TRPC1 regulates skeletal myoblast migration and differentiation

Magali Louis*, Nadège Zanou*, Monique Van Schoor and Philippe Gailly‡

Université catholique de Louvain, Institute of Neuroscience, Laboratory of Cell Physiology, 55/40 avenue Hippocrate, 1200 Brussels, Belgium

*These authors contributed equally to this work
‡Author for correspondence (e-mail: philippe.gailly@uclouvain.be)

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Summary
Myoblast migration is a key step in myogenesis and regeneration. It allows myoblast alignment and their fusion into myotubes. The process has been shown to involve m-calpain or μ-calpain, two Ca2+-dependent cysteine proteases. Here we measure calpain activity in cultured cells and show a peak of activity at the beginning of the differentiation process. We also observed a concomitant and transient increase of the influx of Ca2+ and expression of TRPC1 protein. Calpains are specifically activated by a store-operated entry of Ca2+ in adult skeletal muscle fibres. We therefore repressed the expression of TRPC1 in myoblasts and studied the effects on Ca2+ fluxes and on differentiation. TRPC1-depleted myoblasts presented a largely reduced store-operated entry of Ca2+ and a significantly diminished transient influx of Ca2+ at the beginning of differentiation. The concomitant peak of calpain activity was abolished. TRPC1-knockdown myoblasts also accumulated myristoylated alanine-rich C-kinase substrate (MARCKS), an actin-binding protein and substrate of calpain. Their fusion into myotubes was significantly slowed down as a result of the reduced speed of cell migration. Accordingly, migration of control myoblasts was inhibited by 2-5 μM GsMTx4 toxin, an inhibitor of TRP channels or by 50 μM Z-Leu-Leu, an inhibitor of calpain. By contrast, stimulation of control myoblasts with IGF-1 increased the basal influx of Ca2+, activated calpain and accelerated migration. These effects were not observed in TRPC1-knockdown cells. We therefore suggest that entry of Ca2+ through TRPC1 channels induces a transient activation of calpain and subsequent proteolysis of MARCKS, which allows in turn, myoblast migration and fusion.

Key words: Myoblast, Calcium, Calpain, Differentiation, Migration, TRPC1, MARCKS

Introduction
During myogenesis and muscle regeneration, myogenic stem cells (called satellite cells) proliferate as myoblasts. These myoblasts then withdraw from the cell cycle and begin to express muscle-specific genes. They migrate and align with each other, and finally they fuse either with other myoblasts to form multinucleated myotubes, or with pre-existing muscle fibres. The fusion process is known to be Ca2+ dependent. Indeed, formation of myotubes requires the presence of extracellular Ca2+ (Schmid et al., 1984), and is preceded by an increase of cytosolic concentration of Ca2+ ([Ca2+]cyt) (Przybylski et al., 1994) because of a net influx of Ca2+ into the cell (David et al., 1981). The Ca2+ influx occurs through T-type Ca2+ channels (Bijlenga et al., 2000) and most probably through other not clearly identified Ca2+ channels, possibly including store-operated and stretch-activated channels (Arnaudeau et al., 2006).

During the initial phase of differentiation, Ca2+ activates several myogenic transcription factors such as myogenin and MEF2 (Black and Olson, 1998; Molkentin and Olson, 1996), triggering the expression of muscle-specific genes. Calpain is another possible Ca2+-sensitive target. Indeed, calpains are Ca2+-sensitive intracellular proteases implicated in spreading and locomotion of adherent cells (Glading et al., 2002; Huttenlocher et al., 1997; Mazeres et al., 2006), and it has been reported that calpain inhibition completely prevents myoblast differentiation into myotubes (Dedieu et al., 2004). Sensitivity of calpains to [Ca2+]cyt is quite low (μM to mM range). However, we previously showed that a store-operated entry of Ca2+ was sufficient, in adult skeletal muscle fibres, to activate calpains. Store-dependent entry of Ca2+ is present in adult skeletal muscles (Boitn et al., 2006; Ducret et al., 2006; Vandebrouck et al., 2002) and in myotubes (Gutierrez-Martin et al., 2005; Vandebrouck et al., 2006). The identification of the membrane channels involved in this store-dependent Ca2+ entry has been largely investigated in non-excitable cells (Gailly, 1998; Gailly and Colson-Van Schoor, 2001). The best candidate molecules seem to belong to the superfamily of transient receptor potential (TRP) channels (for reviews, see Owstaniak et al., 2006; Venkatachalam and Montell, 2007). These TRP channels present a similarity of structure (six transmembrane domains) and some sequence homology. In mammals, the TRP superfamily contains six subfamilies. Four of them share substantial sequence identity in the transmembrane domains: classical (TRP), vanilloid (TRPV), melastatin (TRPM) and ANKTM1 (TRPA). The remaining two subgroups, mucolins (TRPML) and polycystins (TRPP) are only distantly related to the other subfamilies.

