



# Caveolae and caveolin-1 are implicated in $1\alpha,25(\text{OH})_2$ -vitamin $\text{D}_3$ -dependent modulation of Src, MAPK cascades and VDR localization in skeletal muscle cells<sup>☆</sup>

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## ABSTRACT

We previously reported that  $1\alpha,25(\text{OH})_2\text{D}_3$  induces non-transcriptional rapid responses through activation of MAPKs in C2C12 skeletal muscle cells. However, there is little information on the molecular mechanism underlying the initiation of  $1\alpha,25(\text{OH})_2\text{D}_3$  signaling through this pathway. Plasma membrane components have been involved in some non-genomic effects. In this work, we investigated the role of caveolae and caveolin-1 (cav-1) in  $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulation of c-Src and MAPKs. When proliferating cells were pretreated with methyl beta cyclodextrin (M $\beta$ CD), a caveolae disrupting agent, under conditions in which cell morphology is not affected and no signs of apoptosis are observed,  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent activation of ERK1/2, p38 MAPK and c-Src was suppressed. Similar results were obtained by siRNA technology whereby silencing of cav-1 expression abolished activation of c-Src and MAPKs induced by the hormone. By confocal immunocytochemistry it was observed that cav-1 colocalizes with c-Src in the periplasma membrane zone at basal conditions. Hormone treatment disrupted the colocalization of these proteins and redistributed them into cytoplasm and nucleus. Co-immunoprecipitation assays corroborated these observations. Changes in VDR localization after  $1\alpha,25(\text{OH})_2\text{D}_3$  exposure were also investigated. Confocal microscopy images showed that the hormone induces VDR translocation to the plasma membrane, and this effect is abolished by M $\beta$ CD. Altogether, these data suggest that caveolae is involved upstream in c-Src–MAPKs activation by  $1\alpha,25(\text{OH})_2\text{D}_3$  and that VDR and cav-1 participate in the rapid signaling elicited by the hormone.

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## 1. Introduction

The steroid hormone  $1\alpha,25$ -dihydroxyvitamin  $\text{D}_3$  [ $1\alpha,25(\text{OH})_2\text{D}_3$ ] acts in target cells by a genomic mode of action where it binds to its receptor (VDR) regulating gene expression [1]. Also, the operation of a non-genomic mechanism associated with rapid hormone regulation of signal transduction pathways has been demonstrated [2]. In many cases, these events appear to be initiated at the plasma membrane level. Particularly, there is growing evidence that VDR localized at the immediate zone of the cell membrane mediates some of these signaling events [3–5]. We previously demonstrated translocation of the VDR to the cellular membrane and its co-immunoprecipitation with c-Src after  $1\alpha,25(\text{OH})_2\text{D}_3$  exposure of chicken skeletal muscle cells [6,7]. Moreover, VDR and c-Src participation in hormone-dependent activation of MAPKs in proliferative skeletal muscle cells by the hormone was also well established [8,9]. But, up to now, membrane

components involved in  $1\alpha,25(\text{OH})_2\text{D}_3$  rapid signaling in muscle have not been established.

Caveolae are invaginations of plasma membrane enriched in sphingolipids and cholesterol. These microdomains are specialized lipid rafts that serve as signaling pathway platforms [10]. Robust information indicates that caveolae concentrate specific signal transducer proteins (e.g. tyrosine kinases of Src family and G proteins) as well as caveolins [11,12]. Caveolins are main structural components of caveolae and also are members of scaffolding cytosolic proteins [13]. In absence of caveolins, no morphologically identifiable caveolae exist [14]. Molecular cloning has identified three caveolins isoforms, caveolin-1, -2 and -3 (cav-1, cav-2 and cav-3) [15]. Although it is known that cav-1 and -2 are present in fibroblasts, endothelial cells and adipocytes whereas cav-3 protein is muscle specific, cav-1 expression was confirmed in undifferentiated skeletal muscle cells in vivo and in vitro [16]. Caveolins play a significant role in different disease phenotypes, specifically in muscular dystrophy. Mice knock-down for cav-1 gene (cav-1  $-/-$ ) present abnormalities in skeletal muscle due to cav-1 expression conditioned later appearance of cav-2, so cav-1 absence promotes the pathogenesis called tubular aggregate formation [17]. Modulation of cav-1 levels also controls satellite cell activation during muscle repair [16]. In parallel, cav-1 plays a role in the

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physiological intracellular signaling network. Cav-1 can bind c-Src via its caveolin-scaffolding domain and thereby “clamp” Src in its inactive configuration [18]. So, the disruption of cav-1–c-Src complex conduces to c-Src activation and this event could be one of the beginning steps of signaling pathway cascades [14].

