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**SYNDECAN-4 DISTRIBUTION DURING THE DIFFERENTIATION OF  
SATELLITE CELLS ISOLATED FROM SOLEUS MUSCLE TREATED  
BY PHORBOL ESTER AND CALPHOSTIN C**

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**Abstract:** It was shown that syndecans have a potential role in muscle development. We focused this study on the role of syndecan-4 distribution and phosphorylation during the differentiation of satellite cells isolated from Soleus muscle. Syndecans are cell surface heparan sulfate proteoglycans (HSPGs) that bind numerous ligands through their HS glycosaminoglycan chains (GAG). They play a role in cell-extracellular matrix and cell-cell adhesion, signal transduction and the targeting of growth factors and other molecules to the cell surface. Syndecan-4 acts as a co-receptor or, along with integrins, is localized to the cell membrane of focal contacts. Syndecan-4 participates in the organization of the structure of focal contacts reacting with extracellular matrix molecules. The interaction of syndecan-4 with protein kinase C (PKC) isoforms is the main mechanism regulating its distribution in cells. Our current study focused on the role of the distribution of syndecan-4, and its interactions with PKC isoforms during the differentiation of activated satellite cells. We used the PKC activator TPA (12-O-tetradecanoyl phorbol 13-acetate) and the PKC inhibitor Calphostin C (Cal C). We concluded that syndecan-4 was important not only in the activation of satellite cells, but also in myoblast differentiation. During our research, we observed the presence of syndecan-4 and changes in its location over the course of that process. We also showed that TPA and Cal C treatment had an influence on the subcellular distribution of syndecan-4, but there was no influence on myoblast differentiation. We speculated that the reason for changes after TPA treatment was the interactions with activated PKC $\alpha$ , which provoked

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Abbreviations used: HSPG - heparan sulfate proteoglycan; GAG - glycosaminoglycan; PKC - protein kinase C; TPA - 12-O-tetradecanoyl phorbol 13-acetate; Cal C - calphostin C; FC - focal contact; ECM - extracellular matrix; FAK - focal adhesion kinase; FGF - fibroblast growth factor; VEGF - vascular endothelial growth factor; HGF - hepatocyte growth factor; PIP<sub>2</sub> - phosphatidyl-inositol-4,5-bisphosphate.

syndecan-4/PKC $\alpha$  complex translocation to integrins. We also supposed that Cal C treatment inhibited PKC $\delta$  activity and probably induced PKC $\alpha$  association to syndecan-4, and syndecan-4 translocation to integrins.

**Key Words:** Differentiation, Satellite cells, Myoblasts, Syndecan-4, Protein kinase C, TPA, Calphostin C, Soleus, Adhesion

## INTRODUCTION

In our investigations, we are particularly interested in changes in the structure of focal contacts during the differentiation of satellite cells *in vitro*. Satellite cells (also called adult myoblasts) are stem myogenic cells located between the basal membrane and the plasmalemma of muscle skeletal fibers in adult skeletal muscles. They are required for skeletal muscle regeneration. Satellite cells dissociated from adult muscle and grown in culture are able to proliferate, fuse and form multinucleate myotubes [1].

Focal contacts (FC) are the areas where cells contact the extracellular matrix (ECM). FC play an essential role in: adhesion to the ECM, migration and signal transduction leading to cell proliferation, growth, differentiation, apoptosis, gene expression, and other processes. Many transmembrane molecules, adhesion molecules and cytoskeletal proteins are involved in the integration of this structure. Some of these proteins participate in the physical linkage of the cytoskeleton (microfilaments) to membrane receptors, while others belong to multiple groups of signal transduction molecules [2, 3]. Many regulatory proteins are engaged in the rearrangement of focal adhesion components: focal adhesion kinases (FAK), PKC, phosphatases, calpain II, paxillin, and others [4, 5]. In addition to the major role of integrins in FC, syndecans are localized to these structures and participate in cell adhesion.