So far, the TRP channels identified as possibly involved in store-operated influxes of Ca2+ belong to the TRPC and TRPV subfamilies. In particular, studies reporting that the endogenous TRPC1 channel constitutes a component of store-operated channels are consistent (for reviews, see Ambudkar, 2007; Beech et al., 2003; Rychkov and Barritt, 2007). TRPC1 might be associated in a ternary complex with another channel called Orai1 (Feske et al., 2006; Soboloff et al., 2006) and with STIM1, an intrareticular Ca2+ sensor, to contribute to store-operated channels (Ambudkar et al., 2007; Hewavitharana et al., 2007; Liao et al., 2008; Worley et al., 2007; Yuan et al., 2007). TRPC1 has also been reported to be mechanosensitive (Maroto et al., 2005), although this is still a matter of controversy (Dietrich et al., 2007; Gottlieb et al., 2008).
In this paper, we studied the involvement of TRPC1 in myoblasts migration and differentiation. We first show that endogenous TRPC1 is indeed a necessary component for store-operated entry of Ca\(^{2+}\) in myoblasts. Second, we show that myoblast differentiation requires a transient increase of expression of TRPC1 channels with a consecutive transient increase of Ca\(^{2+}\) influx in the cytosol, resulting in activation of calpains. Calpain activation in turn allows myoblast migration and fusion into myotubes.

**Results**

TRPC1 repression reduces store-operated entry of Ca\(^{2+}\)

C2C12 myoblasts express several isoforms of the classical subfamily of TRP channels. Among these, TRPC1 is by far the most abundantly expressed (its mRNA content being at least 100 times higher than for any other isoform) (Fig. 1A). Several investigators have reported that TRPC1 is responsible for store-operated entry of Ca\(^{2+}\). Accordingly, we previously showed that in adult skeletal muscle fibres, there was a Ca\(^{2+}\) leak channel that could be activated by store depletion or by membrane stretching (hypo-osmotic shock); this channel is abnormally open in dystrophin-deficient fibres and seems to be constituted of TRPC1 and/or TRPC4 isoforms (Vandebrouck et al., 2002). We therefore repressed the expression of TRPC1 in C2C12 myoblasts using two different siRNAs (see Materials and Methods). By quantitative RT-PCR, we could measure a repression of 72±2% (siRNAs (see Materials and Methods). By quantitative RT-PCR, we could measure a repression of 72±2% (n=5, both siRNAs were similarly effective). Accordingly, western blot analysis showed an important decrease of the expression of TRPC1 protein (Fig. 1B); pooling the results for both TRPC1 siRNAs, we measured a repression of 70.5±11%, n=6. For long-term experiments (such as differentiation for 7 days, see below), we used a shRNA plasmid strategy (bicistronic vector coding for a silencing hairpin RNA and for EGFP) to select the cells stably expressing the shRNA (selection by resistance to antibiotics and by EGFP fluorescence). Myoblasts stably transfected with TRPC1 shRNA showed a significant decrease of TRPC1 expression in comparison with cells transfected with control shRNA (Fig. 1C). These transfected myoblasts were then studied functionally. As expected, we observed that TRPC1 repression significantly reduced the store-operated entry of Ca\(^{2+}\). Indeed, in the absence of external Ca\(^{2+}\), treatment of the cells with 1 μM thapsigargin, a SERCA inhibitor, induced a [Ca\(^{2+}\)] transient (as a result of the release of the stores) that was similar in myoblasts transfected with control shRNA and TRPC1 shRNA. However, re-addition of external Ca\(^{2+}\) triggered a much weaker store-depletion-induced entry of Ca\(^{2+}\) in TRPC1 shRNA transfected cells than in the control (Fig. 2). Similar results were obtained in TRPC1 siRNA knockdown myoblasts and in myoblasts treated with GsMTx4 toxin (Fig. 2), an inhibitor of mechanosensitive and store-operated channels, and in particular, an inhibitor of TRPC1 (Bowman et al., 2007).

