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Induction of c-fos protein by activation of vasopressin receptors in smooth muscle cells

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Stimulation of vasopressin (V_1) receptors of rat aortic smooth muscle cells (A-10, ATCC CRL 1476) results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) with the mobilization of intracellular calcium. When A-10 cells are exposed to arginine vasopressin (AVP), there is an increase in the level of c-fos oncoprotein. The extent of induction of c-fos oncoprotein depends on both the time of exposure of the cells to AVP, reaching a maximum at 60 min after which there is a slow decline, and the concentration of AVP used, with an approximate EC_{50} of 1 nM which corresponds well with the K_d of vasopressin binding to these receptors. This vasopressin-mediated increase in c-fos protein level is inhibited by a V_1/V_2 antagonist (SKF 101498) suggesting that this is a receptor-mediated event. In addition dDAVP, a V_2 selective agonist, is much less effective than AVP in inducing c-fos protein suggesting that AVP mediates its effect via V_1 receptors. Desensitization of vasopressin receptors by prolonged exposure to AVP resulted in no additional induction of c-fos protein level in response to second challenge of AVP. In addition to AVP, phorbol dibutyrate (PDBu), an activator of protein kinase C (PKC), also stimulates the accumulation of c-fos protein although to a lesser extent than AVP. The above data suggest that c-fos protein levels in smooth muscle cells are regulated by AVP and the hormonal effect may be mediated through PI turnover and DAG, IP_3 and Ca^{2+} signals.

Protein, c-fos; Vasopressin receptor; (Smooth muscle cell)

1. INTRODUCTION

Cellular oncogenes have been implicated in the control of normal cell proliferation. Mutation or abnormal or inappropriate expression of these oncogenes can adversely affect the normal control of cell growth and/or differentiation [1]. c-myc and c-fos belong to a class of proto-oncogenes whose products are localized in the nucleus, although their exact function is unknown.

Rapid changes in c-fos, c-myc and actin mRNA levels following addition of serum to quiescent cultures of smooth muscle cells suggest that these genes may be involved in the onset of smooth muscle cell proliferation. Similar observations have been reported in other cell systems [2–7]. Abnormal proliferation of smooth muscle cells has been implicated in the development of atherosclerosis and Benditt et al. [7] have reported that viral infection of smooth muscle cells can also result in these lesions.

Our laboratory has been interested in the signal transduction pathways activated by vasopressin receptors. In an attempt to understand the relationship between vasopressin receptors and cell growth and proliferation we have used a model system A-10 (ATCC CRL 1476), a smooth muscle cell line derived from rat thoracic aorta that displays a high density of vasopressin (V_1) receptors. These vasopressin receptors mediate vascular contraction by interacting with phosphoinositide specific phospholipase C (PI-PLC) generating two

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intracellular second messengers, IP_3 and diacylglycerol. While IP_3 releases intracellular calcium, DAG activates protein kinase C which phosphorylates key proteins resulting in regulation of cellular functions. The data presented here demonstrate that AVP acting through V_1 receptors increases c-fos protein levels.

2. MATERIALS AND METHODS

AVP and dDAVP were from Bachem, California. V_1/V_2 antagonist SK&F 101498 was synthesized in SK&F Laboratories. Phorbol dibutyrate and 4α-phorbol didecanoate were from Sigma, St. Louis. All other chemicals were of reagent grade. The antibodies have been decsribed [8].

2.1. *Cell culture*

Rat aortic smooth muscle cells (A10) were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) plus 20% fetal calf serum (FCS) in 95% air/5% CO_2 [9]. All experiments were carried out with cells passaged for no more than 4 months. Culture flasks (Falcon T-150) were inoculated with 1.5×10^6 cells, which were cultured in 20 ml medium. Experiments were carried out after 3 days unless otherwise mentioned.

2.2. *Treatment of cells with vasopressin and preparation of cell lysates for ELISA*

Cells in monolayers were washed 2 × with 20 ml DPBS (Dulbecco's phosphate buffered saline) at 37°C and then incubated in 10 ml DPBS with the indicated concentrations of test compounds for the times indicated at 37°C. At the end of the incubation, DPBS was removed and the cells were washed with ice cold DPBS, removed with a scraper in ice cold DPBS and collected by centrifugation. The cell lysate for the ELISA was prepared as described [10]. Immediately before use, samples were thawed and insoluble materials were removed by centrifugation at 14000 × g for 2 min.

