Cyclic AMP-mediated Inhibition of Angiotensin II-induced Protein Synthesis Is Associated with Suppression of Tyrosine Phosphorylation Signaling in Vascular Smooth Muscle Cells*

(Received for publication, March 31, 1997, and in revised form, July 3, 1997)

Edith Giasson, Marc J. Servant‡, and Sylvain Meloche§

From the Centre de Recherche, Hôtel-Dieu de Montréal and Department of Pharmacology, University of Montreal, Montreal, Quebec H2W 1T8, Canada

In the present study, we have examined the effect of increased cyclic AMP (cAMP) levels on the stimulatory action of angiotensin II (Ang II) on protein synthesis. Treatment with cAMP-elevating agents potently inhibited Ang II-induced protein synthesis in rat aortic smooth muscle cells and in rat fibroblasts expressing the human AT₁ receptor. The inhibition was dose-dependent and was observed at all concentrations of the peptide. To explore the mechanism of cAMP action, we have analyzed the effects of forskolin and 3-isobutyl-1-methylxanthine on various receptor-mediated responses. Elevation of cAMP did not alter the binding properties of the AT₁ receptor and did not interfere with the activation of phospholipase C or the induction of early growth response genes by Ang II. Likewise, Ang II-dependent activation of the mitogen-activated protein kinases ERK1/ERK2 and p70 S6 kinase was unaffected by cAMP. In contrast, we found that increased concentration of cAMP strongly inhibited the stimulatory effect of Ang II on protein tyrosine phosphorylation. Specifically, cAMP abolished Ang II-induced tyrosine phosphorylation of the focal adhesion-associated protein paxillin and of the tyrosine kinase Tyk2. These results identify a novel mechanism by which the cAMP signaling system may exert growth-inhibitory effects in specific cell types.

Cyclic AMP (cAMP) is a pleiotropic second messenger that has been implicated as a modulator of cell proliferation in several cell types. Intriguingly, depending on the cellular origin and the differentiation state of the cell, cAMP is found to cause either growth inhibition or growth stimulation. For example, elevation of intracellular cAMP stimulates the proliferation of thyrocytes, keratinocytes, epithelial cells, hepatocytes, and Swiss 3T3 cells. On the contrary, elevated cAMP inhibits cell proliferation in fibroblasts, SMC, lymphoid cells, and many tumor cells (for review, see Refs. 1–5). In these cells, cAMP interferes with the mitogenic response to growth factors acting on both receptor tyrosine kinases and protein-coupled receptors (6). In addition to their effect on cell proliferation, cAMP analogs can also partially reverse the phenotype of transformed fibroblasts as well as other cancer cells (5, 7).

The regulatory effects of cAMP are mediated through activation of the multifunctional cAMP-dependent protein kinase (protein kinase A or PKA) (8). These effects are exerted both at the post-translational level and at the transcriptional level through phosphorylation of cAMP-responsive element-binding proteins (CREB/ATF family) (9, 10). Although considerable progress has been made in understanding the mechanism of gene regulation by cAMP, little is known about the molecular mechanisms by which the nucleotide modulates cell growth. A number of studies have proposed that cAMP might inhibit cell proliferation by interfering with Ras-dependent activation of MAP kinases (11–15). Biochemical analysis of the various intermediates in the signaling cascade indicated that cAMP inhibits signal transmission by preventing Ras-dependent activation of the serine/threonine kinase Raf-1 (11, 12, 15). This inhibitory effect of cAMP was mediated by PKA because it was not observed in mutant cells that express a PKA resistant to activation by cAMP (14). However, treatment of CCL39 fibroblasts (16) or interleukin-2-dependent T lymphocytes (17) with cAMP-raising agents was found to block cell proliferation completely without affecting growth factor-induced MAP kinase activation. Another study reported that treatment of murine macrophages with analogs of cAMP raises the overall amount of the inhibitor p27Kip1, thereby increasing its association with cyclin D-Cdk4 and preventing the activation of Cdk4 (18). cAMP was also shown to reduce the accumulation of c-myc mRNA in various cell lines (19–22). In the yeast Saccharomyces cerevisiae, PKA exercises regulatory control on both growth and division, suggesting a role for cAMP in the homeostatic integration of these two processes (23, 24). It is not known whether cAMP exerts similar control on the overall rate of protein synthesis in mammalian cells under conditions of growth factor stimulation or cellular stress.