**TRPC1 repression reduces myoblast migration and differentiation**

Interestingly, we observed that TRPC1 shRNA silenced myoblasts fused much more slowly into myotubes than the controls (Fig. 3). Indeed, after 7 days of differentiation, we observed that the proportion of cellular nuclei in myotubes (being defined as having at least three nuclei) was three times lower in TRPC1-knockdown cells than in controls (Fig. 3B). This effect seemed to be due to the absence of pre-fusion alignment of myoblasts. Therefore, we used a wound-healing assay to investigate the role of TRPC1 in cell migration (Fig. 4). Cells in culture were scraped off with a pipette tip on a 600-μm-wide area and myoblast migration into this acellular area was quantified and expressed as the percentage of the number of cells having migrated in control conditions. We first studied the effect of GsMTx4 toxin. We observed that treatment of C2C12 myoblasts with GsMTx4 dose-dependently reduced cell migration (Fig. 4). We then confirmed the involvement of TRPC1 by showing that repression of TRC1 expression by two different siRNAs or by a shRNA significantly reduced the speed of cell migration (Fig. 5).

Leloup and colleagues have recently demonstrated the involvement of calpains in the migration of myogenic cell lines (Leloup et al., 2006). We confirmed here that inhibition of calpains with 50 μM Z-Leu-Leu-CHO dramatically inhibited migration (Fig. 5).
TRPC1 channels control myoblast migration

Myoblast differentiation is accompanied by a transient increase of calpain activity and a concomitant peak of Ca\(^{2+}\) influx. The possible involvement of calpain in the migration and fusion processes prompted us to measure its activity after inducing differentiation by replacing the proliferation medium (10% fetal calf serum) with a differentiation medium (adult horse serum). In order to measure calpain activity in situ, we used a fluorescent technique based on the detection of the cleavage of Boc-Leu-Met-CMAC peptide, a membrane-permeant fluorogenic substrate of calpain (see Materials and Methods). We observed that the activity of calpain increased from day 0 to day 1 and then return to its basal value (Fig. 6). Interestingly, this peak of activity was not observed in myoblasts transfected with TRPC1 shRNA. Therefore, we investigated whether this transient increase of calpain activity was the result of activation by a transient increase of Ca\(^{2+}\) entry through TRPC1 channels. We evaluated this influx of Ca\(^{2+}\) by measuring the passive influx of Mn\(^{2+}\) ions used as surrogates for Ca\(^{2+}\) ions. This technique is based on the fact that Mn\(^{2+}\) entering the cells through Ca\(^{2+}\) channels is not sequestered by SERCA pumps and quenches Fura-PE3 fluorescence; the rate of quenching therefore directly reflects the rate of Ca\(^{2+}\) influx (Merritt et al., 1989). Using this Mn\(^{2+}\) quenching technique, we observed that indeed, control C2C12 cells presented a differentiation-induced transient increase of Ca\(^{2+}\) influx that was concomitant with the peak of calpain activity (significant increase from day 0 to day 1, then return to basal values) (Fig. 7A). This peak of Ca\(^{2+}\) entry at day 1 was significantly reduced in myoblasts incubated for 10 minutes in the presence of 5 \(\mu\)M GsMTx4 toxin (reduction by a factor of 1.6 in the presence compared with in the absence of GsMTx4, \(n=7\); \(P<0.001\)). In C2C12 myoblasts stably transfected with control plasmid shRNA or transiently transfected with control siRNA, a similar peak of Ca\(^{2+}\) entry was observed, emphasizing the absence of toxicity of transfections. However, this peak of [Ca\(^{2+}\)]\(i\) was abolished in myoblasts transfected with TRPC1 shRNA and TRPC1 siRNA, confirming the involvement of TRPC1 in this differentiation-induced transient increase of Ca\(^{2+}\) influx (Fig. 7B,C). We therefore investigated the possibility that the transient increase of Ca\(^{2+}\) influx observed at day 1 of differentiation might be explained by the presence of a larger number of TRPC1 channels. TRPC1 expression was quantified by real-time RT-PCR. We observed that the expression of TRPC1 increased by 55±12% at day 1 of differentiation in comparison with day 0 (\(n=5\), \(P<0.01\)) and then return to a basal value at day 4 to day 6. Western blot analysis supported this finding (Fig. 8). Incidentally, we also observed a large increase of expression of TRPC3 during differentiation (data not shown), confirming previous observations (Lee et al., 2006).

