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Phorbol ester-induced differentiation of L6 myogenic cells involves phospholipase D activation

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Abstract TPA, a potent PKC activator, inhibits myogenic differentiation and activates phospholipase D (PLD). We evaluated the involvement of PLD in the TPA effects on L6 myoblasts differentiation. TPA, at concentrations inhibiting differentiation of L6 cells, induced a strong, though transient, PLD activation. Surprisingly, at nanomolar concentration, TPA induced both myogenic differentiation and sustained activation of PLD. Differential effect of TPA can be ascribed to PKC downregulation induced by highest TPA concentrations. TPA-induced differentiation was inhibited by 1-butanol, confirming the involvement of PLD in this effect. These data suggest that prolonged elevation of PLD activity is required for myogenic differentiation.

Keywords: Phospholipase D; Phosphatidic acid; Muscle; Myogenic differentiation; Protein kinase C; Phorbol esters

1. Introduction

Myogenic differentiation is a complex process which consists in the conversion of proliferating skeletal myoblasts into polynucleated myotubes expressing the contractile apparatus [1]. Different signaling pathways are involved, including the activation of PKC [2], RhoA [3], PI3K/Akt [4], and p38 MAPkinase [5]. The studies aiming at delineating the role of PKCs in myogenesis have led to conflicting conclusions, due to the multiplicity of the isoenzymes involved, and to the bimodal effects of phorbol ester treatments, which both activate and downregulate the responsive PKCs [6–12]. In view of reported data showing that phospholipase D (PLD) is activated by phorbol esters in L6 cells [13], we asked if PKC-dependent PLD activation was correlated with an induction of myogenesis. A possible involvement of the PLD pathway of signaling in myogenesis had been suggested by our previous observation that in vitro differentiation of L6 myoblasts can be induced by the hormone arginin8-vasopressin (AVP) in the absence of serum or other factors, when present at concentrations which trigger a rapid activation of PLD, without a concomitant activation of phospholipase C [14]. PLD and its product phosphatidic acid have been shown to be involved in the regulation of essential cellular functions, such as vesicular traffic and cytoskeleton rearrangements [15], but the role of this pathway in myogenesis has not yet been thoroughly investigated. PLD is known to be activated through different mechanisms, the most prominent involving PKCs and interaction with small G proteins of the ARF and Rho families [16]. Our observation that phorbol esters are able to induce both PLD activation and myogenic differentiation in the absence of physiological effectors suggests that PKC is positively involved in myogenesis through PLD regulation, and supports the hypothesis that PLD activity is required for the myogenic process.

2. Materials and methods

Cell culture. L6 cells of the C5 subclone [17] were seeded at the density of 10 000/cm^2 in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS. 24 h after plating, cultures were shifted to 5% FBS-containing medium, supplemented with various concentrations of TPA. The cells were cultured for 5 d, the medium was changed every day. Percentage of cell fusion was measured as detailed in [17]. Myosin expression was visualized by immunocytochemistry, using the MF20 antibody directed against sarcomeric myosin (kindly given by Dr. D. Fischman, Cornell University Medical College, New York, NY), and HRP-conjugated secondary antibody, as described in [17]. Creatine kinase activity of the cells was assayed as previously described [17]. Measurement of acetylcholine receptor expression was performed by [^{125}I]-α-bungarotoxin binding, as described in [18]. Alternatively, the cells could be differentiated by culture in serum-free medium supplemented with 1% BSA (Roche Applied Science), and 10^{-7} M AVP (Sigma) or TPA, and myogenin was assessed as described below.

Myogenin immunoblotting and immunofluorescence. After 48 h of culture in BSA medium in the presence of appropriate agents, the cells were homogenized in ice-cold 20 mM Tris/HCl, pH 7.6, containing a protease inhibitor cocktail (Sigma P2714) diluted 1:4, in a Dounce homogenizer (30 strokes). The homogenates were analyzed on a 15% polyacrylamide gel and immunoblotted with F5D anti-myogenin monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) diluted 1:50. After stripping, the membranes were reprobed for normalization, with an anti-tubulin monoclonal antibody (Sigma). Immunoblots were revealed with the ECL detection system (Amersham Biosciences). Videodensitometric

