Mediators of the mitogenic action of human V₁ vascular vasopressin receptors

MARC THIBONNIER, DOREEN M. CONARTY, AND CHRISTINE L. PLESNICHER

Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4951

Received 8 October 1999; accepted in final form 13 June 2000

The intracellular mediators of the mitogenic action of V₁R, signaling pathways that remain to be identified. To explore cell growth and proliferation in different tissues via cellular

10900 Euclid Ave., Cleveland, OH 44106-4951 (E-mail: mxt10@cwr.edu).

Materials of the mitogenic action of human V₁ vascular vasopressin receptors. Am J Physiol Heart Circ Physiol 279: H2529–H2539, 2000.—Arginine vasopressin (AVP) activation of V₁ vascular receptors (V₁Rs) stimulates cell growth and proliferation in different tissues via cellular signaling pathways that remain to be identified. To explore the intracellular mediators of the mitogenic action of V₁R, Chinese hamster ovary (CHO) cells were stably transfected with the human V₁R cDNA clone we isolated previously. We assessed AVP effects on kinase activation (immunoblotting with phosphospecific antibodies), DNA synthesis (tritiated thymidine uptake), cell cycle progression (flow cytometry analysis after nuclear labeling with propidium iodide), and cell proliferation (conversion of the colorimetric reagent MTS) in the presence or absence of various pathway inhibitors. AVP stimulation of V₁Rs leads to the phosphorylation of several kinases, an increase in DNA synthesis, a progression through the S and G₂–M phases of the cell cycle, and an increase in cell proliferation. The mediators of the mitogenic action of V₁R activation included calcium mobilization, coupling to a G₉ protein, and the simultaneous and parallel activation of several kinases, mainly calcium/calmodulin-dependent kinase II, phosphatidylinositol 3-kinase, protein kinase C, and p42/p44 mitogen-activated protein kinase.

kinase activation; cell cycle; cell proliferation; arginine vasopressin

THE NEUROHYOPHYSICAL PEPTIDE HORMONE arginine vasopressin (AVP) is involved in numerous physiological functions, including the regulation of body fluid osmolality, blood volume, vascular tone, and blood pressure (20). In addition, AVP belongs to the family of vasoregulatory and mitogenic peptides involved in physiological and pathological cell growth and differentiation (30). AVP exerts its actions through binding to specific V₁ vascular (V₁R), V₂ renal (V₂R), and V₃ pituitary membrane receptors coupled to distinct G proteins and second messengers (17, 23). V₁R expressed in vascular smooth muscle cells, the liver, and the testes is the product of the same gene undergoing identical splicing (27). V₁R is also expressed in several tissues or organs, including blood platelets, the adrenal cortex, the kidneys, the reproductive organs, the spleen, adipocytes, the brain, and various cell lines (3T3, A10, WRK-1, and A7r5). V₁R activation leads to a mitogenic response in vascular smooth muscle cells, 3T3 cells, renal mesangial cells, hepatocytes, and adrenal glomerulosa cells. These mitogenic responses are specifically blocked by V₁R antagonists of peptide and nonpeptide natures (19). The G proteins coupled to V₁R are mainly members of the G₁₁ family but also of the G₁ family, because some of the signals activated by V₁R stimulation (e.g., phospholipase A₂ activation) are partly reduced by pertussis toxin pretreatment (22). Recently, it was reported (6, 18) in rat vascular smooth muscle cells and 3Y1 fibroblasts that the stimulation of V₁R led to the activation of the mitogen-activated protein (MAP) kinase (MAPK) pathway. The activation of MAPK is pertussis toxin-insensitive, via protein kinase C (PKC)-dependent and independent pathways. The latter seems to involve the phosphatidylinositol 3-kinase (PI3K) (18). Similarly, we (25) recently observed that activation of the human V₁R led to the stimulation of the p42/p44 MAPK pathway.

In mammalian cells, the effects of mitogenic hormones on cell growth, hypertrophy, proliferation, and differentiation are mediated by various sets of cellular pathways, including several kinases (4). Although AVP receptors do not have intrinsic tyrosine kinase activity, they can activate kinases whose nature and role remain to be delineated. Because limited information is available about the pathways mediating the mitogenic action of AVP, we explored that issue in a mammalian cell line stably transfected with human V₁R. We observed that the mitogenic action of V₁R activation requires the mobilization of calcium and the simultaneous and parallel activation of several kinases leading to the stimulation of DNA synthesis, progression through the cell cycle, and cell proliferation.