With respect to the VDR, Norman and collaborators reported that it is present in caveolae-enriched plasma membranes and binds  $1\alpha,25(\text{OH})_2\text{D}_3$  in vivo and in vitro [19]. Plasma membrane requirements for hormone-dependent PKC signaling were observed in chondrocytes and osteoblasts. Specifically, the regulation of growth plate chondrocytes by  $1\alpha,25(\text{OH})_2\text{D}_3$  requires caveolae and cav-1 [4]. Recently it was reported the key role of cav-1 in the compartmentalization of estrogen receptor  $\beta$  (ER $\beta$ ) to the plasma membrane, thus allowing estradiol to control VDR transcription and expression [20]. In view of the data described, our aim was to investigate the role of caveolae and cav-1 in  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent modulation of kinase cascades and VDR localization in skeletal muscle cells.

## 2. Materials and methods

### 2.1. Chemicals

$1\alpha,25(\text{OH})_2\text{D}_3$  was kindly provided by Dr. Jan-Paul van de Velde from Solvay Pharmaceuticals (Weesp, The Netherlands). Dulbecco's modified Eagle's medium (DMEM) low glucose, with L-glutamine and HEPES, without phenol red, was from US Biological (Swampscott, MA, USA). Fetal bovine serum (FBS), protein A sepharose and the compound M $\beta$ CD (methyl beta cyclodextrin) were from Sigma–Aldrich Co. (St. Louis, MO, USA). Primary antibodies: anti-phospho p38, anti-caveolin-1, anti-VDR and 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, USA). Anti-phospho ERK1/2, anti-c-Src, anti-phospho (Tyr 416) c-Src and anti-actin antibodies were acquired in Cell Signaling Technology, Inc. (Beverly, MA, USA). Cell mask and DAPI stains, Alexa Fluor 488 goat anti-rabbit antibody and anti-mouse antibody conjugated with rhodamine were from Invitrogen (Carlsbad, California). The Super Signal CL-HRP substrate system for enhanced chemiluminescence (ECL) was from PerkinElmer (Boston, USA). The C2C12 cell line (American Type Culture Collection, Manassas, VA) was kindly provided by Dr. E. Jaimovich (Universidad de Chile, Santiago, Chile). All other reagents were of analytical grade.

### 2.2. Cell culture

The mouse skeletal myoblastic cell line C2C12 was seeded at an appropriate density (120,000 cells/cm<sup>2</sup>) in Petri dishes (100 mm diameter) with DMEM supplemented with 10% FBS and antibiotic–antimycotic solution. The cells were cultured at 37°C under a humidified atmosphere (95% air/5% CO<sub>2</sub>). Under these conditions, myoblasts divide within the first 48 h and at day sixth these cells become differentiated into myotubes expressing morphological characteristics of adult skeletal muscle fibers [21]. Cells cultured for one or two days (proliferative stage) were used for treatments. The cells were incubated in serum-free medium for 30 min prior to the addition of  $1\alpha,25(\text{OH})_2\text{D}_3$ .

### 2.3. SDS-PAGE and immunoblotting

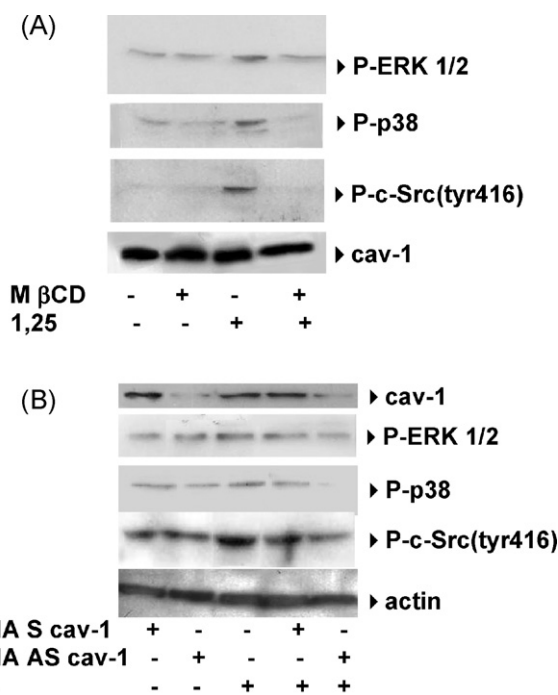
Following treatment, the cells were lysed by sonication, the lysates resolved by one-dimensional SDS-PAGE and then

electrotransferred to polyvinylidene difluoride (PVDF) membranes as previously described [22]. Membranes were subjected to immunoblotting using anti-phospho p38 MAPK, anti-phospho ERK1/2, anti-phospho (tyr 416) c-Src and anti-cav-1 antibodies.