Ang II is a growth factor for a number of cell types, including adrenocortical cells, proximal tubular cells, vascular SMC, cardiac myocytes, and cardiac fibroblasts (for review, see Refs. 25 and 26). In cultured aortic SMC, Ang II induces cellular hypertrophy as a result of increased protein synthesis but not cell proliferation (27–30). The growth-promoting effects of the peptide are mediated by the AT₁ receptor subtype, a member of the superfamily of G protein-coupled receptors. Agonist binding to the AT₁ receptor stimulates the activity of phospholipase C, to generate the second messengers InP₃ and diacylglycerol, and inhibits the activity of adenyl cyclase (for review, see Refs. 31 and 32). One of the immediate consequences of these early
signals is activation of the MAP kinases ERK1/ERK2 (33–35) and the 70/85-kDa S6 protein kinases (referred to as p70S6k) (30). Activation of the AT1 receptor also leads to increased tyrosine phosphorylation of multiple proteins in target cells (36–40). Despite these observations, the nature of the signaling mechanisms coupling the AT1 receptor to the hypertrophic response remains poorly understood.

The aim of this study was to evaluate the effect of increased cAMP levels on Ang II-stimulated protein synthesis in vascular SMC. We show that cAMP-raising agents potently inhibit the hypertrophic effect of Ang II. In addition, we demonstrate that increased cAMP selectively antagonizes the stimulatory effect of Ang II on protein tyrosine phosphorylation in these cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—** I-Labeled [Ser, Ile]Ang II (serile) was prepared by radioliodination of sarile using a solid phase method as described (41). Forskolin, IBMX, Vehirol cholera toxin and 8-bromo-cAMP were obtained from Calbiochem. Forskolin and IBMX were dissolved in dimethyl sulfoxide to give stock solutions of 100 mM and 500 mM, respectively. Cholera toxin was dissolved in water at a concentration of 1 mg/ml, and 8-bromo-cAMP was dissolved in 10 mM Tris-HCl (pH 7.0) at a concentration of 100 mM. Isoproterenol was a gift of Dr. Michel Bouvier (University of Montreal) and was prepared as a 0.5 mM solution in 10% ascorbic acid. The source of other materials has been described (30).

Antisera SM1 and cHcP2 have been described and specifically immunoprecipitate the MAP kinases ERK1 and ERK2, respectively (42, 43). Antiserum S6-24 was produced in rabbits using a synthetic peptide corresponding to amino acids 2–30 of rat p70S6K (Quality Control Biochemicals). The anti-p125 FAK mAb 2A7 was generously provided by Dr. Thomas Parsons (University of Virginia). The anti-Shc serum was provided by Dr. Louise Larose (McGill University). The anti-paxillin and anti-Pyk2 mAbs were purchased from Transduction Laboratories. The anti-phosphotyrosine mAb 4G10 and anti-Tyk2 polyclonal antibody were obtained from Upstate Biotechnology and Santa Cruz Biotechnology, respectively.

**Cell Culture—** Rat aortic SMC were cultured and synchronized as described previously (30). Rat1-AT cells are Rat1 fibroblasts stably expressing the human Ang II AT1 receptor. Receptor Rat1-AT cells were grown in minimum essential medium supplemented with 10% calf serum, 2 mM glutamine, and 0.4 mg/ml Geneticin. They were made quiescent by incubating confluent cell cultures in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 1% FBS, 15 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM leupeptin, and 1% Triton X-100. Aortic SMC were then incubated with 0.2% bovine serum albumin, followed by washing with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM EDTA, 1 mM MgCl2, and 0.1% heat-inactivated bovine serum albumin. Bound 125I-serine was separated from free ligand by rapid filtration through GF/B filters presoaked with 0.2% bovine serum albumin, followed by washing with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl. The filters were counted for radioactivity. Averages of duplicate determinations of bound 125I-serine were used for data analysis. Binding data were analyzed by nonlinear least squares curve fitting using the SCAFT computer program (46).

**Measurement of Inositol Phosphates—** Quiescent aortic SMC were stimulated with 10 nM Ang II for the indicated times at 37 °C. Quiescent aortic SMC were stimulated with 10 nM Ang II for the indicated times at 37 °C. Total RNA was extracted with guanidinium thiocyanate as described (47). Equal amounts of total RNA (10–20 μg) were denatured by heating for 15 min at 65 °C in 2.2 mM formaldehyde and 50% formamide and resolved by electrophoresis in a 1% agarose gel containing 1.8% formaldehyde. The RNA was transferred to Hybond-N (Amersham) nylon membranes by vacuum blotting, fixed, and hybridized with 32P-labeled probes. Hybridization was carried out in hybridization medium (50% formamide, 15 μM SSC, 15 μM NaCl, 5 × Denhardt’s solution (1 × Denhardt’s = 0.01% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 50% formamide, and 100 μM herring sperm DNA) containing the labeled probe (1–2 × 106 cpm/ml) for 16 h at 42 °C. The membranes were washed twice at 25 °C for 15 min in 2 × SSC, 0.1% SDS, and twice at 60 °C for 30 min in 0.5 × SSC, 0.1% SDS. The extent of hybridization was analyzed with a PhosphorImager apparatus (Molecular Dynamics). The results were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