MATERIALS AND METHODS

Materials. Standard reagents, including AVP ([Arg⁸]-vasopressin), unless otherwise stated were from Sigma Chemical (St. Louis, MO). The nonpeptide V₁R antagonist

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
SR-49059 and the nonpeptide V2R antagonist SR-121463A were provided by Dr. C. Serradeil-Le Gal (Sanofi Recherche, Toulouse, France). Chinese hamster ovary (CHO-K1) cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media and genetin were from Gibco-BRL (Grand Island, NY). Fetal bovine serum (FBS) were obtained from the American Type Culture Collection (Toulouse, France). Chinese hamster ovary (CHO)-K1 cells were selected for their resistance to the an
tibiotic geneticin (400 μg/ml) and identified by radioligand binding with SR-49059 and the nonpeptide V2R antagonist SR-121463A. The human V1R cDNA clone we isolated previously (22) was inserted into the pCI-neo mammalian expression vector. Stable transfection of this cDNA clone into CHO cells was performed using the CaPO4 precipitation method. Pure CHO-V1 clones were selected for their resistance to the antibiotic genetin (400 μg/ml), purified by the limiting dilu-
tion technique, and identified by radioligand binding with [3H]AVP.

Radioligand-binding assays. CHO-V1 cells were grown to subconfluence in 24-well dishes and washed twice with PBS plus 10 mM MgCl2 plus 0.2% BSA (pH 7.4). Saturation-binding experiments of AVP receptors of transfected CHO cells were performed in duplicate for 30 min at 30°C in 250 μl of PBS plus 10 mM MgCl2 plus 0.2% BSA (pH 7.4), with increasing concentrations of [3H]AVP ± 1 μM unlabeled AVP. The cells were washed three times with ice-cold PBS and lysed with 250 μl of 0.1 N NaOH/0.1% sodium dodecyl sulfate. Cell-bound [3H]AVP was counted by liquid scintillation spectrometry (Beckman counter LS 5801, yield 64%). The affinity (dissociation constant [Kd]) and capacity (maximum number of binding sites [Bmax]) of the AVP receptors were calculated by a nonlinear least square analysis program (26). Protein concentration was measured with Pierce's bicin-
chonic acid (BCA) reagent using albumin as an internal standard.

Kinase phosphorylation. To assess the effect of V1R stim-
ulation on various kinases activation, CHO-V1 cells were grown in F-12 medium plus 15 mM HEPES and 10% FBS at 37°C in 100-mm dishes until subconfluence and then serum starved for 72 h in F-12 medium plus 15 mM HEPES and 0.1% BSA. After cells were washed twice with PBS plus 10 mM MgCl2 (pH 7.4), they were stimulated by 1 μM AVP for up to 60 min. Stimulation with 10% FBS for 30 and 60 min was used as a positive control. Thereafter, the cells were washed twice with ice-cold PBS plus 50 mM sodium fluoride and scrapped into radioimmunoprecipitation assay buffer (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium vanadate, 50 mM sodium fluoride, 5 mM EDTA, 1 mM phenyl-
methylsulfonyl fluoride, 100 μl/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor). After lysates were incubated on ice for 20 min, they were centrifuged at 15,000 g for 10 min, and the supernatants were saved. Equal amounts of proteins, measured by Pierce's BCA reagent, were mixed with two times Laemmlis's buffer (final concen-
tration 1 mg/ml), and 25-μl aliquots were electrophoresed in 8% SDS-polyacrylamide gels. Proteins were transferred onto PVDF membranes using a semidyed transblot apparatus (Bio-
Rad, Richmond, CA). Uniformity of protein transfer was verified by reprobing the blots with an anti-actin antibody and by colloidal gold staining (Bio-Rad) of the membranes once immunoblotting experiments were completed. The membranes were blocked with 5% nonfat milk in Tris buffered saline (TBS) with Tween 20 (TBST) [20 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.1% Tween 20] overnight at room temperature. The membranes were washed three times in TBST and then incubated for 2 h at room temperature in TBS plus 3% BSA with the primary antibodies at the dilution recommended by the manufacturer. Pro-
tein-bound antibodies were detected by incubation of the membranes with horseradish peroxidase-labeled secondary antibody and an enhanced chemiluminescence detection system (Renaissance kit from NEN Life Science Products). Quantification of kinase phosphorylation was performed by scanning densitometry of the autoradiograms with a SciScan 5000 automated scanning system (USB, Cleveland, OH).

DNA synthesis. Subconfluent monolayer cultures of CHO-V1 cells were grown in F-12 medium plus 15 mM HEPES and 10% FBS at 37°C for 48 h in 24-well plates to measure thymidine uptake as an index of DNA synthesis in the presence of AVP, as described before (25). Cells were washed in 500 μl of F-12 medium and grown for 72 h in 500 μl of F-12 medium plus 15 mM HEPES and 0.1% BSA. Thereafter, AVP (final concentrations 10-12–10-8 M) was added alone or in the presence of various inhibitors, and, 15–18 h later, 0.5 μCi of [3H]thymidine was added for 45 min. The cells were transferred on ice, washed twice with 0.5 ml of ice-cold PBS, fixed with 1 ml of ice-cold 10% TCA for 30 min at 4°C, washed twice with 1 ml of ice-cold 5% TCA solution, and solubilized with 250 μl of 0.1 N NaOH-0.1% SDS. Aliquots were collected, and radioactivity was counted in a scintillation counter.

