Arginine Vasopressin Inhibition of Cyclin D1 Gene Expression Blocks the Cell Cycle and Cell Proliferation in the Mouse Y1 Adrenocortical Tumor Cell Line†

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ABSTRACT: Arginine vasopressin (AVP) is a nonapeptide long known as an endocrine and paracrine regulator of important systemic functions, namely, vasoconstriction, gluconeogenesis, corticosteroidogenesis, and excretion of water and urea. Here we report, for the first time, that AVP specifically inhibits expression of the cyclin D1 gene, leading to cell cycle blockage and halting cell proliferation. In G0/G1-arrested mouse Y1 adrenocortical tumor cells, maintained in serum-free medium (SFM), AVP mimics FGF2, promoting rapid ERK1/2 activation (5 min) followed by c-Fos protein induction (2 h). PKC inhibitor Go6983 and PI3K inhibitors wortmannin and LY294002 all inhibit ERK1/2 activation by AVP, but not by FGF2. Thus, AVP and FGF2 concur to activate ERK1/2 by different regulatory pathways. However, AVP is not a mitogenic factor for Y1 cells. On the contrary, AVP strongly antagonizes FGF2 late induction (2−5 h) of the cyclin D1 gene, down-regulating both cyclin D1 mRNA and protein. AVP inhibition of cyclin D1 expression is sufficient to block G1 phase progression and cell entry into the S phase, monitored by BrdU nuclear labeling. In addition, AVP completely inhibits proliferation of Y1 cells in 10% fetal calf serum (10% FCS) medium. On the other hand, ectopic expression of the cyclin D1 protein renders Y1 cells resistant to AVP for both entry into the S phase in SFM and continuous proliferation in 10% FCS medium. In conclusion, inhibition of cyclin D1 expression by AVP is an efficient mechanism of cell cycle blockage and consequent proliferation inhibition in Y1 adrenocortical cells.

We have been focusing on characterizing antagonistic mitogenic and antimitogenic signals initiated at, respectively, FGF2[1] and ACTH receptors in the mouse Y1 adrenocortical tumor cell line (*1*, *2*), aiming, in the long run, to elucidate the signaling circuitry that controls the cell cycle and cell growth in adrenocortical cells. Y1 is an ACTH-responsive, steroid-secreting, clonal cell line that expresses high levels of ACTH receptors, closely mimicking fasciculata cells from the normal adrenal cortex (*3*, *4*). Although the malignant phenotype of Y1 cells includes evasion from apoptosis and a limitless replicative potential, our strain of this cell line retains control mechanisms of the G0 → G1 → S transition of the cell cycle, being highly responsive to mitogenic stimulation by FGF2.

In Y1 adrenocortical cells G0/G1-arrested by serum deprivation, the mitogenic response elicited by FGF2 includes (a) activation of ERK1/2 and PI3K (2−10 min), (b) transcription activation of the immediate early genes c-fos, c-jun, and c-myc (10−30 min), and (c) the onset of DNA synthesis initiation at 8 h (*5*−*7*). ACTH blocks this FGF2 mitogenic response at the early and middle G1 phase, triggering concerted antimitogenic mechanisms mediated by the cAMP/PKA pathway. These mechanisms include, first, rapid dephosphorylation/deactivation of Akt/PKB (*8*) and degradation of the c-Myc protein (*9*) and, second, a late increase in the levels of the CDK inhibitor $p27^{Kip1}$ (*7*). In addition, we have identified other peptides in pituitary extracts that also antagonize FGF2 mitogenic activity (*1*). Here we report for the first time antimitogenic mechanisms triggered by arginine vasopressin (AVP), antagonizing the mitogenic activity of both FGF2 and bovine fetal calf serum (FCS) in Y1 adrenocortical cells.

The nonapeptide AVP is classically known for being synthesized in the hypothalamus and stored in the anterior hypophysis. But AVP is also synthesized and secreted by cromaffin cells localized in both the cortex and medulla of adrenal glands. AVP exerts its biological effects by binding to three different receptors that belong to the G-protein-

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[1] Abbreviations: AVP, arginine vasopressin; ACTH, adrenocorticotropin; cAMP, adenosine 3′:5′-cyclic monophosphate; PKA, protein kinase A; Akt/PKB, protein kinase B; PKC, protein kinase C; CDK, cyclin-dependent kinase; PI3K, phosphatidylinositol 3-kinase; FGF2, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; GPCR, G-protein-coupled receptor; FCS, fetal calf serum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DME, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; BrdU, bromodeoxyuridine; DAG, diacylglycerol; SFM, serum-free medium; G418, geneticin 418 sulfate; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate conjugate mouse IgG; DAPI, 4,6-diamidino-2-phenylindole; MEK, MAP kinase kinase; PLCβ, phospholipase C β; PKD, protein kinase D; pRb, retinoblastome protein; $G\alpha_{q/11}$, *q* or 11 G protein α subunit; G$\beta\gamma$, G protein $\beta\gamma$ subunits.

