A Bimodal Modulation of the cAMP Pathway Is Involved in the Control of Myogenic Differentiation in L6 Cells*

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We have previously shown that myogenesis induction by Arg²-vasopressin (AVP) in L6 rat myoblasts involves a sustained stimulation of type 4 cAMP-phosphodiesterase. In this model, we observed that a transient cAMP generation occurs in the minutes following AVP addition. Evidence suggests that cAMP generation is due to the prostaglandins produced in response to AVP binding to V1a receptors and subsequent activation of phospholipase A2. The early cAMP increase was effective in activating cAMP-dependent protein kinase (PKA) and increasing phosphorylation of CREB transcription factor. Inhibition of PKA by compound H89 prior to AVP addition led to a significant reduction of expression of the differentiation marker creatine kinase, whereas H89 added 1–5 h after AVP had no significant effect. Furthermore, PKA inhibition 24 h after the beginning of AVP treatment potentiated differentiation. This shows that both an early activation and a later down-regulation of the cAMP pathway are required for AVP induction of myogenesis. Because phosphodiesterase PDE4D3 over-expressed in L6 cells lost its ability to potentiate AVP-induced differentiation when mutated and rendered insensitive to PKA phosphorylation and activation, we hypothesize that the early cAMP increase is required to trigger the down-regulation of cAMP pathway through stimulation of phosphodiesterase.

Myogenic differentiation of myoblasts is a complex process resulting in the formation of multinucleated myotubes expressing an array of muscle-specific proteins. It takes place both in the course of normal embryonic development and during repair of lesions caused by muscle injuries or degeneration occurring in muscular dystrophies (1). Several hormonal factors have been shown to positively influence the myogenic process in vivo or in vitro. Among them, the neuropituitary hormone vasopressin (AVP)† appears particularly interesting, because it induces myogenic differentiation of rat myoblast lines and mouse satellite cells in the absence of other factors (2). The signaling pathways triggered by AVP in L6-C5 myogenic cells include phospholipase C and phospholipase D activation (3, 4). We also reported that AVP-induced differentiation requires a prolonged activation of type 4 phosphodiesterase (PDE4) and a reduction of cAMP levels and PKA activity occurring after several hours of AVP treatment of myogenic cells (5), in agreement with studies showing that a sustained elevation of cAMP levels exerts a potent inhibitory effect on myogenic differentiation (6, 7). Phosphodiesterases of the PDE4 family, and especially the isof orm PDE4D3, ensure a tight control of cAMP levels in L6-C5 cells and are positive effectors of the myogenic response (5, 8). However, the signaling cascades triggered by AVP in myoblasts are still largely unknown, and in particular, the delineation of the complex role played by the cAMP pathway remains incomplete. In fact, despite its recognized inhibitory effect on myogenic differentiation, it has been reported that cAMP intracellular levels and PKA activity rise at the onset of myogenesis (9–11), which suggests that the cAMP pathway may have a dual role in this process. Thus, it seems of importance to better understand the involvement of cAMP in the control of muscle formation. The recent description of alterations in the expression of genes involved in several signaling pathways, including the cAMP pathway, that have been noticed in muscular tissue of patients affected by dystrophic diseases (12) underscores the interest of this question.

In the present work, we describe early AVP effects on cAMP levels in L6-C5 cells and address the possible physiological meanings of the observed changes. Contrary to longer term observations, in the first few minutes of stimulation, AVP induced a marked and transient cAMP accumulation. We identified an indirect mechanism of cyclase stimulation by AVP in these cells involving prostaglandin synthesis, and we made observations indicating that although short-lived, the early cAMP surge has a physiological relevance and exerts a positive influence on the myogenic process. We hypothesize that an important effect of the early cAMP production is to trigger the activation of phosphodiesterase PDE4D3 resulting in the later down-regulation of the cAMP pathway.

† The abbreviations used are: AVP, Arg²-vasopressin; DMEM, Dulbecco’s modified Eagle’s medium; PDE, cAMP-phosphodiesterase; PKA, cAMP-dependent protein kinase; rolipram, 4-(3-[cyclopentyloxy]4-methoxyphenyl)-2-pyrrolidinone; BSA, bovine serum albumin; FBS, fetal bovine serum; IBMX, isobutylmethylxanthine; PBS, phosphate-buffered saline; CREB, cAMP-responsive element-binding protein.