Flow cytometric analysis. Subconfluent cultures of CHO-V1 cells were grown in F-12 medium plus 15 mM HEPES and 10% FBS at 37°C for 48 h in 100-mm dishes to assess cell cycle progression in the presence of AVP. Cells were washed twice with PBS and grown for 5 days in F-12 medium plus 15 mM HEPES and 0.1% BSA. Thereafter, AVP (final concentration 10-8 M) was added alone or in the presence of various inhibitors, and, 24 h later, the cells were harvested. Flow cytometric analysis was performed after labeling the nuclei with propidium iodide. Propidium iodide was excited with an air-cooled argon-ion laser operating at 15 mW at 488 nm (EPICS XL-MCL, Coulter, Miami, FL). Linear and peak propidium iodide fluorescence was collected with a 620-nm band pass (620 BP) optical filter. Linear and peak fluorescence were plotted as bivariate parameters to form a doublet discriminator, which was used to estimate the single cell, cell aggregate, and apoptotic cells in the sample. System II software was used to
acquire the data, and ModFIT 5.2 (Verity Software House, Topsham, ME) was used to model cell cycle phases.

Cell proliferation. Cell proliferation was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega), on the basis of the cellular conversion of the colorimetric reagent MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] into soluble formazan by dehydrogenase enzymes found only on metabolically active, proliferating cells. Subconfluent monolayer cultures of CHO-V1 cells, grown in 96-well plates, were washed with F-12 medium and grown for 72 h in 200 μl of F-12 medium supplemented with 15 mM HEPES and 0.1% BSA. Various inhibitors were added at time 48 h. Thereafter, increasing concentrations of AVP were added for 24 h, followed by incubation with 20 μl of dye solution for 2 h. Subsequently, absorbance was recorded at 490 nm wavelength using an ELISA plate reader (reference wavelength 650 nm). Values recorded for control conditions (cells not stimulated by AVP) were subtracted.

Data analysis. Each experiment was performed three times unless indicated otherwise. Data are expressed as means ± SE, and statistical analysis was performed with the use of Statview software (Abacus Concepts).

RESULTS

Radioligand binding characteristics of human AVP V1R clone expressed in CHO cells. We (22) have previously shown that control CHO-K1 cells do not express endogenous AVP receptors, which could have interfered with our studies of human AVP receptors. We verified that there was no specific binding in cells transfected with the pCI-neo vector alone (data not shown). Saturation binding experiments with [3H]AVP were performed in intact CHO-V1 cells stably transfected with the human V1R cDNA. Nonspecific binding was negligible in intact transfected CHO-V1 cells. A single class of high-affinity binding sites was present in CHO-V1 cells (Kd, 0.54 ± 0.02 nM; Bmax, 23,808 ± 1,183 fmol/mg of protein).

AVP-induced kinase activation. When an agonist binds to G protein-coupled receptors (GPCRs), several kinases have been shown to be activate and translocate to the nucleus, where they activate nuclear transcription factors involved in DNA synthesis and cell division (4, 30). The MAPK cascades are among the cellular kinases that have been shown to modulate cellular proliferation and hypertrophy. Three MAPK cascades have been characterized in mammalian cells, i.e., the p42/p44, p38, and SAPK/JNK MAP kinases. Whereas the stimulation of the V1R vascular AVP receptor activates the p42/p44 MAPK pathway (25), there is no information about the potential activation of the other two MAPK cascades by AVP. Therefore, we looked at AVP stimulation of these three MAPK cascades in CHO cells stably transfected with V1R. As shown in Fig. 1, AVP stimulation of V1R led to a significant (3- to 6-fold), rapid, and sustained phosphorylation of the p42/p44 MAPK but also, to a lesser degree, the p38 MAPK (2- to 3-fold increase). The effect of AVP on SAPK/JNK MAPK was negligible and transient but so was the effect of 10% FBS in CHO cells. We also examined which other kinases could be phosphorylated by activation of human V1R. The p70 S6 kinase, which is downstream of PI3K, was strongly phosphorylated by AVP stimulation (36-fold increase), albeit after a slower time course than the p42/p44 kinase. STAT1 and STAT3 were also phosphorylated after AVP stimulation with a different time course: STAT1 phosphorylation occurred only after 30 min of exposure to AVP, whereas STAT3 phosphorylation took place within 10 min of AVP stimulation. Finally, calcium/calmodulin kinase II was also phosphorylated (3-fold increase) after AVP stimulation of V1R with a peak at 40 min after AVP stimulation.