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Abbreviations: TPA, 12-*O*-Tetradecanoylphorbol 13-acetate; AVP, Arg8-vasopressin; PLD, phospholipase D; PKC, protein kinase C

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quantification of the bands was performed by using a CCD camera system (ImageMaster VSD-CL, Amersham Biosciences) and ImageQuant software. Proteins were assayed by the Bradford method. Alternatively, myogenin nuclear accumulation was detected by immunofluorescence, using F5D monoclonal antibody as described in [17]. The cells were examined by fluorescence microscopy, with a Zeiss Axiovert 200 microscope, an objective LD A-plan, 20×/0.30 PHI ∞/40, a Zeiss Axiocam MRm camera, and Axiovision 4.1 image acquisition software. The total number of nuclei in the considered fields was assessed on phase contrast images.

Actin labeling. The cells were kept in 1% BSA-containing medium for 3 h before being challenged by 1 nM TPA for 15 min. When required, 0.5% 1-butanol or 2-butanol was added to the culture medium 10 min before TPA. The cells were fixed by 3.7% formaldehyde for 10 min at 4 °C, permeabilized with 0.1% Triton for 15 min at 4 °C, and aspecific labeling was blocked in 1% BSA for 20 min. The cells were then stained for 5 min by rhodamine-conjugated phalloidin (Molecular Probes) diluted 1:400, before fluorescence microscopy examination.

Phospholipase D activity assay. PLD was evaluated on the basis of its transphosphatidylation activity, by quantitating phosphatidylbutanol accumulated in intact cells. The cells cultured in 10% FBS-containing medium were shifted to serum-free medium and incubated for 2 h in the presence of 2 μCi/ml of [^{3}H]-palmitic acid (Perkin Elmer Life Sciences). After 2 washes, the labeled cells were shifted to 1% BSA-containing medium, and treated or not for 30 min by 10^{-7} M AVP, or various concentrations of TPA, in the presence of 1% 1-butanol. When required, TPA was added 24 h before labeling. PLD assay incubations were terminated by washing with ice-cold PBS and adding 0.5 ml of 0.1 N HCl in PBS. The cells were scraped and lipids were extracted according to [19], in the presence of 50 μM butylhydroxytoluene.

Phosphatidylbutanol was separated by bidimensional TLC on silica gel G60 plates (Merck) using chloroform/methanol/28% ammonia (65:35:5.5 by volume) as a solvent for the first migration, and ethyl acetate/isooctane/acetic acid (90:50:20 by volume) for migration in the second dimension. Spots stained by Coomassie Brilliant Blue R were scraped off and radioactivity was measured by liquid scintillation counting. The radioactivity associated with phosphatidylbutanol was expressed in percent of total phospholipid radioactivity.

Protein kinase C immunoblotting. Cells were cultured as described, harvested, collected by centrifugation, resuspended in ice-cold homogenization buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 5 mM DTT, 100 mg/ml leupeptin, 100 mg/ml aprotinin and 3.5 mg/ml PMSF), and sonicated. Cell homogenates were centrifuged at $100\,000 \times g$ for 30 min. The supernatant constituted the soluble fraction. The pellet was resuspended in the homogenization buffer containing 0.1% Triton X-100, incubated on ice with repeated vortexing, and centrifuged at $15\,000 \times g$ for 15 min. The final supernatant is referred to as particulate fraction. The fractions were submitted to SDS–PAGE on 10% polyacrylamide gels. The proteins transferred on nitrocellulose sheets were probed with the PKC-$\alpha, \beta 1, \gamma, \iota$ monoclonal antibodies purchased from Transduction Laboratories. Detection was performed using the ECL technique (Amersham). The bands were quantified as stated above. Equal protein content of the samples was assessed by running in parallel a SDS–polyacrylamide gel stained with Coomassie blue.

