[Frontiers in Bioscience 3, d194-207, February 15, 1998]

VASOPRESSIN SIGNALING PATHWAYS IN VASCULAR SMOOTH MUSCLE

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Received 1/21/98 Accepted 2/4/98

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1. ABSTRACT

Arginine vasopressin (AVP) exhibits both acute and long-term effects on vascular smooth muscle cells (VSMC). Acutely, AVP regulates vascular tone and stimulates contraction. Longer term exposure of VSMC to AVP in the absence of other mitogenic agents results in cell hypertrophy without increases in cell number, and increased expression of a number of muscle-specific genes including the smooth muscle form of alpha-actin (SM-alpha-actin). These responses can be distinguished from the proliferative responses seen with growth factors such as platelet-derived growth factor (PDGF), which increase DNA synthesis and cell number and suppress SM-alpha-actin expression. In cultured VSMC, all the effects of AVP are mediated through the V1a receptor which signals through G-proteins. This review examines post-receptor signaling events mediated by AVP in VSMC. AVP rapidly increases intracellular Ca^{2+} via mobilization of intracellular stores and entry of extracellular Ca^{2+} via specific cation channels. This pathway, via activation of myosin light chain kinase, is critical for the early contractile response. Increased intracellular Ca^{2+} also leads to increased arachidonic acid release and eicosanoid production through the action of phospholipase A2. The activation of protein kinases by AVP is examined, focusing on members of the mitogen-activated protein kinase family. These enzymes are likely to play an important role in promoting growth of VSMC as well as modulating their state of differentiation through transcriptional control of muscle-specific gene expression. Recent studies suggesting a role for c-Jun amino terminal kinases in the regulation of smooth muscle-alpha-actin expression are described.

2. INTRODUCTION

Arginine vasopressin (AVP1) plays a major role in the regulation of body fluid volume and the maintenance of blood pressure. The biological effects of AVP are mediated through cell-surface receptors which have been divided into two classes. The renal receptor, designated V2, has been shown to be involved in water reabsorption, and is coupled to adenylyl cyclase. The vascular receptor has been designated V1a, and is expressed in vascular smooth muscle and liver. A third vasopressin receptor, designated V1b, appears to be selectively expressed in pituitary cells. All of these receptors have recently been cloned (1, 2, 3), and belong to the family of "seven membrane spanning" receptors which signal through G-proteins. Non-peptide antagonists have been developed which selectively block individual receptor isoforms (4, 5, 6, 7), allowing assessment of the contributions of these different receptors in different cell types. Following AVP binding, cells undergo homologous desensitization within minutes (8). AVP-receptors are internalized from the cell surface and recycled (9). The role of receptor internalization in AVP signaling remains controversial. Recent studies have indicated that receptor internalization of other receptors signaling through G-proteins is critical for activation of some signaling pathways (10). This has not to date been examined for the V1a receptor in VSMC.

In vascular smooth muscle, AVP has been shown to be a potent vasoconstrictor, both *in vivo*, and in cultured cell preparations. Increases in contractile force are observed within seconds to minutes following exposure to the hormone, and are mediated via the V1a receptor (11).

Perhaps less appreciated is the fact that long-term exposure of vascular smooth muscle cells (VSMC) to AVP promotes the growth of these cells and regulates patterns of gene expression. While it is highly likely that both the acute and the long-term effects are mediated through the V1 receptor, the G-proteins and downstream effectors leading to these pleiotropic responses have only recently begun to be identified. Numerous early signaling events have been described during the past several years. However, linking activation of specific pathways to distinct physiologic responses still represents "work in progress". The goal of this manuscript is to review our current knowledge of post-receptor signaling pathways known to be regulated by AVP in VSMC. The majority of the studies described have been performed in cultured vascular smooth muscle cells. We will briefly describe the physiologic effects of AVP in early passage cells as well as a number of established cell lines which are being used by investigators in this area. Subsequently specific effector systems will be examined. Where data exists, we will attempt to assign a physiologic role to these pathways in mediating the actions of AVP. However, a cautionary disclaimer should be added, which can be applied to other studies on signal transduction. *In vivo*, the responses of vascular smooth muscle cells will reflect a complex interaction from multiple inputs including circulating factors, extracellular matrix, and interaction with other cell types, such as endothelial cells. The work described in this review therefore represent a partial, but growing list of potentially important pathways whose role *in vivo* will need to be studied directly. It is also becoming apparent that VSMC within the vessel wall are not homogenous, and distinct subpopulations have been isolated (12). These cells may well exhibit heterogeneous response to AVP as well as other agonists, and information obtained from "standard" preparations of VSMC may need to be reexamined in phenotypically homogenous preparations.

3. PHYSIOLOGIC EFFECTS OF VASOPRESSIN IN SMOOTH MUSCLE

Cultures of vascular smooth muscle cells have been extensively employed as a model for studying the physiologic and pathophysiologic responses of vascular smooth muscle *in vivo* (13, 14). These preparations have been characterized by monitoring expression of smooth muscle specific genes such as the smooth muscle isoform of alpha-actin (SM- alpha-actin) or smooth muscle myosin (15). In general, freshly isolated primary cultures from adult vessels are slow proliferating and manifest features of highly differentiated vascular smooth muscle. These cells can be subcultured and retain many vascular smooth muscle features for multiple passages. However, eventually they will begin to grow more rapidly and lose many of the characteristics of the differentiated phenotype. In fact, by growing cells, we are selecting for proliferative phenotypes. Most workers have therefore performed experiments in early passage, using some arbitrary cutoff. Studies performed in our laboratory have generally used cells subcultured for less than 8 passages. Most workers using such preparations have isolated cells from large conductance vessels such as aorta. It is likely that VSMC isolated from resistance vessels will exhibit distinct physiologic responses.