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EXPERIMENTAL PROCEDURES

Materials—Synthetic AVP, [deamino-Pen-O-Me-Tyr-*Arg*]-vasopressin 1α antagonist, Kemptide, PKA peptide inhibitor, a creatine kinase immunoblot kit, and lipid standards were purchased from Sigma. Rolipram and H89 were obtained from Calbiochem-Merck KGaA. Fatty acid-free BSA and FuGene 6 were from Roche Applied Science. Anti-Phospho-CREB was from New England Biolabs (Beverly, MA). [γ-32P]ATP, [3H]cAMP, [14C]arachidonic acid, and CAMP-[125I] radioimmunoassay kit were from PerkinElmer Life Sciences. The ECL Western blot detection kit was from Amersham Biosciences.

Cell Culture and Differentiation—Subcloning and characterization of L6 (13) rat myogenic cell clones was previously reported (3). The cells of the subclone C5 (L6-C5), a clone that had shown significant differentiation ability (2, 14), were used throughout this study. The cells were routinely seeded at the density of 10,000/cm² in Dulbecco’s modified Eagle’s medium containing 4500 mg/ml glucose, 10% heat-inactivated FBS, 1% fatty acid-free BSA, 1 mM sodium pyruvate, and 10 mM HEPES. The cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

Preparation of myoblasts—myoblasts were obtained from the subclone C5 (L6-C5), a clone that had shown significant differentiation ability (2, 14), by switching to BSA medium and receiving 0.5 mM IBMX and, after 10 min, 6M AVP. After 15 min of incubation at 37°C, the cell extracts were treated with AVP for different times, washed, and directly extracted in Laemmli buffer. The extracts were probe-sonicated for 2 s. 20 μl of the extract was loaded on a 10% SDS-PAGE gel, and the gel was stained with Coomassie Blue. The bands were scanned and quantified by using NIH Image software. The data are reported as mean ± S.E. Statistical differences were determined by one-way analysis of variance, followed by Tukey’s test for multiple comparisons.

Results—AVP-induced differentiation of L6-C5 cells was monitored by measuring the levels of cAMP and CREB phosphorylation. The results showed that AVP induced a significant increase in cAMP levels within the first 5 min of incubation, followed by a decrease after 10 min. CREB phosphorylation was also induced by AVP, and the maximum phosphorylation was observed after 15 min of incubation.

Vasopressin and cAMP in Myogenesis—Vasopressin and cAMP act synergistically to induce myogenic differentiation. Vasopressin activates PKA, which in turn stimulates the transcription of myogenic genes. cAMP is also essential for the transcription of myogenic genes and is produced by the activation of adenyl cyclase. The results showed that AVP induced a significant increase in cAMP levels within the first 5 min of incubation, followed by a decrease after 10 min. CREB phosphorylation was also induced by AVP, and the maximum phosphorylation was observed after 15 min of incubation.

Type 4 Phosphodiesterase Activity Is Involved in the Limitation of Amplitude and Duration of the Early cAMP Response to AVP—We have previously shown that AVP triggers a biphasic activation of type 4 cAMP-specific phosphodiesterase, with an initial peak after 2 min and a later one after 15 min of hormonal treatment (5). To determine whether the transient nature of the cAMP response is modulated by the type 4 phosphodiesterase, we measured the modulation of cAMP levels by AVP in the presence or absence of a type 4 phosphodiesterase inhibitor. The results showed that the type 4 phosphodiesterase inhibitor significantly reduced the amplitude and duration of the cAMP response to AVP, indicating that the type 4 phosphodiesterase is involved in the limitation of amplitude and duration of the early cAMP response to AVP.
of the cAMP rise triggered by AVP was due to the activity of this phosphodiesterase family. cAMP levels were measured in L6-C5 cells stimulated by AVP in the presence or absence of rolipram, a selective PDE4 inhibitor. Interestingly, we observed that rolipram strongly potentiated the increase in cAMP concentration caused by AVP treatment and allowed the maintenance of elevated cAMP levels for at least 30 min (Fig. 1D). This result indicates that PDE4 activity is required for the rapid return of cAMP to basal levels observed in L6-C5 cells and suggests that AVP controls the level and duration of the cAMP transient by concomitantly stimulating cAMP production and degradation by PDE4 in these cells.

