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Biochemistry

Vasopressin-mediated inhibition of atrial natriuretic factor-stimulated cGMP accumulation in an established smooth muscle cell line

(smooth muscle relaxation/contraction/guanylate cyclase/V_1 vasopressin receptor)

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ABSTRACT **Rat thoracic aortic smooth muscle cells (line A10, ATCC CRL 1476) display a high density of atrial natriuretic factor (ANF) receptors. ANF stimulated the accumulation of cGMP in these cells in a time- and dose-dependent fashion. These cells are known to display a high density of vasopressin receptors of the vascular V_1 subtype. These vasopressin receptors mediate (*i*) inhibition of isoproterenol-stimulated cAMP accumulation and (*ii*) stimulation of inositol phosphate accumulation and calcium fluxes. Addition of [8-arginine]vasopressin ([Arg^8]VP) to these cells inhibited ANF-stimulated cGMP accumulation. Inhibition of cGMP accumulation was dependent on the concentration of [Arg^8]VP, with half-maximal and maximal effects occurring at 0.4 and 10 nM, respectively. [Arg^8]VP did not have significant effects on basal cGMP levels. The inhibition by [Arg^8]VP appears to be mediated by V_1 receptors, since the V_2 renal receptor agonist [1-desaminocysteine,8-D-arginine]vasopressin was ineffective. Also, the selective V_1 antagonist [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyltyrosine),8-arginine]vasopressin and the mixed V_1/V_2 antagonist [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl-D-tyrosine),4-valine,8-arginine]vasopressin blocked the [Arg^8]VP-mediated effect, whereas the selective V_2 antagonist [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-isoleucine,4-valine,8-arginine]vasopressin was minimally effective. These data show that in rat aortic smooth muscle cells, V_1 receptors are negatively coupled to guanylate cyclase. These data also suggest that the vasoconstrictor activity of [Arg^8]VP might involve inhibition of ANF-receptor-mediated vascular relaxation through inhibition of cGMP accumulation in addition to its effects on (*i*) isoproterenol-mediated cAMP accumulation and (*ii*) inositol phosphate accumulation and calcium fluxes.**

Rat and human atrial extracts have been shown to produce diuresis and natriuresis *in vivo* (1, 2) and relaxation of vascular and nonvascular smooth muscles *in vivo* (3–6). These active factors have been identified as peptides of 21–23 amino acids that are reported to be synthesized as larger precursor polypeptides in atrial myocytes and stored within atrial secretory granules (7). Atrial natriuretic factor (ANF) is released into the circulation in response to volume expansion and participates in the maintenance of cardiovascular homeostasis by affecting kidney functions and vascular resistance (1–3). The existence of specific receptors for ANF has been demonstrated in kidney (8), adrenal cortex (9, 10), and vascular smooth muscle cells (11).

Winquist *et al.* (12) have shown that a synthetic ANF produced a profile of relaxation of rabbit aorta and facial vein that was qualitatively similar to that produced by sodium nitroprusside but not by many other vasodilators, including adenosine and papaverine. They suggested that ANF and sodium nitroprusside may share a common biochemical mechanism of action. It was further shown that both produced relaxation, and this correlated well with the formation of cGMP by these agents. The only difference between these agents was that whereas sodium nitroprusside activated soluble guanylate cyclase, ANF stimulated particulate guanylate cyclase (13). We are interested in the molecular mechanisms of the interaction between vasoactive receptors. Studies on the interaction between receptors and signal-transduction process for vasoconstrictors, such as α-adrenergic agonists, and vasodilators, such as β-adrenergic agonists, have focused primarily on the regulation of myosin light chain kinase activity by cAMP-dependent protein kinase (14).