The antibodies were then stripped and the membrane was reprobbed with the corresponding antibody to account for equal loading. Autoradiograms were scanned with a BIORAD densitometer to quantitate hormone signals.

### 2.4. Co-immunoprecipitation assays

Co-immunoprecipitation assays were performed using total cell extracts to analyze putative protein–protein interactions in eukaryotic cells. After treatments, cells were lysed and the protein amounts were measured by the Bradford procedure [23]. One microgram of anti-c-Src antibody was added to 10% (v/v) protein A sepharose and incubated for 2 h at 4°C on a mixer. Lysates containing 150  $\mu\text{g}$  protein were added to this mixture and incubated overnight at 4°C with slow agitation. As a negative control, protein A sepharose was incubated with 150  $\mu\text{g}$  protein in absence of antibody. The precipitated immunocomplexes were washed four times with cold lysis buffer, resuspended in Laemmli sample buffer and subject to SDS-PAGE.



**Fig. 1.** Caveolae disruption suppresses ERK1/2 and p38 MAPK phosphorylation and c-Src activation induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  in C2C12 muscle cells. Silencing of cav-1 expression abolishes  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent activation of c-Src/MAPKs. (A) C2C12 murine skeletal muscle cells were preincubated with M $\beta$ CD (2 mM) for 30 min in serum-free medium. After replacement of the medium, cells were stimulated with 1 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  (1,25) or its vehicle isopropanol (0.01%) for 60 min and then lysed. Cell lysates were clarified followed by Western blotting with anti-phospho ERK1/2, anti-phospho p38 and with anti-phospho (Tyr 416) c-Src antibodies. Membranes were then reblotted with anti-cav-1 antibody as described in Section 2. A representative blot from three independent experiments is shown. Results of next figures were obtained using 1 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  for 60 min. (B) Cells were transfected with either a sense (S) or an antisense (AS) siRNA against cav-1 mRNA and 24 h later the cells were exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$  (1,25) followed by immunoblotting of equal amount of cell lysates with anti-cav-1 antibody. The membrane was stripped and reprobbed with anti-phospho ERK1/2, anti-phospho p38, anti-phospho (Tyr 416) c-Src and anti-actin antibodies. Immunoblots obtained from two independent experiments were quantified by scanning volumetric densitometry. Averages  $\pm$  SD are given. \* $p < 0.05$ .

## 2.5. Immunocytochemistry

C2C12 cells grown onto glass coverslips were fixed in methanol (at  $-20^{\circ}\text{C}$ ) for 20 min, process which also permeabilized the cells. Non-specific sites were blocked with 5% BSA in PBS for 1 h. Samples were then incubated with the appropriate primary antibody prepared in 2% BSA in PBS (1 h, room temperature). After washing with PBS, the samples were incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG or Rhodamine goat anti-mouse conjugated antibodies (1 h, room temperature). The samples were examined using a Leica confocal laser microscope.