The syndecans are transmembrane heparan sulfate proteoglycans expressed on adherent cells [6]. They are a family of four proteins which participate in cell-matrix adhesion, cell-cell adhesion, the regulation of growth factors (FGFs, VEGF, HGF) binding and signaling. Syndecans have a potential role in muscle development. Syndecan-3 and syndecan-1 are expressed in mouse myoblasts C2C12 (a cultured satellite cell line), and the level of protein synthesis decreases in differentiated cells [7, 8]. Transfection with anti-sense mRNA for syndecan-3 accelerates mouse myoblast differentiation [7]. Similar results were obtained for syndecan-1. Transfection with cDNA for syndecan-1 prevents C2C12 cell differentiation [8]. Syndecan-3 and syndecan-4 are also expressed on quiescent satellite cells, and are necessary to activate satellite cells [9].

It is known that syndecan-4 can influence cell adhesion. Syndecan-4 is a transmembrane protein containing an extracellular domain (which has attached GAG), a transmembrane region and a short cytoplasmic domain. A region of the syndecan-4 cytoplasmic domain binds activated PKC $\alpha$  (PKC isoform), phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>), syntenin (a PDZ-containing

protein) and  $\beta$ -catenins [10]. Besides its role in cell adhesion, syndecan-4 binds numerous HS-binding growth factors (FGFs, VEDF, HGF, etc.). In this process, syndecan-4 not only binds growth factors, but also binds their receptors and acts as co-receptor. However, the major role of syndecan-4 is its cooperation with integrins in cell spreading and adhesion. Both integrins and syndecan-4 react with ECM molecules (fibronectin, laminin) in FC [10-12]. The main mechanism that regulates syndecan-4 distribution in cells is its interaction with protein kinase C (PKC) isoforms.

PKC represents a family of serine-threonine kinases. Some PKC isoforms are involved in a wide variety of intracellular signaling events. They play a key role in cellular responses mediated by second messengers [13-15]. PKCs are an important regulator of cytoskeletal function. PKCs have been shown to phosphorylate proteins localized in FC. The substrates for PKC in FC are talin, vinculin and integrins. Some authors suggest that the activation of PKC is indispensable for cell spreading and FC formation [16-18]. To study the involvement of PKC in biological processes, some inhibitors and activators (also isoform specific) are used. One of the activators for PKC is TPA (phorbol ester) [15, 19, 20]. Cal C is a highly specific inhibitor of PKC. Cal C interacts with the protein's regulatory domain and induces irreversible inactivation of PKC [21, 22].

In this study, we investigated the role of syndecan-4 distribution during the differentiation of activated satellite cells. It was shown that syndecan-4 was important in the activation of satellite cells [9]. We supposed that it played a role in myoblast differentiation as well. We noticed the presence of syndecan-4 and changes in its localization during the differentiation of satellite cells. It was shown that interactions of syndecan-4 with PKC isoforms were the main mechanism that regulates its distribution in cells [10]. We decided to investigate the influence of TPA (a PKC activator) and Cal C (a inhibitor of PKC) on the subcellular localization of syndecan-4 during the differentiation of satellite cells. We observed changes in syndecan-4 distribution in treated cells, but there was no influence on myoblast differentiation. We speculated that the causes of changes in syndecan-4 distribution after TPA and Cal C treatment were interactions with activated PKC $\alpha$  and inhibited PKC $\delta$ .

## MATERIALS AND METHODS

### **Isolation and primary culture of satellite cells and cell treatment**

Soleus muscles were excised from the legs of three month old male Wistar rats. Satellite cells were isolated from the muscles by digestion with 0.15% pronase and cultured as previously described [23]. As of the fourth day after plating, some cells were treated daily with freshly prepared solutions of TPA (Sigma) or Cal C (Calbiochem) diluted in DMSO (Sigma) at a final concentration in the medium of  $10^{-7}$  M.

### Immunofluorescent staining

Cultured cells were fixed in 4% PFA/PBS, treated with 0.1% Triton X-100/PBS, and blocked with 0.25% glycine/PBS and 3% BSA/PBS. The cells were incubated with the following primary antibodies (1:100 in 3% BSA/PBS): anti-syndecan-4 and anti-PKC $\alpha$ . The following Secondary antibodies (1:100 in 3% BSA/PBS): anti-goat biotin-conjugated, Extravidin TRITC-conjugated and anti-rabbit FITC-conjugated were used. Each time the cells were washed three times in PBS. After the final wash, the slides were mounted and sealed. All the antibodies were from Santa Cruz. The immunofluorescent stained cells were analyzed using an Axiovert 100M – Zeiss imaging system and the LSM 510 program.