3. Results

TPA is a potent activator of PLD in a number of cell systems, including L6 myoblasts [13]. To test if triggering this pathway is accompanied by myogenic differentiation, proliferating L6 myoblasts were treated by varying concentrations of TPA, in culture conditions (5% FBS-containing medium) which, due to intermediate serum concentration, are expected to allow the detection of either a positive, or a negative effect of TPA on differentiation [18], and several parameters of differentiation were evaluated. Addition of low TPA doses (1–10 nM) efficiently promoted differentiation of L6 cells after 5 days

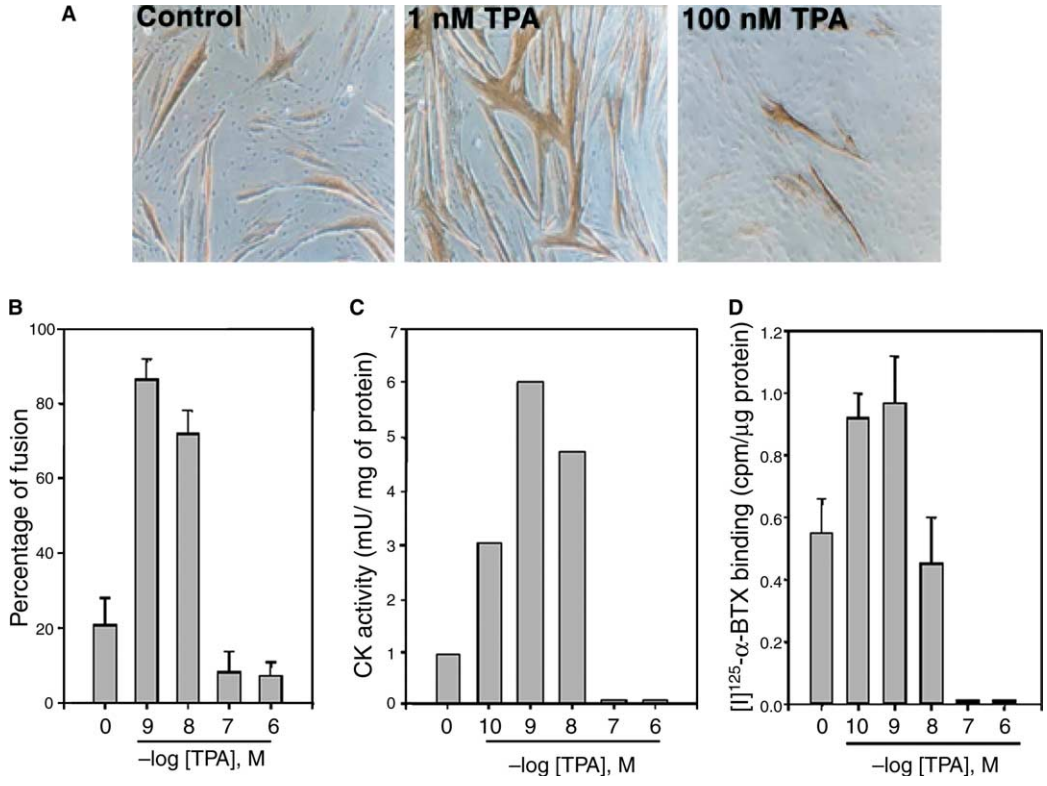

Fig. 1. L6 myoblasts cultured in 5% FBS-containing medium fully differentiate in the presence of nanomolar TPA concentrations. (A) The morphology of cells cultured in the presence of the indicated agent was examined after immunocytochemical visualization of the late differentiation marker myosin heavy chain. (B) Cell fusion was quantitated after culture with various TPA concentrations. (C) The activity of the late differentiation marker creatine kinase was assayed. (D) The late differentiation marker AchR was quantified by [^{125}I]-α-bungarotoxin binding.

of treatment, as evaluated by morphological observation and myosin expression (Fig. 1A), and by quantification of cell fusion (Fig. 1B). By contrast, higher TPA doses (100–1000 nM) did not promote differentiation and even inhibited the low spontaneous rate of differentiation observed in these culture conditions. The differential effect of TPA according to the used dose was further illustrated by quantifying the late differentiation markers creatine kinase activity and α-bungarotoxin binding (which reflects the number of nicotinic acetylcholine receptors in presence) (Fig. 1C and D). In both cases, TPA at a nanomolar range increased the expression of the marker, whereas 100 nM or more concentrated TPA repressed it down to below control values.

