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1α,25(OH)$_2$D$_3$-Dependent Modulation of Akt in Proliferating and Differentiating C2C12 Skeletal Muscle Cells

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ABSTRACT

We previously reported that 1α,25-dihydroxy-vitamin D$_3$ [1α,25(OH)$_2$D$_3$] induces non-transcriptional rapid responses through activation of Src and MAPKs in the skeletal muscle cell line C2C12. In the present study we investigated the modulation of Akt by the secosteroid hormone in C2C12 cells at proliferative stage (myoblasts) and at early differentiation stage. In proliferating cells, 1α,25(OH)$_2$D$_3$ activates Akt by phosphorylation in Ser473 in a time-dependent manner (5–60 min). When these cells were pretreated with methyl-beta-cyclodextrin to disrupt caveolae microdomains, hormone-induced activation of Akt was suppressed. Similar results were obtained by siRNA silencing of caveolin-1 expression, further indicating that hormone effects on cell membrane caveolae are required for downstream signaling. PI3K and p38 MAPK, but not ERK1/2, participate in 1α,25(OH)$_2$D$_3$ activation of Akt in myoblasts. The involvement of p38 MAPK in Akt phosphorylation by the hormone probably occurs through MAPK-activated protein kinase 2 (MK2), which is activated by the steroid. In addition, the participation of Src in Akt phosphorylation by 1α,25(OH)$_2$D$_3$ was demonstrated using the inhibitor PP2 and antisense oligodeoxynucleotides that suppress Src expression. We also observed that PI3K participates in hormone-induced proliferation. During the early phase of C2C12 cell differentiation 1α,25(OH)$_2$D$_3$ also increases Akt phosphorylation and activates Src. Of relevance, Src and PI3K are involved in Akt activation and in MHC and myogenin increased expression by 1α,25(OH)$_2$D$_3$. Altogether, these data suggest that 1α,25(OH)$_2$D$_3$ upregulates Akt through Src, PI$_3$K, and p38 MAPK to stimulate myogenesis in C2C12 cells. J. Cell. Biochem. 113: 1170–1181, 2012.

KEY WORDS: 1α,25(OH)$_2$D$_3$; C2C12 MUSCLE CELLS; Akt AND MYOGENESIS

Akt (also called PKB) is a serine/threonine kinase family with key implications in proliferation, survival, differentiation, and viability of muscle cells [Ceci et al., 2004; Guttridge, 2004]. The mechanism of activation of Akt is complex and has not been fully elucidated [Zhong et al., 2008]. In response to growth factors, Akt upregulation depends on previous PI3K activation. PI3K comprises enzymes which phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to form phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$). Binding of Akt N-terminal pleckstrin homology domain to PIP$_3$ causes its translocation to the plasma membrane. Once there, Akt activation occurs when 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates it in Thr308 and PDK2 (whose identification remains unclear), phosphorylates its Ser473 residue [Ueki et al., 2000; Vanhaesebroeck and Alessi, 2000; Hajduch et al., 2001]. Additional protein kinases, such as integrin-linked kinase-1, can modulate the activity of Akt through their ability to phosphorylate it on Ser473 [Troussard et al., 2006]. Once activated, maintenance of Thr308 phosphorylation of Akt does not appear to be required to keep it active [Yamada et al., 2001], and Akt retains its activity through autophosphorylation at Ser473 [Toker and Newton, 2000].

Cholesterol and sphingomyelin-enriched lipid rafts are plasma membrane microdomains that concentrate a plethora of molecules involved in signal transduction. Caveolae are specialized lipid rafts organized by the cholesterol-binding proteins, caveolins, which have participation in cellular signaling [reviewed by Chidlow and Sessa, 2010]. Of relevance, caveolae intact structure is required for Akt activation by angiotensin II [Ushio-Fukai et al., 2001]. In vascular smooth muscle cells (VSMCs), caveolae and caveolin-1 (cav-1) expression are involved in integrin-mediated activation of Akt [Sedding et al., 2005]. Up to the present, the contribution of caveolae or cav-1 to modulate Akt activity in skeletal muscle cells has not been investigated.

Other molecules have also been implicated in the activation of Akt in different tissues. Kettritz et al. [2002] reported that p38 MAPK-dependent MAPK-activated protein kinase-2 (MK-2) can function as PDK2 and cause phosphorylation of Akt at Ser473 in neutrophils. Furthermore, in osteoclasts, Src has been shown to be

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involved upstream in Akt phosphorylation, and in VSMCs Src mediates PI3K/Akt activation [Wong et al., 1999; Gang et al., 2010].

Of our interest, $1\alpha,25(OH)_2D_3$ actions on Akt activity in non-tumorigenic cells have been scarcely investigated. In keratinocytes, the hormone exerts its protective actions against apoptosis regulating the PI3K/Akt survival pathway [De Haes et al., 2004]. Zhang and Zanello [2008] published that anti-apoptotic effects of the hormone in osteoblasts occur through non-genomic activation of a VDR/PI3K/Akt survival pathway. Furthermore, $1\alpha,25(OH)_2D_3$ modulates Akt activity in human sperm, extending the role of this hormone beyond its conventional physiological actions [Aquila et al., 2009]. Results from our laboratory have shown that the sex steroid hormone 17β-estradiol exerts anti-apoptotic effects in C2C12 proliferative skeletal muscle cells through modulation of the PI3K/Akt pathway [Vasconsuelo et al., 2008]. In addition, it was previously reported that Akt activation is related to survival events in C2C12 differentiated cells [Fujio et al., 2001; Conejo et al., 2002]. In these myotubes, $1\alpha,25(OH)_2D_3$ improves the free-fatty-acid-induced insulin resistance through Akt participation [Zhou et al., 2008]. Nevertheless, the signaling molecules involved in Akt activation by $1\alpha,25(OH)_2D_3$ have not been studied neither in myoblasts (proliferating C2C12 cells) and nor in differentiated C2C12 cells (myotubes). It is important to point out that C2C12 cells live in a proliferation state indefinitely, like satellite myoblasts present in mature muscle, until an adequate stimulus promotes its differentiation to muscle fibres. Considering this and the lack of information about regulation of Akt in these cells, the aim of the present work is to investigate $1\alpha,25(OH)_2D_3$-dependent modulation of Akt activity in proliferating and differentiated C2C12 cells.

MATERIALS AND METHODS

CHEMICALS

Dulbecco's modified Eagle's medium (DMEM) low glucose, with L-glutamine and HEPES, without phenol red, was from US Biological (Swampscott, MA). Fetal bovine serum (FBS), horse serum (HS), $1\alpha,25(OH)_2D_3$ and methyl-beta-cyclodextrin (MβCD) were from Sigma-Aldrich Co. (St. Louis, MO). Primary antibodies anti-caveolin-1, anti-myogenin and anti-myosin heavy chain (MHC), secondary antibodies goat anti-rabbit and goat anti-mouse horse radish peroxidase-conjugated IgG, and caveolin-1 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho Akt, anti-Akt, anti-c-Src, anti-phospho (Tyr 416) c-Src, anti-phospho (Thr 334) MAPK-activated protein kinase 2 (MK-2), and anti-actin antibodies were acquired from Cell Signaling Technology, Inc. (Beverly, MA). Wortmannin and LY294002 were from Alomone Laboratories (Jerusalem, Israel). Antisense oligodeoxynucleotides (ODNs) were synthesized by the DNAgency (Malvern, PA). Enhanced Chemiluminiscence Plus Western blotting detection reagents were from GE Healthcare (Anaheim, CA). Protein size markers were from Amersham Biosciences (Piscataway, NJ). The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay was from PROMEGA Corporation (Madison, WI). The C2C12 cell line (CRL1772) was provided by ATCC (American Type Culture Collection, Manassas, VA). U0126 and SB203580 were from Tocris Cookson Ltd (Bristol, UK). PP2 was from Calbiochem-Novabiochem Corp. (La Jolla, CA).