[8-arginine]Vasopressin ([Arg^8]VP) is a potent vasoconstrictive agent. Two types of [Arg^8]VP receptors (V_1 and V_2) have been reported (15). V_2 receptors are coupled to adenylate cyclase and induce an increase in cAMP accumulation and water permeability in renal collecting duct cells. V_1 receptors mediate vasoconstriction, and there is evidence that these receptors are coupled to inositol phosphate turnover and calcium mobilization (16, 17). It has also been shown that these V_1 receptors are negatively coupled to adenylate cyclase (18). Little is known about the mechanisms of regulation of signal transduction and interactions between other vasoactive hormones. It was of interest to find out whether [Arg^8]VP might also alter ANF-mediated relaxation by affecting the properties of the ANF receptors or sites distal to the receptors. For this purpose, we employed a cultured vascular smooth muscle cell line (A10) derived from rat thoracic aorta, because previous work showed that these cells display V_1 receptors (19).

We report that ANF interaction with these cells results in an increase in cGMP and that the interaction of [Arg^8]VP with V_1 receptors inhibits ANF-induced increases in cGMP.

MATERIALS AND METHODS

Materials. cGMP radioimmunoassay kits and ^{125}I-labeled ANF (^{125}I-ANF, specific activity 2200 Ci/mmol; 1 Ci = 37 GBq) were purchased from New England Nuclear. 3-Isobutyl-1-methylxanthine (iBuMeXan), phorbol 12,13-

Abbreviations: ANF, atrial natriuretic factor; ^{125}I-ANF, ^{125}I-labeled ANF; iBuMeXan, 3-isobutyl-1-methylxanthine; DPBS, Dulbecco's phosphate-buffered saline plus 10 mM $MgCl_2$, 0.1% glucose, and 0.2% bovine serum albumin; VP, vasopressin; dCys, desaminocysteine (i.e., β-mercaptopropionic acid); d$(CH_2)_5$, β-mercapto-β,β-cyclopentamethylenepropionic acid in place of cysteine at residue 1 of VP.

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dibutyrate, and 4α-phorbol 12,13-didecanoate were from Sigma. ANF I, II, and III were from Sigma and Calbiochem–Behring. [Arg^8]VP was purchased from Bachem Fine Chemicals (Torrance, CA). The [Arg^8]VP analogs used were [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),-2-(*O*-methyltyrosine),8-arginine]vasopressin, or [[d(CH$_2$)$_5$]^1,-Tyr(Me)^2,Arg^8]VP; [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl-D-tyrosine),4-valine,8-arginine]vasopressin, or [[d(CH$_2$)$_5$]^1,D-Tyr(Et)^2,Val^4,Arg^8]VP; [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-D-isoleucine,4-valine,8-arginine]vasopressin, or [[d(CH$_2$)$_5$]^1,D-Ile^2,Val^4,Arg^8]VP; and (1-desaminocysteine,8-D-arginine)-vasopressin, or [dCys^1,D-Arg^8]VP. The analogs were synthesized at Smith Kline & French Laboratories.

Culture of Smooth Muscle Cells. Rat aortic vascular smooth muscle cells (line A10, ATCC CRL 1476) were obtained from the American Type Culture-Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) plus 20% fetal bovine serum. Before initiation of the experiments, the cells were subcloned twice by limiting dilution. All experiments were performed with cells passaged once a week for no more than 4 months. Culture wells (35-mm diameter; 6-well Linbro plates) were inoculated with 1 ml of medium containing 75,000 cells. Experiments were performed after 3 days, when there were about 500,000 cells per well.

cGMP Synthesized by Cultured Cells. The cells were washed twice with 1 ml of Dulbecco's phosphate-buffered saline containing 10 mM $MgCl_2$, 0.1% glucose, and 0.2% bovine serum albumin (DPBS). Subsequently, 900 μl of DPBS containing 0.5 mM iBuMeXan with and without the test compounds was added to each well. The cells were preincubated for 5 min at 37°C, and then the reaction was started by addition of 100 μl of DPBS or 10 μM ANF in DPBS (final concentration was 1 μM) and incubation was continued for an additional 10 min, unless indicated otherwise. The reaction was stopped by addition of 100 μl of ice-cold 100% (wt/vol) trichloroacetic acid to each well. The cells were scraped in trichloroacetic acid and the suspension was centrifuged at 1500 × *g* for 20 min. A 500-μl aliquot of the supernatant was extracted five times with 2.5 ml of water-saturated ether, and cGMP was determined by radioimmunoassay after the acetylation procedure.

