V1a AVP receptor is required for neurohypophyseal hormone-dependent differentiation in C2C12 cells

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Abstract

Vasopressin (AVP), oxytocin (OT) and related peptides induce differentiation and hypertrophy in myogenic cells expressing the V1a-vasopressin receptor (V1aR) or the oxytocin receptor (OTR). Either receptor can transduce both ligand signals. Binding of AVP and OT to the V1aR target cells activates phosphatidylinositol hydrolysis, which in turn releases Ca^{2+} from internal stores. The AVP-dependent increase in cytosolic Ca^{2+} induces the activation of calcium/calmodulin-dependent kinase (CaMK) and calcineurin signaling, two pathways required for the full expression of the differentiated phenotype. Here we investigate the role of V1aR in myogenesis and hypertrophy by ectopically restoring V1aR expression and function using the C2C12 cell line, which is an experimental model of satellite cells that do not respond to AVP treatment. Our results show that AVP treatment enhances myogenic differentiation in V1aR-transfected C2C12 cultures alone. Moreover, calcium imaging analyses performed in individual control and V1aR-transfected C2C12 cells demonstrated that the presence of V1aR is sufficient to make C2C12 cells responsive to neurohypophyseal hormones stimulation, as demonstrated by the rapid and sustained release of calcium from internal stores observed in V1aR-transfected cells. These data demonstrate that, despite the high levels of OTR expressed by C2C12 cells, both AVP and OT failed to stimulate the differentiation program, thereby indicating that the presence of V1aR is essential to mediate the effects of neurohypophyseal hormones on myogenic differentiation in C2C12 cells.

Key Words: myogenesis, vasopressin; calcium; V1a receptor

Myogenic differentiation is characterized by the highly regulated transcriptional activation of muscle-specific genes encoding contractile proteins, metabolic enzymes, ion channels and neurotransmitter receptors [2,6,40]. Transcription of muscle-specific genes is regulated by a set of basic helix-loop-helix muscle regulatory factors (bHLH-MRFs) that include Myf-5, MyoD, myogenin and MRF4 [14,15]: Myf-5 and MyoD are responsible for the specification and maintenance of myoblast identity, whereas myogenin regulates the transcriptional activation of skeletal muscle structural genes [54]. The bHLH proteins E12 and E47 and the myocyte enhancer factor 2 (MEF2) are also essential for muscle differentiation [20,27,38]. Calcium acts as an intracellular second messenger and efficiently regulates the activity of many enzymes [4]. Ca^{2+} signaling crosstalks with other signal molecules, e.g. cAMP, in response to a variety of stimuli, such as growth and differentiation factors, hormones, nerve signal and exercise [4]. Calcium signaling plays an important role in skeletal muscle differentiation: calcium/calmodulin-dependent protein kinase (CaMK) is preferentially activated by transient, high-amplitude calcium spikes [30], and promotes myogenesis by disrupting MEF2-HDAC complexes and stimulating HDAC nuclear export [28]. By contrast, calcineurin (CnA), a serine/threonine phosphatase, responds to sustained, low-amplitude calcium transients [13,29,30,53], and has been implicated in hypertrophic cardiomyopathies [7], skeletal muscle differentiation and hypertrophy [32,34,43], as well as specification of slow muscle phenotype [47,55]. Physiologically, myogenic differentiation is regulated by hormones and growth factors [41]. TGF and FGF inhibit differentiation [11,42], whereas IGFs are potent inducers of myogenic proliferation, differentiation and
Calcium mobilization in myogenesis
Basic Applied Myology 19 (5&6): 229-236, 2009