The AVP-induced cAMP Accumulation Is Mediated by V1a Receptor Stimulation—It has been suggested, on the basis of functional observations, that L6 myoblasts do not express the adenylyl cyclase-coupled V2 receptors for AVP. On the other hand, these cells express V1 receptors, which are connected to the phospholipase C signaling pathway (16). We set out to determine whether low levels of the V2 receptor, which could account for the cAMP response to AVP, are expressed by L6-C5 cells. We thus performed reverse transcriptase-PCR experiments using primers designed to amplify specific regions of the V1a and V2 receptor cDNAs. As shown in Fig. 2A, V1a receptor mRNA transcripts are present in both L6-C5 myoblasts and differentiated myotubes. However, we failed to detect even trace amounts of V2 receptor mRNA (Fig. 2A), indicating that AVP does not act through this adenylyl cyclase-coupled receptor in L6-C5 cells.

To test whether the V1 receptor was involved in cAMP generation triggered by AVP, L6-C5 cells were stimulated in the presence of an excess of the selective V1 receptor antagonist [deamino-Pen1,O-Me-Tyr2,Arg8]-vasopressin and cAMP accumulation was measured. We observed that the V1 receptor antagonist completely suppressed the rise in intracellular cAMP concentration induced by AVP in L6-C5 cells (Fig. 1C), indicating that the hormone action requires stimulation of V1 receptors.

To ascertain whether activation of V1a receptors is sufficient to mediate the AVP-induced cAMP rise, we transiently transfected COS-1 cells, which do not respond to AVP, with an expression plasmid carrying the V1a receptor cDNA, and we evaluated the cAMP response of transfected cells. As expected, AVP stimulation did not exert any effect on cAMP levels in mock transfected cells. By contrast, transfection of V1a receptor was sufficient to confer to COS cells the ability to respond to AVP, a 70% increase in intracellular cAMP levels being observed after hormonal stimulation (Fig. 2B).

The mechanism of cAMP Synthesis Activation Induced by AVP in L6 Cells Involves Prostaglandin Synthesis and Secretion—Because V1a receptor is not coupled to adenylyl cyclase, we hypothesized that the activation of cAMP synthesis was due to an indirect mechanism set in motion by AVP. It has been reported that AVP triggers phospholipase A2 activation and liberation of free arachidonic acid in H9c2 cardiac myoblasts (17). Considering that free arachidonate may be further metabolized into prostaglandins that are known activators of cAMP synthesis, we tested the existence of such a pathway in L6-C5 cells. We first determined whether AVP was able to trigger the synthesis of prostaglandins in these cells. L6-C5 myoblasts were labeled by preincubation with [14C]arachidonic acid before the stimulation with the hormone. We observed that a 5-min AVP treatment lead to a 2-fold increase in the total radioactivity released in the culture medium (not shown), indicative of phospholipase A2 stimulation. TLC analysis of lipid extracts of the culture medium (Fig. 3A) showed that AVP promoted the synthesis and release of prostaglandins E2 and D2 principally, and PGF2α in smaller amount, suggesting that these compounds could act as autocrine effectors to activate adenylyl cyclase. To examine this hypothesis, we measured cAMP accumulation in response to AVP in the presence of two different cyclooxygenase inhibitors able to block the conversion of endogenous arachidonate into prostaglandins. Indeed, the cAMP increase induced by AVP was completely suppressed by indomethacin and partially by aspirin (Fig. 3B), confirming that prostaglandin synthesis was required for the production of cAMP.

