1-independent mechanism (Rauch and Loughna, 2005). It should also be mentioned that, in contrast to what occurs in mouse cell lines (Gonzalez et al., 2004; Li et al., 2000; Wu et al., 2000b), concerted activation of both PI3K and p38-MAPK pathways is not sufficient in itself to induce human myoblast differentiation.

Taken together, our results highlight the importance of the calcineurin activation in the control of the early steps of myoblast differentiation. We give for the first time the sequence and the respective roles of the different signaling pathways involved in the differentiation process. We show that p38-MAPK, PI3K and CaMK activity are required for a full human myoblast differentiation but that these three pathways, even together, do not trigger the differentiation process. We also show that these three pathways are or can be activated during proliferation without affecting the proliferation rate. As CaMK is activated before calcineurin, it thus appears that at least two different Ca^{2+} signals may be required for a full differentiation to take place, and that only the signal activating calcineurin is directly linked to the induction of the differentiation leading to myoblast fusion. In our model, the triad constituted by the Kir2.1-linked hyperpolarization, its associated Ca^{2+} signals and the resulting calcineurin activation controls the initiation of myoblast fusion.

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