### SDS-PAGE and Western Blotting

Samples (50  $\mu$ g protein of total protein lysates) were separated on a reducing 7.5% SDS-polyacrylamide minigel, as previously described [24], and transferred electrophoretically to nitrocellulose membranes. Some samples were pretreated with Heparitinase I and Chondroitinase ABC (Sigma) to cut GAG chains from the syndecan-4 core protein. Blots were blocked with 3% BSA/TTBS and incubated with primary antibodies (1:1000 in blocking buffer) and with the secondary antibodies (1:20000) – anti-goat peroxidase-conjugated and anti-rabbit peroxidase-conjugated – and then visualized with Luminol (Santa Cruz). The immunoblots were analyzed with Gel Doc using the Quantity One program (BioRad).

## RESULTS AND DISCUSSION

### Characteristics of the satellite cell culture

Satellite cells isolated from rat skeletal muscles growing in culture are called myoblasts. The myoblasts adhered, proliferated and on day seven of culture began fusing into early 2-3-nuclei myotubes. On day nine, the culture contained long, multinuclei myotubes expressing the ability of spontaneous contraction (Fig. 1). No significant influence of TPA, Cal C or DMSO (solvent) on the proliferation and differentiation of satellite cells was observed.

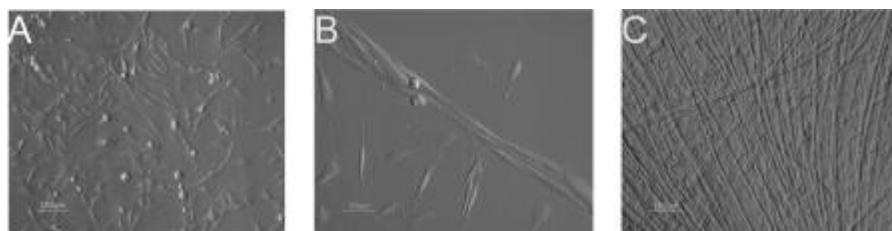


Fig. 1. Differentiation of satellite cells *in vitro* (control culture). A – proliferating myoblasts from day 5 of culture; B – fusing myoblasts from day 7 of culture; C – myotubes from day 9 of culture.

### Immunofluorescent staining of primary cell cultures

The application of confocal microscopy and cytochemical methods allowed the colocalization of syndecan-4 and PKC  $\alpha$  in differentiating satellite cells. On the fifth and seventh days of culture (proliferating and fusing myoblasts) syndecan-4 and PKC $\alpha$  were localized and dispersed near the cell membrane, as well as in the cell-cell and cell-ECM contact area. Syndecan-4 did not form any characteristic focuses under the membrane (Fig. 2A). We observed several changes in the location of both syndecan-4 and PKC $\alpha$  after TPA and Cal C treatment. After a short period of TPA treatment (24, 72 h), the colocalization of syndecan-4 and PKC was much higher (Fig. 2D-G). After a short period of TPA and Cal C treatment, both syndecan-4 and PKC $\alpha$  were found in numerous FC and formed long focuses along the myoblast. They also existed in the cell-cell contact area (Fig. 2B-F). The long focuses of syndecan-4 along the myoblasts were clearly seen in Cal C- (Fig. 2B-C) or TPA- (Fig. 2D-F) treated cells, and they were not noticed in the control cultures. In myotubes (day eleven), both