AVP Induces a Rapid Activation of the cAMP Signaling Pathway—To assess whether, despite its transient feature, the AVP-induced cAMP elevation has a functional significance, we investigated the consequences of AVP treatment on the downstream steps of the cAMP pathway of signaling. We found that PKA was activated 2.3-fold in L6 cells treated for 5 min with AVP and that PKA activity returned to basal levels in 30–45 min (Fig. 4A). Furthermore, as shown in Fig. 4B, AVP stimulation resulted in the phosphorylation of the CREB transcription factor, the PKA phosphorylation of which represents a
critical step in the transcriptional regulation of gene expression by the cAMP pathway (18). CREB was rapidly phosphorylated in response to AVP, with a steady-state phosphorylation reached in 15–30 min (Fig. 4B, upper panel). In the presence of the PKA inhibitor H89, AVP-induced CREB phosphorylation was totally suppressed (Fig. 4B, lower panel), suggesting that it was mediated by activated PKA.

The time course of PKA activation by AVP (Fig. 4A) indicates that its maximal activity is reached before the second phase of PDE4 activation, which peaks at 15–30 min (5). Because the major type 4 phosphodiesterase isofrom expressed in L6 cells is PDE4D3, which can be activated by PKA-mediated phosphorylation (15), it is possible that the late phase of PDE stimulation depends on PKA. Thus, we tested the effects of PKA inhibition on the biphasic activation of PDE by AVP. As shown in Fig. 5, only the late activation of PDE (15 min) was dependent on PKA activity, whereas the early activation (3 min) was not affected in the presence of the PKA inhibitor H89. This result indicates that PKA is involved in the second phase of AVP-dependent PDE stimulation.

The Transient Activation of the cAMP Pathway Induced by AVP Positively Influences Myogenic Differentiation—To evaluate whether the early activation of the cAMP pathway is required for L6-C5 cell myogenic differentiation triggered by AVP, we added H89 at different times before or after the onset of AVP treatment. When PKA activity was blocked before the AVP treatment, it resulted in a marked decrease in the number and size of myotubes formed after 6 days in response to AVP (Fig. 6A). This effect was confirmed by measuring creatine kinase activity, a marker of terminal differentiation (Fig. 6B). H89 added before the onset of AVP stimulation significantly decreased creatine kinase expression (by 65% when added 90 min before AVP), which suggests that early PKA-mediated events have a positive influence on myogenic differentiation. Interestingly, the H89 inhibition of creatine kinase expression was not significant if the inhibitor was added 1–5 h after the onset of AVP treatment (Fig. 6B), demonstrating that a short-lived activation (within 1 h) of the cyclic AMP pathway is sufficient to stimulate myogenic differentiation. Furthermore, we observed that inhibition of PKA activity by H89 24 h after the onset of AVP treatment induced a significant stimulation of terminal differentiation, as evaluated by measuring after 6 days the activity of creatine kinase (+71% with H89 treatment) and the expression of myosin heavy chain (+96% with H89 treatment) (Fig. 6C).

A Mutated Form of Phosphodiesterase PDE4D3 That Is Insensitive to PKA Phosphorylation Is Less Efficient at Enhancing AVP-induced Differentiation than Wild-type Enzyme—We have
were pretreated or not for 15 min with 20 M AVP. The cells were extracted after different times of treatment by 10−7 M AVP, and the proteins were immunoblotted with an antibody specific for phosphorylated CREB. Lower panel, PKA inhibition by H89 suppresses CREB phosphorylation induced by AVP. The cells were pretreated or not for 15 min with 20 M H89 and then treated at different times with 10−7 M AVP. All of the samples were harvested at the same time and analyzed as above. The experiment was repeated twice with similar results.

Results are the means ± S.E. of four to six samples from two experiments. *, different from control (p = 0.02); **, p = 0.009. ***, p = 0.001. #, different from AVP alone (p = 0.006).

Previously shown that overexpression of the PDE4D3 phosphodiesterase isoform is able to potentiate AVP-induced differentiation of L6-C5 cells and, in particular, to enhance the nuclear accumulation of myogenin, an early and crucial step of the myogenic process (5). Based on the results of Fig. 5 suggesting that AVP induces a PKA-mediated activation of phosphodiesterase, it is possible that PDE4D3 phosphorylation is involved in AVP differentiating effects. To test the requirement of PKA-mediated phosphorylation and activation of PDE4D3 for the onset of myogenic differentiation, we took advantage of a PDE4D3 point mutant, S54A-PDE4D3, which is insensitive to PKA activation (15). L6-C5 cells were transfected with either wild-type or S54A-PDE4D3, stimulated with AVP, and monitored for nuclear translocation of myogenin. As shown in Fig. 7, the PKA-insensitive mutant, in contrast with the wild-type form, did not activate the basal level of differentiation, nor did it enhance the effect of AVP, suggesting that PKA-dependent phosphorylation of PDE4D3 has an important role in the AVP-induced myogenic response.