Since the above experiments showed that TPA was able to induce differentiation, we asked whether it could act by itself, in the absence of serum components. We thus investigated the effects of TPA on differentiation of L6 cells cultured in serum-free, 1% BSA-containing medium. An early step in myogenic process is the expression and nuclear accumulation of the muscle-specific transcription factor myogenin, that governs the expression of an array of proteins typical of differentiated myocytes [1]. Cells treated with 1 nM TPA expressed myogenin, in a manner similar to cells treated by the differentiating agent AVP taken as a positive control, which shows that they were undergoing differentiation. By contrast, 100 nM TPA did not induce myogenin expression (Fig. 2A). Differentiation was further evaluated by assessment of nuclear accumulation of myogenin by immunofluorescence (Fig. 2B). These experiments showed the ability of 1 nM TPA, but not of 100 nM TPA to induce myogenin nuclear accumulation in BSA-cultured cells. It can thus be concluded that TPA, at a low concentration, was sufficient by itself to trigger differentiation, in the absence of other stimuli.

To further characterize its effects on myogenesis, we investigated the consequences of TPA addition on myogenin expression and nuclear accumulation induced by the differentiating agent AVP. Whereas 1 nM TPA did not significantly modify either myogenin expression or nuclear accumulation

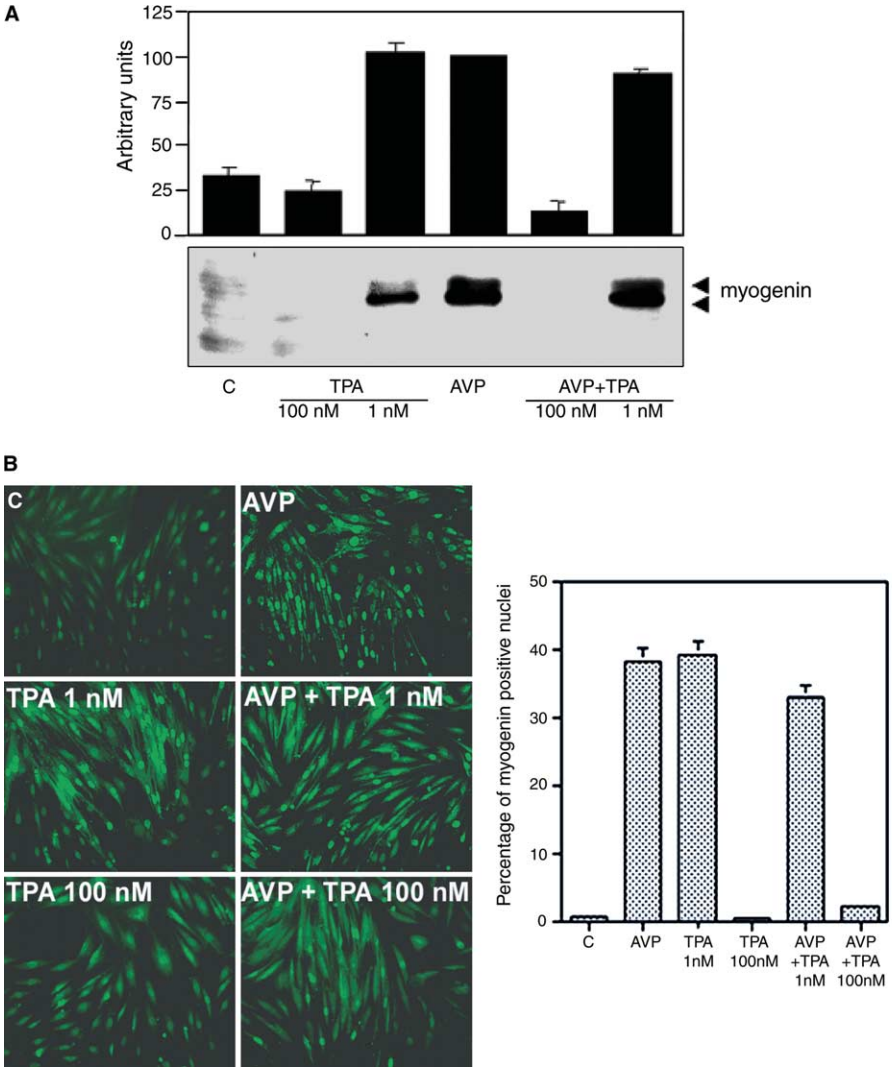