Radioimmunoassay for cGMP. The amount of cGMP in each well was determined in duplicate. Each experimental point was represented by triplicate wells. Each experiment was repeated two or three times. The mean ± SEM values of data derived from one experiment are presented.

Binding of ^{125}I-ANF to A10 Cells. This was done according to the procedure of Schenk *et al.* (20) with slight modifications. Cells in 6-well plates were washed with DPBS and then incubated with ^{125}I-ANF in 1 ml of DPBS for 30 min at 37°C. The reaction was stopped by aspirating the medium and washing the cells three times with 1 ml of ice-cold DPBS. The cells were then solubilized in 0.5 ml of 0.5 M NaOH and the radioactivity was measured in a gamma counter. Nonspecific binding was measured in the presence of 1 μM unlabeled ANF II.

RESULTS

Exposure of A10 cells to 1 μM ANF in the presence of 0.5 mM iBuMeXan resulted in an 8- to 12-fold increase in cGMP levels. This increase in cGMP levels in response to ANF was time-dependent, being observed as early as 1 min, and increased with time up to 60 min of incubation (Fig. 1*A*). It was observed that ^{125}I-ANF bound to these cells specifically (70–85% specific binding) and in a saturable fashion with an approximate K_d and B_{max} of 2.13 nM and 4 pmol/mg of protein, respectively (data not shown). The ANF analogs ANF I, II, and III competed for the binding sites in a dose-dependent fashion with an approximate EC_{50} of 6, 6, and 3.5 nM, respectively (Fig. 1*B*). All three ANF analogs stimulated cGMP accumulation in a dose-dependent fashion with an approximate EC_{50} of 300, 200, and 250 nM, respec-