**DISCUSSION**

We have previously established that the neuropituitary hormone AVP is able to strongly stimulate the in vitro myogenic differentiation of L6 myoblasts, as well as of other myogenic cell lines and primary cells. AVP myogenic effects involve the sustained activation of a type 4 phosphodiesterase and a prolonged decrease in both cAMP levels and PKA activity, occurring after a few hours of AVP treatment. In the present study, we examined the modifications of the cAMP pathway induced by AVP in L6 cells in the first minutes of the treatment. We found that, in contrast to what is observed in longer term studies, AVP produced an elevation of cAMP levels, which peaked at 1–3 min and then rapidly returned to basal levels.

Activation of the cAMP pathway by AVP classically involves the stimulation of V2 receptors linked to the activation of adenyl cyclase through interaction with the Gs protein, as observed for example in kidney collecting duct (19). However, functional observations suggest that L6 myoblasts only express the V1 receptors for AVP, which are related to phospholipase C activation. We have used the reverse transcriptase-PCR approach to clearly define the expression profile of AVP receptors in L6 cells, and we observed that only V1a receptor mRNA transcripts are detectable in L6 myoblasts, as well as in differentiated myotubes. That AVP is able to indirectly stimulate adenyl cyclase through the activation of V1 receptors was confirmed by showing that a selective V1 receptor antagonist inhibited the AVP-induced cAMP accumulation. Furthermore, we observed that overexpression of V1a receptor in COS-1 cells confers to these cells the ability to respond to AVP stimulation by an increase in cAMP, which demonstrated that a signaling pathway triggered by V1a receptor stimulation indirectly causes the activation of adenyl cyclase.

The indirect mechanism of cyclase stimulation involves prostaglandin synthesis, because it was inhibited by two prostaglandin synthesis inhibitors, indomethacin and aspirin. This is consistent with our observation of a stimulation of arachidionate release and synthesis of prostaglandins E2, D2, and F2α under AVP stimulation of L6 myoblasts. Arachidionate release has been reported to occur in AVP-treated cardiac myoblasts through the stimulation of V1 receptors (17), and AVP-induced prostaglandin synthesis in vascular smooth muscle cells has also been reported (20). As determined in the H9c2 cardiac myoblasts, the cascade leading to AVP-induced arachidionate release includes the following steps: AVP binding to V1 receptors triggers the activation of phospholipase C and leads to increased intracellular Ca2+, mitogen-activated protein kinase activation, and cPLA2 phosphorylation. In L6 skeletal myoblasts, a similar activation of mitogen-activated protein kinases by AVP has been observed.2 In these cells, free arachidonate could then ultimately be metabolized into prostaglandins E2, D2, and secondarily F2α, which, after release into the extracellular medium, could activate adenyl cyclase through binding to their receptors. Despite the multiplicity of the steps involved in

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this process, it can likely take place in a short time span, as shown in other systems. It has been reported that in tracheal smooth muscle cells bradykinin induces cAMP synthesis through a similar pathway, the maximal cAMP concentration being reached in 2 min (21, 22).

Although transient and of modest amplitude compared with the one elicited by epinephrine, the AVP-induced cAMP increase was able to trigger the activation of downstream effectors, as evidenced by PKA activation and H89-sensitive CREB transcription factor phosphorylation. Because H89 is known not to be a fully specific PKA inhibitor (23), we cannot exclude that other kinases, together with PKA, are involved in AVP-induced CREB phosphorylation. Nevertheless, this last observation suggests that the early steps of the myogenic process induced by AVP could include the expression of cAMP-regulated genes. In addition, activation of PKA seems to be involved in the delayed phase of PDE4 stimulation by AVP, as shown by its marked reduction under PKA inhibition.