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coupled receptor (GPCRs) superfamily (*10*). Type V1 receptor induces breakdown of phosphoinositides, generating inositol phosphates and diacylglycerol (DAG) to initiate mechanisms of vasoconstriction, gluconeogenesis, and corticosteroidogenesis. V1a receptors are expressed in the liver, smooth muscle cells from blood vessels, and certain peripheral tissues, whereas V1b receptors are found in the adenohypophysis. Type V2 receptor, localized in the kidney, acts via the cAMP/PKA pathway and is involved in the regulation of water and urea excretion (*11*−*13*). Y1 adrenocortical cells express V1a receptors (P. F. Asprino and H. Armelin, unpublished results).

Reports from several laboratories have shown that AVP initiates mitogenic signaling, via V1a receptors, in different cell types, eventually leading to stimulation of DNA synthesis and cell proliferation. In intestinal epithelial cells (*14*), mesangial cells (*15*), cardiomyocytes (*16*), smooth muscle cells (*17*), and Swiss 3T3 fibroblasts (*18*, *19*), addition of AVP triggers a cascade of events including (a) activation of phospholipase C β, (b) an increase in cytosolic Ca^{2+}, (c) activation of PKC, (d) activation of protein kinase D (PKD), (e) activation of PI3K, and (f) phosphorylation of ERK1/2−MAP kinases. Thus, signals initiated in AVP−V1a receptors and in tyrosine kinase receptors converge to main mitogenic pathways, namely, the ERK1/2−MAP kinases and the PI3K (*15*, *20*).

In G0/G1-arrested Y1 cells, AVP rapidly promotes phosphorylation of ERK1/2 and induction of c-Fos protein, resembling the first steps of the mitogenic response triggered by FGF2. However, AVP inhibits G1 phase progression in a step after induction of the immediate early genes, specifically blocking expression of the cyclin D1 gene induced by FGF2. Altogether these results demonstrate, for the first time, that AVP inhibits the cell cycle and cell proliferation of Y1 adrenocortical cells initiating antimitogenic mechanisms different from those triggered by ACTH.

MATERIALS AND METHODS

Cell Line Culture. An HSR subline (*21*) of mouse Y1 adrenocortical carcinoma cells (*2*) was purchased from ATCC in 1973 and adapted to grow in 90% Dulbecco's modified Eagle's medium (DME), 10% FCS in a 5% CO_2–95% air atmosphere. Stocks are kept frozen in liquid nitrogen and periodically thawed. This Y1 cell line displays (a) c-Ki-ras gene amplification according to the original report of Schwab et al. (*22*), as monitored by Southern blots and in situ hybridization (*23*), (b) an average of 39 chromosomes/cell, within a very narrow range, and (c) marker chromosomes m1 and m2 (*21*) in 100% of the metaphase spreads. To arrest the cell cycle at the G0/G1 boundary, Y1 cells growing exponentially in 10% FCS−DME were incubated for 48 h in SFM (serum-free medium), where they are viable and fully responsive to both FGF2 and AVP.

Y1 Clonal Lines Expressing Cyclin D1 Protein (*Y1*−*D1 Lines*). A mouse cyclin D1 cDNA clone, obtained from Dr. C. J. Sherr (St. Jude Children's Research Hospital, Memphis, TN), was inserted into pSVK3 vector to yield the pSVD1 construct, which was cotransfected into Y1 cells with a neo gene marker vector (pSVNeo) and neutrally selected with G418 to generate stable Y1−D1 clonal lines constitutively expressing cyclin D1 protein. Stocks of Y1−D1 clonal lines were kept frozen in liquid nitrogen and periodically thawed to grow in 10% FCS−DME plus 100 μg/mL G418. All the Y1−D1 clones arrested the cell cycle at the G0/G1 interface upon serum deprivation, maintaining response to FGF2 and AVP, despite expressing high levels of cyclin D1 (T. T. Schwindt and H. A. Armelin, unpublished results).

Synthetic or Recombinant Peptides. Synthetic arginine vasopressin peptide was purchased from Sigma or synthesized in L. Juliano's laboratory, Escola Paulista de Medicina (Universidade Federal de São Paulo, São Paulo, Brazil). Recombinant bovine FGF2 was prepared in the laboratory of Dr. Angelo Gambarini, Instituto de Química (Universidade de São Paulo, São Paulo, Brazil).