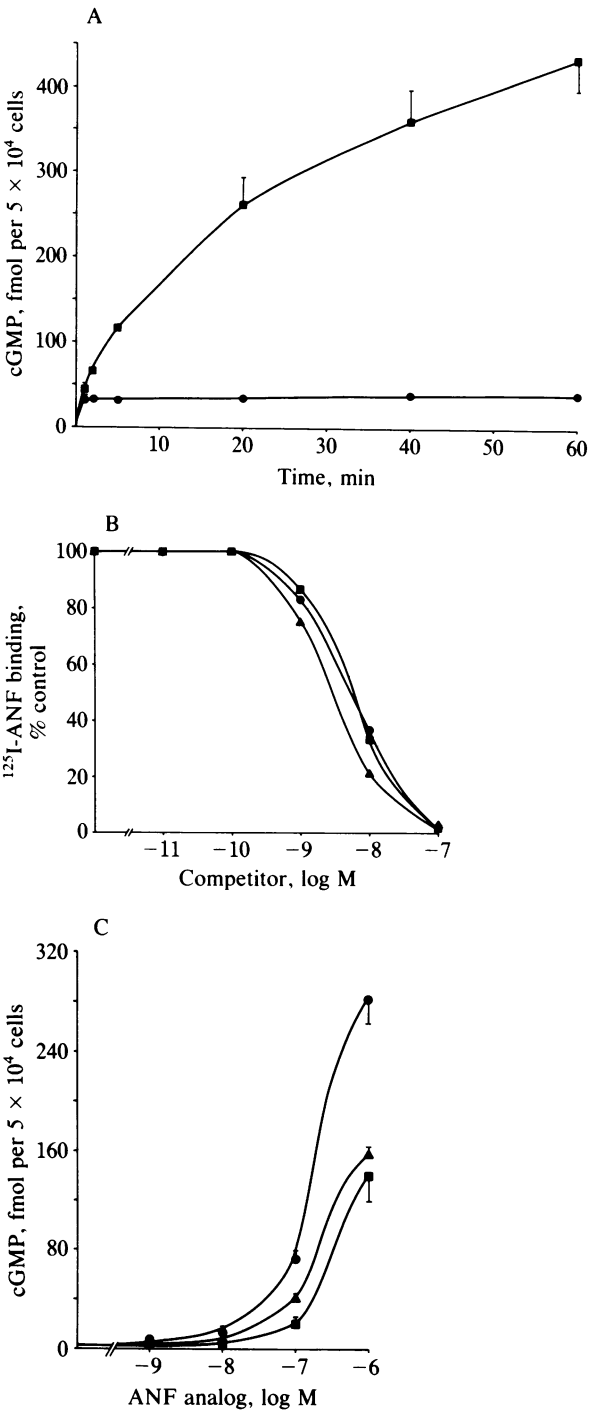

FIG. 1. (*A*) Time course of cGMP accumulation in response to ANF in rat A10 aortic smooth muscle cells. Cells were incubated in the presence of 1 μM ANF and 0.5 mM iBuMeXan for the times indicated, and cGMP levels were measured by radioimmunoassay as explained in *Materials and Methods*. ●, Basal; ■, 1 μM ANF. (*B*) Competition of ^{125}I-ANF binding by ANF I (■), II (●), and III (▲) in A10 cells. Cells were incubated with 5 nM ^{125}I-ANF and various concentrations of competitor at 22°C for 60 min. Binding was measured as explained in *Materials and Methods*. (*C*) Dose–response relationship of ANF I (■), II (●), and III (▲) and cGMP accumulation in A10 cells. Cells were incubated with various concentrations of ANF I, II, or III for 10 min at 37°C, and cGMP was measured by radioimmunoassay.

tively (Fig. 1*C*). Oxytocin, vasopressin, and bradykinin did not affect the binding of ^{125}I-ANF to these cells (data not shown).

Incubation of these cells with 10 nM [Arg^8]VP in the presence of 0.5 mM iBuMeXan had no significant effect on basal cGMP accumulation. However, [Arg^8]VP inhibited cGMP accumulation induced by ANF, and this inhibition was dependent on the concentration of [Arg^8]VP used (Fig. 2). The maximal inhibition was 50% and occurred at ≈10 nM [Arg^8]VP. The half-maximal response occurred at ≈0.4 nM [Arg^8]VP (Fig. 2). This value corresponded well with the dissociation constant (2.5 nM) of [Arg^8]VP for binding to these cells in monolayer (19) and with the concentration (0.8 nM) of [Arg^8]VP required for half-maximal inhibition of isoproterenol-stimulated cAMP accumulation (18) and the concentration (2.0 nM) of [Arg^8]VP required for the half-maximal stimulation of inositol phosphate formation (16) in these cells.

To determine whether the [Arg^8]VP-induced inhibition was receptor-mediated, the effects of various [Arg^8]VP antagonists were studied. As shown in Fig. 3, the selective V_1 antagonist [[d(CH$_2$)$_5$]^1,Tyr(Me)^2,Arg^8]VP (21) and the V_1/V_2 antagonist [[d(CH$_2$)$_5$]^1,D-Tyr(Et)^2,Val^4,Arg^8]VP (22) induced inhibition of the [Arg^8]VP effect in a concentration-dependent fashion, whereas the selective V_2 antagonist [[d-(CH$_2$)$_5$]^1,D-Ile^2,Val^4,Arg^8]VP (23) was ineffective in blocking the [Arg^8]VP-mediated effect. The selective V_2 agonist [dCys^1,D-Arg^8]VP did not significantly inhibit the ANF-induced cGMP accumulation at any concentration tested (Table 1), whereas at 10 nM this compound caused maximal activation (stimulation of adenylate cyclase) of the V_2 receptors of the medullary membranes of rat kidney (24).

To test whether the vasopressin receptor is directly coupled to guanylate cyclase or whether the [Arg^8]VP-mediated inhibition of ANF-stimulated cGMP accumulation is secondary to its activation of the phosphatidylinositol turnover pathway, the cells were pretreated with phorbol 12,13-dibutyrate (an activator of protein kinase C) and the levels of cGMP in response to ANF were measured. Pretreatment of the cells with 10 nM phorbol dibutyrate for 30 min at 37°C resulted in 30–35% inhibition of ANF-stimulated cGMP accumulation. The inactive compound 4α-phorbol 12,13-didecanoate, which does not activate protein kinase C, did not inhibit ANF-mediated cGMP accumulation.