2.3. *Absorption and detection of bound fos proteins*

The absorption of a fos 5 antibody and capture of the c-fos protein from the cell lysates were done following the procedure of Moore et al. [10,11]. Bound fos proteins were recognized by incubation with 100 μl/well of antibody/alkaline phosphatase conjugate (a fos 1-AP) diluted in TMT [4% non-fat milk powder, 0.5% Tween in TBS (Tris buffered saline, pH 8.0)] and incubated on a rocker for 1 h at room temperature. The incubation was terminated by washing the wells thoroughly with TBS (6 × with 200 μl/well).

Bound alkaline phosphatase was detected with the ELISA amplification system (BRL). The design of the system has been described as has its use in ELISAs for myc oncoproteins [10,11]. In the present assay the wells were incubated for 30 min at room temperature with 100 μl of 'substrate solution', followed by 30 min with 100 μl of 'amplifier solution'. The reactions were stopped by addition of 50 μl of 0.5 M HCl and the absorbance (OD_{492}) determined using a conventional automated plate-reader (Titertek).

2.4. *Desensitization of A-10 cells in response to AVP*

The cells in T-150 flasks were treated with 1 μM AVP for 30 min or 2 h at 37°C. At the end of the pretreatment the medium was removed, fresh medium added and the incubation continued for another 2 h to wash off any bound AVP. At the end of this incubation, the medium was removed and the cells were washed with DPBS 4 × (10 ml each time) and processed as described above.

Each experiment was performed in quadruplicate and repeated at least twice. The data presented are from one representative experiment.

3. RESULTS AND DISCUSSION

Incubation of rat thoracic aortic smooth muscle cells (A10) with 1 μM AVP elicited a time-dependent increase in c-fos protein in these cells (fig.1). Maximal stimulation was observed at 60 min after which the level started to decline slowly. Tumor promoting phorbol ester (PDBu), an activator of protein kinase C, also caused a time-dependent increase in c-fos protein although to a lesser extent than AVP (fig.1). 4α-12,13-didecanoate (αPDD), which does not activate protein kinase C, did not induce an increase in c-fos protein (fig.1).

The AVP-mediated increase in c-fos protein level was dependent on the concentration of AVP used (fig.2). Even at concentrations as low as 0.1 nM, there was a significant increase in c-fos

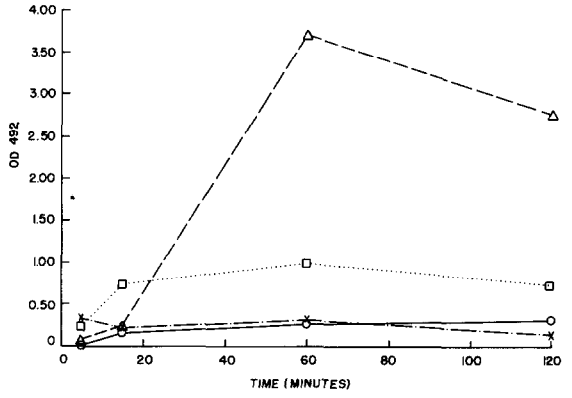

Fig.1. Time course of c-fos protein appearance in response to AVP and phorbol dibutyrate: smooth muscle cells in T-150 flasks were treated with the medium above, 1 μM AVP or 1 μM PDBu for indicated time periods and processed as explained in section 2. Basal (○); AVP (△); PDBu (□) and αPDD (×). The data presented are the mean of four independent determinations from one experiment with < 10% variation between the values. The experiment was repeated with similar results.

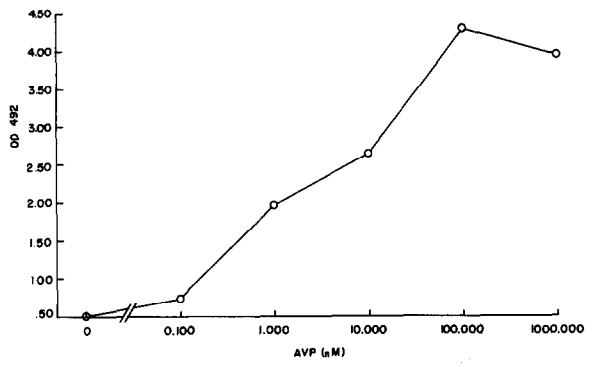

Fig.2. C-fos protein appearance in response to increasing concentrations of AVP: A-10 cells in T-150 flasks were incubated with medium alone or increasing concentrations of AVP for 1 h. At the end of incubation, the cells were washed and c-fos protein levels were quantitated as explained in section 2. The data presented are the mean of four independent determinations from one experiment with <5% variation between the values. The experiment was repeated with similar results.