Fig. 2. L6 myoblasts cultured in the absence of serum differentiate in the presence of 1 nM TPA, but not 100 nM TPA. (A) Immunoblotting of myogenin expressed in cells cultured for 48 h in the presence of the indicated agent. The diagram shows the videodensitometric quantification of myogenin bands, as normalized by the amount of tubulin. The mean ± S.E. of 3 independent experiments is shown. (B) Immunofluorescence of myogenin in cells cultured as above. The diagram shows the mean ± S.E. of the percentages of myogenin positive nuclei as determined in 5 fields.

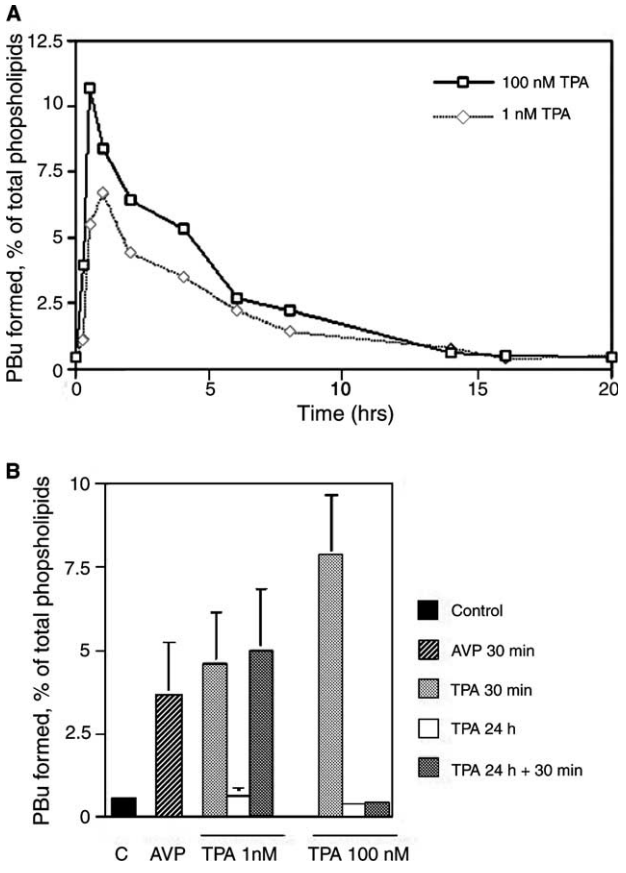

Fig. 3. PLD activity in TPA-treated cells. (A) Time-course of PLD activation by 1 and 100 nM TPA. (B) PLD activity in cells activated for 30 min by TPA and previously treated, or untreated for 24 h by TPA. The mean ± S.E. of 3 or 4 independent assays is shown.

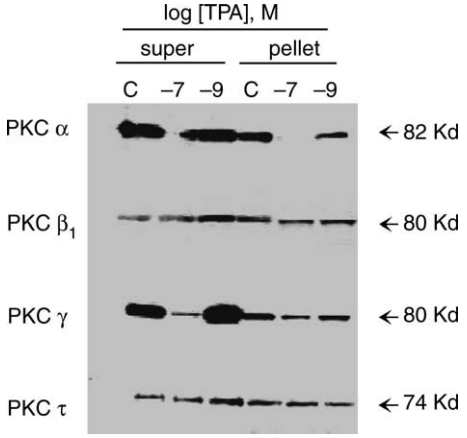

Fig. 4. Prolonged treatments by 1 and 100 nM TPA have different effects on PKC isoform downregulation. The supernatant and membrane fractions of cells treated or not (C) for 24 h by TPA at the indicated concentrations were immunoblotted for different PKC isoforms. The observed bands were quantified by videodensitometry. The intensity of the bands, normalized by the amount of PKCι in the considered sample, showed significant decreases only for the PKCα and γ isoforms, in fractions from 100 nM TPA-treated cells (−72 ± 3% and −91 ± 6% for PKCα, respectively, in supernatant and pellet; −81 ± 4% and −55 ± 9% for PKCγ, respectively, in supernatant and pellet, as compared to the control in the same fraction, $n = 3$).

induced by AVP, 100 nM TPA treatment was able to totally suppress both responses (Fig. 2A and B), showing that at a high concentration the phorbol ester was a differentiation inhibitor.