**Fig. 6.** Effect of PKA inhibition on the AVP-induced myogenic differentiation of L6-C5 cells. A, morphological examination of the effects of H89 pretreatment on myotube formation induced by AVP. The cells cultured in 1% BSA medium were pretreated (panels c and d) or not (panels a and b) with 10 μM H89 for 20 min and then stimulated (panels b and d) or not (panels a and c) with 10^{-7} M AVP. After 6 days of culture, the cells were stained, and the formation of myotubes was assessed by microscopy. B, the effect of H89 on myogenic differentiation depends on the time of inhibitor addition. The cells cultured in 1% BSA medium were treated by 5 μM H89 at different times before or after the addition of 10^{-7} M AVP. After 6 days of culture, the cells were harvested, and creatine kinase activity was assayed. The results are the means ± S.E. of five to eleven independent measurements performed in triplicate. *, significantly different from control with AVP (p < 0.01). †, significantly different from H89 added 90 min before AVP (p < 0.01). C, H89 added 24 h after AVP potentiates AVP differentiating effect. The cells, treated or not by AVP as above, received 5 μM H89 24 h later. After 6 days of culture, the cells were harvested, and creatine kinase activity was assayed, or myosin content was evaluated by enzyme-linked immunosorbent assay. The results are expressed as percentages of the value of control with AVP and are the means ± S.E. of nine samples, each assayed in triplicate. *, significantly different from the control with AVP (p < 0.01).

**Fig. 7.** A PKA-insensitive mutant of the PDE4D3 phosphodiesterase isoform is less efficient than the wild-type counterpart at enhancing the AVP-induced nuclear accumulation of myogenin. A, analysis of myogenin nuclear accumulation by immunofluorescence in PDE-transfected L6-C5 cells. L6-C5 myoblasts were transiently transfected either with pCMV5 containing wild-type PDE4D3 cDNA, pCMV5 containing mutant S54A-PDE4D3 cDNA (15), or the empty vector (mock). After 16 h, the cells were shifted to 1% BSA medium with or without the addition of 10^{-7} M AVP. After 6 h, myogenin was detected by immunofluorescence, by using the anti-myogenin antibody F5D and a secondary antibody coupled to rhodamin. All the nuclei in presence were visualized by UV microscopy using Hoechst 33342 nuclear dye. ser54ala mut., S54A-PDE4D3. B, quantification of myogenin positive nuclei in the above experiment. The results are expressed as percentages of myogenin-positive nuclei. Normalization of the efficiency of transfection was obtained by cotransflecting a GFP-carrying plasmid. The results are the means ± S.E. of 10 fields counted in a typical experiment, representative of four performed. The basal specific activities of PDE in the homogenates of cells transfected with wild-type PDE4D3 and S54A mutant were very similar (5–6-fold the activity of mock transfected cell homogenates). *, different from mock without AVP (p < 0.05). ***, different from mock with AVP (p < 0.001). ###, different from wt-PDE4D3 transfected with AVP (p < 0.001).
The essential role of early PKA activation in the AVP-induced differentiation process is supported by experiments showing that differentiation is impaired if PKA activity is inhibited by H89 added before the differentiating agent AVP. Because we have shown in a previous study that the AVP-induced differentiation process requires an elevation of cAMP-phosphodiesterase activity resulting in a prolonged lowering of cAMP levels and PKA activity (from 3 to 48 h after AVP addition), the above observations might appear contradictory. However, in view of the transitory feature of the cAMP pathway activation triggered by AVP, a model reconciling our different observations can be proposed. In the first period of L6 response, an elevated PKA activity would have a positive influence on initiation of the myogenic process. Then, the progress of differentiation would require a rapid deactivation of the cAMP pathway insured by cAMP-PDE stimulation, a sustained high level of cAMP being strongly inhibitory to further differentiation steps. This notion is in agreement with the present observation that the PKA inhibitor H89 added 24 h after the onset of AVP treatment stimulated myogenesis. It can be noticed that opposite effects were obtained under treatment by a cAMP-elevating agent. The addition of rolipram to the culture medium up to 24 h after AVP induced a complete inhibition of differentiation (5), later treatments being ineffective, which supports the conclusion that H89 effects at 24 h were actually due to PKA inhibition. It is thus likely that molecular event(s) negatively regulated by cAMP and taking place at 24 h is (are) responsible for the cAMP-induced blockade of myogenesis. We can hypothesize that one such event is the nuclear accumulation of the muscle-specific transcription factor myogenin, because we have previously shown that it is totally inhibited if cAMP levels are kept elevated by the presence of the phosphodiesterase inhibitor rolipram (5, 8).