DISCUSSION

Smooth muscle relaxation in response to many agents has been shown to be associated with an elevation of the intracellular concentration of cAMP and activation of cAMP-dependent protein kinase (25–27) or with an elevation of cGMP concentration and activation of cGMP-dependent protein kinase (28, 29). These biochemical changes mediate relaxant responses by causing cellular protein phosphorylation that regulates contraction of smooth muscle. ANF has been shown to stimulate the accumulation of cGMP and cause relaxation of rat thoracic aorta (30). In the present study with A10 cultured vascular smooth muscle cells, ANF II stimulated the accumulation of cGMP in a time-dependent fashion and ^{125}I-ANF bound to these cells in a saturable fashion with an approximate K_d of 2.1 nM. This compares very well with the K_d values reported by Schenk *et al.* (20) and Hirata *et al.* (11) for cultured bovine aortic smooth muscle cells (2.1 nM) and cultured vascular smooth muscle cells of rat aorta (1–2 nM), respectively. The concentrations of ANF I, II, and III required for half-maximal cGMP accumulation are approximately 50-, 30-, and 60-fold higher than that required to inhibit binding. The reason for this difference is not known. It appears that ANF stimulated only particulate guanylate cyclase, since there was no measurable soluble guanylate cyclase in these cells. Sodium nitroprusside and sodium azide, which are known to stimulate soluble

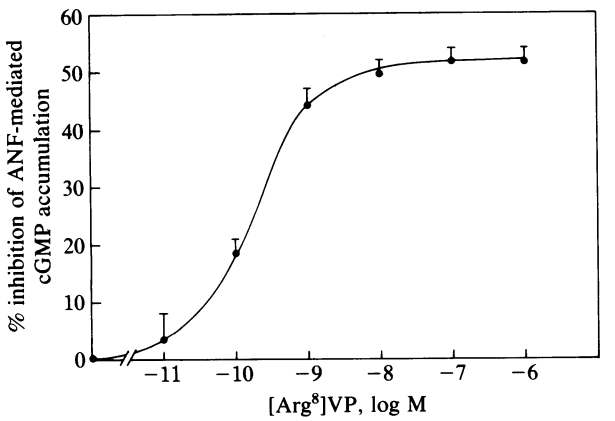

FIG. 2. [Arg^8]VP inhibition of 1 μM ANF-stimulated cGMP accumulation in rat aortic smooth muscle cells.

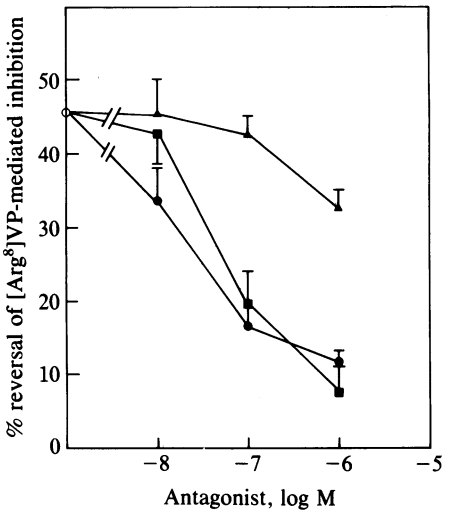

FIG. 3. Reversal by various [Arg^8]VP antagonists of the [Arg^8]VP (0.01 μM) inhibition of 1 μM ANF-stimulated cGMP accumulation in A10 cells. ○, [Arg^8]VP alone; ●, [[d(CH$_2$)$_5$]^1,Tyr(Me)^2,Arg^8]VP; ■, [[d(CH$_2$)$_5$]^1,D-Tyr(Et)^2,Val^4,Arg^8]VP; ▲, [[d(CH$_2$)$_5$]^1,D-Ile^2,Val^4,-Arg^8]VP.