Acute stimulation of early passage VSMC by AVP as well as angiotensin II results in contraction (16, 17, 18). Many groups have studied contraction in cultured cells by measuring shape change following agonist stimulation. While this is easily quantitated, the relationship of a change in shape of a cell attached to a plastic substratum to contraction of a vessel is not clear. Growth of VSMC on polymerized silicone-coated dishes allows measurement of contractile force by the appearance of wrinkles (19). However, this technique is more difficult to quantitate. Care also needs to be taken that growth of these cells on silicone dishes does not induce phenotypic changes in the cells, making it difficult to compare signaling studies done under standard tissue culture conditions.

Long-term exposure of VSMC to AVP in the absence of other mitogens, or in the presence of low concentrations of serum, results in an increase in protein content per cell, which we will define as hypertrophy (20, 21). This induction requires several days of continuous exposure to AVP, and is also observed with other vasoconstrictors such as angiotensin II (22). No increases in DNA synthesis as assessed by ^{3}H-thymidine incorporation into DNA is observed, and there is no increase in cell number. At higher concentrations of serum, or in the presence of other mitogens, AVP potentiates DNA synthesis and cell proliferation, acting as a progression factor. These results can be contrasted with exposure of VSMC to growth factors such as PDGF, which as a sole factor causes increases 3H-thymidine uptake into DNA and promotes mitogenesis, characteristic of a competence factor. We have recently examined the effects of AVP on progression of VSMC through the cell cycle using flow cytometry. AVP stimulation resulted in cells which accumulated in G1, and did not enter S phase[2].

Perhaps suprisingly, AVP and PDGF stimulate many of the same early post-receptor signaling events. These include increases in intracellular Ca^{2+}, activation of protein kinases and phospholipases, and induction of immediate early response genes. The factors mediating the distinct hypertrophic response seen with AVP versus the proliferative response of PDGF is therefore not clear. We propose three conceptual models to account for these findings (figure 1). One possibility is that AVP only gives a partial mitogenic signal. In this model, PDGF would engage some specific effector(s), critical for mitogenesis, which is not activated by AVP (figure 1A). Alternatively, it has been proposed that vasoconstrictors induce both a mitogenic and anti-mitogenic signal (figure 1B). To that end it has been shown that angiotensin II induces the expression of TGF-beta in VSMC, which has antimitogenic effects (23). The combination of the two signals (mitogenic and anti-mitogenic) would be predicted to result in hypertrophy of the cells. Finally, a third model would propose that vasoconstrictors such as AVP engage distinct signaling pathways not controlled by PDGF which lead to hypertrophy (figure 1C). We and others have recently

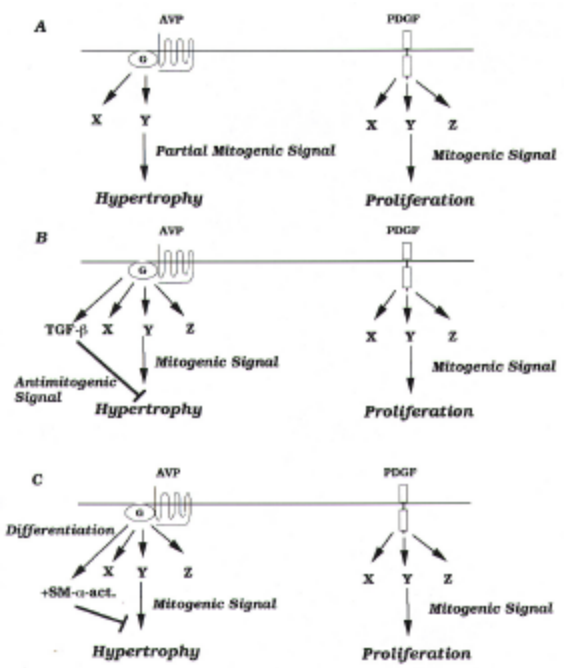

Figure 1. Models of AVP Action in VSMC. We propose three specific models to account for the hypertrophic response seen with AVP, as opposed to the proliferative response seen with PDGF. In the first model (Panel A), PDGF stimulates three hypothetical pathways (X,Y, and Z) required for proliferation. AVP, through a G protein only stimulates 2 of these (X and Y), leading to an incomplete mitogenic signal. In model 2 (Panel B), AVP stimulates the same three hypothetical mitogenic effectors as PDGF, but in addition also stimulates production of an anti-mitogenic agent, in this case TGF-b. The simultaneous engagement of mitogenic and anti-mitogenic pathways results in hypertrophy. In model 3 (Panel C), AVP stimulates a mitogenic response plus induces differentiation of the cells into a more highly differentiated contractile phenotype, as indicated by the induction of SM-alpha-actin (SM-alpha-act.). This combination of proliferation and conversion to a non-proliferative phenotype results in hypertrophy.