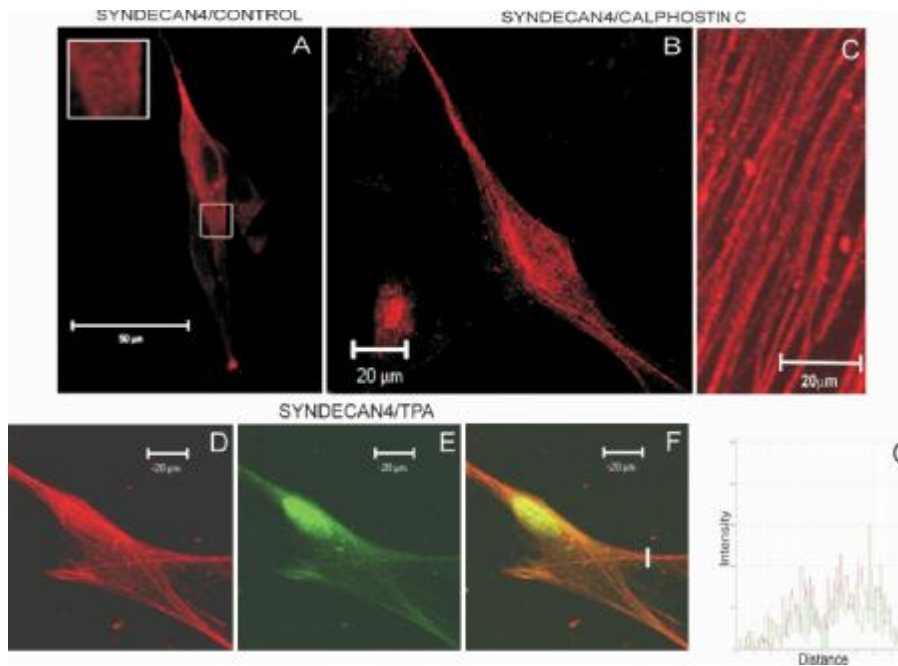


Fig 2. Immunolocalization of syndecan-4 and PKC $\alpha$  in cells from day 5 of the control culture. Color red – the areas where syndecan-4 exists; color green – the areas where PKC $\alpha$  is present; color yellow – the areas of colocalization. The chart (referring to picture F) presents the intensity of the signal for both fluorochromes along the white line shown on the picture. The cells were obtained from four independent experiments (description in the text).

syndecan-4 and PKC $\alpha$  were found dispersed along the whole length of the myotubes, but were present in very low amounts. Syndecan-4 was also concentrated at the end of the myotubes. We did not detect any changes between the control and long-term TPA- or Cal C-treated myotubes.

#### Analysis of Western Blots

We performed electrophoretic separation of proteins from cell lysates prepared on days 5, 7 and 9 of the control and TPA- or Cal C-treated cultures. The presence of two syndecan-4 bands was detected. The upper band (200 kD) is a form of syndecan-4 with GAG chains, while the lower band (40 kD) corresponds to the syndecan-4 core protein after the enzymatic cleavage of GAG chains. The bands were present at almost the same level in homogenates from all the stages of the control, TPA- and CalC-treated cultures (Fig. 3 A,B). On the basis of immunoblotting, we noted two PKC $\alpha$  bands. The upper one (70 kD) was a native form of PKC and the lower (45kD) was formed as the result of PKC $\alpha$  cleavage after activation (the form called Protein Kinase M, PKM) (Fig. 3C,D). In the lysates from the short-term TPA-treated cultures (days 5 and 7), almost only the 45 kD active form of PKC $\alpha$  (PKM) was present.

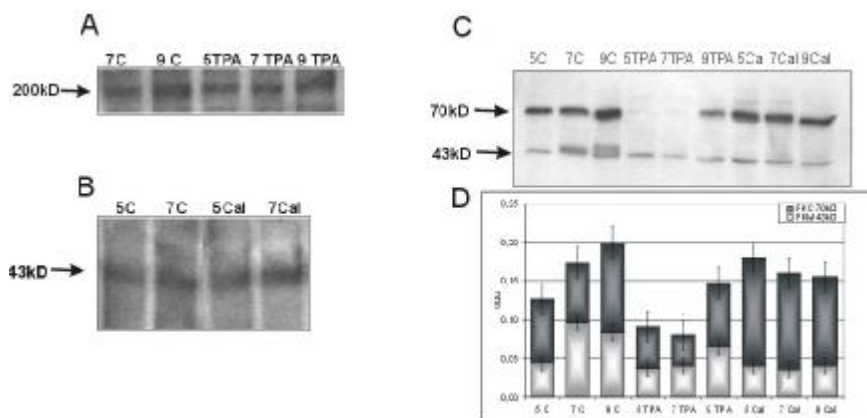


Fig. 3. Western blot analysis for syndecan-4 (A,B) and PKC $\alpha$  (C), lysates from days 5, 7, 9 of the control (C), TPA- and Calphostin C- (Cal) treated cultures. The chart (D) presents average density of PKC $\alpha$  bands on picture C. Cells were isolated from Soleus muscles in four independent experiments (description in the text).