hypertrophy [16,18,19,34,35,44]. We have previously demonstrated that the neurohyphophysal nonapeptide Arg8-Vasopressin (AVP) and related peptides, such as oxytocin (OT), constitute a novel family of positive regulators of terminal differentiation in myogenic cell lines (L5 and L6), and primary satellite cells [17,31,39]. AVP receptors are coupled with G proteins [51] and have been classified in three types (V1a, V2, and V1b, which has also been termed V3). These receptors have been cloned [12,25,26,33,52] and show a high degree of homology [3,22]. The oxytocin receptor (OTR), another seven-transmembrane receptor of the same subfamily, is expressed in various tissues, including skeletal muscle [5,8,9]. The OT receptor shows the same affinity for AVP as its cognate receptor V1a [8] and, by interacting with AVP, activates the phosphatidylinositol cascade and calcium release from internal stores in CHO cells [8]. V1a (vascular/hepatic) and V1b (anterior pituitary) receptors act through phosphatidylinositol hydrolysis to mobilize intracellular Ca2+ [23,24]. V1b receptor is expressed in the anterior pituitary, where it stimulates ACTH release [48]. V2 receptors are found primarily in the kidney, are functionally linked to adenylate cyclase and are responsible for the antidiuretic effect of AVP [50]. V1aR is the only vasopressin receptor expressed in skeletal muscle [10,52]; our recent data demonstrated that its expression is modulated during myogenic differentiation in L6 cells [1]. By interacting with V1aR, AVP and analogues activate phospholipases C and D, increase cytosolic Ca2+ concentrations, regulate cAMP levels by activating a CaMK dependent pathway in cytosolic calcium activates CaMK and calcineurin [45,46]. The stimulation of the CaMK pathway induces the cytosolic compartmentalization of histone deacetylase 4, which in turn stimulates the transcription of muscle specific genes. The activation of calcineurin is responsible for the dephosphorylation of the NFAT transcription factor and its translocation into the nucleus [46]. The combined activation of both pathways by AVP results in the formation of multifactor complexes on the promotor of muscle specific genes, stimulates muscle differentiation and is required for the full expression of the differentiated phenotype.

We noted that the murine myogenic cell line C2C12 is unique inasmuch as these cells do not, unlike rat myogenic cell lines such as the L6 cells and primary cultures of murine muscle cells, respond to AVP [49]. We exploited C2C12 as a myogenic cell system to ectopically study the role of V1aR in myogenesis and hypertrophy and demonstrated that the V1aR (and not the OTR) mediates neurohypophysal hormone dependent differentiation in C2C12 cells. This findings shed new lights on the mechanisms of action of neurohypophysal hormones on myogenesis.

Materials and Methods
Cell cultures. C2C12 cells were seeded at 12,000/cm2 and cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (DMEM) and 10% heat-inactivated fetal bovine serum (FBS; growth medium [GM]). Where indicated, cultures were extensively washed with DMEM, shifted to low-serum medium, consisting of 2% horse serum (HS) DMEM, and treated with synthetic AVP 0.1 µM or oxytocin 0.1 µM as indicated (Sigma-Aldrich Chemical Co). Morphological analyses of cultures were performed after Giemsa staining (Merck).

Plasmid construction. p-IRES-EGFP-V1aR plasmid was derived from pRES-EGFP2 (Clontech) and PC3D-V1aR (Lolait) expression vectors. A PCR-based strategy was used because of the absence of compatible restriction sites in the containing vectors and in PC3D-V1aR polylinkers. Briefly, the V1aR-base pair fragment was obtained by PCR from the original plasmid using oligonucleotides containing tails with the appropriate restriction sites. The PCR product were purified, digested and subcloned in pIRES-EGFP. The constructs were analyzed by sequencing to avoid PCR-introduced mutations.

Cell transfections. For transient transfections, C2C12 cells were plated in 35-mm dishes at a density of 2.5 x 105 cells/well in DMEM supplemented with 10% FBS. Twenty-four hours later, cells were shifted to serum- and antibody-free DMEM, and transfected using the lipid-based reagent Lipofectamine LTX supplemented with Plus Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, using 4µg of each of the following reporter plasmids/plate: pIRES-EGFP-V1aR for V1aR-C2C12, and pRES-GFP for mock-C2C12. We used GFP-conjugated vector to monitor the transfection efficiency and to select transfected cells by fluorescence analyses. After 5h, the transfection mixture was removed and replaced with DMEM 10% FBS (for calcium imaging), or DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (DMEM) and 10% heat-inactivated fetal bovine serum (FBS; growth medium [GM]).