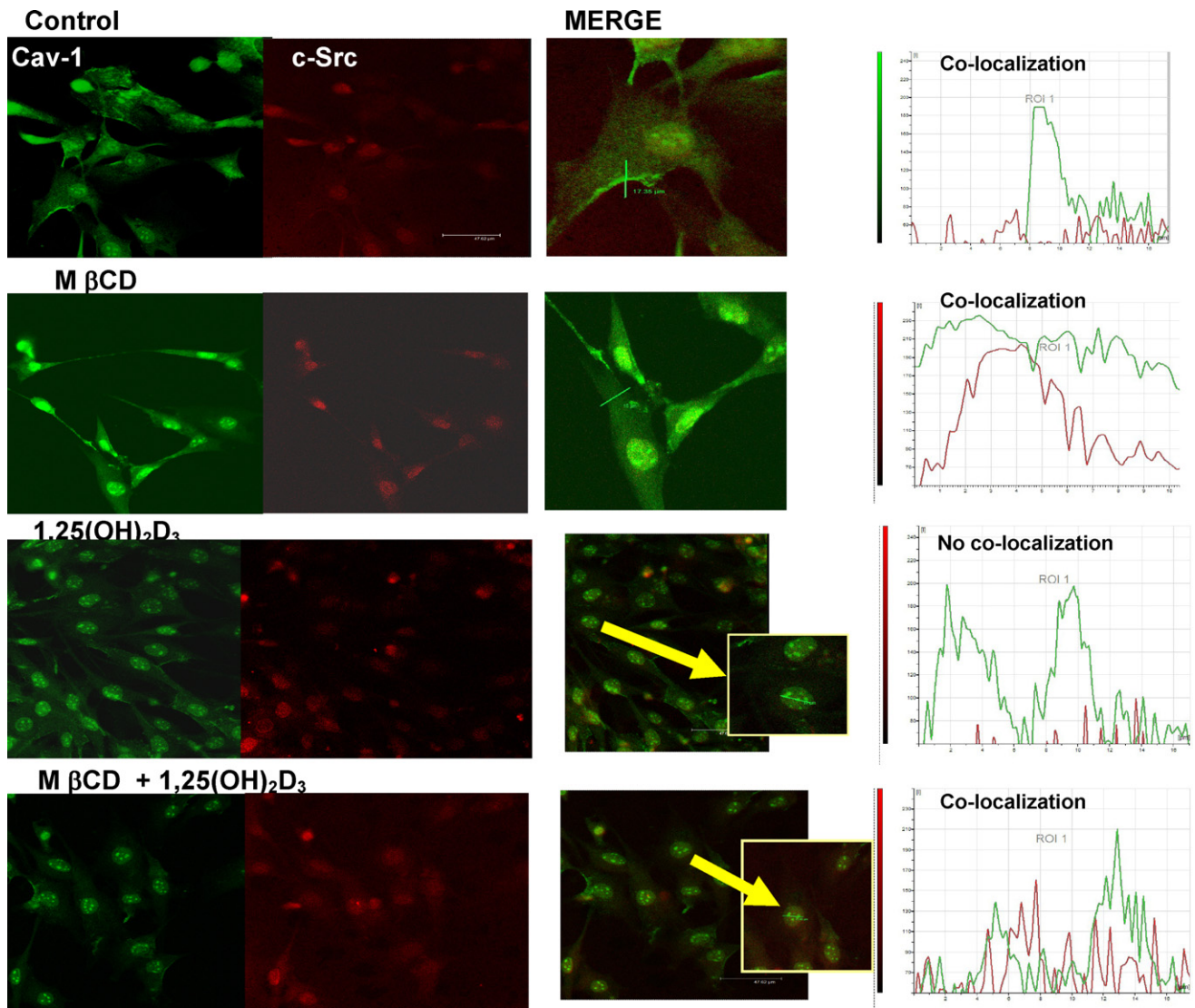
## 2.6. siRNA technology

Six-well tissue culture plates with  $2 \times 10^5$  cells/well in antibiotic-free normal growth medium supplemented with FBS were incubated at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator until the cells were 60–80% confluent (usually 24 h). Then the following solutions were used. Solution A: for each transfection, 5  $\mu\text{l}$  of siRNA duplex (i.e.

0.5  $\mu\text{g}$  siRNA) into 100  $\mu\text{l}$  siRNA transfection medium. Solution B: for each transfection, 5  $\mu\text{l}$  of siRNA transfection reagent into 100  $\mu\text{l}$  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  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator. The transfection mixture was removed and replaced with normal growth medium. Cells were incubated for an additional 18 h until used for treatments.

## 2.7. Statistical evaluation

The statistical significance of the results was evaluated by Student's *t*-test [24] and probability values below 0.05 ( $p < 0.05$ ) were considered significant. Results are expressed as means  $\pm$  standard deviation (SD) from the indicated set of experiments.



**Fig. 2.** Colocalization of cav-1 and c-Src is disrupted by  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment of C2C12 cells. Muscle cell growth over coverslips was stimulated with  $1\alpha,25(\text{OH})_2\text{D}_3$  or vehicle as explained before. Immunocytochemistry assays were performed as described in Section 2. Confocal fluorescence digital images of proliferating C2C12 cells incubated with vehicle (control), M $\beta$ CD,  $1\alpha,25(\text{OH})_2\text{D}_3$ , and M $\beta$ CD +  $1\alpha,25(\text{OH})_2\text{D}_3$  are shown. All conditions were double labeled with anti-cav-1 antibody (green) and anti-c-Src antibody (red). On the right side panels the images are merged and the fluorescence intensity across a traced line over the images quantified by histograms. Representative images of different fields of all coverslips analyzed are shown.