In view of the well-described PLD-stimulating effect of TPA, PLD activity was evaluated in intact L6 cells treated by 1 and 100 nM TPA (Fig. 3). At the highest concentration, TPA strongly stimulated PLD (by 14.7-fold). At 1 nM, the stimulation was lower, but still marked (8.6-fold), and similar to

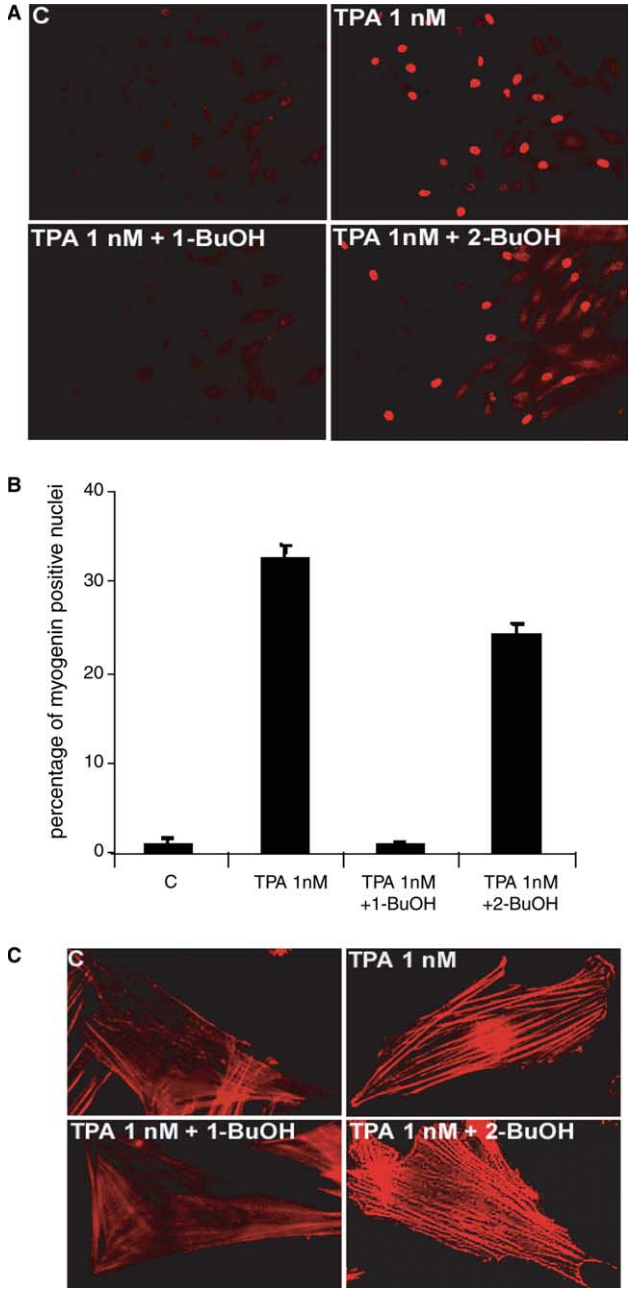

Fig. 5. 1-Butanol selectively inhibits the myogenic response to 1 nM TPA. (A) Cells were cultured in 1% BSA medium, without addition (control), or in the presence of 1 nM TPA, with or without 0.5% 1-butanol or 2-butanol. After 48 h, myogenin immunofluorescence was assessed. (B) The means ± S.E. of the percentages of myogenin positive nuclei counted in 10 fields were calculated. (C) Actin fibers were stained with rhodamine-phalloidin in cells kept for 15 min without addition (control), or in the presence of 1 nM TPA, with or without 0.5% 1-butanol or 2-butanol.

what was observed after AVP stimulation. The time-courses of PLD activation were determined: at both TPA concentrations, the activity reached a maximum at 30–60 min, and then progressively declined (Fig. 3A). After 24 h of treatment, PLD activity was near starting values. Re-stimulation of the cells by the same TPA concentration had a very different effect according to the concentration used: after a first treatment by 1 nM TPA, this agonist induced again a similar PLD response, whereas stimulation of PLD was no longer observed after a first 100 nM TPA treatment (Fig. 3B). This shows that in the latter conditions PLD effectors were downregulated, but not after a 24 h 1 nM TPA treatment.