Calcium Imaging. C2C12 cells were grown and transfected, as described above, on collagen-coated glass coverslips. Forty-eight hours after transfection, C2C12 cells were incubated in MEM containing 3 mM fura-2-AM for 1 h at 37°C. The cells were then rinsed with Krebs-Henseleit-HEPES (KHH) buffer (140 mM Na+, 5.3 mM K+, 132.4 mM Cl-, 0.98 mM PO42-, 1.25 mM Ca2+, 0.81 mM Mg2+, 5.5 mM glucose and 20 mM HEPES) supplemented with 0.2% fatty acid free BSA (Sigma-Aldrich Chemical Co). The coverslips were placed in a culture chamber, kept at a constant temperature of 37°C, on the stage of an inverted fluorescence microscope (TE2000E Nikon,
Calcium mobilization in myogenesis
Basic Applied Myology 19 (5&6): 229-236, 2009

Fig. 1 Expression of vasopressin and oxytocin receptor mRNA in C2C12 cells.
(A) RT-PCR analysis of V1aR and OTR expression was performed on RNA obtained from differentiating C2C12 cells. Positive controls were obtained from L6E9 cells for V1aR expression and from rat uterus for OTR. (B) RT-PCR analysis of V1aR expression was performed on RNA obtained from differentiating C2C12 cells, either untransfected (U) or transfected with pIRES-V1aR-EGFP, and from L6E9 cells as a positive control for V1aR (C+). The GAPDH gene was used to normalize the quantification of expression. (C) WB analyses of MHC expression in differentiating C2C12 cells in the control condition or after 3 days of 0.1 μM OT stimulation. The expression of α-tubulin was used to normalize the quantification of expression.

Florence, Italy), connected to a cooled charge-coupled device 512B Cascade camera (Princeton Instruments, Monmounth Junction, NJ). Both V1aR-transfected and untransfected cells were stimulated alternatively with AVP or OT and [Ca2+]i was measured by dual wavelength fluorescence in single cells loaded with the Ca2+-sensitive indicator Fura-2 (Invitrogen, Carlsbad, CA). Samples were illuminated alternately at 340 and 380 nm using a random access monochromator (Photon Technology International, Birmingham, NJ), while emission was detected using a 510 nm emission filter. Images were acquired using Metafluor software (Universal Imaging Corporation, West Chester, PA). Calibration of the signal was obtained at the end of each experiment by maximally increasing intracellular Ca2+-dependent fura-2 fluorescence with 5 μM ionomycin (Fluka), followed by the recording of minimal fluorescence in Ca2+-free medium. [Ca2+]i was calculated according to formulas described by Grynkiewicz [21].

Immunofluorescence C2C12 cells, cultured for 48h in DMEM 2% HS, were fixed for 10 min in 4% paraformaldehyde on ice, permeabilized with 0.1% Triton X-100 in PBS for 2 min, and incubated for 30 min with 10% goat serum in PBS. Cells were then incubated overnight at 4°C with anti-MHC (MF20) (Developmental Studies Hybridoma Bank at University of Iowa, IA). Cells were then washed with PBS containing 1% BSA and incubated for 60 min with an appropriate secondary antibody, i.e. AlexaFluor568-conjugated anti-mouse 1:500 in PBS (Molecular Probes, Eugene, OR). Nuclei were visualized with Hoechst dye. The dishes were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined under a Zeiss Axioplan (Thornwood, NY) fluorescence microscope.

RT-PCR. Total RNA was extracted from C2C12 cells using Trizol Reagent (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. RT-PCR was performed using 1 μg of total RNA reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen, Carlsbad, CA). PCR reactions were carried out in a final volume of 50 μl in a buffer containing 1 μl of RT reaction, 200 μM dNTP, 1.5 mM MgCl2, 0.2 μM of each primer and 1 U of Taq-DNA polymerase (Invitrogen, Carlsbad, CA). The PCR products were analyzed in 2% agarose gel. The following specific primers were used: V1aR-for: 5′-GTACGAATTCACCGACAGCATGAGTTTCC-3′, V1aR-rev: 5′-GATCAAGCTCTGGGCCTCAAGTGAAGACAG-3′, OTR-for: 5′-GGCGCGCGTGGAGGTGGCGG-3′, OTR-rev: 3′-GTCGGGACCCCTGGGTTCCGG-5′.

The following oligonucleotides were used to detect glyceraldehyde 3-phosphate dehydrogenase transcript (used as an internal control):
GAPDH-forward: 5′-AACATCAAATGGGGTGAGGCC-3′,
GAPDH-rev: 5′-GTTGTCATGGATGACCTTGGC-3′.