**Analysis of Tyrosine-Phosphorylated Proteins—** Quiescent aortic SMC were incubated with 10 nM Ang II for the indicated times at 37 °C. The cells were then washed twice in ice-cold phosphate-buffered saline and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 M phenylmethylsulfonyl fluoride, 10 M sodium orthovanadate, 10 M phenylmethylsulfonyl fluoride, 10 M leupeptin, 10 M pepstatin A, 1 % Triton X-100) for 30 min at 4 °C. Cell lysates were centrifuged at 12,000 × g for 10 min, and the supernatants were used for immunoprecipitation. Immunoprecipitates were washed three times with SDS-gel electrophoresis on 7.5% acrylamide gels and electroeluted transferred to Hybond-C nitrocellulose membranes (Amersham) in 25 mM Tris, 192 mM glycine. After fixation for 15 min in 40% methanol, 7% acetic acid, 3% glyceral, the membrane was blocked for 1 h at 37 °C in Tris-buffered saline containing 0.1% Tween 20 and 1% bovine serum albumin and then incubated for 2 h at 25 °C with mAb 4G10 (1.4,000) in blocking solution. The membrane was washed three times in Tris-buffered saline, 0.1% Tween 20 prior to incubation for 1 h with horseradish peroxidase-conjugated anti-IgG diluted 1:10,000 in Tris-buffered saline containing 0.1% Tween 20 and 3% non-fat dry milk. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

**Protein Synthesis Measurements—** Quiescent aortic SMC or Rat1-AT cells in triplicate wells of 24-well plates were stimulated with the...
indicated concentrations of Ang II in serum-free medium containing 0.5 μCi/ml [3H]leucine. After 24 h of stimulation, the medium was aspirated, and the cells were incubated for a minimum of 30 min in cold 5% trichloroacetic acid. The wells were then washed once with trichloroacetic acid and three times with tap water. The radioactivity incorporated into trichloroacetic acid-precipitable material was measured by liquid scintillation counting after solubilization in 0.1 M NaOH. Where indicated, the cells were stimulated for 24 h with Ang II in the continuous presence of cAMP-elevating agents.

Other Methods—Protein concentrations were measured using the BCA protein assay kit (Pierce) with bovine serum albumin as standard. Dose-response curves were analyzed according to a four-parameter logistic equation using the ALLFIT computer program (48).

RESULTS

Increased cAMP Inhibits Ang II-stimulated Protein Synthesis in Aortic SMC—Ang II is a hypertrophic factor that potently stimulates protein synthesis in rat aortic SMC but has no effect on DNA synthesis or cell proliferation (27–30). To examine the effect of cAMP on the growth response to Ang II, aortic SMC were treated with a variety of agents known to increase intracellular cAMP, and the rate of protein synthesis was determined by [3H]leucine incorporation. As shown in Fig. 1, the addition of cAMP-raising agents strongly inhibited the stimulatory effect of Ang II on protein synthesis, without affecting the basal rate of protein synthesis. All of these agents were found to raise the intracellular concentration of cAMP significantly (data not shown). The growth-inhibitory effect of cAMP was reversible, and no sign of long term cytotoxicity was observed at the concentrations of agents used. The cAMP phosphodiesterase inhibitor IBMX and the adenyl cyclase activator forskolin were the most effective inhibitors, reducing the hypertrophic effect of Ang II by 100 and 70%, respectively. These two compounds were therefore used in all subsequent experiments.

Pharmacological studies revealed that forskolin and IBMX block Ang II-induced leucine incorporation in a dose-dependent manner. Half-maximal inhibition was observed at a concentration of 0.5 ± 0.2 μM forskolin and 79 ± 18 μM IBMX (Fig. 2). We also analyzed the effect of the two inhibitors on the rate of protein synthesis at different concentrations of Ang II. Fig. 3 shows that treatment with forskolin or IBMX inhibited the induction of protein synthesis by every concentration of the peptide. The half-maximal effect of Ang II on protein synthesis was found to be similar in the absence or in the presence of either forskolin or IBMX. Taken together, these results demonstrate that elevation of intracellular levels of cAMP, through different cellular mechanisms, antagonizes the stimulatory effect of Ang II on protein synthesis. This inhibitory effect of cAMP is presumably mediated by activation of PKA.