reported that AVP as well as angiotensin II increase the expression of muscle-specific genes such as the smooth muscle isoform of alpha-actin (SM-alpha-actin). This effect is mediated through increased transcription of this gene (see below). SM-alpha-actin has been used as a marker for the state of differentiation of VSMC (15). Developmentally, expression is low in embryonic and developing vessels, and increases as the vessels mature and the cells convert from a proliferative to a contractile phenotype. In the setting of atherosclerosis, VSMC undergo a "de-differentiation" to a more proliferative phenotype and this is associated with suppression of SM-alpha-actin expression. Importantly, exposure of VSMC to PDGF suppresses expression of SM-alpha-actin, and is able to inhibit induction in response to vasoconstrictors (24). Thus the regulation of SM-alpha-actin expression represents a physiologic action which is regulated in opposite directions by vasoconstrictors and PDGF. A simplistic model would propose that AVP or angiotensin II result in a mitogenic stimulation together with a signal promoting differentiation of the cells to a contractile, non-proliferative phenotype (High SM-alpha-actin), whereas PDGF results in a mitogenic signal combined with promotion to a proliferative phenotype (low SM-alpha-actin). Delineating the upstream signaling events regulating SM-alpha-actin expression may therefore provide a better understanding of the specific signaling pathways engaged by AVP, leading to hypertrophy. Finally, it should be emphasized that these three models proposed are not mutually exclusive. To that end it has been reported that TGF-beta induces hypertrophy in VSMC (25).

More recently a number of established cell lines derived from vascular smooth muscle cells by immortilization techniques have become available. These include the A7r5 and A10 cell lines. While these cells are responsive to AVP (26), and have been useful in defining early post-receptor events, care must be taken in extrapolating results obtained with these cell lines to the biology of vascular smooth muscle *in vivo*. In particular, we have noted that chronic effects of AVP observed in early passage rat aortic VSMC are not reproduced in either of these cell lines[2]. AVP-induced hypertrophy and induction of smooth muscle alpha-actin expression did not occur in either cell line. Furthermore, phenotypic modulation achieved by growing VSMC on specific extracellular matrices is also not achieved in these cells. Growth of early passage VSMC on Matrigel, an extracellular matrix preparation rich in laminin promotes conversion to the contractile phenotype (27), which is characterized by elevated SM-alpha-actin expression. Established cell lines fail to mimic this response. Thus data on specific signaling pathways obtained in these cell lines needs to be confirmed in a more "physiologic" preparation.

With the cloning of many signaling enzymes and effectors, a powerful technique to study post-receptor signaling has been the use of cell transfection experiments. Expressing mutations encoding either constitutively active or dominant-negative mutants of individual signaling molecules has been widely employed in other cell types such as fibroblasts. These studies enable investigators to examine the role of signaling pathways for which specific pharmacological agents are not currently available. The development of stable cell lines expressing foreign genes requires growth of individual clones in selectable media. This process entails many cell divisions, and raises the concern that transfection of VSMC will lead to dedifferentiation and loss of vascular smooth muscle features. In our studies we have successfully used cells transfected with a plasmid lacking a cDNA insert as a control for the transfection process (28, 29). As with all techniques, use of molecular biological approaches is not a panacea. Effects of expression of foreign genes in these cells can be antagonized by the cells, through activation of compensatory mechanisms. This problem can be addressed using both stable and transient expression of foreign genes, or by placing them under the control of inducible promoters.

4. POST-RECEPTOR SIGNALING PATHWAYS

4.1. Mobilization of intracellular Ca^{2+}

Exposure of VSMC to AVP rapidly results in increases in intracellular Ca^{2+}($[Ca^{2+}]i$). This increase is mediated through the V1 receptor, since several studies have shown that specific inhibitors of the V1 receptor antagonize the effect (6, 11). The rise in $[Ca^{2+}]i$ is not inhibited by pretreatment of cells with pertussis toxin, indicating that the V1 receptor, via a pertussis-insensitive G-protein, probably Gq, causes activation of phosphatidylinositol-specific phospholipase C-beta (PLC-beta) (30), resulting in production of inositol trisphosphate, which releases Ca^{2+} from intracellular stores, (31, 32, 33) and diacylglycerol which activates protein kinase C (PKC). While this rapid increase in $[Ca^{2+}]i$ is due to release of intracellular stores, a second phase of Ca^{2+} increase involves receptor-mediated Ca^{2+} influx through cation channels. This phase is slower and more sustained and appears to involve activation of Ca^{2+}-permeable nonselective cation channels (34). Consistent with this finding, several groups have shown that AVP stimulates Ca^{2+} entry as assessed by 45 Ca^{2+} uptake (35, 36). One area of future research will be to determine the post-receptor signaling events leading to Ca^{2+} entry in these cells. Ca^{2+}-mediated Ca^{2+} entry is likely to play a role, but other effectors also contribute.

The rapid increase in $[Ca^{2+}]i$ is the critical event in the initial contractile response to AVP. Elevated Ca^{2+} levels, through the action of calmodulin, activate myosin light chain kinase (MLCK), which in turn phosphorylates myosin light chain (37), leading to enhanced actinomyosin ATPase activity and contraction. In fact, myosin phosphorylation has been employed as a quantitative marker for contraction (38). While the rapid increase in $[Ca^{2+}]i$ is transient, contractile force is maintained for longer periods of time. The molecular events controlling this so-called "latch" state (37) are still poorly understood. An additional control of contractile force involves alterations in the force/Ca^{2+} ratio, or Ca^{2+} sensitization. A role for low molecular weight G-proteins of the Rho family has been proposed to play a role in this process. Activation of Rho leads to increased Ca^{2+} sensitization through novel downstream kinases which phosphorylate and inactivate myosin light chain phosphatase (39). The ability of vasoconstrictors such as AVP to modulate this pathway remains to be examined, and it is likely that AVP will regulate contraction at multiple points.