Immunoblotting analyses. For total homogenates, C2C12 cells stimulated for 72 h with 0.1 μM OT were lysed in ice-cold RIPA buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5 M NaCl, 0.5% sodium deoxycholate, 1% NP40) containing protease inhibitor cocktail (Mini Complete, Roche Molecular Biochemicals). Equal amount of proteins (15-20 μg), determined according to the BCA protocol, were separated by SDS-PAGE and transferred electrophoretically to Hybond-P Extra membranes (Amersham, Piscataway, NJ). Non-specific binding was blocked in TBST containing 5% non-fat milk for 1 h, and the membrane was then incubated overnight in TBST containing primary antibodies. The primary antibodies used were undiluted anti-MHC (MF20) (Developmental Studies Hybridoma Bank at University of Iowa, IA) and monoclonal anti α-tubulin 1:500 (Sigma-Aldrich Chemical Co). Blots were then washed extensively in TBST and incubated with goat anti-mouse HRP-conjugated secondary antibody (Bio-Rad...
Calcium mobilization in myogenesis

Basic Applied Myology 19 (5&6): 229-236, 2009

Fig. 2 AVP treatment induces Ca2+ mobilization in V1aR-transfected C2C12 cells. (A) Fluorescence and phase contrast images of pIRES-V1aR-EGFP transfected cultures. A representative GFP positive-V1aR-transfected cell is circled in red and a GFP negative untransfected cell in green. (B) Intracellular [Ca2+]i, expressed in nM, obtained by fluorescence trace of fura-2 loaded individual C2C12 cells, either transfected with pIRES-V1aR-EGFP (red line) or not transfected (green line), after AVP stimulation. Only the transfected cells respond to AVP stimulation with a calcium transient.

Laboratories, Hercules, CA) in TBST containing 0.5% non-fat milk. Blots were washed in TBST and antibody binding was detected using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Results

Expression of vasopressin and oxytocin receptor mRNA in C2C12 cells.

Here we demonstrate, for the first time, that AVP exerts no significant effect on the morphological differentiation of C2C12 cells (Fig. 3A), which is in keeping with a previous study we conducted [49] in which we showed that AVP does not elicit Ca2+ mobilization in C2C12 cells. These findings prompted us to verify the expression of both V1aR and OTR in C2C12 cells. RT-PCR analysis revealed that V1aR is not expressed in these cells (Figure 1A). Interestingly, in the same cells, we detected high expression levels of the related OTR (Figure 1A), which is known to display a high binding affinity for AVP [8]. However 0.1 μM OT treatment did not stimulate myogenic differentiation in C2C12 cells, as demonstrated by the expression levels of MHC (Figure 1 C).

AVP treatment induces Ca2+ mobilization in V1aR-transfected C2C12 cells

Since V1aR is not expressed in C2C12 cells, we used p-IRES-V1aR-EGFP to ectopically express this receptor. The percentage of GFP positive cells (Fig. 2A) indicated high transfection efficiency and allowed cell-based calcium imaging assay to determine the effects of AVP on calcium homeostasis. We also verified V1aR expression levels in C2C12 transfected culture by means of RT-PCR analysis (Fig. 1B).
Table 1. Calcium mobilization in C2C12 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Treatment</th>
<th>[Ca2+]i, nM</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Peak</td>
</tr>
<tr>
<td>V1aR-transfected</td>
<td>0.1 μM AVP</td>
<td>41 ± 39</td>
</tr>
<tr>
<td>Untransfected</td>
<td>0.1 μM AVP</td>
<td>51 ± 27</td>
</tr>
<tr>
<td>Untransfected</td>
<td>0.1 μM OT</td>
<td>67 ± 34</td>
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Values are means ± SD. [Ca2+]i, intracellular free calcium concentration. (*), p < 0.001 vs. basal. (§), p < 0.001 vs. peak.

We found that only pRES-V1aR-EGFP transfected cells (i.e. GFP positive cells, Fig. 2A) responded to 0.1 μM AVP stimulation, with a rapid and sustained increase in [Ca2+]i followed by a stable lower plateau (Fig. 2B and Table 1). By contrast, both AVP and OT failed to induce significant [Ca2+]i changes in GFP negative cells (Fig. 2B and Table 1), in spite of the presence of OTR. However, both GFP positive and GFP negative cells responded to 0.5 μM Ca2+ ionophore ionomycin treatment (data not shown).