Elevation of cAMP Does Not Interfere with Ang II Early Signaling Events—To explore the mechanism by which cAMP interferes with the activation of protein synthesis by Ang II, we examined the effects of forskolin and IBMX on various recep-
cAMP Inhibits Ang II-stimulated Tyrosine Phosphorylation

Ang II has been shown to exert its hypertrophic effect through activation of the AT₁ receptor subtype, a member of the superfamily of G protein-coupled receptors (29, 30). We first tested the effect of increased cAMP levels on the agonist binding properties of the AT₁ receptor by performing competition binding studies in membranes derived from either control cells or cells treated with forskolin or IBMX. As shown in Fig. 4, treatment with cAMP-raising agents did not change the number of high affinity sites of the receptor. The proportion of high affinity sites and the affinity for Ang II (control, $K_d = 2.1$ nM; forskolin, $K_d = 1.1$ nM; IBMX, $K_d = 1.6$ nM) was similar in control cells and cAMP-treated cells, indicating that elevation of cAMP does not interfere with the initial coupling of the AT₁ receptor with G proteins.

We next analyzed the effect of cAMP on phospholipase C activation by measuring the intracellular mass of InsP₃, Ang II binding to the AT₁ receptor has been shown to stimulate the activity of phospholipase C in aortic SMC and in many other target cells rapidly (31, 32). Pretreatment of the cells with either forskolin or IBMX did not prevent the rapid increase in the production of InsP₃ induced by Ang II (Fig. 5). These results indicate that cAMP does not inhibit the growth effect of Ang II by interfering with early receptor-mediated signaling events.

**Elevation of cAMP Does Not Inhibit Ang II-dependent Activation of ERK1/ERK2 and p70S6K**—In common with growth factors, Ang II potently stimulates the enzymatic activity of the MAP kinase isofoms ERK1/ERK2 in vascular SMC (30, 33–35). In view of the demonstration that PKA antagonizes growth factor-induced Ras-dependent activation of MAP kinases in a number of cell types (11–15), we sought to determine if the inhibitory effect of cAMP on Ang II-induced protein synthesis resulted from a negative regulatory effect on the MAP kinases ERK1/ERK2. Quiescent aortic SMC were pretreated with forskolin or IBMX prior to Ang II stimulation, and the activity of ERK isoforms was determined by specific immune complex kinase assays. In contrast to the above mentioned results, elevation of cAMP levels did not affect Ang II-dependent activation of ERK1 and ERK2 isoforms in rat aortic SMC (Fig. 6). Detailed kinetic analysis also confirmed that cAMP does not alter the time course of ERK1/ERK2 activation by Ang II (data not shown). We also examined the effect of forskolin and IBMX on the activity of MEK isoforms in Ang II-stimulated cells. Ang II has been shown to activate both MEK1 and MEK2 isoforms in rat aortic SMC (33). As expected, the response to Ang II was not altered in cells treated with cAMP-raising agents (data not shown).

Another potential candidate for the inhibitory action of cAMP is the serine/threonine kinase p70S6K, the major S6 protein kinase in vivo. We have demonstrated previously that...
Ang II stimulates the phosphotransferase activity of p70^{S6K} in aortic SMC and that inhibition of this enzyme with rapamycin correlates with inhibition of Ang II–induced protein synthesis (30). To determine if cAMP antagonizes Ang II–stimulated p70^{S6K} activation, cells were treated with forskolin or IBMX, and the activity of p70^{S6K} was measured by immune complex kinase assay. Fig. 7 shows that treatment of cells with either compound had no effect on Ang II–mediated activation of p70^{S6K} in rat aortic SMC. These results indicate that cAMP does not interfere with the two major protein kinase signaling cascades to exert its inhibitory action on protein synthesis.