CULTURE OF PROLIFERATING C2C12 CELLS

The murine skeletal muscle cell line C2C12 is a good model system for studying myogenesis. These myoblasts proliferate in medium containing fetal serum with high growth factor concentrations (growth medium, GM), but are induced to differentiate upon incubation of preconfluent cells in medium with low growth factor quantities (horse serum). C2C12 cells were seeded at an appropriate density (120,000 cells/cm^2) in Petri dishes (100 mm diameter) with DMEM without phenol red, supplemented with 10% FBS (GM medium) and antibiotic-antimycotic solution at 37°C under a humidified atmosphere (95% air/5% CO_2). Undifferentiated cells cultured for 2 days were used to perform the experiments during the proliferation stage. Before each treatment, cells were deprived of serum for 60 min. During this preincubation the cells were exposed to inhibitors when indicated in experiments. All cell treatments were carried out in phenol red-free medium without serum.

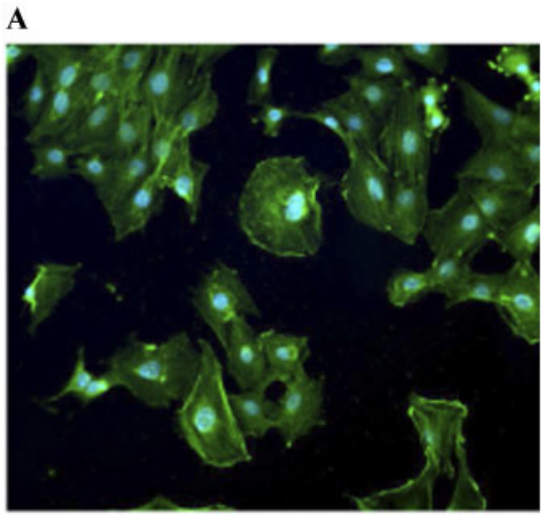
DIFFERENTIATION OF C2C12 MYOBLASTS

To promote myoblast differentiation, GM of C2C12 cells grown up to 70% of confluence was replaced by DMEM without phenol red, supplemented with 1% HS (differentiation medium, DM) and cells were used after 24, 48, and 72 h of culture. As for proliferative myoblasts (2.2), cells were preincubated without serum for 60 min in the presence of inhibitors when indicated followed by treatments in phenol red-free and serum-free medium.

We obtained digital images by immunofluorescence where whole cells were green stained with anti-actin antibody and nuclei were visualized by DAPI, at 40× objective magnification. As shown in the micrografs of muscle cell cultures, under the above conditions, a differentiation pattern of the C2C12 mouse cell line typical of the onset of myogenesis was observed [Kubo, 1991]. Differentiation of C2C12 myoblasts was revealed by their morphological changes such as alignment, elongation and fusion of mononucleated cells to multinucleated myotubes after switching cells from GM to DM media. By day 2 in GM, non-confluent proliferating C2C12 cells were polygonal and had only one nucleus (A). By day 4 in DM, 70% of C2C12 cells were elongated, multinucleated and aligned, indicative of cell fusion (B), whereas a subpopulation of cells remained undifferentiated as reserve cells [Yoshida et al., 1998]. Accompanying these morphological changes, the expression of muscle-specific proteins, myogenin and MHC was upregulated (please see Fig. 8, Results Section). MHC, a marker for mature muscle cells, was only detected in myotubes, as reported before [Sun et al., 2005].

MTS PROLIFERATION ASSAY

The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay is a homogeneous, colorimetric method for determining the number of viable cells in proliferation, cytotoxicity, or chemosensitivity assays. This assay is based upon the use of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The conversion of MTS into formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of aqueous soluble formazan



product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

Ninety-six-well tissue culture plates with 1,200 cells/well in antibiotic-free normal GM supplemented with 10% FBS were incubated at 37°C in a CO_2 incubator for 24 h. Then, the medium was removed and replaced by 1% FBS supplemented medium for 4 h. Cells were incubated with 10^{-9} M $1\alpha,25(OH)_2D_3$ or its vehicle (isopropanol, 0.001%) for the indicated times. The medium was aspirated and MTS was added following manufacturer's instructions and after 1 h the absorbance at 490 nm was measured.

SDS–PAGE AND IMMUNOBLOTTING

Cells were lysed using a buffer made of 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 1% NP40, leupeptin 20 μg/ml, and aprotinin 20 μg/ml. Lysates were collected by aspiration and centrifuged at 12,000*g* during 15 min. The protein content of the supernatant was quantified by the Bradford procedure [Bradford, 1976]. Lysate proteins dissolved in Laemmli sample buffer [Laemmli et al., 1970] were separated on SDS–polyacrylamide (10%) gels and electro-transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked 1 h at room temperature in TBST buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0.1% Tween-20) containing 5% dry milk. Membranes were subjected to immunoblotting using different primary antibodies overnight at 4°C. Membranes were then washed three times in TBST, incubated in TBST containing 1% dry milk with peroxidase-conjugated secondary antibody for 1 h at room temperature and washed again three times with TBST. Next, membranes were visualized using an enhanced chemiluminiscent technique (ECL) according to the manufacturer's instructions. Images were obtained with a GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA) by scanning at 600 dpi. Bands were quantified using the Molecular Analyst program (Bio-Rad). To strip the membranes for reprobing with other antibodies, the membranes were washed 10 min in TBST, incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 50 mM mercaptoethanol) for 30 min at 55°C, washed 10 min in TBST and then blocked and blotted as described above.

TRANSFECTION OF SMALL INTERFERING RNA (siRNA)

Six-well tissue culture plates with 2×10^5 cells/well in antibiotic-free normal GM supplemented with FBS were incubated at 37°C in a CO_2 incubator until the cells were 60–80% confluent (usually 24 h). Then the following solutions were used. Solution A: for each transfection, 5 μl of siRNA duplex (i.e., 0.5 μg siRNA) into 100 μl siRNA transfection medium. Solution B: for each transfection, 5 μl of siRNA transfection reagent into 100 μl siRNA transfection medium. Afterwards, solution A was directly added to solution B, mixed gently and incubated 30 min at room temperature. Cells were washed with 2 ml of siRNA transfection medium. For each transfection, 0.8 ml siRNA transfection medium containing the siRNA mixture (Solution A + Solution B) were added and the cells incubated 6 h at 37°C in a CO_2 incubator. The transfection mixture was removed and replaced with normal GM. Cells were incubated for an additional 18 h until used for treatments.