Analysis of ERK1/2 Phosphorylation. Cells were lysed in ice cold 62.5 mM Tris−HCl, pH 6.8, 2% SDS (w/v), 10% glycerol, 50 mM DTT, and 1% bromophenol blue (w/v). The lysates were sonicated for 2 min, boiled for 5 min, and clarified by centrifugation (14000 rpm, 5 min, 4 °C). A 40 μL sample of the total extract containing 100 μg of protein was resolved by 10% SDS−PAGE. After electrophoresis the gel was electroblotted onto Hybond-C nitrocellulose membranes, using a semidry Bio-Rad apparatus, total ERK1/2 or Thr 202- and Tyr 204-phosphorylated ERK1/2 isoforms were detected with monospecific rabbit polyclonal antibodies (New England Biolabs), followed by secondary peroxidase-conjugated antirabbit polyclonal antibodies for chemiluminescent detection (ECL, Amersham-Pharmacia). The results are representative of three independent experiments.

Levels of Cyclin D1 Protein. Cells were lysed in cold NP-40 buffer (20 mM Tris−HCl, pH 8.0, 135 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM sodium orthovanadate, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 2 μg/mL pepstatin). After quantitation by Bradford assay, 150 μg aliquots of the total protein were mixed with SDS−PAGE sample buffer, resolved by 10% SDS−PAGE, and processed for Western blotting as described above for ERK1/2 proteins, using a rabbit polyclonal antibody monospecific for mouse cyclin D1 (Santa Cruz). The results are representative of three independent experiments, and Ponceau staining of electroblotted membrane was used to visually monitor protein transfer.

Northern Blot for Cyclin D1 mRNA. G0/G1-arrested Y1 cells were stimulated with FGF2 and/or AVP and lysed with Trizol reagent, and the total extracted RNA was quantified by absorbance at A_{260}. Aliquots of 15 μg of the total RNA were loaded and resolved in denaturing agarose gel electrophoresis and blotted in a Hybond-N+ nylon membrane. A mouse cyclin D1 cDNA-containing vector was radioactively labeled with α-^{32}P-dCTP using the kit Random Primers DNA Labeling System to yield the cyclin D1-^{32}P probe. Hybridization of blotted membranes with cyclin D1-^{32}P probes was carried out overnight at 42 °C, and after successive washes with SSC−SDS mixtures, the membranes were exposed to X-ray films at −20 °C for 24 h. A mouse GAPDH cDNA probe was used to reprobe the membranes under the same conditions. The results are representative of two independent experiments.

BrdU Incorporation. G0/G1-arrested Y1 cells, seeded on coverslips, were incubated initially with hormone at zero time, followed by 100 μM BrdU between 12 and 24 h. The cells were then fixed with cold methanol for 10 min, washed

three times with PBS, and subsequently stored in PBS overnight at 4 °C. The coverslips with fixed cells were incubated in 1.5 N HCl for 30 min with agitation, washed three times with PBS, inverted onto a drop (40 μL) of mouse anti-BrdU antibody (Amersham/Pharmacia), and incubated for 30 min at room temperature. After the coverslips were washed with PBS, immune complexes were detected with FITC secondary antibody (fluorescein isothiocyanate conjugate mouse IgG, Sigma). The coverslips were then incubated with 5 μg/mL DAPI (4′,6-diamidino-2-phenylindole, Sigma) for 20 min, and fluorescently stained nuclei were examined under a Nikon Fluophot microscope using two excitation filters: UV 330-380 to visualize DAPI-stained nuclei and IF 420-490 to visualize the BrdU-labeled nuclei stained with FITC. The coverslips (two per condition in each experiment) were randomly coded, and 500−600 nuclei per coverslip were blindly counted. The raw data of independent experiments (at least two) were pooled to derive a final value of the percent labeled nuclei under each condition and statistically analyzed by χ^2 with 1 degree of freedom. In this analysis *n* (number of independent events) is the pooled number of cells counted per each condition in all experiments.

Cellular Growth Curves. Parental Y1 cells and Y1−D1 transfectant clonal lines were plated in 35 mm plates containing 10% FCS−DME without or with 100 μM G418, respectively, and maintained in culture for five to six days, with or without AVP stimulation. Duplicate plates from each cell line were removed daily, trypsinized, and counted in a Neubauer chamber. On the third day, the cultures were refed with fresh medium (±AVP). The results are presented as the average of three independent experiments.