**Elevation of cAMP Does Not Prevent Ang II–stimulated Early Growth Response Genes Induction**—Ang II has been shown to increase mRNA expression of the c-fos (49–51), c-jun (52), egr-1 (53), fosB (54), and c-myc (55) genes in vascular SMC. However, the role of these early gene products in the stimulatory effect of Ang II on protein synthesis remains to be established. In this study, we analyzed the effect of raising cAMP levels on mRNA expression of the c-fos, fosB, and egr-1 genes in rat aortic SMC. Treatment of the cells with either forskolin or IBMX had no effect on the peak induction of these genes in response to Ang II stimulation (Fig. 8A). We also examined the effect of cAMP on the induction of the c-myc gene. Previous studies in diverse cell types have shown that elevation of cAMP can inhibit growth factor–dependent expression of c-myc mRNA by down-regulating the transcription of the gene (19–22). Fig. 8B shows that Ang II induced the expression of the c-myc gene in aortic SMC, with a peak induction observed after 2–4 h. Neither forskolin or IBMX prevented the induction of the c-myc gene in response to Ang II. Moreover, incubation with either compound had no effect on the time course of c-myc mRNA expression in Ang II–stimulated cells (data not shown). Together, these data suggest that cAMP does not inhibit the trophic effect of Ang II by blocking early growth response genes induction.

**Increased cAMP Inhibits Ang II–stimulated Tyrosine Phosphorylation of Cellular Proteins**—Ang II stimulates tyrosine phosphorylation of multiple proteins in vascular SMC and other target cells (36–40). This signaling pathway appears to play a critical role in the hypertrophic action of Ang II because treatment of aortic SMC with selective tyrosine kinase inhibitors completely abrogates Ang II–dependent increase in protein synthesis (40). These observations led us to examine the effect of cAMP elevation on Ang II–dependent activation of tyrosine phosphorylation. Preincubation of aortic SMC with forskolin or IBMX resulted in a complete inhibition of Ang II–stimulated tyrosine phosphorylation of the 120–125- and 65–75-kDa bands, the two major substrates detected in these cells (Fig. 9A). As predicted from the enzymatic assays (see Fig. 6), treatment of cells with either compound had no influence on tyrosine phosphorylation of the MAP kinase isoforms ERK1/ERK2, clearly showing the selectivity of the cAMP inhibitory action (Fig. 9B).

We and others have identified the major 65–75-kDa tyrosine phosphorylated substrate in Ang II–treated cells as the focal adhesion–associated protein paxillin (56, 57). We therefore examined more specifically the effect of cAMP on tyrosine phosphorylation of paxillin. Addition of Ang II increased tyrosine phosphorylation of paxillin in quiescent aortic SMC, and this response was blocked completely by preincubation with either forskolin or IBMX (Fig. 9C). In contrast, neither compound prevented tyrosine phosphorylation of the adaptor protein Sbc, another major target of Ang II signaling (39, 58). These observations are consistent with the idea that Ang II activates at least two tyrosine kinase pathways, one of which is sensitive to cAMP. To explore further the mechanism by which cAMP interferes with tyrosine phosphorylation signaling, we examined the effect of cAMP elevation on the tyrosine phosphorylation level of Ang II–regulated cellular tyrosine kinases. Ang II significantly increased the phosphotyrosine content of the focal adhesion–associated kinases p125^{FAK} and Pyk2 (also known as...
CAK beta and RAFTK) and of the Janus kinase Tyk2 in aortic SMC (Fig. 9D). Treatment of the cells with forskolin or IBMX did not affect Ang II-induced tyrosine phosphorylation of p125FAK and Pyk2, thereby indicating that these tyrosine kinases are not the major paxillin kinases. However, both agents completely blocked the increased tyrosine phosphorylation of Tyk2 in response to Ang II (Fig. 9D). Thus, these results provide strong evidence that raising intracellular cAMP levels interferes with Ang II-stimulated tyrosine phosphorylation in vascular SMC.

**Increased cAMP Inhibits Ang II-stimulated Protein Synthesis in a Heterologous Cell Line Expressing the Human AT1 Receptor**—We have established a rat fibroblast cell line expressing a physiological number of human AT1 receptors which shows an increased rate of protein synthesis in response to Ang II. To determine whether the growth-inhibitory effect of cAMP was specific to the cellular context, we examined the effect of cAMP elevation on Ang II-induced protein synthesis in Rat1-AT1 cells. As shown in Fig. 10, treatment of Rat1-AT1 cells with forskolin completely abolished the stimulatory effect of Ang II on protein synthesis. Forskolin was found to be more potent in inhibiting the trophic effect of Ang II in Rat1-AT1 cells compared with aortic SMC, with half-maximal inhibition observed at 0.044 ± 0.006 μM concentration. Notably, forskolin also inhibited Ang II-induced tyrosine phosphorylation in Rat1-AT1 cells (data not shown).