Several other Ca^{2+}-dependent enzymes will also be activated in response to elevations in $[Ca^{2+}]i$. Ca^{2+} dependent forms of adenylyl cyclase have been described in VSMC (40). In the setting of AVP-mediated rises in $[Ca^{2+}]i$, cAMP levels will be increased, leading to activation of protein kinase A, which phosphorylates MLCK and decreases the affinity of this enzyme for calmodulin(38) This will result in a decreased contraction at a given Ca^{2+} concentration, antagonizing the contractile effect of AVP. In addition, cAMP which has antimitogenic activity and decreases expression of muscle-specific genes in VSMC (41), will act as a negative feedback to counteract the hypertrophic response to AVP.

Increased Ca^{2+} will also, via calmodulin, activate other Ca^{2+}-dependent kinases such as Ca^{2+}/CaM kinase II (42). The physiologic role of this kinase in VSMC is not well understood. However, at least one study has shown that CaM kinase II is involved in the activation of the extracellular-regulated kinases (ERKs), which are members of the mitogen-activated protein kinase family (see below). Other targets of this pathway may include transcription factors which remain to be identified. Lastly, elevations in $[Ca^{2+}]i$ are critical for the activation of phospholipase A2 (43), which is the rate limiting enzyme in the production of eicosanoids. We will consider this pathway in greater detail below. In summary, AVP rapidly engages multiple pathways leading to both acute and sustained increases in $[Ca^{2+}]i$ which are critical events in the vasoconstrictor effects of this hormone. Moreover, activation of multiple Ca^{2+}-dependent pathways will in all likelihood also impinge on the longer term effects of AVP on growth and differentiation.

4.2. Regulation of protein kinase pathways

Protein phosphorylation is perhaps the most common post-translational modification in eukaryotic cells, and is regulated through multiple families of protein kinases and phosphatases. In VSMC, AVP rapidly activates multiple protein kinase cascades. Much work has recently focused on the mitogen activated protein (MAP) kinase family of kinases. This family of proline-directed kinases are highly conserved from yeast to man (44). Three major branches of the MAP kinase family have been identified. These include the extracellular regulated kinases (ERKs), the first members identified, which were previously designated p42/p44 MAP kinase; the stress-activated protein kinases/c-Jun amino terminal kinases (SAPK/JNK), and the p38 MAP kinase family which have homology to the HOG enzymes in yeast. A large number of review articles have recently appeared describing the regulation of these kinases (45, 46, 47, 48, 49, 50, 51, 52) , so this article will summarize these findings and focus on these pathways in VSMC.

ERK activation by receptor tyrosine kinases is mediated through the low molecular weight G protein Ras. Phosphorylation of receptors such as the PDGF receptor recruits specific effector molecules through binding of SH2 domains to specific phosphotyrosine residues on the cytoplasmic tail of the receptor. The binding to SOS, via the linker GRB-2 promotes displacement of GDP by GTP on the low molecular weight G-protein Ras, resulting in activation (53). Activation of Ras then initiates a cascade of protein kinases including the protooncogene serine kinase Raf-1, and the dual specificity kinase MEK-1, culminating in the activation of ERK family members p42/44 MAP kinase (49, 54, 55, 56, 57, 58). We have demonstrated that this pathway is operative in VSMC stimulated by PDGF or EGF (28).

Vasoconstrictors also activate ERK. In VSMC, AVP stimulates ERK activity to the same extent as PDGF

(5-10 fold), and with similar kinetics. ERK activation is detectable within 2 minutes after stimulation, is maximal by 5-10 minutes, and has returned to basal levels by 30 minutes (27, 59). However, direct measurement of Ras activation assessed by the ratio of GTP/GDP bound, found that AVP did not significantly activate Ras compared to EGF or PDGF(28). Similarly, activation of Raf as determined by phosphorylation of MEK-1, was much weaker with AVP than with receptor tyrosine kinases. Activation of ERKs in VSMC by AVP did require activation of protein kinase C (59, 60). Inhibition of PKC by either pharmacological agents, or by down regulation following chronic exposure to high concentrations of phorbol esters, completely abolished the ability of AVP to activate ERKs, while not affecting activation by PDGF or EGF. Thus while activation of ERKs in response to AVP is similar to that seen with PDGF, the signaling pathways are different.

In other cell types ERK activation is critical for cell growth (61). Following cell stimulation, ERKs translocate to the nucleus where they presumably phosphorylate transcription factors. It has been shown that Elk-1, a member of the ets family of transcription factors is a substrate of ERKs (52, 62). ERKs also phsophorylate cytoplasmic enzymes including cytosolic PLA2 (63). Based on analogies with other cell types, it appears reasonable to assume that ERK activation by AVP contributes to the hypertrophic growth response of these cells. Use of recently developed specific MEK-1 inhibitors (64) will be able to clarify this role.