### 3. Results

#### 3.1. Effects of cholesterol depletion by M $\beta$ CD on proliferating skeletal muscle C2C12 cells

M $\beta$ CD (methyl beta cyclodextrin), a cyclic oligosaccharide employed to remove cholesterol of membrane lipid rafts [25] disrupts caveolae [26]. It is known that cholesterol has a key role on myoblast fusion [27] but there are not reports about the effects of cholesterol depletion by M $\beta$ CD on proliferative C2C12 cell physiology. We studied then cellular morphology and size and nucleus integrity in cells preincubated for 30 min with this agent. We used the nuclear marker DAPI and the cellular stain *cell mask* to perform immunocytochemical assays. Confocal images obtained were employed to measure the nucleus diameter and cellular size of control and M $\beta$ CD (2 and 4 mM) pretreated cells and then incubated in the presence and absence of  $1\alpha,25(\text{OH})_2\text{D}_3$  (1 nM, 60 min). There were no significant changes in cellular measures and neither in size and shape of nuclei caused by cholesterol depletion (data not shown). We used 2 mM of M $\beta$ CD in successive experiments.

#### 3.2. M $\beta$ CD treatment abolishes ERK1/2 and p38 MAPK phosphorylation and c-Src activation induced by the hormone

As mentioned above, plasma membrane is required for  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent PKC signaling in chondrocytes [4]. In skeletal muscle cells it is unknown whether hormone signaling through kinase cascades involves membrane components.

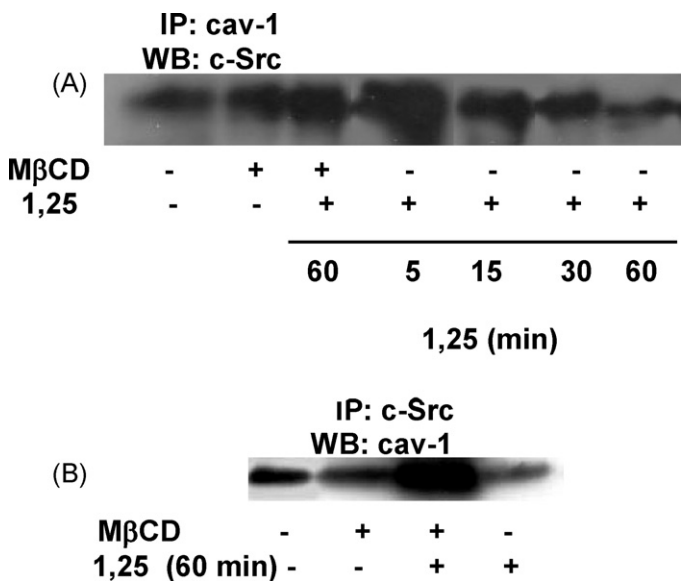
Therefore, we examined the participation of caveolae in phosphorylation of MAPKs and c-Src activation in C2C12 cells treated with  $1\alpha,25(\text{OH})_2\text{D}_3$ . Cells were preincubated with M $\beta$ CD before exposure to the hormone. Fig. 1 (panel A) shows that pretreatment carried out to remove cholesterol suppressed the  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent ERK1/2 and p38 MAPK phosphorylation and c-Src activation. This result shows that intact caveolae are necessary for ERK1/2, p38 MAPK and c-Src-hormone-dependent activation.

#### 3.3. Silencing of cav-1 expression abolishes $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent MAPKs and c-Src activation

It has been established that cav-1 protein is essential for caveolae maintenance [13]. We significantly reduced cav-1 expression in C2C12 cells using a specific siRNA. Cav-1 silencing blocked ERK1/2 and p38 MAPK phosphorylation and diminished markedly c-Src activation induced by  $1\alpha,25(\text{OH})_2\text{D}_3$ , showing again the importance of caveolae in stimulation of MAPKs and c-Src by the hormone (Fig. 1, panel B).