**DISCUSSION**

The role of cAMP as a regulator of cell growth has received considerable attention since the demonstration that elevation of the intracellular level of the nucleotide can stimulate or inhibit cell proliferation (1–5). However, until recently, the mechanism by which cAMP negatively affects the proliferation of normal and transformed cells has remained largely unknown. More recent work in various cellular models has led to new hypotheses to explain cAMP action (see below). In particular, cAMP has been shown to induce G1 arrest in macrophages by interfering with the cell cycle machinery at the level of Cdk4 activation (18). All of these studies have examined the consequences of cAMP elevation on mitogen-induced cell cycle progression and cell division. Here, we have investigated the effect of cAMP on the induction of protein synthesis by the hypertrophic factor Ang II in aortic SMC and in fibroblasts expressing the human AT1 receptor.

We report that a variety of agents known to elevate the intracellular concentration of cAMP potently inhibit the stimulatory effect of Ang II on protein synthesis (Fig. 1). The inhibition was dose-dependent and was observed at all concentrations of Ang II. Because these agents increase cAMP by...
different mechanisms of action, the above data indicate that the growth-inhibitory response is mediated by the elevation of intracellular cAMP levels. The effect of cAMP is most presumably dependent on the activity of PKA, as virtually all of the effects of the nucleotide in mammalian cells are attributed to this multifunctional enzyme. Interestingly, we show that the inhibitory effect of cAMP on Ang II-induced protein synthesis is not restricted to vascular SMC but is also observed in heterologous rat fibroblasts expressing the human AT$_2$ receptor. These data are consistent with the idea that cAMP has a generalized effect on the signaling machinery linking the AT$_2$, receptor to the regulation of protein synthesis. Indeed, previous studies have shown that cAMP-elevating agents exert similar inhibitory effect on the trophic action of Ang II in epithelial cell lines of renal origin (59, 60). Similar to vascular SMC, Ang II was found to stimulate the rate of protein synthesis and to induce cellular hypertrophy in these cells, without increasing DNA content. However, the mechanism by which cAMP interferes with the hypertrophic effect of Ang II was not addressed in the latter studies. Thus, in addition to its well defined modulatory role on cell proliferation and cellular morphology, cAMP also exerts negative regulatory effects on the stimulation of protein synthesis by hypertrophic factors.

The molecular basis of the hypertrophic action of Ang II is still largely undefined. Activation of the AT$_1$ receptor is known to trigger multiple G protein-mediated signaling pathways, including the inhibition of adenylyl cyclase (31, 32). Based on the observation that Ang II decreases the intracellular concentration of cAMP in the renal epithelial cell lines of renal origin (59, 60), Similar to vascular SMC, Ang II was found to stimulate the rate of protein synthesis and to induce cellular hypertrophy in these cells, without increasing DNA content. However, the mechanism by which cAMP interferes with the hypertrophic effect of Ang II was not addressed in the latter studies. Thus, in addition to its well defined modulatory role on cell proliferation and cellular morphology, cAMP also exerts negative regulatory effects on the stimulation of protein synthesis by hypertrophic factors.

The molecular basis of the hypertrophic action of Ang II is still largely undefined. Activation of the AT$_1$ receptor is known to trigger multiple G protein-mediated signaling pathways, including the inhibition of adenylyl cyclase (31, 32). Based on the observation that Ang II decreases the intracellular concentration of cAMP in the renal epithelial cell lines of renal origin (59, 60). Similar to vascular SMC, Ang II was found to stimulate the rate of protein synthesis and to induce cellular hypertrophy in these cells, without increasing DNA content. However, the mechanism by which cAMP interferes with the hypertrophic effect of Ang II was not addressed in the latter studies. Thus, in addition to its well defined modulatory role on cell proliferation and cellular morphology, cAMP also exerts negative regulatory effects on the stimulation of protein synthesis by hypertrophic factors.

The molecular basis of the hypertrophic action of Ang II is still largely undefined. Activation of the AT$_1$ receptor is known to trigger multiple G protein-mediated signaling pathways, including the inhibition of adenylyl cyclase (31, 32). Based on the observation that Ang II decreases the intracellular concentration of cAMP in the renal epithelial cell lines of renal origin (59, 60). Similar to vascular SMC, Ang II was found to stimulate the rate of protein synthesis and to induce cellular hypertrophy in these cells, without increasing DNA content. However, the mechanism by which cAMP interferes with the hypertrophic effect of Ang II was not addressed in the latter studies. Thus, in addition to its well defined modulatory role on cell proliferation and cellular morphology, cAMP also exerts negative regulatory effects on the stimulation of protein synthesis by hypertrophic factors.