The involvement of PKC in the regulation of PLD activity being well documented and the ability of PLD to be re-stimulated after a prolonged treatment with 1 nM TPA suggested that this TPA concentration did not induce the downregulation of PKC. We have thus evaluated the amounts of immunoreactive proteins corresponding to major PKC isoforms present in L6 myoblasts, in both the soluble and the membrane fractions. As shown in Fig. 4, a 24 h-treatment by 100 nM TPA strongly lowered the PKC α and γ isoform amounts in both compartments, whereas β1 and ι isoforms were not notably altered. By contrast, a prolonged treatment with 1 nM TPA did not downregulate either of the PKC isoforms.

Mammalian PLDs are characterized by their ability to preferentially catalyze a transphosphatidylation reaction in the presence of primary alcohols, thus producing phosphatidylalcohol at the expense of the normal reaction product phosphatidic acid. Addition of a primary alcohol such as 1-butanol to the culture medium thus allows to straightforwardly evaluate the role of PLD activity in a given cell function [15]. It is shown in Fig. 5A and B that L6 cells treated by 1 nM TPA in the presence of 0.5% 1-butanol totally failed to accumulate nuclear myogenin, whereas a control treatment by 0.5% 2-butanol, an isomer which is not recognized by PLDs, had only a moderate effect. This shows that the myogenic response to 1 nM TPA required PLD activity. The role of PLD in cytoskeleton rearrangements being documented in some cell types, we examined the effects of 1 nM TPA treatment of L6 myoblasts on the organization of actin filaments. A 15 min-TPA treatment induced the formation of stress fiber-like actin structures, as evidenced by labeling with phalloidin-rhodamine conjugate, and 1-butanol selectively inhibited this response, showing that it depended on PLD activity (Fig. 5C).

4. Discussion

This study shows a correlation between PLD stimulation and induction of the myogenic response in L6 myoblasts, suggesting that PLD activity is requested for the differentiation process. Firstly, PLD was similarly activated by two different inducers of differentiation, AVP [14] and TPA used at a nanomolar concentration (Fig. 3). Furthermore, the observation that the effects of AVP and 1 nM TPA upon differentiation were not additive suggests that the two agents acted through the same signaling pathway. The ability of phorbol ester to induce by itself a myogenic response at a low concentration is a new finding, since TPA is commonly considered as an inhibitor of myogenesis [6,7,10–12]. However, it is in line with data showing that chick myoblasts treated with PKC inhibitors fail to fuse [8]. By using a number of differentiation criteria (morphological examination, myogenin expression, nuclear accumulation, creatine kinase activity, and nicotinic receptor binding), we determined that 1–10 nM (but not 100–1000 nM) TPA allowed a complete myogenic differentiation of L6 cells, including cell fusion and expression of late markers. This shows that activation of PKC is able to bypass the requirement for a physiological effector to induce both PLD activation and myogenesis. It is noteworthy that since PLD can also be activated through various PKC-independent pathways [16], the involvement of other mechanisms of PLD activation during physiologically occurring myogenesis cannot be ruled out. Secondly, high concentrations of TPA that prevented PLD from further responding to stimulation after an initial transient activation were clearly inhibitory to the differentiation process. The prevention of PLD-restimulation can be attributed to the observed downregulation of PKC, and in particular PKCα, the role of which as a regulator of the PLD1 isoform is well documented [20]. Other PKC isoforms are downregulated by prolonged treatment of L6 myoblasts by high doses of phorbol ester, such as PKCγ (this article) and PKCε and δ, as reported in [21]. Interestingly, the PLD2 isoform has been shown to be activatable by PKCδ-dependent phosphorylation [22] and may thus be also affected by the elimination of TPA-sensitive PKCs. Since L6 cells express both PLD1 and PLD2 isoforms (our unpublished results), TPA regulation may concern the whole PLD set of these cells. The time-courses of PLD activation in response to the initial challenge by both high and low TPA concentrations were similar. However, in view of the fact that a new challenge by high TPA concentration could not re-activate PLD, it can be deduced that periodical renewal of the 100 nM TPA-containing culture medium during the whole culture period maintained PLD activity at a near basal level after 24 h, whereas in culture conditions involving nanomolar TPA, high levels of PLD activity could be restored. It can thus be hypothesized that maintenance of PLD under an activation-responsive state is required for the completion of myogenic differentiation. As a confirmation of PLD involvement in the myogenic response to nanomolar TPA, 1-butanol was able to totally suppress myogenin nuclear accumulation.