#### 3.4. Basal colocalization and co-immunoprecipitation of c-Src with caveolin-1 is disrupted by $1\alpha,25(\text{OH})_2\text{D}_3$ treatment

c-Src mediates phosphorylation of MAPKs by the hormone in C2C12 cells [8]. The activation mechanism of c-Src is complex and it may involve the cav-1 protein [18]. We studied in this work if there exists an association between cav-1 and c-Src. In Fig. 2, confocal images obtained from immunocytochemical assays and their corresponding histograms show that cav-1 colocalizes with c-Src in the periplasma membrane zone under basal conditions. When cells are exposed to M $\beta$ CD, the c-Src/cav-1 colocalization is observed in cytosol.  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment disrupted their colocalization and redistributed these proteins into cytoplasm and nucleus. Preincubation with M $\beta$ CD before hormone stimulation maintains the colocalization of c-Src and cav-1 in the nucleus. Co-immunoprecipitation assays with specific antibodies corroborated these observations. In addition we observe that, as exposure



**Fig. 3.**  $1\alpha,25(\text{OH})_2\text{D}_3$  diminishes co-immunoprecipitation of cav-1 and c-Src in muscle cells. (A) After preincubation with M $\beta$ CD, C2C12 cell cultures were stimulated with  $1\alpha,25(\text{OH})_2\text{D}_3$  for different times (5–60 min). Cav-1 was immunoprecipitated with anti-cav-1 antibody followed by Western blotting with anti-c-Src antibody. (B) Cells preincubated with M $\beta$ CD were treated with the hormone for 60 min. c-Src was immunoprecipitated with anti-c-Src antibody followed by Western blotting with anti-cav-1 antibody. Representative immunoblots from two independent experiments are shown.

time to the hormone (in absence of M $\beta$ CD) augmented, the co-immunoprecipitation of c-Src and cav-1 diminishes (Fig. 3).

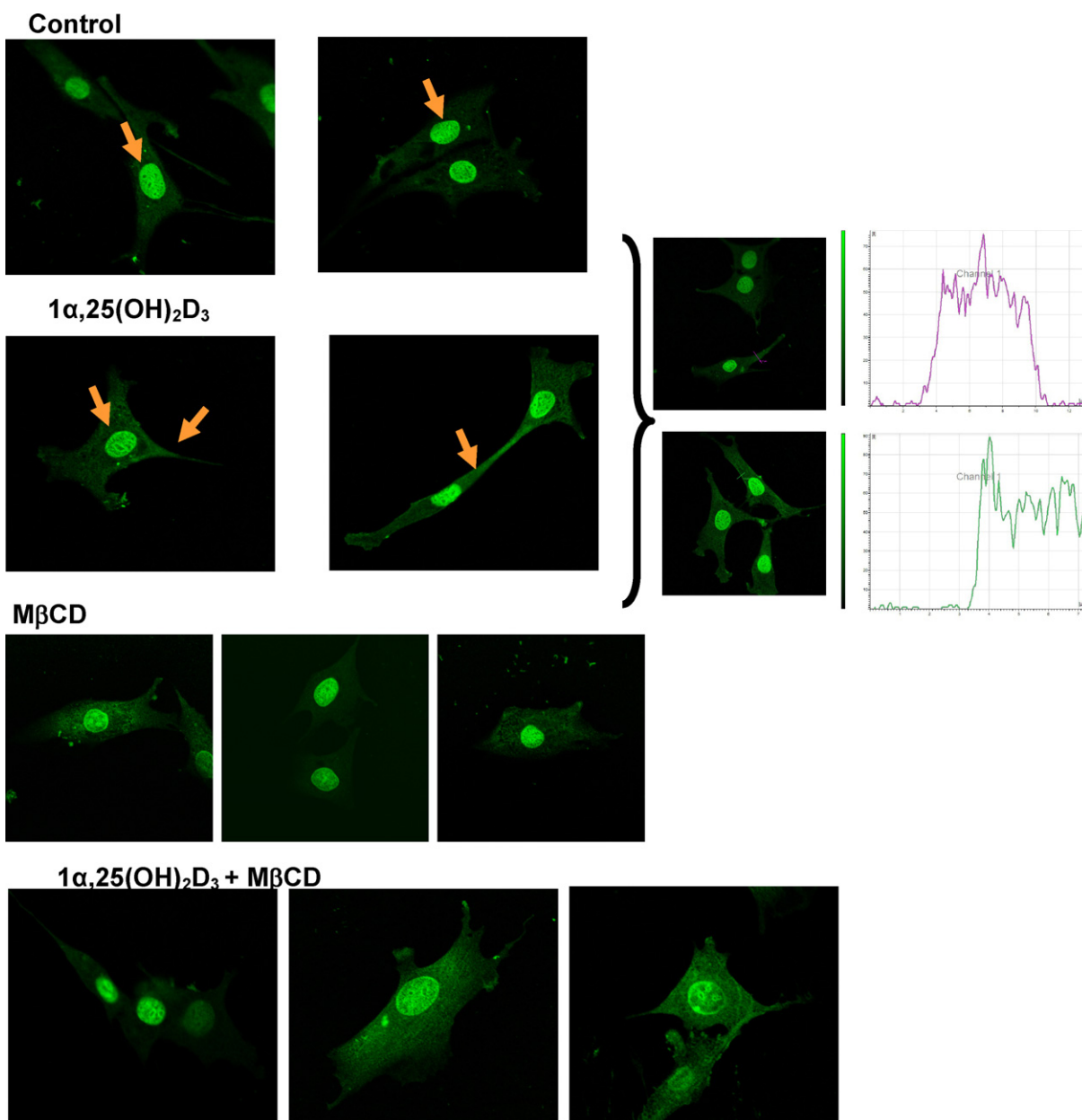
#### 3.5. Plasma membrane translocation of VDR after hormone treatment is abolished in cells pretreated with M $\beta$ CD