We have investigated the biochemical mechanism by which cAMP elevation antagonizes Ang II-induced protein synthesis in aortic SMC. We first excluded the possibility that cAMP was acting very early at the level of receptor activation. No significant effect of forskolin or IBMX was found on the total number of AT$_1$, receptors, the formation of high affinity receptor sites, and the activation of phospholipase C. We then examined the possibility that cAMP elevation might interfere with MAP kinase or p70$^S6K$ signaling cascades. As mentioned, a number of reports have shown that cAMP-raising agents inhibit Ang II-induced protein synthesis, it has been proposed that the hypertrophic action of the hormone may be mediated by the decrease in intracellular cAMP (59, 60). However, several lines of evidence do not support this hypothesis but rather indicate that G$_i$-regulated pathways are not important for the hypertrophic effect of Ang II. We have demonstrated that pretreatment of aortic SMC or Rat1-AT$_1$ cells with concentrations of pertussis toxin which completely prevent the decrease in cAMP levels have no effect on Ang II-induced protein synthesis. In addition, Ang II has been shown to increase slightly the cAMP content in cardiac myocytes where it induces cellular hypertrophy potently (61). The physiological significance of the inhibition of adenylyl cyclase remains to be determined.

We also provide evidence that cAMP does not affect the induction of immediate-early genes in aortic SMC. It is interesting to note that in contrast to the results presented here, increased cAMP has been shown to down-regulate c-myc mRNA expression in several cell types, including normal and neoplastic B cells (19), leukemic cells (20), fibroblasts (21), and a macrophage cell line (22). The cAMP-mediated reduction in c-myc RNA levels results from a decrease in c-myc transcription (20, 22). Despite these observations, the significance of c-myc down-regulation is not known because constitutive expression of c-myc was insufficient to override the growth-inhibitory effect of cAMP in murine macrophages (22).

Accumulating evidence suggests that tyrosine phosphorylation may play a significant role in the growth response to G protein-coupled receptor agonists. Numerous studies have shown that growth factors such as thrombin, bombesin, vasopressin, endothelin, and lysophosphatidic acid stimulate tyrosine phosphorylation of multiple substrates in their target cells (36, 62, 63). The observation that selective tyrosine kinase inhibitors can block the stimulation of DNA synthesis induced by thrombin (64), endothelin (65), and bombesin (66) has provided strong evidence for the importance of this signaling pathway in the mitogenic response. Ang II was also reported to induce tyrosine phosphorylation of multiple proteins (36–40) and to stimulate the activity of cytosolic tyrosine kinases (39, 67–69) in target cells. Most importantly, treatment of aortic SMC with the tyrosine kinase inhibitors genistein and herbimycin A was found to abolish completely the stimulatory effect of Ang II on protein synthesis (40). In this study, we demonstrated that cAMP elevation interferes with tyrosine phosphorylation signaling as revealed by inhibition of Ang II-dependent tyrosine phosphorylation of the Janus kinase Tyk2 and of the focal adhesion protein paxillin. Interestingly, the effect of cAMP is selective with regard to the tyrosine kinase or the target substrate, clearly indicating that Ang II activates more than one tyrosine kinase pathway in vascular SMC. Thus, agents that raise cAMP levels may provide very useful tools to dissect the individual roles of Ang II-regulated tyrosine kinases in the induction of protein synthesis. In this regard, we are currently investigating the specific role of Janus kinases in the nuclear and cytoplasmic events controlling the rate of protein synthesis. Preliminary pharmacological data suggest that activation of the Janus kinase/Stat pathway is an essential component of Ang II hypertrophic action.

In summary, our results reveal a functional cross-talk between tyrosine phosphorylation, and more specifically Tyk2, and the cAMP signaling system. During the course of this work, two other groups reported that activation of PKA inhibits

---

3 M. J. Servant and S. Meloche, unpublished results.
cAMP Inhibits Ang II-stimulated Tyrosine Phosphorylation

cytokine-dependent activation of the Janus kinase/Stat pathway in the myeloma cell line U266 (70) and in monocytes (71). Most importantly, these observations identify a novel mechanism by which cAMP may exert growth-inhibitory effects in specific cell types.

Acknowledgments—We thank Drs. F. Hall, M. Dunn, G. L’Allemain, J. Pouyssegur, L. Larose, and T. Parsons for a generous supply of antisera; E. Pérès for preparation of the figures; and I. Rémillard for secretarial assistance. We are also grateful to Dr. A. De Léan and N. McNicoll for access to the inoculation and computer facilities.