TRPC1-knockdown myoblasts accumulate MARCKS

Myristoylated alanine-rich C kinase substrate (MARCKS) is an actin-binding protein that is reported to be involved in myoblast fusion through its regulation by calpain proteolytic cleavage (Dulong et al., 2004). It accumulates in cells showing a reduced calpain activity, such as in calpastatin-overexpressing cells (Dedieu Fig. 2. Store-operated entry of Ca\(^{2+}\) in control and TRPC1-knockdown myoblasts. Influence of GsMTx4. (A) Store-operated entry of Ca\(^{2+}\) was triggered by depletion of the stores with 1 \(\mu\)M thapsigargin (TG) in the absence of external Ca\(^{2+}\) (0.1 mM EGTA). 1.8 mM Ca\(^{2+}\) was then re-added to the extracellular medium as indicated, inducing a second peak of [Ca\(^{2+}\)]. Comparison of the response in C2C12 myoblasts (1 day post differentiation) transfected with control shRNA or TRPC1 shRNA (efficiency of transfection checked by EGFP fluorescence emission). Traces are representative of up to 11 experiments. (B-D) Results of similar experiments obtained in myoblasts treated with TRPC1 shRNA or TRPC1 siRNA and myoblasts treated with 5 \(\mu\)M GsMTx4. SOCE, store-operated Ca\(^{2+}\) entry. Results are mean ± s.e.m. Student’s \(t\)-test: *\(P<0.05\) vs control; \(n=6\)–12.
et al., 2004). We therefore compared the amount of MARCKS in control and TRPC1-knockdown myoblasts. Using western blot analysis (Fig. 1B), we detected a large band around 80 kDa and showed that the expression was increased by a factor of 2.25 in myoblasts transfected with siRNA-TRPC1 compared with myoblasts treated with control siRNA (n=6, Mann-Whitney Rank Sum Test P<0.01). Fig. 9 shows MARCKS localization in myoblasts treated with control siRNA and TRPC1 siRNA (1 day of differentiation). MARCKS presented a diffuse cytosolic pattern in control myoblasts. Interestingly, in TRPC1-knockdown myoblasts, MARCKS expression increased and appeared both as punctate structures and diffusely in the cytosol.

**IGF-1 increases the influx of Ca^{2+} and the activity of calpain and accelerates migration**

Stimulation of myoblasts with growth factors such as IGF-1 is known to accelerate their migration. The effect requires calpain activity because it is inhibited by 80 μM calpeptin, a calpain inhibitor.
myoblasts overexpressing calpastatin, Dedieu and collaborators investigated whether IGF-1 stimulation influenced transmembrane Ca\(^{2+}\) influxes and calpain activity. Stimulation of control myoblasts maintained in proliferation medium with 5 nM IGF-1 for 10 minutes significantly increased the rate of Mn\(^{2+}\) quenching of Fura-PE3 from 0.18±0.01%, n=18 to 0.453±0.030%, n=7 (P<0.001). When stimulated for longer periods of time (1-4 hours), the effect was less pronounced but the influx of Mn\(^{2+}\) nevertheless remained significantly higher than in basal conditions (Fig. 10B). A similar effect was observed in myoblasts transfected with control siRNA or shRNA, but the effect was completely lost in cells treated with TRPC1 siRNA and shRNA (not shown). In parallel, stimulation of C2C12 cells with 5 nM IGF-1 significantly increased calpain activity (Fig. 10C). A trend was observed within 10 minutes, but the effect was significant only after 4 hours. In myoblasts transfected with TRPC1 siRNA, the effect of IGF-1 on calpain activity was completely abolished (not shown), again suggesting a causal relation between the influx of Ca\(^{2+}\) through TRPC1 channel and the activation of calpain.

Discussion

Calpains are members of a large family of Ca\(^{2+}\)-dependent cysteine proteases. Both \(\mu\)-calpain and \(m\)-calpain, which are activated by micromolar or millimolar ranges of [Ca\(^{2+}\)], respectively, have been implicated in cell spreading by modifying adhesion sites, and in promoting locomotion of adherent cells by facilitating rear-end detachment (Glading et al., 2002; Huttenlocher et al., 1997; Mazeres et al., 2006). In particular, calpains are implicated in growth-factor-mediated migration of myoblasts (Leloup et al., 2006). Migration is inhibited by pharmacological inhibitors of calpains, such as calpeptin, leupeptin or calpain inhibitor II, and migration speed is reduced in cells overexpressing calpastatin, an endogenous inhibitor of calpains (Dedieu et al., 2004). When myoblast differentiation is promoted by removing fetal serum from the culture medium, the expression of \(m\)-calpain increases; conversely, the expression of \(\mu\)-calpain decreases (Leloup et al., 2006). The resulting intracellular calpain activity is unpredictable because it depends not only on the expression of the proteins but also on the presence of intracellular regulators (calpastatin, Ca\(^{2+}\)).