RESULTS

AVP Blocks G1 Phase Progression in G0/G1-Arrested Y1 Adrenocortical Cells Stimulated by FGF2. G0/G1-arrested Y1 cells were treated with FGF2 and AVP, for short pulses (2 h) or sustained treatments (24 h). Cell entry into the S phase, within 24 h, was estimated by BrdU nuclear labeling, incorporating BrdU into DNA between 12 and 24 h. A 2 h FGF2 pulse was sufficient to cause 3.5-fold relative stimulation, whereas sustained FGF2 treatment led to 4.3-fold relative stimulation (Table 1), in agreement with previously published results (*5*, *6*). AVP treatments, on the other hand, displayed a different pattern of DNA synthesis stimulation (Table 1): 2 h pulses caused moderate stimulation (1.7-fold relative stimulation) that significantly decreased in sustained treatment (0.9-fold relative stimulation), showing that AVP does not function as an FGF2-like mitogen. On the contrary, AVP is indeed an inhibitor of FGF2 mitogenic activity: (a) combination of FGF2 and AVP in 2 h pulses yielded only half (51%) of the stimulation obtained by FGF2 alone (Table 1); (b) in sustained treatment the AVP inhibitory effect was significantly greater; stimulation of FGF2 plus AVP was 79% less than that of FGF2 alone (Table 1); (c) finally, 2 h FGF2 stimulation followed by AVP stimulation between 6 and 24 h was 84% less than 2 h FGF2 stimulation alone (Table 1). Altogether, these results suggest that AVP inhibits G1 phase progression induced by FGF2 blocking reactions in the middle of the G1 phase.

AVP Mimics FGF2, Activating ERK1/2, but in a PKC- and PI3K-Dependent Manner. To probe into the molecular mechanisms by which AVP could regulate G0/G1 → S transition in Y1 cells, we began by asking whether AVP would activate ERK1/2. This seemed to be a reasonable question since, as a rule, mitogen receptors rapidly activate ERK1/2 and AVP 2 h pulses significantly stimulated cell entry into the S phase, resembling mitogens. In fact, AVP mimicked FGF2, promoting rapid activation of ERK1/2 (Figure 1A). In addition, a combination of FGF2 and AVP, at saturating concentrations, promoted maximal activation of ERK1/2 (Figure 1B,C), indicating that there is no antagonism between FGF2 and AVP as far as ERK1/2 activation goes. However, the mechanisms of ERK1/2 activation triggered by FGF2 and AVP are different. The PKC inhibitor Go6983 strongly inhibited ERK1/2 activation by AVP, but not by FGF2 (Figure 1B). Furthermore, PI3K inhibitors wortmmanin and LY294002 also inhibited ERK1/2 activation by AVP, but not by FGF2 (Figure 1C). These results imply that, first, AVP activation of ERK1/2 is mediated by PKC and PI3K and, second, AVP and FGF2 activate ERK1/2 by different regulatory pathways in Y1 adrenocortical cells. Consistent with this conclusion, 2 h pulses of both AVP and FGF2 induced c-Fos protein (Figure 2). c-fos is an immediate early gene long recognized as a nuclear target of ERK1/2, whose induction is mainly regulated at the level of transcription and whose expression is required for G1 phase progression stimulated by mitogens. Thus, the event that AVP inhibits to block G1 phase progression, antagonizing FGF2 mitogenic activity, is likely to occur after induction of the immediate early genes.

AVP Blocks Cyclin D1 Expression Induced by FGF2 in Y1 Adrenocortical Cells. In mitogen-induced G1 phase progression, the D-type cyclins provide a link between the immediate early genes and the cell cycle machinery (*14*). In G0/G1-arrested Y1 cells, FGF2 triggered G1 phase progression, leading to cyclin D1 protein induction at 5−6 h (Figure 3B,C) via a process dependent on protein synthesis (not shown) and inhibited by inhibitors of ERK1/2 and PI3K, respectively, PD98059 (Figure 4A) and wortmmanin (Figure 4B), but not by the inhibitor of PKA, H89 (Figure 4B). We

Table 1: DNA Synthesis of G0/G1-Arrested Y1 Adrenocortical Cells Stimulated by FGF2 and/or AVP[a]

period of treatment (h)			
FGF2 (2×10^{-10} M)	AVP (10^{-6} **M**)	% BrdU-labeled nuclei	rel DNA synthesis stimulation
		13	0
0−2		58	3.5
0−24		69	4.3
	0−2	35	1.7
	0−24	25*	0.9
0−2	0−2	40	2.1
0−24	0−24	26*	1.0
0−2	6−24	22	0.7

[a] G0/G1-arrested adrenocortical cells, maintained in SFM, were treated with FGF2 and/or AVP, according to the time course indicated, and DNA synthesis stimulation was estimated by BrdU incorporation into DNA between 12 and 24 h. Values of percent BrdU-labeled nuclei from two independent experiments were pooled such that 2000−2400 cells were counted per condition. As analyzed by χ^2 with 1 degree of freedom, all differences between percentage values were significant ($p < 0.001\%$), except for the pair marked with asterisks. Relative DNA synthesis stimulation was derived from values of percent BrdU-labeled nuclei as a ratio of experimental minus control divided by control, where the control is untreated cells.