Table 1. [Arg^8]VP, but not the V_2 subtype agonist [dCys^1,Arg^8]VP, inhibits 1 μM ANF-stimulated cGMP accumulation in rat aortic smooth muscle cells

Addition(s)	cGMP accumulation, fmol per 5 × 10^4 cells	% inhibition of ANF-induced cGMP accumulation
None	12.0 ± 0.3	—
ANF	138.0 ± 6.3	—
ANF + 10^{-8} M [Arg^8]VP	67.0 ± 3.1	51
ANF + 10^{-8} M [dCys^1,Arg^8]VP	147.0 ± 6.6	0
ANF + 10^{-7} M [dCys^1,Arg^8]VP	133.0 ± 4.3	4
ANF + 10^{-6} M [dCys^1,Arg^8]VP	128.0 ± 2.7	7
ANF + 10^{-5} M [dCys^1,Arg^8]VP	121.0 ± 4.4	12
10^{-8} M [Arg^8]VP	11.8 ± 1.0	—
10^{-5} M [dCys^1,Arg^8]VP	13.0 ± 0.5	—

The mean values ± SEM of data derived from one experiment are presented. The experiment was repeated with similar results.

guanylate cyclase, had no stimulatory effect on cGMP formation (data not shown).

Previous work showed that these cultured smooth muscle cells display vasopressin receptors of V_1 subtype (19). Addition of [Arg^8]VP to these cells resulted in inhibition of isoproterenol-induced cAMP accumulation (18) and stimulation of inositol phosphate accumulation and calcium fluxes (16), and these responses followed V_1 receptor specificity. The present data indicate that although [Arg^8]VP had no effect on the basal level of cGMP, it inhibited ANF-induced cGMP accumulation in a concentration-dependent fashion (half-maximal and maximal inhibition at $\approx$0.4 nM and $\approx$10 nM, respectively). The maximal inhibition by [Arg^8]VP was 45–50%, and this partial inhibition compares well with the inhibition of adenylate cyclase by other inhibitory hormones (18, 32). The reason for this partial inhibition is unknown at present. This inhibitory effect of [Arg^8]VP appears to be mediated through V_1 receptors, since V_1 and V_1/V_2 antagonists were more effective than V_2 antagonists in blocking the [Arg^8]VP effect. Moreover a V_2-specific agonist was found to be ineffective in inhibiting ANF-induced cGMP accumulation. The inhibition by [Arg^8]VP of ANF-induced cGMP accumulation did not result from stimulation of phosphodiesterase activity, since all the experiments were performed in the presence of iBuMeXan, a potent phosphodiesterase inhibitor. Nor could it be due to the inhibition of cAMP accumulation, as no effect of [Arg^8]VP on basal cAMP levels was observed. It is possible that [Arg^8]VP inhibition of ANF-stimulated cGMP accumulation may be secondary to its effect on the phosphatidylinositol pathway, since incubation of these cells with phorbol dibutyrate (an activator of protein kinase C), similar to diacyglycerol (one of the products of the phosphatidylinositol pathway), resulted in 30–35% inhibition of ANF-induced cGMP accumulation.

We have shown that [Arg^8]VP binding to these cell membranes is modulated by guanine nucleotide (33), suggesting that [Arg^8]VP might mediate its responses through guanine nucleotide-binding proteins. The mechanism by which ANF stimulates particulate guanylate cyclase is not known. Kuno *et al.* (31) have shown that, in rat lung, ANF receptor and particulate guanylate cyclase are contained in a single transmembrane glycoprotein. It is possible that ANF might mediate its effect through a guanine nucleotide-binding protein that could be part of this transmembrane glycoprotein and could be negatively modulated by [Arg^8]VP. Since [Arg^8]VP did not affect the basal level of cGMP, the locale of [Arg^8]VP action is probably not at the catalytic moiety of guanylate cyclase.

Thus, in the A10 smooth muscle cell line, [Arg^8]VP might induce contraction via one positive (stimulation of inositol phosphate accumulation and calcium fluxes) and at least two negative (inhibition of β-adrenergic receptor-mediated cAMP accumulation and ANF receptor-mediated cGMP accumulation) pathways.

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