The reverse traffic of VDR from nucleus/cytosol to plasma membrane was described in our laboratory using avian muscle cells [6]. We also reported VDR–c-Src association dependent on  $1\alpha,25(\text{OH})_2\text{D}_3$  [8]. In this work, confocal microscopy showed that the hormone induces VDR translocation to the plasma membrane and, of relevance, this effect was abolished when cholesterol was sequestered of plasma membrane by M $\beta$ CD (Fig. 4).

### 4. Discussion

Most processes of signal transduction involve activation of sequential cascades of kinases. Among them are the ubiquitous MAPKs that regulate a plethora of responses. Different molecules are required to interact in an orderly manner and activate MAPK pathways. Several reports have shown that some of these components are concentrated within caveolae membranes [28–30]. Particularly, Src kinases are early key intermediates of ERK1/2 and p38 MAPK pathways that can be compartmentalized into lipid rafts and caveolae [31].

Previously, we established the role of c-Src in  $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent MAPK activation in avian and murine skeletal muscle cells [8,9]. But we failed to investigate hormone-dependent upstream c-Src events. The results of this work contribute to elucidate the molecular mechanism underlying the initiation of  $1\alpha,25(\text{OH})_2\text{D}_3$  signaling in skeletal muscle cells. Data presented here, show that pretreatment with M $\beta$ CD (sequesters cholesterol of plasma membrane disrupting caveolae) abolishes ERK1/2 and p38 MAPK phosphorylation and c-Src activation induced by the hormone. It was shown before that caveolae play a key function in ERK1/2 modulation in smooth muscle [32]. In fact, compart-



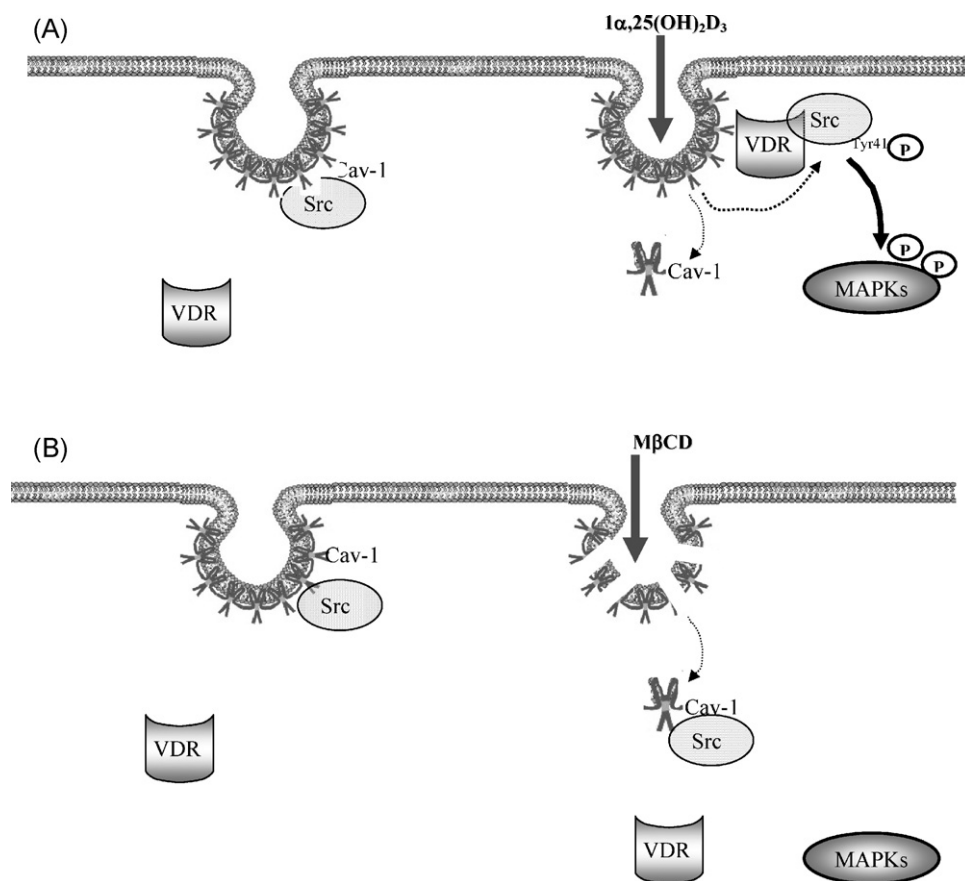
**Fig. 4.** VDR translocation to the plasma membrane after  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment is abolished when C2C12 cells are exposed to M $\beta$ CD. Monolayers of cells over coverslips were treated with  $1\alpha,25(\text{OH})_2\text{D}_3$  or vehicle as explained before. Immunocytochemistry assays were performed as described in Section 2. Confocal fluorescence digital images of proliferating C2C12 cells incubated with vehicle (control),  $1\alpha,25(\text{OH})_2\text{D}_3$ , M $\beta$ CD and M $\beta$ CD +  $1\alpha,25(\text{OH})_2\text{D}_3$  are shown. Cells were then fixed and labeled with anti-VDR antibody (green). Representative images of different fields of all coverslips analyzed. On the right of the second panel there are histograms showing fluorescence intensity changes in a line traced on the cell width. Arrows indicate the nuclear expression of VDR in control cells and the VDR presence in plasma membrane of  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated cells.