protein levels. The concentration of AVP required to increase the protein level half-maximally (1 nM) corresponds well with both the kDa of this hormone for binding to these receptors [12] and the concentration required to half-maximally stimulate inositol phosphates accumulation in these cells [13], suggesting that both processes may be mediated by the same receptors. To further confirm that this is a receptor-mediated event, we studied the effects of SK&F 101498, a V_1/V_2 antagonist on c-fos protein levels. While SK&F 101498 had no effect on c-fos protein levels by itself, it markedly inhibited the stimulation mediated by AVP (table 1). Furthermore, dDAVP, a V_2 selective agonist, was much less effective than AVP in stimulating c-fos protein, suggesting that the effects of AVP are mediated by V_1 receptors (not shown).

We also studied the effect of regulating vasopressin receptor response on c-fos protein. One of the mechanisms of regulating receptors is desensitization. Prolonged treatment of these cells with AVP results in an attenuation of both inositol phosphates accumulation and intracellular calcium release in response to a second challenge of AVP (submitted). These reduced responses are accompanied by a loss in cell surface receptors for vasopressin. Under the conditions where the accumulation of inositol phosphate was attenuated, there was no c-fos protein induction in response to AVP in desensitized cells (table 2). The basal levels of c-fos protein in the desensitized cells was greater than that in control cells (208 ± 5.5 vs 51 ± 5.1). This is attributable to induction of c-fos protein by the initial exposure to AVP (cf. fig.1, 120 min data point). Note that whereas the control cells were fully responsive to AVP (396 ± 25), desensitized cells were not responsive to a second challenge with AVP (173 ± 19). This time course (2 h pretreatment) compares very well with desensitization of inositol phosphate turnover (submitted).

Taken together the above data suggest that in smooth muscle cells, vasopressin acting through V_1 receptors induces c-fos protein expression in a time- and dose-dependent manner which is blocked by a V_1/V_2 antagonist. Desensitization of the vasopressin receptors by prolonged treatment with AVP results in an attenuated response to further stimulation, supporting the relationship between AVP receptors and c-fos protein levels. In addition

Table 1

Effect of V_1/V_2 antagonist (SK&F 101498) on vasopressin (AVP)-mediated c-fos protein expression in vascular smooth muscle cells (A-10)

Time of exposure (min)	OD 492			
	Basal	SK&F 101498	AVP	SK&F 101498 + AVP
60	0.27 ± 0.053	0.18 ± 0.052	3.73 ± 0.087	0.91 ± 0.087
120	0.32 ± 0.037	0.18 ± 0.046	2.79 ± 0.028	1.02 ± 0.076

A-10 cells were incubated with medium alone (basal), 1 μM each of SK&F 101498 or AVP or both together for 60 min and 120 min. At the end of incubation, the cells were washed and assayed for c-fos protein

Table 2

Effect of vasopressin-induced desensitization on c-fos protein expression in A-10 cells

Condition	OD 492	
	Basal	AVP
Control	0.51 ± 0.051	3.96 ± 0.25
Desensitized	2.08 ± 0.055	1.73 ± 0.19

A-10 cells were pretreated with medium alone (control) or 1 μM AVP (desensitized) for 2 h at 37°C. At the end of the pretreatment, the medium was removed, fresh medium was added and the incubation continued for another 2 h to reverse any bound AVP from the receptors. At the end of second incubation, the cells were washed and challenged with medium alone (basal) or 1 μM AVP for 60 min at 37°C and assayed for c-fos protein expression

to AVP, tumor promoters such as PDBu also stimulate c-fos protein levels although to a much lesser extent. This suggests that mechanism(s) other than PKC activation may also be involved in the increase of c-fos protein level. At present it is not known whether the AVP-induced increase in c-fos protein levels is due to activation of transcription, translation or stabilization of the protein, or a combination of these factors. In contrast to serum-stimulation of quiescent fibroblasts, where both c-fos and c-myc are induced, in smooth muscle cells activation of vasopressin receptors results in induction of c-fos protein without any change in c-myc protein levels (unpublished).

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