TRANSFECTION OF OLIGODEOXYNUCLEOTIDES

Transfections with ODNs against c-Src mRNA and ODNs with scramble sequence were performed using Lipofectin according to the manufacturer's instructions. As in previous studies [Capiati et al., 2000, 2001], ODNs were incubated with Lipofectin in DMEM without serum for 15 min at room temperature. Plates of subconfluent cells were washed to remove serum before addition of ODN–Lipofectin mixtures and incubation was performed for 12 h at 37°C. The ODN solution was removed, DMEM (1% serum) was added and the plates were placed into a metabolic incubator for a further 24 h. Control treatments including DMEM or Lipofectin only were also carried out. Dose- and time-response curves for Lipofectin and ODN were previously optimized [Buitrago et al., 2002]. The following ODN sequence with phosphorothioate linkages throughout the entire molecule was used: anti-(c-Src), 5′-CACCACCATGGGGAGCAGCA-3′ (antisense against the 95–114 nucleotide sequence containing the AUG region from *Gallus gallus* c-Src mRNA).

STATISTICAL ANALYSIS

Statistical significance of the data was evaluated using Student's *t*-test [Snedecor and Cochran, 1967] and probability values below 0.05 ($P < 0.05$) were considered significant. Results are expressed as means ± standard deviation (SD) from the indicated set of experiments.

RESULTS

TIME-DEPENDENT PHOSPHORYLATION OF Akt (Ser473) BY $1\alpha,25(OH)_2D_3$ IN PROLIFERATIVE C2C12 MUSCLE CELLS

We have previously shown that 10^{-9} M $1\alpha,25(OH)_2D_3$ is the optimal dose to promote activation of MAPKs in C2C12 myoblastic cells [Buitrago et al., 2006]. We used then this hormone concentration, within the physiological range, to investigate the time course of its effects on Akt phosphorylation and related experiments in the present work. To evaluate if $1\alpha,25(OH)_2D_3$ was able to modulate Akt activity we employed an antibody that recognizes Akt only when it is phosphorylated in Ser473 (indicative of Akt activation). C2C12 myoblasts were incubated with $1\alpha,25(OH)_2D_3$ or its vehicle (isopropanol 0.001%) for 5, 15, and 60 min followed by Western blot analysis with the antibody selected. The immunoblots shown in Figure 1 reveal that, whereas equivalent Akt protein levels are expressed, $1\alpha,25(OH)_2D_3$ induced a steady time-dependent increase in the phosphorylation of Akt (Ser473) until 60 min.

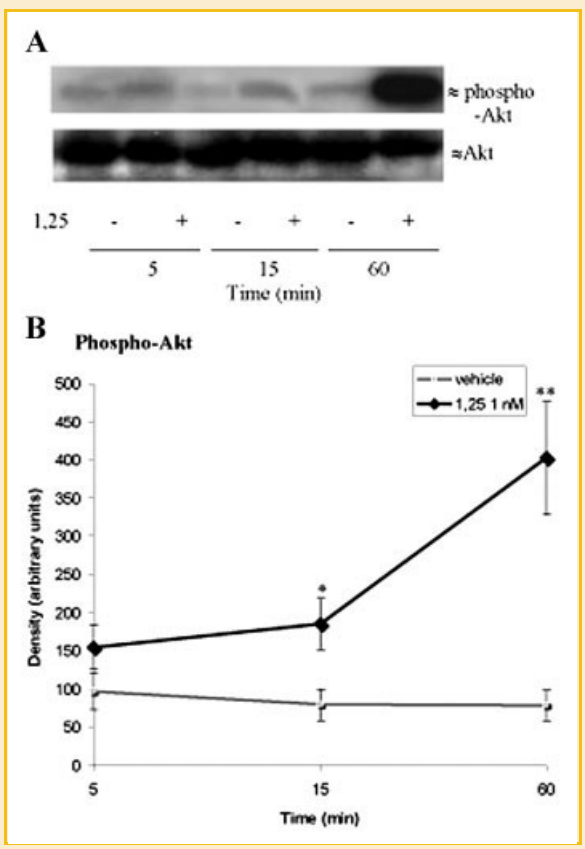


Fig. 1. Time-course of Akt activation in response to $1\alpha,25(OH)_2D_3$ in proliferating skeletal muscle cells (myoblasts). C2C12 myoblasts incubated in serum-free medium were treated with 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle (0.001% isopropanol) at the indicated times. A: Western blot analysis was performed using anti-phospho-Akt antibody. Akt levels are shown as loading control. B: Immunoblots quantified by scanning volumetric densitometry were normalized with Akt levels. Averages ± SD from three independent experiments are given. $^{*}P < 0.05$ and $^{**}P < 0.01$ respect to the respective control.

PI3K MEDIATES ACTIVATION OF Akt BY $1\alpha,25(OH)_2D_3$ IN MYOBLASTS

The activation of Akt mostly occurs via PI3K [Frost et al., 2002; Wang et al., 2009], although in some cases a PI3K-independent pathway may be involved in Akt phosphorylation [Woods Ignatoski et al., 2003; see the p38 MAPK Is Involved in the Phosphorylation of Akt by $1\alpha,25(OH)_2D_3$ in Myoblasts Section]. We investigated then the role of PI3K in Akt Ser473 phosphorylation by $1\alpha,25(OH)_2D_3$ using Ly294002 (10 μM) and Wortmannin (100 nM), both specific inhibitors of PI3K. These compounds were present both during the preincubation period without serum and subsequent hormone treatment (60 min) performed afterwards. At the dose and exposure time used in this work, Ly294002 was shown to effectively block activation of PI3K in C2C12 cells by other authors [Gamell et al., 2008]. The fungal metabolite Wortmannin, which acts on PI3K by irreversibly binding to its catalytic subunit, at a similar concentration as in our study (100 nM), has been previously demonstrated to inhibit the enzyme in the C2C12 muscle cell line [Milasincic et al., 1996]. As shown in Figure 2, $1\alpha,25(OH)_2D_3$ did not stimulate Akt phosphorylation when PI3K was inhibited with Ly294002 or Wortmannin.

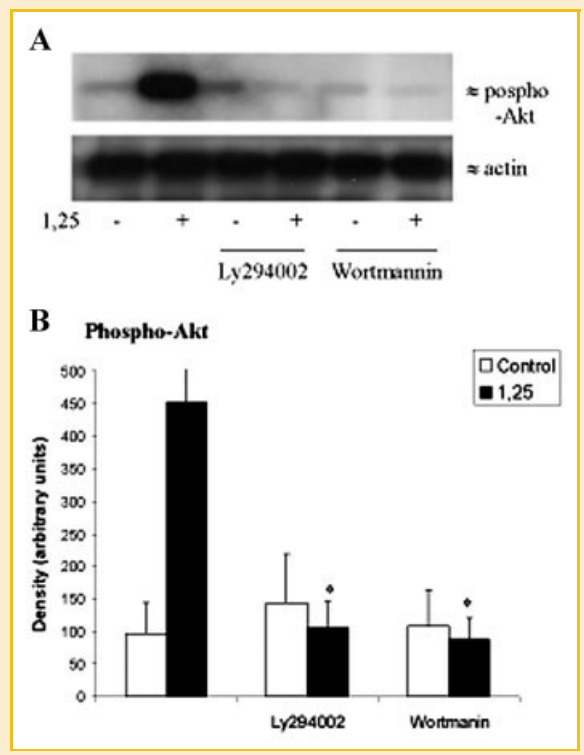


Fig. 2. PI3K participates in Akt activation induced by $1\alpha,25(OH)_2D_3$ in proliferating myoblasts. Cultures of C2C12 proliferating cells were preincubated in presence or absence of 10 μM Ly294002 or 100 nM Wortmannin for 60 min. Then, cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or its vehicle during 60 min. A: Western blot analysis was carried out using anti-phospho-Akt and anti-actin antibodies. Actin levels are shown as loading control. B: Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independent experiments are given. $^{*}P < 0.01$ respect to the corresponding control.