The SAPK/JNK family of kinases was originally identified as kinases activated by external stresses such as UV light or hyperosmolarity (65, 66, 67, 68, 69, 70). By molecular cloning three genes encoding distinct JNK family members have been characterized (69, 71, 72, 73). Due to the expression of splice variants, at least 10 distinct JNKs have been identified (74). As their name indicates, these kinases phosphorylate the amino terminal of the transcription factor c-Jun, which results in activation of transcription at AP-1 sites. In addition, JNKs can phosphorylate other transcription factors such as ATF-2 and Elk-1(75). Analogously to the ERKs, JNKs are activated by upstream dual specificity kinases which phosphorylate threonine and tyrosine residues in the sequence TPF, conserved in all the JNKs (66, 76). AVP, as well as angiotensin II rapidly activate JNKs in VSMC (29). Activation of JNKs has a slower time course than that for the ERK pathway, with maximal activation (4-6-fold) detected at approximately 15 minutes, and values returning to basal levels by 60 minutes. The activation appears to be mediated by members of the Gq family of G-proteins, since stable expression of constitutive forms of Gq family alpha-subunits resulted in constitutive activation of JNKs (29). In contrast to ERK activation, stimulation of JNK activity does not appear to be mediated through a PKC dependent pathway. Phorbol esters, which potently stimulate ERKs are weak activators of JNK in VSMC. Inhibition of PKC using pharmacological agents or down regulation by chronic exposure to high concentrations of phorbol esters did not significantly affect JNK activation. However, inhibiting agonist-mediated increases in $[Ca^{2+}]i$ by pretreatment of cells with the Ca^{2+}chelator BAPTA blocked AVP-stimulated JNK activity. Exposure to ionomycin did not by itself activate JNKs, indicating that Ca^{2+} is necessary but not sufficient for JNK activation. The physiologic role of JNK remain to be determined. We will describe recent studies from our laboratory below suggesting a role for this pathway in mediating increased transcription of SM-alpha-actin.

A third branch of the MAP kinase family is represented by p38 MAP kinase. The yeast homologue of this enzyme is involved in response to hyperosmotic shock (77). The effects of vasoconstrictors and growth factors on p38 activation in VSMC has not been extensively examined. However, G-protein coupled receptors activate p38 MAP kinase in other cell types (78). The physiologic targets of p38 are not well characterized. The availability of specific inhibitors of this pathway (79) will facilitate understanding the role of these kinases in VSMC.

4.3. Regulation of arachidonic acid release and eicosanoid production

Arachidonic acid and its metabolites have been shown to have a variety of roles in different cell types. In VSMC, stimulation by AVP or other vasoconstrictors results in the production of vasodilatory prostaglandins, principally prostaglandin E1(PGE1) and prostacyclin (PGI2) (80). These eicosanoids are released from the cell and act in an autocrine fashion through specific G-protein coupled receptors. In VSMC, these prostaglandins through Gs activate adenylyl cyclase and stimulate production of cAMP (81). Elevated cAMP levels, as discussed above, inhibit contraction and proliferation of VSMC. Thus these eicosanoids provide a negative feedback mechanism to counteract the effects of vasoconstrictors such as AVP.

The rate limiting step in eicosanoid production is the release of free arachidonic acid from membrane phospholipids. The bulk of arachidonic acid in a resting cell is esterified to the sn-2 position of membrane phospholipids. While multiple pathways can mediate hydrolysis of the ester bond to produce free arachidonic acid, the dominant pathway in most cells including VSMC is through the action of phospholipase A2(PLA2). Multiple forms of this enzyme exist which can be dissociated into secreted forms and intracellular forms. Several years ago we and others identified a major form of PLA2 which controls agonist-induced arachidonic acid in many cell types(82, 83, 84, 85.) This enzyme, now designated cPLA2, is an 85 kDa enzyme which is specific for arachidonic acid. cPLA2 requires Ca^{2+} for enzymatic activity and translocates to an intracellular compartment in response to elevation in $[Ca^{2+}]i$ (43, 86). By immunofluorescence and electron microscopy it has been show that cPLA2 localizes to the nuclear envelope(87). In addition to Ca^{2+}, cPLA2 is regulated by protein phosphorylation, and is phosphorylated and activated by ERKs (63, 88). In VSMC, cPLA2 is constitutively expressed, and acutely activated by AVP as well as other growth factors such as PDGF. However, recent data indicate that other forms of PLA2 may play a role in eicosanoid production in VSMC. Gross

and coworkers have described a Ca^{2+}-independent form of PLA2(89). Employing a specific inhibitor of this enzyme, these workers have reported that approximately two thirds of AVP-induced arachidonic acid release in VSMC is contributed by this enzyme (90). The signals mediating AVP-regulation of this enzyme remain to be fully determined. However, depletion of intracellular Ca^{2+} stores may play a role (91). The relative contributions of these distinct isoforms of PLA2 to prostaglandin production and vascular smooth muscle physiology is also an area for future research.