Immunoblots were also performed after cell stimulation by 1 μM AVP alone or in the presence of various kinase inhibitors to verify the selectivity of these compounds. As shown in Fig. 2, the MEK1 inhibitor PD-98059 (10 μM) selectively reduced the AVP effect on p42/p44 MAPK phosphorylation without altering the phosphorylation of the other kinases. The p38 MAPK inhibitor SB-20358 (10 μM) reduced AVP-induced phosphorylation of p38 kinase but also JNK phosphorylation. This compound was recently shown to inhibit both p38 MAPK and JNKs (3). The PI3K inhibitor wortmannin (100 nM) significantly reduced p70 S6 kinase phosphorylation, thus confirming that p70 S6 kinase is directly downstream of PI3K. A mild reduction of AVP-induced phosphorylation of p42/p44 MAPK was also noted in the presence of wortmannin. Finally, the calcium/calmodulin-dependent protein kinase II inhibitor KN-93 (10 μM) specifically and selectively blocked AVP induced phosphorylation of calcium/calmodulin-dependent protein kinase II. The PKC inhibitor bisindolylmaleimide I, the JAK2 phosphotyrosine kinase inhibitor AG-490, and the protein kinase A inhibitor H-89 did not alter the phosphorylation pattern of p42/p44, p38, phosphorylated JNK, p70, and calcium/calmodulin-dependent kinase II.

AVP stimulation of DNA synthesis in CHO cells expressing human AVP V1R. Kinase phosphorylation and translocation to the nucleus leads to increased nucleic acid synthesis that can be assessed by measurement of [3H]thymidine uptake. Thus AVP-induced [3H]thymidine uptake was measured in CHO cells stably transfected with V1R cDNA. In these CHO-V1R cells, 10% FBS produced a significant stimulation of [3H]thymidine uptake (+470%; data not shown). AVP also stimulated [3H]thymidine uptake in CHO-V1 cells (+339%; Fig. 3). The AVP-induced increase in [3H]thymidine uptake was dose dependent (EC50, 1.48 nM) and specifically blocked by the nonpeptide V1R antagonist SR-49059. In addition, the effect of AVP on DNA synthesis was blocked by a Gq protein antagonist, whereas a G12/13 protein antagonist did not alter AVP effect on [3H]thymidine uptake (Fig. 3).

The intracellular pathways mediating the action of AVP on DNA synthesis were explored by adding various inhibitors to the [3H]thymidine uptake assay. First, we assessed the role of calcium and calcium-dependent pathways in the mediation of AVP action on DNA synthesis, because calcium mobilization is a typical initial feature of V1R activation. As shown in Fig. 4A, calcium is absolutely required for AVP stimulation.
of [³H]thymidine uptake because calcium chelation by EGTA abolished the AVP effect. Furthermore, the calcium/calmodulin-dependent protein kinase II is involved in the process: the calmodulin antagonist W-7, used at a concentration close to its IC₅₀ for displacement of tritiated W-7 (31 µM), reduced the effect of AVP on [³H]thymidine uptake by 46%. Finally, KN-93, a selective and potent inhibitor of calcium/calmodulin-
dependent protein kinase II, used at a concentration close to its inhibitor constant, also reduced the AVP effect on [3H]thymidine uptake in a dose-dependent fashion.

We subsequently examined the role of other kinase pathways in the mediation of AVP action on DNA synthesis, and it appears that several kinase pathways are involved. As shown in Fig. 4A, the cell-permeable PKC inhibitor bisindolylmaleimide I, used at a concentration close to its IC50 (2 μM), reduced the AVP action by 32%. Similarly, the selective MEK1 inhibitor PD-98059, used at a concentration close to its IC50 (2 μM), reduced the AVP action by 34%. No additive inhibition was noted by blocking simultaneously PKC and MEK1 by bisindolylmaleimide I and PD-98059 (data not shown), thus suggesting that the PKC effect is mediated by the MAPK pathway. The selective and cell-permeable PI3K inhibitor wortmannin, used at a concentration close to its IC50 (200–300 nM), reduced the AVP effect on DNA synthesis by 96%. Simultaneous inhibition of the PKC/MEK1 and PI3K pathways completely abolished the effect of AVP on DNA synthesis (data not shown). The p38 MAP kinase blocker SB-203580, used at a concentration close to its IC50 (1 μM), reduced the AVP action on DNA synthesis by 68%. The specific JAK2 phosphotyrosine kinase inhibitor AG-490 reduced AVP action by 77%, whereas the protein kinase A inhibitor H-89, used at a concentration that inhibits PKA but not PKC or calmodulin kinase II, decreased the AVP effect on [3H]thymidine uptake by only 27%.
**Cell cycle progression.** To study the effect of AVP on cell cycle progression, flow cytometry analysis was performed in CHO-V1 cells after AVP stimulation for 24 h. The results of a typical experiment are shown in Fig. 5. In control conditions, most (79%) of the serum-starved cells were arrested in the G0–G1 phases of the cell cycle, with only 5% of cells in the S phase and 7% of cells in the G2–M phases (Table 1). AVP stimulation of V1R produced a significant increase of the proportion of cells that progressed through both the S and G2–M phases with a simultaneous reduction of the percentage of cells in the G0–G1 phases. The fraction of apoptotic cells was small in control conditions (6%) and was not altered by AVP stimulation of V1R. The effect of AVP on cell cycle progression was specifically blocked by the nonpeptide V1R antagonist SR-49059 but not by the nonpeptide V2R antagonist SR-121463A (Table 1).