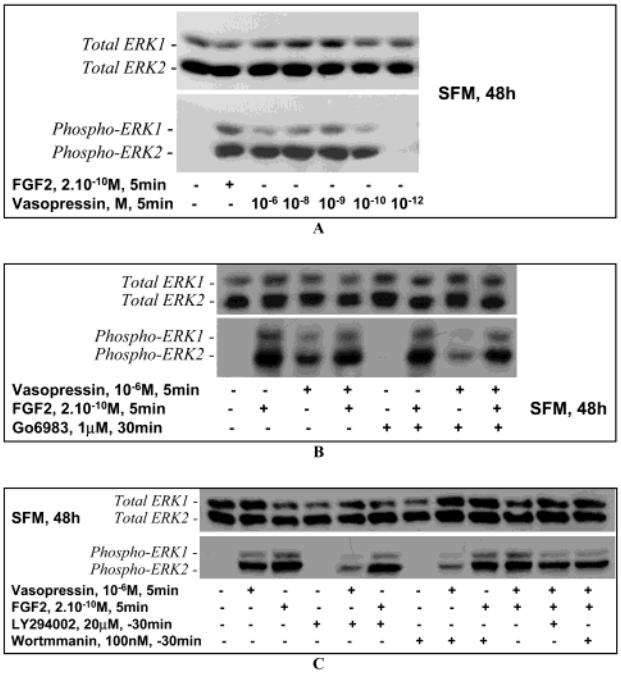

FIGURE 1: Phosphorylation of ERK1/2 promoted by AVP in Y1 adrenocortical cells and inhibition by PKC and PI3K inhibitors. G0/G1-arrested Y1 cells, maintained in SFM, were previously treated (−30 min), or not, with PKC and PI3K inhibitors and stimulated with FGF2 and/or AVP (0−5 min). Cellular lysates were assayed by Western blot using total or phospho-ERK1/2-specific antibodies. These results are representative of three independent experiments. (A) AVP dose−response curve. (B) Effect of PKC inhibitor Go6983. (C) Effect of the PI3K inhibitors LY294002 and wortmannin.

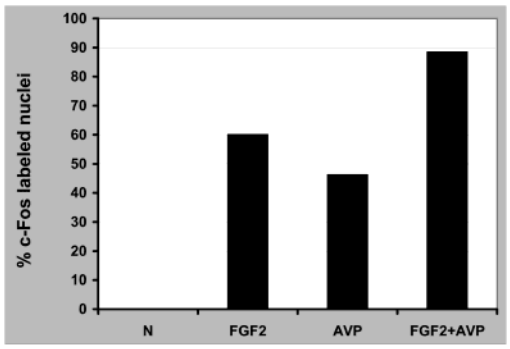

FIGURE 2: Immunocytochemistry assay showing the induction of c-Fos protein by AVP and/or FGF2. G0/G1-arrested Y1 cells plated on coverslips and maintained in SFM for 48 h were treated with FGF2 and/or AVP for 2 h and assayed by immunocytochemistry for the c-Fos protein as previously described (*6*). Briefly, nuclear c-Fos immune complex was visualized by immunoperoxidase staining using the Vectastain Elite ABC kit and diaminobenzidine. Values of percent c-Fos-labeled nuclei from three independent experiments were pooled such that about 3000 cells were counted per condition. As analyzed by χ^2 with 1 degree of freedom, all differences between percentage values were significant ($p <$ 0.001%).

investigated whether AVP would interfere with cyclin D1 gene induction by analyzing mRNA steady-state levels by Northern blot (Figure 3A) and protein levels by Western blot (Figure 3B,C) to show that AVP, in fact, blocked induction of the cyclin D1 protein.

Northern blot assays, in G0/G1-arrested Y1 cells, displayed low levels of cyclin D1 mRNA that increased with FGF2 treatment (Figure 3A). On the other hand, AVP caused