Such a model implying a bimodal modulation of the cAMP pathway allows integrating apparently conflicting results obtained with different myogenic cell systems. First, it is consistent with data demonstrating that maintaining high cAMP levels throughout the experiment or overexpressing PKA catalytic subunit inhibits the differentiation of mouse and rat myoblasts (7, 24). This model also takes into account the data showing that increasing cAMP before the onset of differentiation (i.e., before switching cells to differentiating conditions) induces a potent stimulation of avian myoblast terminal differentiation (10). In this last study, the authors observed an amplifying effect of triiodothyronin on a transient elevation of cAMP levels preceding the onset of myoblast fusion. They propose that at least part of the positive effect of triiodothyronin on quail myoblasts terminal differentiation is mediated by cAMP, through enhanced withdrawal of the cells from the replicative state, because both triiodothyronin and cAMP inhibit avian myoblast proliferation. However, this hormone does not influence proliferation in a murine myoblast model (25), and cAMP has been shown to have no influence on the proliferation of a variety of myogenic cells of mammalian origin (24, 26–28). In our hands, the cAMP-elevating agents rolipram and forskolin had very little effect on L6-C5 cell proliferation. It is thus likely that in the L6-C5 rat myoblasts the positive effect of cAMP on the myogenic response does not involve an accelerated withdrawal from the cell cycle. cAMP effectors such as PKA and CREB might target a still undefined element of the complex machinery, which insures the control of skeletal muscle-specific gene expression by the Myo D family of transcription factors. In C2 mouse myoblasts, phosphorylated CREB is increased at the onset of differentiation and associates with MyoD and other protein partners in a complex targeting a cyclic AMP-responsive element in the promoter of RB gene, which results in an enhancement of RB expression, RB protein being involved not only in cell cycle arrest but also in the expression of late stage muscle-specific genes and in prevention of apoptotic cell death during differentiation (29).

Additionally, it is possible that moderate and transient early activation of the cAMP pathway is required to induce a later inactivation of this pathway, through PKA phosphorylation and activation of the type 4 phosphodiesterase PDE4D3, which is in agreement with our observation that PKA inhibition reduces the increase in phosphodiesterase activity induced by a 15-min AVP stimulation. This would ensure the prolonged lowering of cAMP levels that is essential to the progress of myogenesis. In strong support to this hypothesis, we observed (Fig. 7) that overexpression in L6 cells of a mutated PDE4D3 phosphodiesterase isoform lacking the serine 54 target of PKA phosphorylation was much less effective than overexpression of the wild-type enzyme at enhancing the AVP-induced nuclear import of myogenin, a crucial step of the myogenic process. Thus, PKA-dependent phosphorylation and activation of PDE4D3 seem to play important roles in the progress of AVP-induced myogenesis. In this regard, the recently reported dramatic underexpression of the PDE4D gene, that encodes phosphodiesterase PDE4D3, in the muscle of patients affected by Duchenne muscular dystrophy (12), illustrates the involvement of this enzyme in the preservation of muscle function and stresses the importance of the control of cAMP signaling by phosphodiesterase in muscle pathophysiology.

The present study supports the existence of an autocrine loop responsible for a bimodal modulation of the cAMP pathway and its physiological involvement in myogenesis. This autocrine loop involves prostaglandin formation, which underlines the role of arachidonic acid metabolism in muscle differentiation. Such a role of prostanoids had been proposed in early pioneer work (30). More recently, the importance of the inflammatory component in muscular dystrophy pathogenesis has been evidenced by gene expression studies that pointed out, in particular, an overexpression of phospholipase A2 likely to increase prostanoid content in human dystrophic muscle (12, 31). In addition, it has been recently reported that PGE2α stimulates muscle cell growth and nuclear accretion by increasing intracellular calcium concentration (32). The delineation of the role of prostaglandins in the formation and regeneration of muscle thus appears to deserve further investigation.

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