### Table 1. Effect of AVP on cell cycle progression

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis (%)</th>
<th>G0–G1 (%)</th>
<th>S (%)</th>
<th>G2–M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 ± 1</td>
<td>79 ± 5</td>
<td>5 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>AVP</td>
<td>5 ± 1</td>
<td>64 ± 2a</td>
<td>16 ± 1a</td>
<td>16 ± 1a</td>
</tr>
<tr>
<td>AVP + SR-49059</td>
<td>6 ± 1</td>
<td>78 ± 5</td>
<td>8 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>AVP + SR-121463A</td>
<td>5 ± 1</td>
<td>67 ± 2a</td>
<td>16 ± 1a</td>
<td>17 ± 1a</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. The percentage of Chinese hamster ovary (CHO) stably transfected with the V1 vascular receptor (CHO-V1 cells) within each phase of the cell cycle is shown after 24 h of stimulation with arginine vasopressin (AVP; 10 nM) or in the presence of the nonpeptide V1 receptor antagonist SR-49059 (1 µM) or the nonpeptide V2 receptor agonist SR-121463A (1 µM). *P < 0.0001 when compared with the corresponding cell cycle phase in the control condition.

The intracellular pathways mediating the action of AVP on cell cycle progression were explored by adding various inhibitors to the flow cytometric assay. As shown in Table 2, chelation of calcium by EGTA completely abolished the AVP action on cell cycle progression while inducing a dramatic increase of the percentage of apoptotic cells. The calmodulin antagonist W-7 also increased the percentage of apo-

### Table 2. Effect of various inhibitors on AVP effect on cell cycle progression

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cell Cycle Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>APV (10 nM)</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>EGTA (4 mM) + AVP</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>KN-93 (10 µM) + AVP</td>
<td>33 ± 6a</td>
</tr>
<tr>
<td>W-7 (50 µM) + AVP</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Bisindolylmaleimide I</td>
<td>13 ± 1a</td>
</tr>
<tr>
<td>Bisindolylmaleimide I + PD-98059 + AVP</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>Wortmannin (300 nM) + AVP</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Bisindolylmaleimide I + PD-98059 + Wortmannin</td>
<td>25 ± 1a</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. The percentage of CHO-V1 cells within each phase of the cell cycle is shown after 24-h stimulation by AVP alone (10 nM) or in the presence of various inhibitors. *P < 0.05 when compared with the corresponding cell cycle phase in the presence of AVP alone.
optotic cells. The PI3K inhibitor wortmannin also reduced the AVP action on cell cycle progression. Further blockade of the AVP action on cell cycle progression and increase of the apoptotic fraction was produced by the simultaneous blockade of PKC, MEK, and PI3K. The p38 kinase inhibitor SB-203580 and the protein kinase A inhibitor H-89 had no effect on the AVP action on cell cycle progression. Interestingly, the JAK2 inhibitor AG-490 blocked AVP-induced progression from the S phase to the G2-M phases of the cell cycle.

**AVP stimulation of cell proliferation in CHO cells expressing V1R.** We directly explored the effect of AVP on cellular proliferation by using the MTS colorimetric assay in CHO cells stably transfected with the human V1R. As shown in Fig. 6, AVP produced a dose-dependent stimulation of CHO-V1 cell proliferation (EC50 1.10 nM), which was specifically blocked by the non-peptide V1R antagonist SR-49059 (data not shown).

The intracellular pathways mediating the action of AVP on cell proliferation were explored by adding various inhibitors to the cell proliferation assay. First, we examined the role calcium might play in the mediation of AVP mitogenic action. As shown in Fig. 7A, chelation of extracellular calcium by EGTA completely abolished the AVP mitogenic action. AVP mitogenic action was also reduced in a dose-dependent fashion by the calmodulin antagonist W-7 and by the calcium/calmodulin-dependent protein kinase II inhibitor KN-93.