AVP treatment stimulates myogenic differentiation in V1aR-transfected C2C12 cells.

We investigated whether AVP-dependent calcium mobilization has a positive effect on differentiation in V1aR-transfected C2C12 cells. Morphological analysis (Fig. 3 A) demonstrated that AVP increased the formation of larger myotubes in V1aR-transfected cultures alone. No morphological effects were exerted either by AVP upon mock transfected C2C12 cells, or upon V1aR transfected cells in the absence of AVP treatment. Furthermore, V1aR-transfected C2C12 culture, stimulated by AVP, displayed an increased MHC accumulation, whereas other treatments (MOCK, MOCK+AVP, V1aR) did not (Figure 3B).

Discussion

Vasopressin, oxytocin and related peptides induce differentiation in myogenic cells [31,39,49]. The myogenic cells that respond to AVP express V1αR (as occurs in L6 cells) or OTR (as occurs) in human satellite cells [1,8]. Binding of AVP to the specific membrane receptor V1a in the target cells activates phosphatidylinositol hydrolysis, which in turn releases Ca2+ from internal stores. The AVP-dependent increase in cytosolic Ca2+ induces the activation of CaM kinases and calcineurin signaling [45,46], two pathways required for the full expression of the differentiated phenotype. We investigated the role of V1αR in myogenesis and hypertrophy by ectopically restoring V1αR expression and function using the C2C12 cell line, which is an experimental model of satellite cells that do not respond to AVP treatment. The lack of V1αR expression in C2C12 cells is in agreement with the absence of AVP-dependent Ca2+ mobilization in C2C12 cells [49].

Interestingly, although C2C12 cells express high OTR levels, neither AVP nor OT stimulation had any effect on either [Ca2+]i or morphological differentiation; molecular analysis of the expression of MHC showed that OT treatment did not have any significant effect either.

Therefore, C2C12 cells may serve as a useful model to evaluate the role of V1αR in mediating neurohypophyseal-dependent myogenic differentiation.

Our results show that AVP treatment enhances myogenic differentiation in V1αR-transfected C2C12 cultures alone, as demonstrated by the presence of larger multinucleated myofibers, which were absent in mock-transfected cells, and by the up-regulation of MHC expression. These data demonstrate that, although C2C12 cells express high OTR levels, both AVP and OT failed to stimulate the differentiation program, and suggest that the presence of V1α receptor is required to mediate the effects of neurohypophyseal hormones in these cells.

Although high transfection efficiency was predictable, the specific construct we set up and transfected allowed us to analyze the response of individual control and V1αR-transfected C2C12 cells by means of calcium imaging analyses. We demonstrated that no changes in [Ca2+]i are detectable after stimulation with either AVP or OT in control C2C12. These data are in keeping with the absence of an AVP receptor and suggest a failure in OTR function. The inability of OTR to respond to OT in C2C12 cells, which may be due to a defect either in translational or post-translational events, or in the early phases of transduction, is currently under investigation.

By contrast, we demonstrated that the presence of V1αR suffices to render C2C12 cells responsive to neurohypophyseal hormone stimulation, as demonstrated by the rapid and sustained calcium release from internal stores observed in V1αR-transfected cells alone. The AVP-dependent Ca2+ increase obtained in these cells, however, differs from...
that observed in L6. In conclusion, all the results obtained in this work suggest that the presence of V1a receptor is essential to mediate the effects of neurohypophysial hormones on myogenic differentiation in C2C12 cells. Further studies are warranted to clarify whether the positive effects of AVP mediated by V1aR observed in these cells are due to the activation of calcium-dependent signalling pathways involved in myogenic differentiation.

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Abbreviations

AVP, arg 8-vasopressin; CaMK, Ca 2+/calmodulin-dependent kinase; CnA, calcineurin A; HDAC, histone deacetylase; MHC, myosin heavy chain; OT, oxytocin; OTR, oxytocin receptor; V1aR, V1a vasopressin receptor.

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Calcium mobilization in myogenesis
Basic Applied Myology 19 (5&6): 229-236, 2009


Calcium mobilization in myogenesis
Basic Applied Myology 19 (5&6): 229-236, 2009