mentalization of ERK1/2 within the caveolae, has been reported [33,34]. Our studies provide for the first time evidence on caveolae participation in  $1\alpha,25(\text{OH})_2\text{D}_3$  non-genomic signaling in skeletal muscle.

Cav-1 plays an important role in the regulation of MAPK activity in some tissues [35]. In the present work, silencing the expression of cav-1 in C2C12 cells blocked ERK1/2 and p38 MAPK phosphorylation and c-Src activation by  $1\alpha,25(\text{OH})_2\text{D}_3$ . It is known that the absence of cav-1 expression prevents caveolae formation and maintenance [10]. The above results show then that caveolae are necessary to transduce the hormone signal to MAPK cascades in skeletal muscle cells. On the other hand, it has been reported a negative action of cav-1 on c-Src activation [36,37]. Therefore, we expected to observe an increase in

c-Src activation when cav-1 expression was silenced. We suggest that the absence of cav-1 prevents caveolae formation and this event avoids the hormone-dependent c-Src activation. The results obtained by immunocytochemistry and co-immunoprecipitation assays revealed the existence of cav-1/c-Src association under basal conditions where c-Src is inactive, as has been previously reported [36].  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment dissociates the cav-1/c-Src complex and activates c-Src. When the caveolae structure is disrupted by M $\beta$ CD, the hormone is not able to separate cav-1 from c-Src preventing its activation.

As mentioned before, we demonstrated in avian skeletal muscle cells reverse traffic of the VDR and VDR/c-Src complex formation induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  [6,7]. In this work, we also show in murine C2C12 cells hormone-dependent VDR translocation



**Fig. 5.** Proposed model of upstream signaling mechanism triggered by  $1\alpha,25(\text{OH})_2\text{D}_3$  in skeletal muscle cells. (A) At basal condition we observed the cav-1 interaction with c-Src, event that clamped this kinase in an inactive state. On the right,  $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulation promotes VDR translocation to the plasma membrane and dissociates the cav-1/c-Src complex allowing c-Src activation and phosphorylation of MAPKs by the hormone. It is suggested that  $1\alpha,25(\text{OH})_2\text{D}_3$  would promote VDR/c-Src association like in avian skeletal muscle cells [7]. (B) Left: basal condition described above (A, left). Right: when cells were preincubated with M $\beta$ CD, caveolae was disrupted and the cav-1/c-Src association is maintained with concomitant lack of MAPKs activation.

to plasma membrane, altogether consistent with the concept that VDR is implicated in non-genomic responses triggered by  $1\alpha,25(\text{OH})_2\text{D}_3$  [5]. We are now investigating in further depth these findings.

Altogether, these data suggest that in skeletal muscle cells intact caveolae participate in early upstream steps of  $1\alpha,25(\text{OH})_2\text{D}_3$  signal transduction via c-Src–MAPKs and that VDR and cav-1 are involved in the rapid events elicited by the hormone (Fig. 5).

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