We subsequently examined the role of other kinase pathways in the mediation of AVP action on cell proliferation. Like DNA synthesis and cell cycle progression, several kinase pathways seem to be involved. As shown in Fig. 7B, the cell-permeable PKC inhibitor bisindolylmaleimide I reduced the mitogenic effect of AVP in CHO-V1 cells by 49%. Similarly, the selective MEK1 blocker PD-98059 reduced the action of AVP by 34%. No additive inhibition was noted by simultaneously blocking PKC and MEK1 by bisindolylmaleimide I and PD-98059, thus suggesting that PKC effect is mediated by the MAPK pathway. The selective and cell-permeable PI3K inhibitor wortmannin reduced the mitogenic effect of AVP by 51%, thus confirming the involvement of this kinase in the mitogenic action of AVP through activation of V1R. Simultaneous inhibition of PKC/MEK1 and PI3K pathways completely abolished the effect of AVP on cell proliferation. The protein kinase A inhibitor H-89 had little effect on the mitogenic effect of AVP (−16%). Finally, the p38 MAPK blocker SB-203580 and the specific JAK2 phosphotyrosine kinase inhibitor AG-490 did not modify AVP action on cell proliferation.

Fig. 6. AVP-induced proliferation of CHO cells transfected with the human V1R. Transfected CHO cells were grown to subconfluency in 96-well dishes and incubated in serum-free F-12 medium for 72 h. Thereafter, the cells were stimulated by AVP for 24 h, followed by addition of the dye MTS for 2 h. Absorbance was recorded at 490 nm (n = 8 for each series).

Fig. 7. A: effect of calcium chelation and CaM kinase inhibition on AVP-induced proliferation of CHO cells transfected with the human V1R. Transfected CHO cells were grown to subconfluence in 96-well dishes and incubated in serum-free F-12 medium for 72 h, and the inhibitors were added at time 48 h. Thereafter, the cells were stimulated by AVP for 24 h, followed by addition of the dye MTS for 2 h. Absorbance was recorded at 490 nm (n = 8 for each series). *P < 0.05; **P < 0.01; ***P < 0.001. B: effect of various kinase inhibitors on AVP-induced proliferation of CHO cells transfected with the human V1-vascular AVP receptor. Transfected CHO cells were grown to subconfluence in 96-well dishes and incubated in serum-free F-12 medium for 72 h. The inhibitors were added at time 48 h. The cells were stimulated by AVP for 24 h, followed by addition of the dye MTS for 2 h. Absorbance was recorded at 490 nm (n = 8 for each series). *P < 0.05; **P < 0.01; ***P < 0.001.
DISCUSSION

AVP not only modulates blood pressure and blood volume through its vasoconstrictor and antidiuretic properties but also alters cell growth and proliferation. As a matter of fact, AVP has been shown to elicit mitogenic responses, including stimulation of DNA synthesis, expression of oncogenes, and cell proliferation in different cell types (5, 21). These mitogenic effects of AVP are triggered by the activation of the \( V_1 \) vascular receptor, which belongs to the superfamily of seven membrane-spanning GPCRs. Typically, the \( V_1 \) vascular AVP receptor activates phospholipase \( C_\beta \) through the heterotrimERIC \( G_q \) protein, causing production of inositol trisphosphate and diacylglycerol. Although GPCRs have no intrinsic tyrosine kinase activity, agonist activation of GPCRs triggers the recruitment of various cytosolic tyrosine kinases (2, 7).

To further understand the pathways mediating AVP mitogenic properties, we utilized in this study CHO cells stably transfected with human \( V_1 \)R. We (24) reported previously that AVP effect on DNA synthesis (appreciated by measurement of thymidine uptake) was a function of the receptor subtype expressed by the same cell type. Whereas a significant increase in DNA synthesis was observed in cells transfected with human \( V_1 \)R or \( V_3 \) pituitary AVP receptors, a reduction of thymidine uptake was noted in cells transfected with human \( V_2 \) renal AVP receptors. Thus structurally close-related GPCR subtypes can elicit opposite effects on DNA synthesis within the same cellular phenotypical environment, presumably in relation to distinct \( G \) protein coupling (\( G_\beta \) for the \( V_2 \) renal receptor vs. \( G_i \) and \( G_q \) for the \( V_1 \) vascular and \( V_3 \) pituitary receptors). The involvement of \( G_q \) in mediating the mitogenic action of \( V_1 \)R was confirmed in this study by the inhibitory effect of the \( G_q \) protein antagonist on AVP-induced thymidine uptake, whereas the \( G_i \) protein antagonist was ineffective. At the level of \( V_1 \)R expression in the clone used in the present study, we did not observe any promiscuous coupling of other \( G \) proteins besides \( G_q \) in the mediation of the mitogenic signal, a phenomenon that might occur at very high levels of receptor expression (28). AVP stimulation of DNA synthesis after occupancy of the human \( V_1 \)-vascular receptor is physiologically relevant because the observed \( EC_{50} \) value in the thymidine uptake assay is in agreement with the \( K_d \) measured in ligand binding experiments.