PLD has been suggested to play a role in the differentiation of several cell types, although the mechanisms of PLD participation into the differentiation processes remained speculative. In keratinocytes, a sustained PLD activation is associated with differentiation. It is supposed to provide a prolonged supply of diacylglycerol resulting from phosphatidic acid hydrolysis and induce the late activation of certain PKC isoforms [23]. PLD1 is upregulated during differentiation of C6 glioma cells and PC12 pheochromocytoma cells [24]. Concerning myogenesis, it is tempting to infer from the ability of PLD to affect the actin cytoskeleton, which has been established in endothelial cells [25] and in fibroblasts [26], that PLD-dependent cytoskeletal rearrangements might play a pivotal role. Indeed, it has been demonstrated in cardiomyocytes [27] and in smooth muscle cells [28] that the status of actin in the cell governs the transcription of muscle-specific genes. In agreement with this hypothesis, we observed that stimulation of L6 myoblasts by a myogenic concentration of TPA induced the formation of stress fiber-like actin structures, in a PLD-dependent way as shown by selective inhibition by 1-butanol. It thus appears that PLD may be an essential factor of myogenic differentiation through its involvement in the reorganization of cytoskeleton.

An alternative interpretation of the present data could be that a strong activation of some PKC isoforms by high TPA concentration induced the mobilization of factors exerting a blocking effect on the myogenic process, thereby preventing the triggering of differentiation insured by transient PLD activation. Thus, the TPA-responsive PKCθ has been implicated in inhibiting chick myotube formation by virtue of its ability to phosphorylate the PKC substrate MARCKS [11]. Furthermore, PKC may induce the inhibition of essential components of the myogenic machinery. When expressed in mouse C2C12 myoblasts, a constitutively active PKCα phosphorylates and inactivates myogenin, thus repressing the myogenic program [29]. In line with this observation, in chick embryo myoblasts the positive effect of dihydroxy-vitamin D3 on differentiation correlates with a decrease in PKC activity and PKCα expression [30]. Conversely, high TPA concentrations might exert negative effects by eliminating PKC and thus preventing the delayed activation of PKC targets essential to the completion of differentiation, in addition to PLD. Several PKC-dependent events exerting a positive influence on myogenesis have been reported. For example, RhoA, a well-identified positive effector of myogenic differentiation [3], undergoes PKCα-mediated activation and translocation in C2C12 myoblasts, and PKC inhibition or downregulation prevents these effects [31]. In rhabdomyosarcoma RD cells, it has been shown that the myogenic response is mediated by PKCα-induced transient activation of JNK and sustained activation of p38 kinase and ERKs, PKC inhibition preventing the accumulation of the late differentiation marker sarcomeric myosin heavy chain [2]. In addition, chronic exposure of differentiated avian skeletal muscle cells to TPA, that induces a drastic PKC downregulation, results in the loss of expression of muscle-specific contractile proteins and disassembly of sarcomeric structures, showing that sustained PKC activity is required for the maintenance of the differentiated myocyte phenotype [9].

The present study shows that low concentrations of the phorbol ester TPA, allowing a sustained increase in PLD activity, are able to bring myoblasts to complete differentiation. The involvement of PLD in the myogenic response to TPA was confirmed by the complete blockade of the response in the presence of a primary alcohol. The PLD pathway thus appears to play an important role in the myogenic process. The delineation of the mechanism by which PLD might participate in myogenesis will deserve further investigations.

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