Prostaglandin production is mediated through the cyclooxygenase (COX) family of enzymes. COX-1 is generally localized to the endoplasmic reticulum and is constitutively expressed in most cell types including VSMC. COX-2, which was originally identified as an immediate early response gene (92), is induced by mitogenic stimuli. The rapid production of prostaglandins in response to AVP is likely mediated through COX-1, since COX-2 levels are very low in resting VSMC. In fibroblasts, PDGF stimulates induction of COX-2 expression through increased transcription (93). We have observed that PDGF also induces COX-2 expression and constitutive increases in prostaglandin production in VSMC (28); this induction is not observed with AVP treatment, and may play a role in the suppression of muscle-specific gene expression mediated by PDGF. It is therefore apparent that multiple enzyme pathways mediate prostaglandin production in VSMC, and that these are differentially regulated by AVP vis a vis PDGF.

Like many cells, VSMC produce a spectrum of eicosanoids, of which PGE1 and PGI-2 are the major secreted products. Eicosanoids produced in lesser quantities may have important physiologic roles in these cells. To that end, it has been reported that prostaglandins A and J are potent antimitogenic agents in these cells (94). Production of these molecules in response to AVP or other agonists has not been examined. An additional complexity in this field is the recent finding that activation of cPLA2 appears to be critical for growth of VSMC (95). Exposure of cells to antisense oligonucleotides inhibited growth. To date, no eicosanoids have been detected in VSMC which promote growth of these cells, therefore the critical role for PLA2 in proliferation is not well understood. It is conceivable that activation of PLA2 has effects distinct from eicosanoid production. One report has indicated that arachidonic acid activates mitogen activated protein kinase pathways in VSMC (96). Thus PLA2 may lie both upstream and downstream of the MAP kinase pathway.

Besides activating phospholipases A2 and C, AVP also stimulates phospholipase D (PLD) activity in VSMC (97). This enzyme hydrolyzes membrane phospholipids to produce phosphatidic acid, which in turn can be converted to diglyceride through the sequential action of phosphatidic acid phosphohydrolases (98, 99). Diglyceride production in VSMC and other cells is composed of an acute and a sustained phase. PLD is likely to be responsible for the sustained phase of diglyceride production, and may thus contribute to long-term activation of PKC. A number of physiologic roles have been attributed to phosphatidic acid. Both phosphatidic acid and lysophosphatidic acid have mitogenic effects in fibroblasts (100, 101) . The role that PLD products play in VSMC physiology has not been established. Similarly very little is known about the PLD isoforms or its mechanisms of regulation in VSMC.

5. EFFECTS ON GENE EXPRESSION

5.1.Immediate early response genes

AVP has been shown to induce expression of selective genes. Both AVP and angiotensin II have been shown to increase transcription of the immediate early response genes egr-1 and c-fos (102, 103). These genes are also induced by growth factors such as PDGF as well as serum. Increased transcription of c-fos in response to mitogens is mediated through a specific cis-acting element of the c-fos promoter, which has been designated the serum response element (SRE) (104). The core of this element has the sequence $CC(A/T)^6GG$, referred to as a CArG Box. The transcription factor serum response factor (SRF) binds specifically to this sequence (105). Transcriptional regulation of the SRE is mediated through SRF and members of the ets family such as the transcription factor Elk-1 (106). Phosphorylation of Elk-1 by the ERK family of kinases promotes association with SRF and the SRE, leading to activation of the promoter and increased transcription. This pathway has been shown to be operative for growth factors signaling through receptor tyrosine kinases as well as G-proteins. While, not proven, it is likely that the effects on induction of c-fos by AVP in VSMC is mediated through this pathway, as part of the growth promoting response.

Vasoconstrictors also increase expression of TGF-beta 1 (23) and PDGF-AA (107). TGF-beta 1 has been shown to have anti-mitogenic effects in VSMC, and it has been suggested that stimulation of TGF-beta 1 is responsible for the hypertrophic response to vasoconstrictors (23). Increased expression of PDGF-AA may establish an autocrine loop for growth of VSMC. The post-receptor events mediating induction of these genes are not well understood, although the appropriate promoter constructs are available.

5.2. Smooth muscle-alpha-actin

As discussed above, the induction of SM-alpha-actin by AVP as well as angiotensin II are post-receptor events that distinguish these agents from complete mitogens such as PDGF. A number of laboratories have focused on the regulation of this protein using rat VSMC and the rat SM-alpha-actin promoter. AVP increases mRNA levels for SM-alpha-actin. The increase in mRNA is not blocked by treatment of cells with cycloheximide, an inhibitor of protein synthesis[2], indicating that induction is not mediated through synthesis of other factors. Examination of the 5' region of this promoter reveals a number of putative regulatory elements (108). These includes two E-boxes. E-boxes have been implicated in the regulation of muscle specific gene expression in skeletal muscle and are involved in the binding of myogenic

transcriptional factors of the MyoD family. In addition the promoter contains three CArG boxes. Using transient transfection experiments in which the 750 bp region of the promoter was linked to a chloramphenicol acetyltransferase reporter (CAT), we demonstrated that AVP induced promoter activity 6-10 fold in rat aortic VSMC (109). This induction was inhibited by PDGF. Similar inductions have been observed with angiotensin II (110) and TGF-b(111). These data strongly argue that at least a component of the induction seen with AVP is due to increased transcription.

To define the regulatory elements mediating the regulation of SM-alpha-actin by hormones and growth factors, cells were transfected with a series of truncation mutants of the promoter (109). Removal of both E-boxes and the first CArG box did not alter the ability of vasoconstrictors to stimulate promoter activity or of PDGF to inhibit activity. However, removal of the second CArG box resulted in a construct which was not sensitive to regulation by either class of agents. Point mutations in either of these CArG boxes markedly reduced the ability of AVP to increase promoter activity in VSMC, and the construct encoding the double mutant was completely inactive. Thus the induction of SM-alpha-actin is mediated through these elements.