In the present study, we measured the activity of calpain in situ and show that differentiation of myoblasts is accompanied by a transient increase of calpain activity. This activity peaks after 1 day of differentiation and then returns to basal values. As expected, stimulation with IGF-1, which increases migration speed, also increases calpain activity, whereas Z-Leu-Leu-CHO, which inhibits calpain activity, reduces migration accordingly.

Calpain exerts its action on cell motility by cleaving different substrates of the adhesion complex (e.g. desmin, vinculin, talin, focal adhesion kinase) (Glading et al., 2002). However, comparing the proteolytic pattern of these proteins in normal myoblasts and in myoblasts overexpressing calpastatin, Dedieu and collaborators found accumulation of MARCKS only in cells showing a reduced calpain activity, suggesting the involvement of this actin-binding protein in cell migration (Dedieu et al., 2003; Dedieu et al., 2004). Dulong and colleagues demonstrated the direct cleavage of MARCKS by calpain and its involvement in myoblast migration (Dulon et al., 2004). Calpain-dependent MARCKS proteolysis was also shown to activate its actin binding activity and therefore its ability to modulate actin dynamics and cell migration (Tapp et al., 2005). This proteolysis seems to be PKC\(\alpha\) dependent (Goudenege et al., 2005). In the present study, we show that MARCKS accumulates in TRPC1-knockdown myoblasts. In these cells, MARCKS partially appears as spotted structures. We propose that accumulation of MARCKS only in cells showing a reduced calpain activity, suggesting the involvement of this actin-binding protein in cell migration (Dedieu et al., 2003; Dedieu et al., 2004). Dulon and colleagues demonstrated the direct cleavage of MARCKS by calpain and its involvement in myoblast migration (Dulon et al., 2004). Calpain-dependent MARCKS proteolysis was also shown to activate its actin binding activity and therefore its ability to modulate actin dynamics and cell migration (Tapp et al., 2005). This proteolysis seems to be PKC\(\alpha\) dependent (Goudenege et al., 2005). In the present study, we show that MARCKS accumulates in TRPC1-knockdown myoblasts. In these cells, MARCKS partially appears as spotted structures. We propose that accumulation of MARCKS could stabilize actin structures, possibly focal adhesion plaques, and that the entry of Ca\(^{2+}\) through TRPC1 (see below) and the subsequent activation of calpain would induce MARCKS proteolysis and allow migration.

If the substrate targets of calpain are identified, a question remains concerning the mechanisms involved in their activation at the beginning of differentiation. The most important regulator of calpain is Ca\(^{2+}\). The \(m\)-calpain isoform requires millimolar levels of [Ca\(^{2+}\)] for activation, a concentration unattainable inside cells under physiological conditions. The \(\mu\)-calpain isoform is more sensitive to Ca\(^{2+}\) but nevertheless requires a half-activating [Ca\(^{2+}\)] as high as 34 \(\mu\)M (Kapprell and Goll, 1989), which is also largely
channels are involved in the deregulation of intracellular Ca\(^{2+}\) (Vandebrouck et al., 2002). We also showed that these belong to the TRPC family (TRPC1 and/or TRPC4) (Ducret et al., 2007). Previously, we had suggested the necessity of a prolonged and localized (probably above the [Ca\(^{2+}\)], occurring within cells in physiological conditions. However, conditions that lower the Ca\(^{2+}\) requirement have been identified: binding of calpain to membrane phosphatidylinositol (Arthur and Crawford, 1996; Melloni et al., 1996) and autolysis that occurs in activated calpain (Baki et al., 1996; Suzuki and Sorimachi, 1998). In addition, we recently reported that calpain was partially autolyzed and specifically activated by a store operated and/or mechanosensitive entry of Ca\(^{2+}\) in adult skeletal muscle fibres (Gailly et al., 2007). Previously, we had suggested that the channels responsible for store-dependent and mechanosensitive entry of Ca\(^{2+}\) in adult skeletal muscle might belong to the TRPC family (TRPC1 and/or TRPC4) (Ducret et al., 2006; Vandebrouck et al., 2002). We also showed that these channels are involved in the deregulation of intracellular Ca\(^{2+}\) homeostasis observed in dystrophin-deficient fibres (Gailly, 2002; Gailly et al., 1993a; Gailly et al., 1993b). Here, we therefore repressed the expression of TRPC1 in C2C12 myoblasts. As expected, we observed an important decrease of store-operated entry of Ca\(^{2+}\). Interestingly, these TRPC1-knockdown myoblasts migrated more slowly and therefore presented a retarded fusion process into myotubes.