It was shown that syndecan-3 and syndecan-4 were expressed on quiescent and activated satellite cells [9]. Both syndecan-3 and syndecan-4 act as co-receptors binding FGF (fibroblast growth factor) and HGF (hepatocyte growth factor). FGF and HGF signaling is required for muscle tissue development [9]. We noticed that there was a constant expression of syndecan-4 in activated and differentiating myoblasts. Syndecan-4 expression was also observed in

myotubes. We concluded that syndecan-4 was important in the activation of satellite cells, and furthermore that it played a role in myoblast differentiation. We also observed several changes in the location of syndecan-4 during differentiation. In proliferating myoblasts and myotubes, it was dispersed near the cell membrane and present in FC, but in fusing myoblasts, it existed in the cell-cell contact area as well.

Because the participation of syndecan-4 in the differentiation of myoblasts is significant, we decided to study the role of syndecan-4 interactions with PKC isoforms in its translocation during this process. We treated cells with TPA (PKC activator) and Cal C (PKC inhibitor).

Syndecan-4 binds  $\text{PIP}_2$  and activated  $\text{PKC}\alpha$ . In this form, the proteins generate a stable complex. Only in association with  $\text{PIP}_2$  and  $\text{PKC}\alpha$  can syndecan-4 form dimers and oligomers and translocate close to integrins and FCs [11, 12, 25, 26]. In the short-term TPA-treated (24, 72h) cells, we noticed translocation of both syndecan-4 and  $\text{PKC}\alpha$  to FCs (Fig. 2D-G). We also observed the formation of long focuses of syndecan-4 and  $\text{PKC}\alpha$ , probably with integrins along actin stress fibers. Syndecan-4 did not form any focuses (except FCs) under the membrane in myoblasts in the control culture (Fig. 2A). In the short term TPA-treated myoblasts, we noticed the presence of the  $\text{PKC}\alpha$  form with constitutive activity (45kD, PKM) (Fig. 3C). It was recently shown that only active  $\text{PKC}\alpha$  and its PKM form could bind with syndecan-4;  $\text{PKC}\delta$  and  $\text{PKC}\epsilon$  did not interact with syndecan-4 [27]. The syndecan-4/active  $\text{PKC}\alpha$  complex was able to translocate to FC and integrins [27]. TPA treatment induced an increase of  $\text{PKC}\alpha$  activity and its translocation to the plasma membrane. Probably, in short term treated myoblasts, the increase of  $\text{PKC}\alpha$  activity stimulated the translocation of syndecan-4 and  $\text{PKC}\alpha$  to FCs and to integrins, which anchor actin fibers to the cell membrane. In this case, syndecan-4 formed long focuses. Chronic stimulation of PKC with activators (TPA) leads to its down-regulation [28, 29]. Activation of PKC induces some conformational changes that provides an increase in PKC sensitivity to proteolysis. As a result of chronic stimulation with TPA, the activity of PKC decreases. For that reason, we did not observe any changes in the long term treated cultures.

We also noticed translocation of syndecan-4 to FCs and long sizeable focuses after short-term Cal C treatment (Fig. 2). This was probably caused by  $\text{PKC}\delta$  inhibition, because Cal C is a more efficient inhibitor of  $\text{PKC}\delta$  than  $\text{PKC}\alpha$ , and TPA is a more effective activator of  $\text{PKC}\alpha$  [30]. Only  $\text{PKC}\delta$  (not  $\text{PKC}\alpha$ ) is responsible for syndecan-4 phosphorylation. The cytoplasmic domain of syndecan-4 phosphorylated by  $\text{PKC}\delta$  at  $\text{Ser}^{183}$  showed a reduced ability to bind  $\text{PIP}_2$  and  $\text{PKC}\alpha$  [31]. We speculate that inhibition of  $\text{PKC}\delta$  activity could induce  $\text{PKC}\alpha$  association to syndecan-4 and syndecan-4 translocation to integrins. In spite of the significant influence of TPA and Cal C on syndecan-4 location during the differentiation of myoblasts, we did not observe an effect of TPA or Cal C on the proliferation or differentiation of satellite cells.

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