Functional CArG boxes are critical elements for expression of muscle-specific genes including skeletal and cardiac alpha-actin (112, 113) myosin light chain (114) as well as smooth muscle alpha-actin (109, 111, 115). SRF appears to also bind to these CArG boxes (116, 117, 118), and is required for increased expression. However, paradoxically, these genes are generally repressed by mitogenic stimuli. Conversely, induction of these genes is associated with conversion to the contractile phenotype,withdrawal from the cell cycle and growth arrest. The mechanism whereby SRF acts on these two opposing patterns of gene expression (c-fos and SM-alpha-actin) remains to be determined, but is likely to involve interactions with other transcriptional factors. In ventricular myocytes induction of skeletal alpha-actin requires SRF, and this is antagonized by the transcription factor YY1(119). One recent study has described physical associations between SRF and members of the helix-loop-helix family (120). In all of these studies, it is necessary to distinguish between elements and factors required for cell-specific expression and those involved in regulation by external signals.

We have begun to examine the early post-receptor events mediating the induction of SM-alpha-actin by AVP. To determine whether coupling of the V1 receptor to Gq family members is required, rat aortic VSMC were stably transfected with a constitutively active alpha-subunit of the Galphaq family- Galpha16Q212L (29). Initial studies used a constitutively active alphaq(GalphaqQ209L), but expression of this construct was not tolerated by VSMC and no stable transfectants were obtained. Expression of Galpha16Q212L mimicked the effects of AVP on induction of SM-alpha-actin, with steady state protein levels and promoter activity markedly induced. Interestingly, these cells also had a higher protein/cell content than wild-type cells or VSMC transfected with a control plasmid, suggesting that common signals emanating from Gq family members mediate both hypertrophy and induction of SM-alpha-actin. Cells expressing Galpha16Q212L did not show alterations in basal ERK activity, and agonist-stimulation was unimpaired. Consistent with this finding, a specific MEK inhibitor did not affect the ability of AVP to induce SM-alpha-actin expression, nor the ability of PDGF to inhibit. In contrast, basal JNK activity was markedly elevated in cells expressing Galpha16Q212L. In transient transfection experiments, co-transfection of a dominant-negative JNK kinase (SEK-1) blunted the ability of AVP to induce the SM-alpha-actin promoter. Taken together, these data suggest that AVP stimulation of JNKs, through a Gq mediated pathway contributes to the transcriptional activation of the SM-alpha-actin promoter. The presence of similar CArG boxes in the promoters of other smooth muscle-specific genes suggest that these pathways may lead to a coordinated increase in a number of markers of the differentiated phenotype of vascular smooth muscle.

The suppression of SM-alpha-actin induction by PDGF appears to involve Ras-dependent pathways (28). Expression of constitutively active Ras completely blocked the abilitiy of AVP to activate the promoter. The effects of Ras are not mediated through the classic Raf/MEK/Erk pathway, but appear to involve induction of cPLA2 and COX-2. It therefore appears activation of Ras represents a critical difference which may account for the distinct physiologic responses to AVP compared to PDGF.

6. OTHER EFFECTORS

AVP and other vasoconstrictors have been shown to rapidly activate the Na^+/H^+ exchanger in VSMC (20, 121). In media not containing bicarbonate, activation of this exchanger results in increases in intracellular Na^+, and alkalinization of the cells. Alkalinization has been associated with mitogenic stimuli; however recent studies have demonstrated that in bicarbonate-containing buffers this phenomenon does not occur (122). The physiologic importance of activation of the Na^+/H^+ exchanger therefore remains to be established. It is possible that increased intracellular Na^+ will regulate intracellular free Ca^{2+} through the Na^+/Ca^{2+} exchanger. Intracellular Na^+ levels may also regulate other intracellular pathways.

Intensive interest has recently been focused on nitric oxide as a potent vasodilator and second messenger in vascular cells (see (123) for review). In response to cytokines such as interleukin 1 beta, VSMC increase expression of an inducible form of nitric oxide synthase (iNOS). AVP has been shown to inhibit this induction (124, 125), by inhibiting transcriptional activation. This finding suggests that in addition to directly stimulating contractile pathways, AVP inhibits vasodilatory pathways. Further studies are required to dissect the post-receptor pathways mediating this effect.

A number of studies have examined abnormalities in vasoconstrictor responsiveness of VSMC