By contrast, stimulation of control cells with IGF-1, which is known to increase store-operated influx of Ca\(^{2+}\) (Ju et al., 2003), increased the basal influx of Ca\(^{2+}\), activated calpain activity and accelerated migration. These effects were not observed in TRPC1-knockdown cells, confirming the involvement of TRPC1 in this process. The rapidity of the effect of IGF-1 on Ca\(^{2+}\) influx suggests a translocation of TRPC1 protein from a pre-existing intracellular pool to the plasma membrane, as shown for other TRP channels (Iwata et al., 2003; Rolland et al., 2006). Alternatively, IGF-1 stimulation might activate or induce translocation of K\(^+\) channels, leading to a rapid hyperpolarization. The time lag between the IGF-1-induced influx of Ca\(^{2+}\) and the activation of calpain might indicate the necessity of a prolonged and localized (probably subsarcolemmal) increase of [Ca\(^{2+}\)], as recently suggested (Gailly et al., 2007).

Finally, we showed that normal differentiation of myoblasts is accompanied by a transient overexpression of TRPC1 and by concomitant TRPC1-dependent transient increases of Ca\(^{2+}\) influxes and of calpain activity. We therefore suggest that the following sequence of events takes place during myoblasts differentiation: (1) transient increase of the expression of TRPC1 channels; (2) influx of Ca\(^{2+}\); (3) activation of calpains; (4) cleavage of MARCKS (and possibly other substrates); (5) migration of myoblasts; (6) alignment; and (7) fusion into myotubes.

Ca\(^{2+}\) influx has long been known to be important for triggering myoblast differentiation or fusion. This influx of Ca\(^{2+}\) is activated by a hyperpolarization of the plasma membrane induced by a progressive expression of the Kir2.1 K\(^+\) channel (Bernheim and Bader, 2002; Lory et al., 2006; Park et al., 2002). The hyperpolarization induces a very small but permanent current of Ca\(^{2+}\) through T-type Ca\(^{2+}\) channels in human myoblasts (Bijlen et al., 2000). However, it was recently shown that T-type Ca\(^{2+}\) currents are not detectable in C2C12 cells and are detected only subsequent to differentiation process in mouse primary satellite cells (Bidaud et al., 2006). RT-PCR confirms that T-type Ca\(^{2+}\) channels

![Calpain activity during differentiation of control and TRPC1-knockdown myoblasts.](Image)

Fig. 6. Calpain activity during differentiation of control and TRPC1-knockdown myoblasts. Calpain activity was measured as the rate of fluorescence increase of cells incubated with 10μM Boc-Leu-Met-CMAC, a permeant and nonfluorescent substrate that, upon cleavage by calpain, becomes fluorescent. Calpain activity was measured at different stages of differentiation (day 0 to day 4) and all measurements were expressed relative to the calpain activity measured in control myoblasts at day 0 of differentiation, during the same session of measurements (n=10 or more controls per session). Two-way ANOVA followed by a Student-Newmann-Keuls test: **P<0.01 for controls at D1 vs control at D0, D2, D3 and D4; §P<0.05 for TRPC1-knockdown at D1 vs control cells at D1; n=12-48 measurements from four different cultures.

![Mn\(^{2+}\) influxes in control and in TRPC1-knockdown myoblasts.](Image)

Fig. 7. Mn\(^{2+}\) influxes in control and in TRPC1-knockdown myoblasts. (A-C) The rate of quenching of Fura-PE3 by Mn\(^{2+}\) was used to estimate the influx of Ca\(^{2+}\) at different stages of differentiation (from day 0 to day 4) in control C2C12 myoblasts (A), in control shRNA (B, black columns) and TRPC1 shRNA-transfected cells (B, grey columns) and in control siRNA (C, black columns) and TRPC1 siRNA-transfected cells (C, grey columns). Statistical analysis: in A, one-way ANOVA, **P<0.01 for D1 vs D0, D2, D3 and D4; in B,C, two-way ANOVA followed by Student-Newmann-Keuls tests, *P<0.05 D1 control vs control at D0 and D2; §P<0.05 TRPC1-knockdown myoblasts at D1 vs controls at D1.
are not expressed in these cells; they are therefore not involved in the early stages of myogenic differentiation (Bidaud et al., 2006). Other channels might be involved, such as nicotinic acetylcholine receptors (Constantin et al., 1996; Krause et al., 1995), store-operated channels (Arnaudeau et al., 2006) or even stretch-activated receptors (Constantin et al., 1996; Krause et al., 1995), store-operated channels (Arnaudeau et al., 2006) or even stretch-activated receptors (Constantin et al., 1996; Krause et al., 1995). Here, we show the involvement of TRPC1 protein, a component of the store-operated channel, in the influx of Ca2+ linked to the initial phase of differentiation of C2C12 cells. Interestingly, the group of Bernheim recently demonstrated that the hyperpolarization-induced Ca2+ signal was linked to myoblast differentiation through a calcineurin pathway. We showed previously that the expression of TRPC1 was under control of the calcineurin-NFAT pathway. We showed previously that the expression of TRPC1 was under control of the calcineurin-NFAT pathway. We showed previously that the expression of TRPC1 was under control of the calcineurin-NFAT pathway.