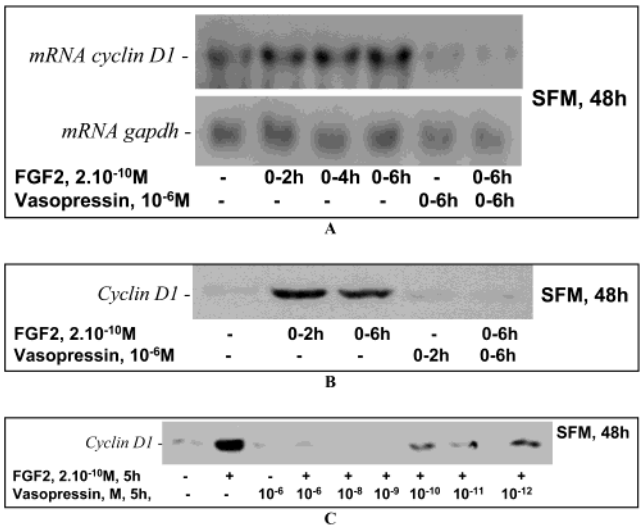

FIGURE 3: AVP inhibits cyclin D1 expression (mRNA and protein) induced by FGF2 in Y1 adrenocortical cells. G0/G1-arrested Y1 cells, maintained in SFM, were treated with FGF2 and/or AVP according to the time periods and concentrations indicated in the panels. (A) Northern blot for cyclin D1 and GAPDH mRNA (control). (B) Western blot for cyclin D1 protein. (C) AVP dose−response curve in a Western blot assay for cyclin D1 protein. The results are representative of three independent experiments; Ponceau staining of blotted membranes was used to monitor protein transfer (not shown).

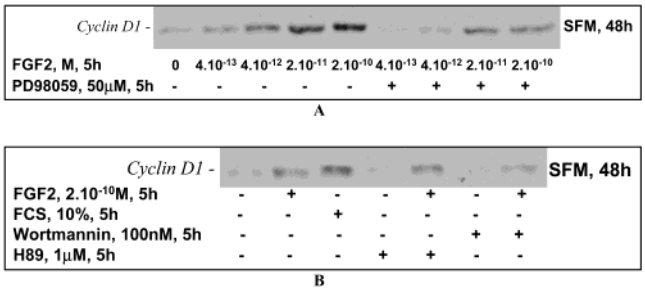

FIGURE 4: The induction of cyclin D1 by FGF2 is dependent on MEK1 and PI3K pathways and independent of the PKA pathway. G0/G1-arrested Y1 cells in SFM for 48 h were stimulated by FGF2 after 30 min of pretreatment with MEK1 inhibitor PD98059 (A) and PI3K inhibitor wortmannin and PKA inhibitor H89 (B). The results are representative of three independent experiments; Ponceau staining of blotted membranes was used to monitor protein transfer (not shown).

drastic reduction in the basal levels of cyclin D1 mRNA and completely abolished its induction by FGF2 (Figure 3A). Cyclin D1 protein was barely detectable in Western blot assays, but was strongly induced by FGF2, (Figure 3B) whereas AVP, at physiological concentrations (Figure 3C), entirely blocked cyclin D1 protein induction (Figure 3B,C). It is very likely that AVP blocks cyclin D1 protein expression by inhibiting transcription of the cyclin D1 gene or by promoting cyclin D1 mRNA degradation or both.

Ectopic Expression of Cyclin D1 Is Sufficient To Render Y1 Adrenocortical Cells Resistant to Growth Inhibition by AVP. To test the hypothesis that inhibition of the cyclin D1 gene expression is the mechanism by which AVP blocks G1 phase progression, we transfected a cyclin D1 cDNA construct into Y1 cells, generating stable transfectant clones to ask whether sustained expression of cyclin D1 protein would render Y1 cells resistant to cell cycle inhibition by AVP. The transfectant clone (D1G) cell cycle arrested at

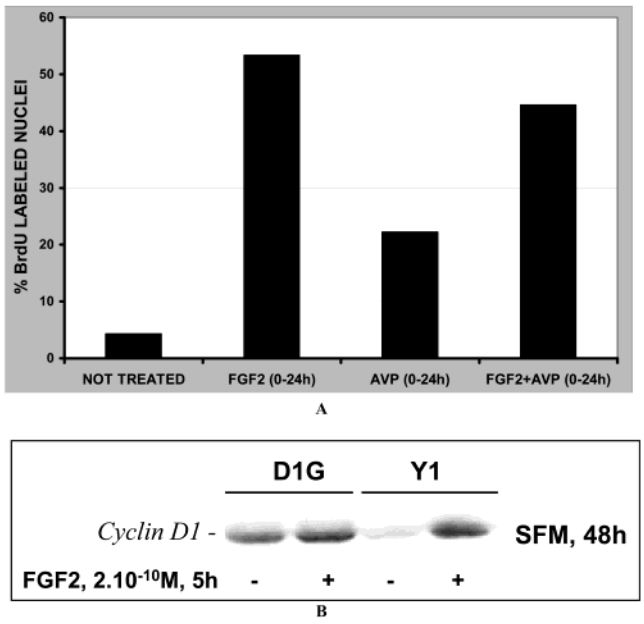

FIGURE 5: S phase entry promoted by FGF2 and/or AVP in G0/G1-arrested Y1−D1G cells, a clone constitutively expressing cyclin D1 protein. G0/G1-arrested Y1−D1G cells, maintained in SFM, were treated with FGF2 (2×10^{-10} M) and/or AVP (10^{-6} M) for the time periods indicated in the panels; BrdU incorporation between 12 and 24 h. (A) Values of percent BrdU-labeled nuclei from three independent experiments were pooled such that about 3000 cells were counted per condition. As analyzed by χ^2 with 1 degree of freedom, all differences between percentage values were significant ($p < 0.001\%$). (B) Western blot immunoassay for cyclin D1 protein in lysates of Y1−D1G cells and Y1 parental cells. The results are representative of two independent experiments; Ponceau staining of blotted membranes was used to monitor protein transfer (not shown).