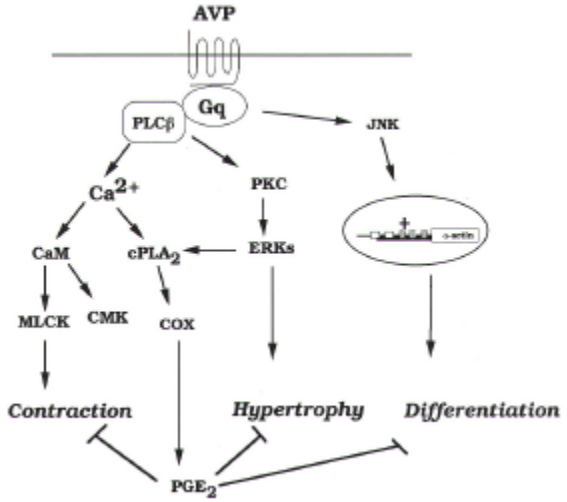

Figure 2. AVP Signaling Pathways in VSMC. AVP binds to its receptor, which is coupled to Gq. Gq activates phospholipase C(PLCb), resulting in increased Ca^{2+}, via inositol trisphosphate, and activation of protein kinase C (PKC). Gq also increases JNK activity through an unidentified pathway. Increases in Ca^{2+} activate Ca^{2+} dependent enzymes. Via calmodulin (CaM), myosin light chain kinase (MLCK) and Ca^{2+}/calmodulin dependent kinases (CMK) are activated. Ca^{2+} also activates cytosolic phospholipase A2 (cPLA2). PKC is critical for ERK activation, which phosphorylates cPLA2 as well as contributing to hypertrophy. JNK activation participates in the positive induction of SM-alpha-actin, a marker for the differentiated phenotype of VSMC.

from SHR rats. Aortic smooth muscle cells from spontaneously hypertensive rats (SHR) rats proliferate more rapidly, and are more numerous at confluency than cells from the control WKY rats (126). In contrast to normal VSMC, angiotensin has been shown to have some mitogenic activity in these cells (127). The magnitude of the Ca^{2+} increase observed after vasoconstrictor stimulation appears to be greater in the SHR rats (128). However, this abnormality in itself does not appear to account for the enhanced proliferation (129). The role of phenotypic state in the responsiveness of VSMC from SHR rats has not been examined and is one area for future research.

7. CONCLUSION AND FURTHER DIRECTIONS

During the past ten years we have acquired a greater understanding of some of the early events following AVP binding to its receptor in VSMC. Many of the relevant molecules have been cloned including the AVP receptors and the G-proteins to which it couples. Our current understanding of AVP-mediated signaling in VSMC is depicted in figure 2. A critical event mediating the contractile responses to AVP in these cells is the increase in $[Ca^{2+}]i$, which subsequently activates a number of Ca^{2+} dependent pathways. This increase in Ca^{2+} involves both mobilization of intracellular stores, as well as entry of extracellular Ca^{2+} via cation channels. Sophisticated techniques have examined the kinetics and spatial distribution of Ca^{2+} in great detail, and further progress will be achieved in the next several years. The mechanisms determining contractile sensitivity as well as events controlling the "latch state" are less well understood. It is likely that a critical biological response such as contraction is controlled by multiple mechanisms.

Longer term exposure of VSMC to AVP or other vasoconstrictors has important effects on the growth and differentiation of these cells. Activation of a number of kinase pathways is involved in the induction of families of genes, including immediate early response genes, as well as muscle specific genes. The former family of genes including c-fos, are also induced by mitogenic stimuli such as PDGF. However, the induction of muscle-specific genes is a selective effect of vasoconstrictors, and is in fact antagonized by PDGF. As such understanding the molecular events mediating this induction appears to be a fruitful area for further research. Elucidating events mediating control of muscle specific genes in VSMC will increase our knowledge of control of differentiation. This has direct relevance to pathophysiologic states such as atherosclerosis, where VSMC in the neointima manifest many of the characteristics of the embryonic or proliferative phenotype. By working back from events occurring at the level of muscle-specific promoters such as the SM-alpha-actin promoter, we will reveal pathways that are differently engaged by AVP compared to complete mitogens such as PDGF. These pathways are presumably critical to the hypertrophic response seen with this agent. Much of the focus here will be on downstream events intermediate between the early stimulation of phospholipases and MAP kinase family members, and subsequent activation of specific transcription factors. Two potential attractive candidates are the cytoplasmic tyrosine kinases such as src, and the low molecular weight G-proteins of the Rho, Rac and cdc42 family (130) . These molecules have already been implicated in control of cytoskeletal function (131, 132). In addition to elucidating the signaling pathways regulated by AVP, these studies will in a broader context help define pathways mediated by other hormones which engage Gq proteins.

Finally, it is becoming apparent that there is greater heterogeneity among VSMC *in vivo*, with multiple cell populations that can be distinguished by differential expression of smooth muscle markers (12). Recently, these distinct populations have been isolated in culture and show distinct phenotypic features and proliferative responses (133). The effects of AVP as well as other agonists is likely to be cell type specific, with functional AVP receptors likely to be expressed on only a subpopulation of these cells. Since most of the studies described in this article have employed preparations which are a mixture of distinct cell types, it may be necessary to revisit the actions of AVP in individual cell populations.

8. ACKNOWLEDGMENTS

This work was supported by grants from the NIH (DK 19928, DK 39902).

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Text footnotes:

1. The abbreviations used are: VSMC, vascular smooth muscle cells; MEM, minimal essential media; SM-alpha-actin, smooth muscle- alpha-actin; PCR, polymerase chain reaction; CAT, chloramphenicol acetyl transferase; FCS, fetal calf serum; PAGE, polyacrylaminde gel electrophoresis; PDGF, platelet derived growth factor; PMA, phorbol 12-myristate 13-acetate; AVP, arginine vasopressin; AII, angiotensin II; JNK, cJun NH2-terminal kinase.

2. R.A. Nemenoff, unpublished observations

Key words: Smooth muscle, Vasopressin, Vessel, Smooth muscle actin, Signal transduction, Protein kinases, c-Jun

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