CAVEOLAE DISRUPTION BY MβCD TREATMENT AND SILENCING OF CAVEOLIN-1 EXPRESSION ABOLISH Akt PHOSPHORYLATION INDUCED BY $1\alpha,25(OH)_2D_3$ IN C2C12 MYOBLASTS

As mentioned before, intact caveolae microdomains are required for $1\alpha,25(OH)_2D_3$-dependent activation of Src and MAPKs in C2C12 myoblasts [Buitrago and Boland, 2010]. Therefore, we examined the participation of caveolae in the phosphorylation of Akt triggered by the hormone. Cells were preincubated with MβCD to disrupt caveolae structure before exposure to the steroid as previously described [Buitrago and Boland, 2010]. Figure 3A shows that cholesterol removal from the plasma membrane suppressed the stimulation of Akt phosphorylation on Ser473 by $1\alpha,25(OH)_2D_3$, implying that intact caveolae are required for hormone modulation of Akt activity.

It has been established that cav-1 protein is essential for caveolae maintenance [Rothberg et al., 1992]. To corroborate the caveolae requirement in Akt activation, we used a previous experiment where we significantly reduced cav-1 expression in C2C12 cells using a specific siRNA [Buitrago and Boland, 2010]. The re-probing of these membranes shows that Cav-1 silencing blocked Akt phosphorylation induced by $1\alpha,25(OH)_2D_3$, proving again the importance of caveolae in the upregulation of Akt activity by the hormone (Fig. 3B).

p38 MAPK IS INVOLVED IN THE PHOSPHORYLATION OF Akt BY $1\alpha,25(OH)_2D_3$ IN MYOBLASTS

We previously reported that MAPK pathways are activated by $1\alpha,25(OH)_2D_3$ in C2C12 myoblasts [Ronda et al., 2007]. In view of these results and evidence demonstrating that p38 MAPK is involved in Akt (Ser473) phosphorylation in VSMCs [Ushio-Fukai et al., 2001] and human mammary epithelial (HME) cells [Woods Ignatoski et al., 2003], we investigated the role of ERK1/2 and p38 MAPK in Akt activation in response to the hormone in C2C12 myoblasts. For this purpose, we used U0126 and SB203580 to inhibit ERK1/2 and p38 MAPK, respectively. Various doses of each inhibitor were previously tested to establish its most effective concentration (data not shown). As seen in Figure 4, 10 μM SB203580 markedly blocked both basal and $1\alpha,25(OH)_2D_3$-induced Akt phosphorylation in Ser473, whereas 20 μM U0126 did not exert a significant effect. These data reveal that p38 MAPK and not ERK1/2 mediates hormone activation of Akt. Exposure of cells to H_2O_2 (50 μM, 10 min) did not affect the phosphorylation of Akt, as previously reported in VSMCs [Ushio-Fukai et al., 2001], whereas incubation with 10% FBS for 5 min activated Akt in C2C12 muscle cells, as reported for fibroblasts [Lim, 2008].

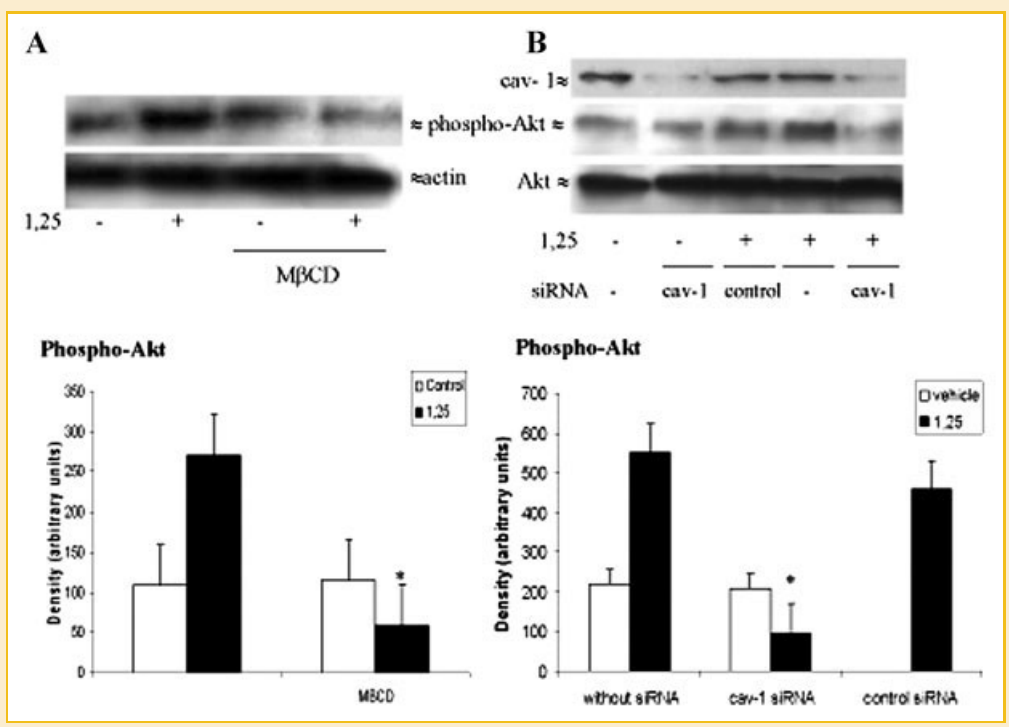


Fig. 3. Caveolae and caveolin-1 are implicated in hormone-dependent Akt activation in proliferating myoblasts. A: Cultures of C2C12 myoblasts were preincubated in presence or absence of 4 nM MβCD for 30 min. Then, cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle during 60 min in fresh free-serum medium. Western blot analyses were carried out using anti-phospho Akt and anti-actin antibodies. Actin levels are shown as loading control. Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^*P < 0.01$ respect to its respective control. B: Cells were transfected with control-siRNA or cav-1-siRNA as described in the Materials and Methods Section. Twenty-four hours later cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ or its vehicle during 60 min. Western blot analyses were performed using anti-cav-1, anti-phospho-Akt, and anti-Akt antibodies. Total Akt levels are shown as loading control. Immunoblots quantified by scanning volumetric densitometry were normalized with Akt levels. Averages ± SD from three independent experiments are given. $^*P < 0.01$ respect to the corresponding control.