In the present study, we used a four-pronged approach to tease out the intracellular pathways mediating the mitogenic properties of human \( V_1 \)R, i.e., the assessment of kinases phosphorylation, DNA synthesis, cell cycle progression, and cell proliferation after AVP stimulation. From our observations, one can conclude that several intracellular mediators are involved in the transmission of the mitogenic effect of the AVP \( V_1 \) vascular receptor. Not unexpectedly, calcium is absolutely required because its chelation abolishes AVP actions. Involvement of the calcium/calmodulin-dependent protein kinase II in the process is demonstrated by the experiments done in the presence of the calmodulin antagonist W-7 and the calcium/calmodulin-dependent kinase II inhibitor KN-93. In addition, calcium is certainly instrumental in the activation of other pathways, such as PKC, p42/p44 MAPK and JNK, because it was shown for another vasoactive and mitogenic peptide, angiotensin II (29, 35).

Besides calcium and calcium/calmodulin-dependent kinase II, other pathways are involved in the mediation of the AVP mitogenic action. Our present data confirm that AVP stimulation of human \( V_1 \)R activates the p42/p44 MAPK pathway. This is demonstrated by the inhibition of AVP stimulation of DNA synthesis, cell cycle progression, and cell proliferation by the selective MEKI inhibitor PD-98059 as well as by the direct evidence of p42/p44 MAPK phosphorylation after AVP stimulation. The inhibitory effect of PD-98059 is only partial, thus suggesting the implication of additional and parallel pathways mediating the AVP mitogenic action. Furthermore, it appears that AVP activates p42/p44 MAPK via PKC because the simultaneous blockade of PKC by bisindolylmaleimide I and MEKI1 by PD-98059 did not inhibit further AVP mitogenic action compared with the inhibitory effect of each compound tested alone. Distinct pathways of \( G_i \) and \( G_q \)-mediated MAPK activation have been reported (10). In the case of \( G_i \)-coupled receptors, MAPK activation seems to be pertussis toxin sensitive and PKC independent, presumably via PI3K stimulation (9). At variance, in the case of \( G_q \)-coupled receptors (including the AVP \( V_1 \)R), MAPK activation is secondary to phospholipase C-PKC stimulation. Similarly, angiotensin II activation of p42/p44 MAPK occurs via PKC (1, 29, 32).

Thus p42/p44 MAPK can be activated by various members of the GPCR superfamily via different pathways. Moreover, it appears that there is a significant feedback mechanism between p42/p44 MAPK and GPCRs because angiotensin II phosphorylation of the neuronal angiotensin I receptor was shown to involve MAPK (33).

Phosphorylation of p38 MAPK by AVP and reduction of AVP-induced thymidine uptake by the p38 kinase inhibitor SB-203580 in CHO cells transfected with the human \( V_1 \) vascular receptor suggest that the p38 MAPK pathway is activated by AVP. However, the lack of inhibition of AVP-induced cell cycle progression and cell proliferation by SB-203580 implies that this kinase is not involved in mediating the AVP final mitogenic action. As a matter of fact, p38 MAPK has been implicated in cellular apoptosis (8, 13). Recently, p42/p44 and p38 kinases and JNK have been shown to exert opposite effects on apoptosis of cultured cardiac myocytes: p38 kinase and JNK mediate apoptosis, whereas p42/p44 kinase plays a protective role (34). Thus one may hypothesize that AVP stimulates simultaneously survival and apoptotic pathways, but, in the case of \( V_1 \)R, the survival pathways activation prevail.

In the present study, we also looked at the possible activation of the SAPK/JNK MAP kinase pathway by AVP. The immunoblotting experiments performed with the phosphospecific SAPK/JNK antibody showed a weak and transient activation of this kinase pathway.
by AVP. However, in the absence of specific inhibitors of this kinase, it is premature to conclude about the role played by this kinase in mediating AVP mitogenic action. The effect of angiotensin II to activate JNK was studied by Nadler and co-workers (31) in CHO cells overexpressing the rat vascular type 1 angiotensin II receptor. These authors observed that angiotensin II stimulated JNK activity with a peak at 30 min and that JNK activation by angiotensin II was mediated by a G₁ protein and the lipoxygenase pathway.