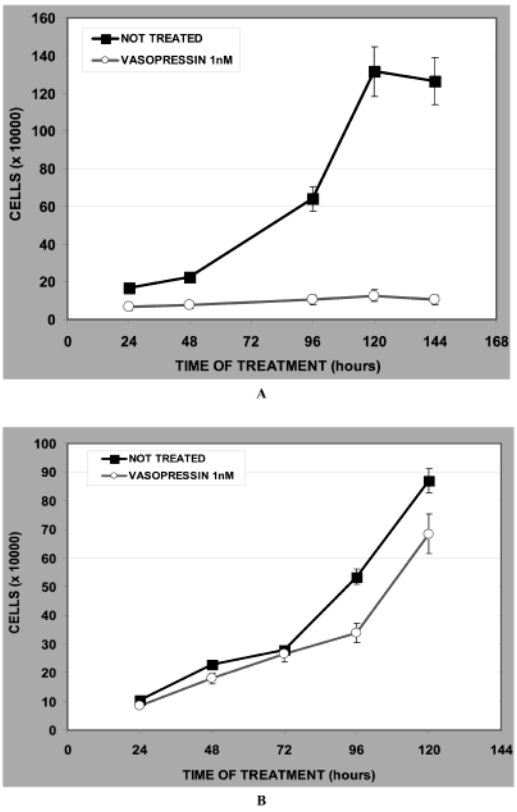

FIGURE 6: Growth curves of Y1 adrenocortical parental cells and the Y1−D1G transfectant clone in 10% FCS medium: inhibitory effect of AVP. Y1 (A) and Y1−D1G (B) cells were plated in 35 mm dishes and daily counted in a Neubauer chamber immediately after trypsinization. The results are averages (±SD) of three independent experiments.

the G0/G1 boundary by 48 h of starvation in SFM displayed constitutive expression of the cyclin D1 protein (Figure 5B) and presented very low levels of DNA synthesis (Figure 5A). These G0/G1-arrested D1G cells responded to FGF2 with a strong increase in DNA synthesis, reaching 54% BrdU labeling that represented a 13.3-fold relative stimulation (Figure 5A). This DNA synthesis stimulation by FGF2 was poorly inhibited by AVP: compare 18% inhibition shown in Figure 5A for the D1G transfectant with 79−84% inhibition in the parental Y1 cells (Table 1; 24 h treatment). Altogether, these results support the proposal that AVP blocks the cell cycle of Y1 adrenocortical cells by inhibition of cyclin D1 expression. In addition, Figure 5A also shows that AVP remained a weak mitogen even for cells displaying constitutive expression of the cyclin D1 protein. Two other Y1−D1 clonal lines were tested and also proved to be resistant to AVP inhibition of DNA synthesis stimulated by FGF2 in SFM (not shown).

Finally we asked whether AVP would also block exponential growth of Y1 cells maintained in 10% FCS medium and whether sustained expression of cyclin D1 would render Y1 cells resistant to AVP growth inhibition. The growth curves shown in Figure 6 clearly indicate that AVP completely blocked growth of Y1 parental cells (Figure 6A), but not growth of the D1G transfectants (Figure 6B). Growth curves for two Y1−D1 clones other than D1G have shown the same pattern displayed in Figure 5B (data not shown), indicating that resistance to AVP for growth among Y1−D1 clones is independent of clonal variation. In conclusion, inhibition of cyclin D1 expression by AVP, detected in Northern and Western blot assays, seems to be an efficient mechanism of growth inhibition in Y1 adrenocortical cells.

DISCUSSION

We have previously shown that Y1 adrenocortical cells display ERK−MAP kinases under remarkably tight control, whose FGF2- or FCS-dependent stimulation is absolutely required for triggering the G0/G1 → S transition (*1*, *5*, *6*). Here we demonstrate that induction of the cyclin D1 protein is obligatory for G1 phase progression in G0/G1-arrested Y1 cells stimulated by FGF2 or FCS. AVP inhibits cyclin D1 induction (Figure 3), an inhibitory effect sufficient to block G1 phase progression in serum-free medium containing FGF2 (Table 1) and to halt cell proliferation in FCS-supplemented culture medium (Figure 6A). This conclusion is supported by the fact that ectopic expression of cyclin D1 protein is sufficient to render Y1 cells resistant to the growth inhibitory effect of AVP (Figures 5 and 6B). However, cyclin D1 ectopic expression is not sufficient to abrogate the FGF2 requirement for triggering the G0/G1 → S transition (Figure 5A), implying that cyclin D1 is not a dominant factor to per se warrant G1 phase progression without mitogen stimulation.