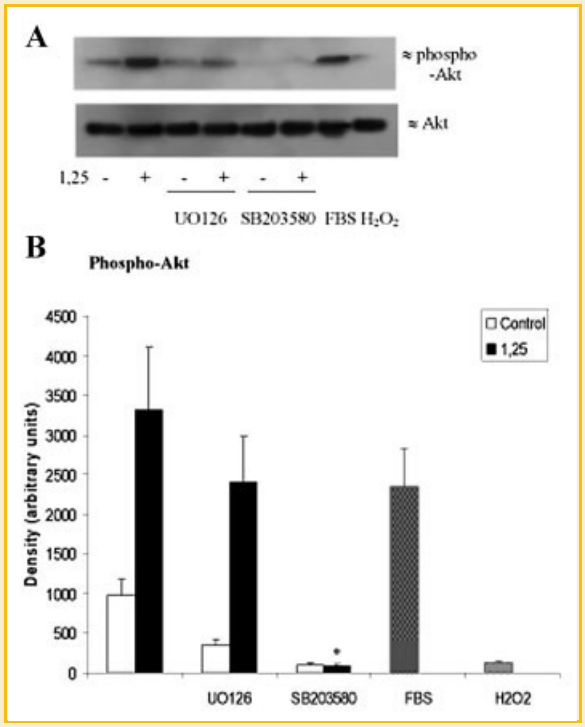


Fig. 4. Involvement of p38 MAPK and not ERK1/2 in $1\alpha,25(OH)_2D_3$-dependent Akt phosphorylation in proliferating skeletal muscle cells. C2C12 myoblasts were preincubated with or without 10 μM U0126 or 20 μM SB203580 for 60 min. Then, cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle during 60 min. As positive and negative control of Akt activation, cells were exposed to FBS (10%, 5 min) and H_2O_2 (50 μM, 10 min), respectively. A: Western blot assays were carried out using anti-phospho-Akt and anti-Akt antibodies. B: Akt levels are shown as loading control. Immunoblots quantified by scanning volumetric densitometry were normalized with Akt levels. Averages ± SD from three independent experiments are given. $^*P < 0.01$ respect to $1\alpha,25(OH)_2D_3$ without inhibitor.

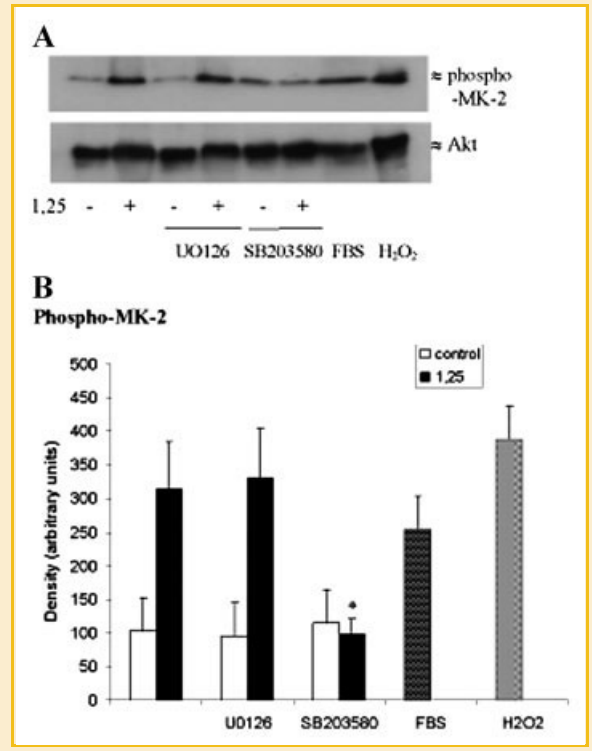


Fig. 5. $1\alpha,25(OH)_2D_3$ modulates p38 MAPK-dependent MK-2 in C2C12 myoblasts. C2C12 proliferating myoblasts were preincubated with or without 10 μM U0126 and 20 μM SB203580 for 60 min. Then, cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle for 60 min. Myoblasts were also exposed to FBS and H_2O_2 as mentioned in this legend. A: Western blot assays were performed with anti-phospho-MK-2 and anti-Akt antibodies. Akt levels are shown as loading control. B: Immunoblots quantified by scanning volumetric densitometry were normalized with Akt levels. Averages ± SD from three independent experiments are given. $^*P < 0.05$ respect to $1\alpha,25(OH)_2D_3$ without inhibitor.

STIMULATION OF MAPK-ACTIVATING PROTEIN KINASE-2 (MK-2) BY $1\alpha,25(OH)_2D_3$

Our result above shows that p38 MAPK is implicated in Akt activation by $1\alpha,25(OH)_2D_3$ in proliferating C2C12 cells. It has been reported that p38 MAPK-dependent MAPK-activated protein kinase 2 (MK-2) phosphorylates Akt in neutrophils [Kettritz et al., 2002]. Also, the p38 MAPK/MK-2 pathway mediates Akt stimulation by angiotensin II in VSMCs [Taniyama et al., 2004]. We previously demonstrated that $1\alpha,25(OH)_2D_3$ rapidly activates MK-2 (at 1 min) in C2C12 cells [Buitrago et al., 2006]. In view that, in the preceding experiments C2C12 cells were exposed to $1\alpha,25(OH)_2D_3$ for 60 min we investigated the changes in MK-2 Thr 334 phosphorylation (activation) at this hormone treatment interval. C2C12 myoblasts were preincubated with 10 μM U0126 or 20 μM SB203580 to inhibit ERK1/2 and p38 MAPK, respectively. Our results show that $1\alpha,25(OH)_2D_3$-induced MK-2 activation is dependent on p38 MAPK, whereas ERK1/2 seems not to be involved (Fig. 5). These data suggest then that p38 MAPK-dependent MK-2 mediates Akt activation by $1\alpha,25(OH)_2D_3$.

Src MEDIATES Akt PHOSPHORYLATION INDUCED BY $1\alpha,25(OH)_2D_3$ IN PROLIFERATING SKELETAL MUSCLE CELLS

Src is a key molecule in the regulation of several signal transduction pathways. Regarding the present work, it has been demonstrated that Src mediates PI3K/Akt activation in endothelial cells exposed to estrogen [Haynes et al., 2003] and more recently in IGF-I-stimulated VSMCs [Gang et al., 2010]. Interestingly, there is evidence that $1\alpha,25(OH)_2D_3$ activates Src in proliferating C2C12 cells [Buitrago and Boland, 2010]. To investigate the role of Src in the stimulation of Akt by the hormone in myoblasts we used PP2, a specific inhibitor for all members of the Src family. Various doses of the inhibitor were tested to establish its most effective concentration (data not shown). As observed in Figure 6A,B, Akt phosphorylation induced by the steroid was completely inhibited when the cells were incubated with 25 μM PP2. To confirm the participation of Src in hormone-dependent Akt (Ser473) phosphorylation, we markedly diminished the expression of c-Src by transfecting cells with a pool of antisense ODNs against c-Src mRNA (Fig. 6C, upper blot). Decreased Src expression abolished the phosphorylation of Akt induced by