Our studies revealed that another kinase pathway, i.e., PI3K, mediates AVP mitogenic actions. Indeed, the PI3K inhibitor wortmannin, used at a low concentration of 100 nM, did reduce AVP effect on DNA synthesis, cell cycle progression, and cell proliferation. Activation of PI3K by AVP seems to occur in parallel to the activation of the PKC-p42/p44 MAPK pathway because the simultaneous presence of wortmannin and bisindolylmaleimide I or PD-98059 reduced further the effects of AVP compared with the inhibitory action of each compound alone. The p42/p44 MAPK and PI3K pathways can be activated in parallel or in sequence as a function of the type of GPCR involved. For instance, PI3K mediates G₁₁,₂-dependent regulation of the MAPK signaling pathway after stimulation of the m₁ muscarinic receptor in COS-7 cells (14). In NIH3T3 cells, stimulation of the α₁A-adrenergic receptor activates PI3K and p42/p44 MAPK in parallel, whereas for the α₁B-adrenergic receptor, PI3K is critical for activation of p42/p44 MAPK (11). In the same cell line, the α₁D-adrenergic receptor does not stimulate PI3K. In our study, the simultaneous blockade of the p42/p44 MAPK and PI3K pathways is required for complete inhibition of AVP-induced cell proliferation, thus suggesting that these two pathways function in parallel rather than in sequence in response to AVP receptor activation.

In our study, we directly assessed by flow cytometry the effect of AVP stimulation of V₁R on cell cycle progression. We found that V₁R activation led to complete progression of cells through both S and G₂–M phases. This progression through the full cell cycle is in agreement with the positive effect of AVP on cell proliferation. Progression through the cell cycle and cell proliferation triggered by AVP stimulation are mediated by the simultaneous and parallel activation of several kinase pathways, mainly calcium/calmodulin kinase II, PI3K, and PKC-p42/p44 MAPK. As a matter of fact, several signal transduction pathways have been implicated in the regulation of cell survival and apoptosis, and receptor agonists may activate several signal transduction pathways with opposite effects on cell growth (8). It appears that both the PI3K and the p42/p44 MAPK pathways favor cell survival, whereas p38 kinase and SAPK/JNK are proapoptotic. On the basis of these facts and our findings, one may conclude.

Fig. 8. Schematic representation of the proposed intracellular pathways mediating the mitogenic effect of V₁R. PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; MEK1/2, MAP or extracellular signal-regulated kinase 1 or 2; MAPK, mitogen-activated protein kinase; PI3 kinase, Ca₃, intracellular calcium.
that AVP stimulation of V1R will support cell survival instead of cell death. Indeed, direct assessment of the effect of V1R stimulation on cell cycle progression clearly demonstrates a mitogenic effect and no apoptotic transition.

Recent studies (12, 15, 16) have revealed that not only growth factors but also vasoactive and mitogenic peptide hormones can stimulate the JAK/STAT pathway. In our study, the stimulation of STAT1 and STAT3 phosphorylation by AVP, as well as the reduction by the JAK2 inhibitor AG-490 of AVP stimulation of DNA synthesis and progression to the G1–M phases of the cell cycle, suggest that the JAK/STAT pathway is involved in mediating AVP actions. Similarly, AG-490 has been shown to reduce thymidine incorporation and JAK2 tyrosine phosphorylation in response to angiotensin II or platelet-derived growth factor (15). However, we observed that AVP stimulation of cell proliferation was not altered by AG-490, which may indicate that MAPK and PI3K are the main pathways mediating the mitogenic action of AVP in a fibroblast cell line, whereas the JAK/STAT pathway seems to play a secondary role.

The limited inhibitory effect of the protein kinase A inhibitor H-89 on AVP mitogenic action suggests that the cAMP/protein kinase A pathway does not play a significant role in this regard, a finding that should be expected for a receptor that is not coupled to Gαs.

In summary, we demonstrated that AVP stimulation of V1R elicits the stimulation of DNA synthesis, progression through the cell cycle, and cell proliferation. Only the pathways that could be consistently blocked in the various functional assays we tested were deemed to be of physiological significance. Thus we concluded that the trophic effect of AVP occurred mainly through the simultaneous and parallel activation of calcium/calmodulin kinase II, PI3K, and PKC-p42/p44 MAPK in a calcium-dependent manner. The main intracellular pathways coupled to the activation of V1R are depicted in Fig. 8.

This work was supported by National Heart, Lung, and Blood Institute Grants RO1 HL-39757 and PO1 HL-41618. The Ireland Cancer Research Center flow cytometry core facility was supported by National Institutes of Health Grant P30 CA-43703.

REFERENCES