**Materials and Methods**

**Cell culture**

C2C12 mouse skeletal myoblasts were obtained from American Type Culture Collection and grown in DMEM supplemented with 10% fetal bovine serum and 1% non essential amino-acids, and maintained at 37°C in a humidified atmosphere of 5% CO2. To induce differentiation, myoblasts were grown to about 50% confluency; the growth medium was then replaced by DMEM supplemented with 1% horse serum (differentiation medium). For [Ca2+]i measurements, cells were cultured on glass coverslips.

**Transfection and mRNA quantification**

For transient transfections, two different TRPC1 siRNAs were used: (1) 5'-GCCUGACUUUGUAUGAAGAU-3' and (2) 5'-GAUAGAAGUUGCCACCUGUUA-3'. A final concentration of 100 nM of each siRNA and their controls (sequence with no homology with any known eukaryotic gene; Eurogentec, Seraing, Belgium) were transfected into C2C12 myoblasts using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). For stable transfections, we used a bicistronic shRNA-TRPC1 plasmid encoding a short hairpin silencing sequence (5'-GATGGATGTCGCACCTGTTA-3') and a luciferase-silencing sequence and a GFP (psiRNA-Luc-GL3, Invitrogen). The cells were selected for a few days using 300 μM zeocin. For calpain and [Ca2+]i measurements, GFP-related fluorescence emission was used to select transfected cells. For mRNA quantification, C2C12 cells were extracted with a Ribopure kit (Ambion) and reversed-transcribed using SuperScript II RNase H (Invitrogen). Gene-specific PCR primers were designed using Primer3 (http://biotools.umassmed.edu/biapps/primer3_www.cgi). To avoid amplification of genomic DNA, primers were chosen in different exons: TRPC1 (NM_011643) F, 5'-CTGAGATGTCGCCACTCTGA-3' and R, 5'-CTCCCAAAGGGCTGTAGTCTT-3'; TRPC2 (NM_011644) 5'-ACTTCTGGAGCGGTTCATC-3' and R, 5'-TGAGACATGGTGCTGACGT-3'; TRPC3 (NM_019510) 5'-TGGATTGACCTTGAGCA-3' and R, 5'-ACGTG-
After incubation for 24 hours in a culture medium containing 0.1% fetal bovine serum, myoblasts were loaded with 1 μM Fura-PE3/AM for 60 minutes at room temperature. Fura-2-loaded cells were alternatively excited at 340 nm and 380 nm and fluorescence emission was monitored at 510 nm using a Deltascan spectrofluorimeter (Photon Technology International, dichroic mirror at 400 nm) coupled to an inverted immersion) on a Zeiss S100 inverted microscope equipped with an Axiocam HR camera. The settings for fluorescence recordings were maintained rigorously constant for all measurements. To avoid photobleaching, the cells were illuminated for only 6 seconds every minute for a period of 10-20 minutes. The significant interference could be detected during Fura-2 and EGFP fluorescence (De Backer et al., 2002; Gailly et al., 1993a).

Mn2+ quenching measurements

Passive Ca2+ influx was estimated by measuring the passive influx of Mn2+ ions used as a replacement for Ca2+ ions. Influx of Mn2+ into fibres loaded with Fura-PE3, quenches the fluorescence of the dye and the quenching rate reflects the influx rate (Gailly et al., 1996; Merritt et al., 1989). To avoid any interference of Ca2+, Fura-2<sup>18</sup>-fluorescence was excited at 360 nm where the dye is insensitive to changes of [Ca2+]i (isobestic point) and the intensity obtained before Mn2+ perfusion (500 μM) was set to 100%.