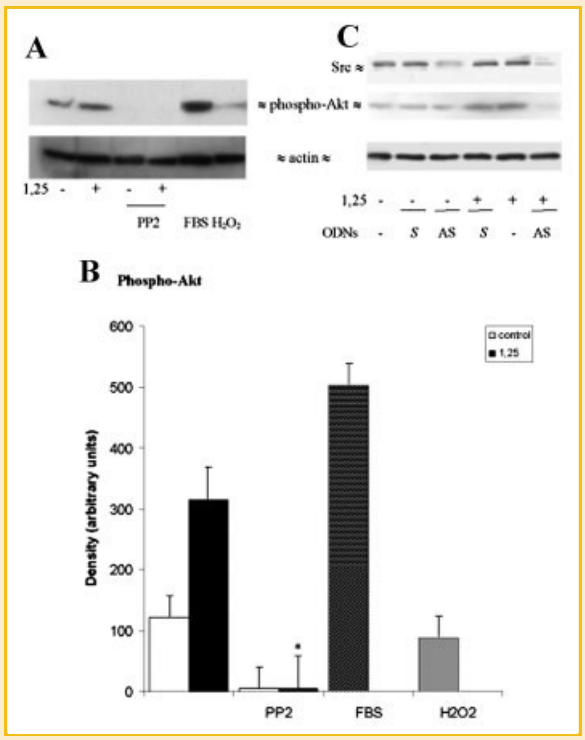


Fig. 6. Src mediates Akt phosphorylation induced by $1\alpha,25(OH)_2D_3$ in proliferative skeletal muscle cells. A: Proliferating C2C12 myoblasts were preincubated with or without 25 μM PP2. As positive and negative control of Akt activation, cells were exposed to FBS (10%, 5 min) and H_2O_2 (50 μM, 10 min), respectively. Then, cultures were treated with 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle for 60 min. Western blot assays were carried out using anti-phospho-Akt and anti-actin antibodies. Actin levels are shown as loading control. B: Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^*P < 0.05$ respect to hormone without PP2. C: Cells were transfected with sense (S) or antisense (AS) c-Src ODNs as described in the Materials and Methods Section. Eighteen hours later cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ or its vehicle during 60 min. Western blot analysis was performed using anti-Src, anti-phospho-Akt, and anti-actin antibodies. Actin levels are shown as loading control. The immunoblots are representative from three independent experiments.

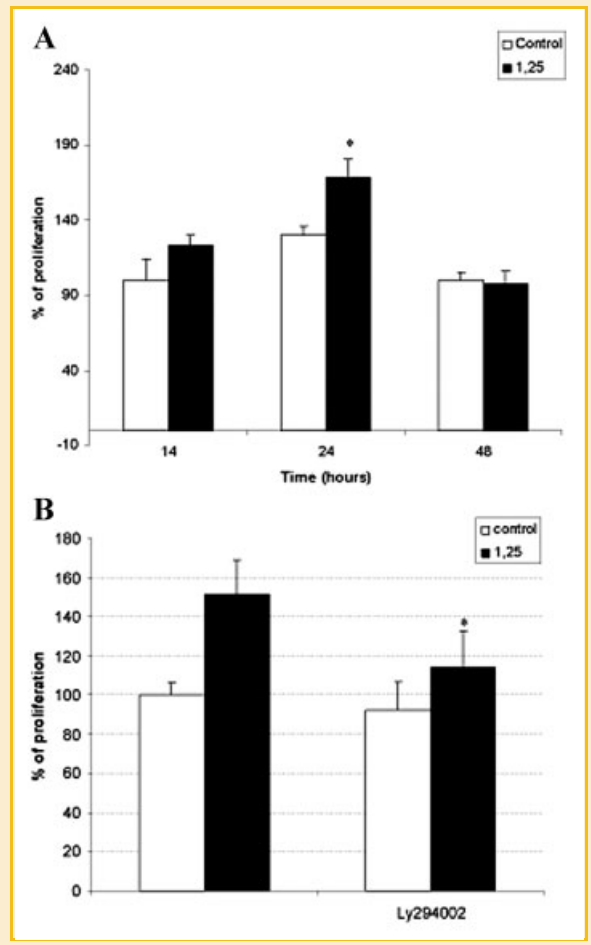


Fig. 7. PI3K is involved in $1\alpha,25(OH)_2D_3$-dependent C2C12 myoblasts proliferation. A: C2C12 myoblasts were exposed to 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle for 14, 24, and 48 h. At these times, MTS proliferation assays were carried out using a kit according to manufacturer's instructions. Percentage (%) of proliferation was calculated from five independent experiments. Averages ± SD are given. $^*P < 0.01$ respect to the respective control. B: C2C12 proliferating cells were preincubated with or without 10 μM Ly294002. Then, cultures were treated with 1 nM $1\alpha,25(OH)_2D_3$ or vehicle for 24 h. At this time, MTS proliferation assays were carried out. Proliferation (%) was calculated from five independent experiments. Averages ± SD are given. $^*P < 0.05$ respect to $1\alpha,25(OH)_2D_3$ without inhibitor.

$1\alpha,25(OH)_2D_3$ (Fig. 6C, center blot). The data from these experiments provide strong evidence indicating that Src is necessary for Akt activation by the hormone in myoblasts.

1α,25(OH)2D3 STIMULATION OF C2C12 MYOBLAST CELL PROLIFERATION IS DEPENDENT ON PI3K

Knowing that PI3K has been involved in C2C12 cell proliferation [Spangenburg and Booth, 2002], we tested if PI3K and/or Src are implicated in the mitogenic effects of $1\alpha,25(OH)_2D_3$ on these muscle cells. First, to determine changes in proliferation in C2C12 myoblast cultures stimulated with the hormone, we used a MTS proliferation assay as described in the Materials and Methods Section. Figure 7A shows that 24 h of treatment with $1\alpha,25(OH)_2D_3$ stimulates C2C12 cell proliferation by 40%. Using the PI3K inhibitor Ly294002 during the 24 h incubation with the hormone, the increase in proliferation was no longer observed (Fig. 7B). Our data indicate then that PI3K is involved in the mitogenic action of $1\alpha,25(OH)_2D_3$ on C2C12 myoblasts.

$1\alpha,25(OH)_2D_3$ STIMULATES PHOSPHORYLATION OF Akt (Ser473) AND THE EXPRESSION OF MYOSIN HEAVY CHAIN (MHC) AND MYOGENIN IN THE EARLY STAGE OF DIFFERENTIATION OF C2C12 CELLS

Consistent with the idea that Akt signaling is essential for myoblast differentiation [Wilson et al., 2004; Wilson and Rotwein, 2007], it was recently demonstrated that API-2, an Akt inhibitor, blocks C2C12 myoblast cell differentiation [Shu and Houghton, 2009].

Our laboratory described for first time that $1\alpha,25(OH)_2D_3$ regulates chick myoblast differentiation [Capiati et al., 1999]. Subsequently, Endo et al. [2003] demonstrated that the VDR is

necessary for normal skeletal muscle development and the correct expression of myoregulatory transcription factors in mice. Except the work of the latter authors, the action of $1\alpha,25(OH)_2D_3$ on the differentiation of mammalian skeletal muscle cells has not been investigated. To gain insights into the regulatory mechanism of this process by $1\alpha,25(OH)_2D_3$, the effects of the hormone on Akt activation during C2C12 myoblast differentiation were studied, evaluating the expression of MHC and myogenin, marker proteins of early muscle cell maturation. C2C12 cells grown in DM for 24, 48, and 72 h were stimulated with $1\alpha,25(OH)_2D_3$ or its vehicle isopropanol every 24 h. Figure 8 shows that, although the expression pattern of total Akt was not altered, phosphorylation of Akt Ser473 augments during differentiation, as previously reported [Fujio et al., 2001; Conejo et al., 2002]. $1\alpha,25(OH)_2D_3$ further increases Akt (Ser473) phosphorylation and the expression levels of MHC and myogenin at the three time intervals studied. These results suggest that the hormone stimulates myoblast differentiation in an Akt-dependent manner.