Cyclin D1 is a recognized critical regulator of G1 phase progression, activating CDK4/6 to phosphorylate pRb and to sequester $p27^{Kip1}$ (*24*, *25*). Despite the functional redundancy within the cyclin D family, cyclin D1 is well-known

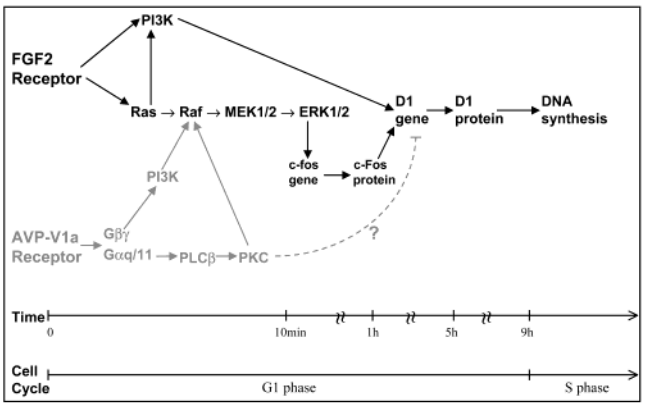

FIGURE 7: Scheme of the proposed pathways that integrate signals initiated in FGF2 and AVP receptors.

for normal and pathological effects in the regulation of the cell cycle and cell growth. Overexpression of cyclin D1 causes G1 phase shortening and reduces growth factor dependency in cultured fibroblasts (*26*−*28*). In addition, cyclin D1 is amplified and overexpressed in a large proportion of human mammary carcinomas and also has a causative role in breast cancer formation in transgenic mice. However, cyclin D1 pathways are not completely elucidated, displaying exquisite cell-type specificity (*29*). This report shows that, in Y1 adrenocortical cells, cyclin D1 protein is an efficient target for negative regulators of the cell cycle, but G1 phase progression in G0/G1-arrested Y1 cells requires more than cyclin D1 expression.

AVP, through V1a receptors, triggers mitogenic pathways in intestinal epithelial cells (*14*), mesangial cells (*15*), cardiomyocytes (*16*), smooth muscle cells (*17*), and Swiss 3T3 fibroblasts (*18*, *19*). Our results with Y1 adrenocortical cells confirm that AVP elicits reactions typical of mitogenic stimulation, namely, activation of ERK1/2 (Figure 1A), induction of the c-fos proto-oncogene (Figure 2), and incorporation of BrdU into DNA (Table 1 and Figure 5A). Inhibitors of PKC (Figure 1B) and PI3K (Figure 1C) inhibit ERK1/2 activation by AVP but not by FGF2. These results are consistent with the notion that, in Y1 cells, AVP also acts through V1a receptors as summarized in the scheme of Figure 7. However, in Y1 cells, AVP−V1a receptors also initiate antimitogenic signals that block expression of the cyclin D1 gene, very likely at the level of transcription but by still undefined routes (Figure 7). In addition, Y1 cells ectopically expressing cyclin D1 are resistant to growth inhibition by AVP (Figures 5A and 6B), but remain poorly responsive to DNA synthesis stimulation by AVP (Figure 5A). This last observation implies that AVP−V1a receptors lack intrinsic potential to trigger a strong mitogenic response in Y1 adrenocortical cells.

We have previously reported that ACTH blocks G1 phase progression, triggering concerted antimitogenic mechanisms mediated by the cAMP/PKA pathway and characterized by, first, rapid dephosphorylation/deactivation of Akt/PKB (*8*) and degradation of the c-Myc protein (*9*) and, second, a late increase in the levels of the CDK inhibitor $p27^{Kip1}$ (*7*). In addition, ACTH does not interfere, positively or negatively, with the expression of cyclin D1. Furthermore, ectopic expression of cyclin D1 renders Y1 cells resistant to growth inhibition by AVP, but not by ACTH (T. T. Schwindt and H. A. Armelin, unpublished results). Thus, ACTH (*7*−*9*) and AVP block cell cycle progression in Y1 adrenocortical cells at the G1 phase by entirely different mechanisms. Although we do not know exactly how AVP blocks expression of the cyclin D1 gene, transcription is very likely inhibited and, perhaps, degradation of mRNA is also promoted (Figure 7).

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