In situ measurements of calpain activation

The activities of m-calpain and μ-calpain were measured in situ as described (Gailly et al., 2007). Briefly, myoblasts were incubated in a Krebs medium containing 10 mM Boc-Leu-Met-CMAC (7-amino-4-chloromethylcoumarin-butoxycarbonyl-L-methionine amide), a permeant and nonfluorescent synthetic peptide that becomes impermeant and fluorescent upon cleavage of the Boc-Leu-Met moiety by calpain. The rate at which fluorescence increases therefore reflects the intracellular accumulation rate of the cleavage product resulting from the enzymatic activity (excitation and emission wavelengths: 380 and 480 nm, respectively). Fluorescence was detected with a photon counter and restricted to an adjustable rectangular aperture of the size of a single cell. The settings for fluorescence recordings were maintained rigorously constant for all measurements. To avoid photobleaching, the cells were illuminated for only 6 seconds every minute for a period of 10-20 minutes. The quasilinear increase of the signal made the calculation of the slope straightforward. The first-order kinetics allowed the determination of the activation of mu-calpain.

In vitro wound-healing assay

After incubation for 24 hours in a culture medium containing 0.1% fetal bovine serum, some cells were scraped off with a pipette tip to obtain a 600-μm-wide acellular area (controlled under DIC microscopy) (Fig. 4A). The medium was then replaced and, after 15 hours, the cells were stained with Hansen’s hemalum for 10 minutes and cells that migrated into the acellular area were counted.

Western blot analysis

Cells of six-well tissue culture dishes were rinsed twice with PBS, scraped off in PBS and centrifuged for 5 minutes at 5000 g. Dry pellets were kept at –80°C until use. Pellets were extracted, sonicated once for 2 seconds and incubated for 30 minutes at 0°C in 100-200 μl lysis buffer pH 7.2 containing 150 mM NaCl, 1 mM Tris, 1 mM EGTA, 1 mM PMSF, 0.1% SDS, 1% deoxycholate, 1% NP40 and a protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma P8340).

Protein concentration was determined by the BCA protein assay kit (Pierce) on 10 μl sample in a total reaction volume of 50 μl and concentration was measured on 2 μl with a nanodrop spectrophotometer. Samples were heated for 5 minutes at 95°C in Laemmli sample buffer containing SDS and β-mercaptoethanol. Equal protein quantities (5-20 μg) were separated on 7.5% SDS-polyacrylamide gels and transferred on nitrocellulose membranes for 7 minutes at 20 V with the dry iBlotting system (Invitrogen). After blocking with 5% non-fat milk, blots were incubated with anti-TRPC1 (Alomone labs; dilution 1:200) or anti β-actin antibodies (Sigma). After incubation with the secondary antibody coupled to peroxidase (Dako), peroxidase was detected with ECL+ (Amersham) on ECL hyperfilm.

TRPC1 and MARCKS expression was quantified by densitometry. Three batches of polyclonal anti-TRPC1 antibodies from Alomone were tested. Only the batch number AN-66 allowed the detection of a single band (between 100 kDa and 130 kDa), which disappeared in TRPC1 shRNA-treated cells (Fig. 1). Moreover, TRPC1 protein seemed very sensitive to contaminant proteases. Indeed, in the absence of protease inhibitors, two degradation products were regularly observed (around 65 kDa and 50 kDa). Actin staining was used as a control of gel loading (Fig. 1). In case of differentiation, where the expression of β-actin and β-actin is upregulated and downregulated, respectively, Ponceau Red staining was used to check gel loading and transfer.

Immunohistochemistry

siRNA-transfected cells were cultured on glass coverslips. Cells were fixed with 4% paraformaldehyde (w/v) in phosphate-buffered saline, permeabilized with Triton X-100 (0.5% w/v in phosphate-buffered saline) and stained with MARCKS antibody (goat polyclonal sc-6454, Santa Cruz Biotechnology). Primary antibody was revealed with donkey anti goat IgG-FITC. Images were obtained using a ×63 objective (water immersion) on a Zeiss S100 inverted microscope equipped with an Axiocam HR camera.

Solutions and drugs

The Krebs solution contained 135 mM NaCl, 5.9 mM KCl, 1.8 mM CaCl2, 12 mM MgCl2, 11.6 mM HEPES and 10 mM glucose (pH 7.3). In Ca2+ free solution, CaCl2 was omitted and 0.1 mM EGTA was added. Thapsigargin and ionomycin were purchased from Sigma. Fura-PE3/AM was obtained from Calbiochem (Darmstadt, Germany) DMEM, serum and streptomycin-penicillin solutions were purchased from Invitrogen. The GitTx-4 toxin, isolated from Grammostola spatulata spider (Suchyna et al., 2000), was obtained from PeptaNova (Sandhausen, Germany), Boc-Leu-Met-CMAC from Molecular Probes; Z-Leu-Leu-CHO from Bio Mol Labs and SKF-96365 from Alexis Corporation (Lausen, Switzerland).

Statistical analysis

Data are presented as mean ± s.e.m. ANOVA and Student’s t-tests were used to determine statistical significance.

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