$1\alpha,25(OH)_2D_3$ PROMOTES ACTIVATION OF Src AT EARLY STEPS OF C2C12 CELL DIFFERENTIATION. SRC AND PI3K MEDIATE MHC AND MYOGENIN EXPRESSION AND PHOSPHORYLATION OF AKT INDUCED BY THE HORMONE

It has been previously demonstrated that the differentiation of L6 rat skeletal muscle cells involves a PTPα-mediated Src pathway [Lu et al., 2002]. In the C2C12 cell line, Src could also be involved in the enhancement of FAK activity and in the activation of downstream pathways which lead to the differentiation of myoblastic cells [Clemente et al., 2005]. We investigated here the role of Src in $1\alpha,25(OH)_2D_3$-dependent enhancement of Akt activation during the early stages of C2C12 differentiation. As Figure 9A shows, hormone treatment for 48 h induces activation of Src, which was efficaciously inhibited by 25 μM PP2. C2C12 proliferating cells (grown in GM) and C2C12 differentiating cells (in DM) do not show differences in basal Src phosphorylation levels (Fig. 9A, lanes 1 and 2). In differentiating cells exposed for 48 h to the hormone, the expression

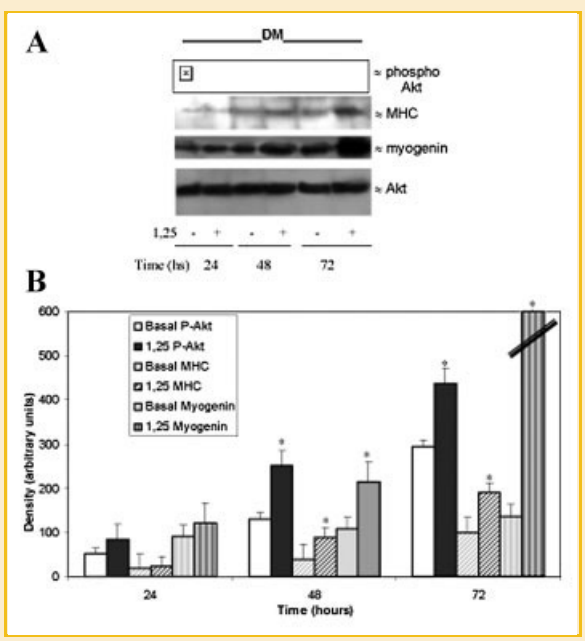


Fig. 8. $1\alpha,25(OH)_2D_3$ promotes Akt phosphorylation and the expression of MHC and myogenin in early steps of C2C12 cell differentiation. C2C12 myoblasts induced to differentiate as described in the Materials and Methods Section were incubated with 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle for 24, 48, and 72 h. Western blot assays were carried out using anti-phospho-Akt, anti-myosin heavy chain (MHC), anti-myogenin, and anti-Akt antibodies. Akt bands correspond to loading controls showing that protein levels do not significantly change in any tested condition. The immunoblots are representative from three independent experiments.

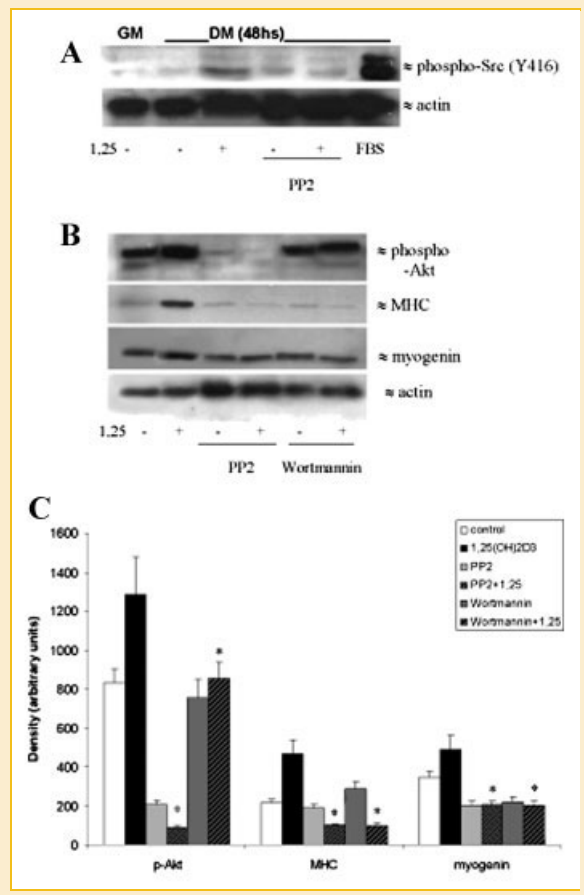


Fig. 9. At early stages of C2C12 cell differentiation, $1\alpha,25(OH)_2D_3$ stimulates Src phosphorylation. Src and PI3K participate in MHC and myogenin expression and Akt activation induced by the hormone. A: C2C12 proliferating myoblasts (lane 1) and C2C12 differentiating cells (lanes 2–6) were incubated with or without 25 μM PP2 and exposed to 1 nM $1\alpha,25(OH)_2D_3$ (1,25) or vehicle for 48 h. As positive control of Src activation, 10% FBS (5 min) was used. Western blot assays were carried out using anti-phospho-Src (Tyr416) and anti-actin antibodies. The immunoblots showed are representative from three independent experiments. B: C2C12 differentiating cells were incubated with or without 25 μM PP2 or 100 nM Wortmannin and exposed to 1 nM $1\alpha,25(OH)_2D_3$ or vehicle for 48 h. Western blot assays were carried out using anti-phospho-Akt, anti-MHC, anti-myogenin, and anti-actin antibodies. The immunoblots showed are representative from three independent experiments. C: Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^*P < 0.05$ respect to the respective control.

of MHC and myogenin as well as Akt activation were suppressed by PP2 and Wortmannin (Fig. 9B). These results suggest that Src and PI3K act upstream Akt activation which is involved in MHC and myogenin expression.

DISCUSSION

Myogenesis involves withdrawal of myoblasts from the cellular cycle, subsequent expression of myotube-specific genes and formation of multinucleated myotubes [McKinsey et al., 2002; Buckingham et al., 2003; Parker et al., 2003]. This process is largely regulated by the myogenic basic helix-loop-helix family of transcription factors (myogenin, MyoD, myf5, and MRF4) and MEF2, which regulate the expression of many muscle-specific genes, such as the MHC [Olson et al., 1995]. The presence of myogenin guarantees MHC expression and correct myotube development, demonstrating that myogenin acts early determining myoblasts to be differentiated into myotubes [Davie et al., 2007]. In mature skeletal muscle, a pool of satellite myoblasts stay quiescent until a tissue injury triggers their proliferation and subsequent differentiation to replace the loss of functional muscle fibers.

Akt is a key molecule involved in signaling pathways which regulate myogenesis. On the one hand, this kinase takes part in the control of skeletal muscle cell proliferation [Frost et al., 2002; Glass, 2003; Guttridge, 2004]. Specifically, proliferation of C2C12 myoblasts induced by insulin depends on Akt activity [Conejo and Lorenzo, 2001]. On the other hand, related to the skeletal muscle differentiation process, PI3K/Akt signaling modulates muscle gene expression during myogenic differentiation [White, 2003]. Of relevance, PI3K has been shown to participate in MHC expression induced by insulin in C2C12 cells [Sumitani et al., 2002] and the IGF-PI3K-Akt signaling pathway regulates myogenin expression in normal myogenic cells [Xu and Wu, 2000]. Therefore, Akt plays an important role in muscle proliferation and differentiation, controlling the number, size and survival of muscle cells. However, the signaling pathways underlying the participation of Akt in the regulation of these processes in muscle have not been investigated in detail.

Some of the actions of $1\alpha,25(OH)_2D_3$ are related to the normal development of skeletal muscle [Endo et al., 2003], in keeping with the importance of this hormone in the control of skeletal muscle metabolism and contractility [Boland, 1986; Boland et al., 2005; Buitrago et al., 2009]. Of relevance, physiological doses of $1\alpha,25(OH)_2D_3$ stimulate proliferation of chicken myoblasts and also their differentiation into myotubes [Capiati et al., 1999]. Our study shows that treatment with 1 nM $1\alpha,25(OH)_2D_3$ for 24 h promotes proliferation of murine C2C12 cells in a PI3K-dependent manner. In addition, at the proliferative cell stage, $1\alpha,25(OH)_2D_3$ induces Akt (Ser473) phosphorylation, implying its activation, in a time-dependent way. The time course of Akt activation by the hormone was similar to that observed in fibroblasts by Park et al. [2003]. Also, 17β-estradiol elicits a similar pattern of Akt phosphorylation in endometrial cancer cells [Guoa et al., 2006] and hypoxia has been shown to activate Akt at comparable times in artery endothelium [Chen and Meyrick, 2004]. In agreement with other results reporting that Akt activation in muscle is PI3K-dependent [Vasconsuelo et al., 2008; Gorelick-Feldman et al., 2010], we report here the involvement of PI3K in hormone-induced Akt activation. These data were obtained using LY 294002 and Wortmannin, two different blockers of PI3K. Regarding the use of both pharmacological inhibitors, their specificity and possible undesirable effects deserve consideration. LY 294002 was reported to inhibit all isoforms of PI3K but not to affect other kinases such as PKC and PKA, MAPK family, S6 kinase and Src kinases [Vlahos et al., 1994]. Wortmannin, although affecting myosin light chain kinase, is widely recognized as a selective and specific PI3K inhibitor too, with reportedly no effects on PKC and protein-tyrosine kinases like Src [Powis et al., 1994].

We asked ourselves which other mechanisms, independent of PI3K, could be used by $1\alpha,25(OH)_2D_3$ to activate Akt. In this study, experiments with C2C12 myoblasts in which caveolae were disrupted by MβCD treatment or caveolin-1 expression was silenced with specific siRNA, revealed that Akt phosphorylation induced by $1\alpha,25(OH)_2D_3$ was abolished, demonstrating that caveolae and cav-1 are required in Akt Ser473 phosphorylation by the hormone. In agreement with this interpretation, it was previously observed that cav-1 and caveolae participates in Akt activation in messangial cells [Zhang et al., 2007] and in VSMCs [Sedding et al., 2005]. Moreover, it was reported that caveolin-enriched microdomains play a crucial role in the mechanism by which ceramide impairs the activation of Akt in adipocytes and in cultured rat skeletal-muscle cells [Hajduch et al., 2008]. Akt function is complex and it has been also related to p38 MAPK-dependent MAPKAPK-2 (MK-2) activation [Taniyama et al., 2004]. Our results show that p38 MAPK inhibition abolishes Akt and MK-2 activation induced by the hormone, suggesting that the sequential cascade of p38 MAPK/MK-2/Akt is modulated by $1\alpha,25(OH)_2D_3$ in proliferating C2C12 cells. In earlier work we reported that in these cells Src is required for p38 MAPK activation by the hormone [Buitrago et al., 2006]. In myoblasts and myotubes of L6 and L8 cell lines, Src appears to upregulate Akt activation by insulin [Jacob et al., 2009]. In the present investigation we obtained evidence that Src is needed for hormone-induced Akt activation both in C2C12 myoblasts and in C2C12 differentiating cells, by inhibiting either its activity or its expression. To inhibit Src, the

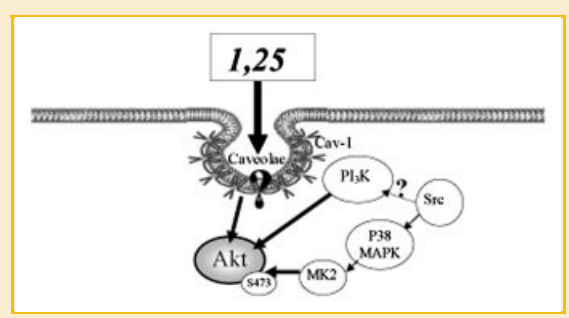


Fig. 10. Proposed schematic diagram of Akt activation by $1\alpha,25(OH)_2D_3$ in C2C12 skeletal muscle cells. $1\alpha,25(OH)_2D_3$ (1,25) may act through caveolae microdomains and after that, the hormone triggers rapid signaling cascades including activation of PKC/PTPα/Src/p38 MAPK/MK2 leading to Akt activation. An alternative mechanism of regulation of Akt implicates PI3K action which could be activated by Src action.

selective inhibitor PP2 is widely used [Hanke et al., 1996]. The inhibitory action of this compound on $1\alpha,25(OH)_2D_3$-induced Akt phosphorylation is most likely to be the result of its main pharmacological effect on the corresponding Src kinase family rather than an unspecific effect. Supporting this contention, we could abolish Akt phosphorylation by the hormone using Src antisense ODNs which successfully blocked Src expression in C2C12 cells.

Modulation of Akt by $1\alpha,25(OH)_2D_3$ was also investigated in the early stages of differentiation of C2C12 cells. It has been shown that MHC and myogenin expression requires Akt activation in chicken embryo myoblasts induced to differentiation [Jiang et al., 1999]. Moreover, PI3K-dependent MHC and myogenin expression in C2C12 cells was previously reported [Xu and Wu, 2000; Sumitani et al., 2002]. Correlated to this information, this study revealed that the steroid hormone $1\alpha,25(OH)_2D_3$ increases MHC and myogenin expression and also activates Akt in differentiating myoblasts. Of relevance, Src and PI3K were required for $1\alpha,25(OH)_2D_3$ to upregulate MHC and myogenin expression and Akt phosphorylation in these cells. In accord with our data, Lu et al. [2002] previously informed that Src activation is needed for C2C12 muscle cell differentiation. However, it has been observed that the Src inhibitor SU6656 has no effect on MHC and myogenin expression in C2C12 cells after 6 days of initiation of the differentiation program [Lim et al., 2007]. We speculate that Src activation is necessary in the early stages of C2C12 myoblast differentiation (first 3 days) but might be dispensable in mature muscle fibers.

Finally, the data obtained in this work lead us to conclude that Akt may represent a key intermediate in $1\alpha,25(OH)_2D_3$ regulation of myoblast proliferation, survival and myogenic differentiation. One important finding in this study is that Src takes part in Akt-mediated $1\alpha,25(OH)_2D_3$ regulation of signaling pathways involved in skeletal muscle proliferation and differentiation. Moreover, our results suggest the operation of another mechanism of Akt activation that is independent of PI3K action, that is, the involvement of p38 MAPK in Akt phosphorylation by the hormone through MK2 (Fig. 10).

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