REFERENCES

1. Pastan, I. H., Johnson, G. S., and Anderson, W. B. (1975) Annu. Rev. Biochem. 44, 491–522.
2. Boynton, A. L., and Whitfield, J. F. (1983) Adv. Cyclic Nucleotide Res. 15, 193–294.
3. Rosomoff, H. L. (1986) Science 233, 161–166.
4. Dumont, J. F., Jauniaux, J. C., and Roger, P. P. (1989) Trends Biochem. Sci. 14, 67–71.
5. Che-Chung, Y. S. (1990) Cancer Res. 50, 7093–7100.
6. Magnaldo, J., Pouyssegur, J., and París, S. (1989) FEBS Lett. 245, 65–69.
7. Pastan, I. and Willingham, M. (1978) Nature 274, 645–650.
8. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005.
9. Roessler, W. J., Vandenbark, G. R., and Hanson, R. W. (1988) J. Biol. Chem. 263, 9063–9066.
10. Lalli, E., and Sassone-Corsi, P. (1994) J. Biol. Chem. 269, 17359–17362.
11. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9933–9937.
12. Cooke, R. J., and McCarthy, F. (1993) Science 260, 1069–1072.
13. Graves, L. M., Borndorf, K. E., Raines, E. W., Potts, B. C., MacDonald, S. G., Ross, R., and Krebs, E. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10300–10304.
14. Sevros, B., Kong X., and Lawrence, J. C. Jr. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10305–10309.
15. Burgering, B. M. T., Pronk, G. J., van Weeren, P. C., Charding, P., and Bos, J. L. (1993) EMBO J. 12, 4211–4220.
16. Kahan, C., Seuwen, K., Molbe, S., and Pouyssegur, J. (1992) J. Biol. Chem. 267, 13369–13375.
17. Monfar, M., Lemon, K. P., Grammer, T. C., Cheatham, L., Chung, J., Vlahos, C. J., and Blenis, J. (1995) Mol. Cell. Biol. 15, 537–543.
18. Tsuda, T., Kawahara, Y., Ishida, Y., Koide, M., Shii, K., and Yokoyama, M. (1993) J. Biol. Chem. 268, 7338–7345.
19. Schorh, W., Feeler, T. C., Madigan, N. N., Konrad, K. M., and Baker, K. M. (1994) J. Biol. Chem. 269, 19626–19632.
20. Leduc, I., Haddad, P., Giasson, E., and Meloche, S. (1995) Mol. Pharmacol. 48, 582–592.
21. Lambert, C., Massillon, Y., and Meloche, S. (1995) Circ. Res. 77, 1001–1007.
22. Meloche, S. (1995) J. Cell. Physiol. 163, 577–588.
23. Wang, Y., Simonson, M. S., Pouyssegur, J., and Dunn, M. J. (1992) Biochem. J. 287, 589–594.
24. Toey, K. C., O’Donagh, K. G., and Whelan, J. A. M. (1974) Clin. Chim. Acta 56, 221–234.
25. Meloche, S., Ong, H., Cantin, M., and De Léan, A. (1986) Mol. Pharmacol. 30, 537–543.
26. De Léan, A., Hancock, A. A., and Leftkowitz, R. J. (1982) Mol. Pharmacol. 21, 5–16.
27. Czaczosz, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
28. De Léan, A., Munson, P. J., and Rodbard, D. (1978) Am. J. Physiol. 237, E102–E102.
29. Kawahara, Y., Sunako, M., Tsuda, T., Fujikawa, H., Fujimoto, Y., and Takai, Y. (1986) Biochem. Biophys. Res. Commun. 139, 52–59.
30. Taubman, M. B., Burk, B. C., Izumo, S., Tsuda, T., Alexander, R. W., and Nadal-Ginard, B. (1989) J. Biol. Chem. 264, 526–530.
31. Naftilan, A. J., Pratt, R. E., Elderidge, C. S., Lin, H. L., and Dzau, V. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 26, 1039–1043.
32. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) J. Biol. Chem. 270, 12563–12568.
33. Hordijk, P. L., Van Corven, E. J., and Moolenaar, W. H. (1994) J. Biol. Chem. 269, 2645–2651.
34. Tsuda, T., Kawahara, Y., Ishida, Y., Koide, M., Shii, K., and Yokoyama, M. (1992) J. Biol. Chem. 267, 6650–6656.
35. Huckle, W. R., Prokop, C. A., Dye, R. C., Herman, B., and Earp, S. (1990) Mol. Cell. Biol. 10, 6290–6298.
36. Molloy, C. J., Taylor, D. S., and Weber, H. (1993) J. Biol. Chem. 268, 109–112.
37. Timmermans, P. B. M. M., Wong, P. C., Chiu, A. T., Herbst, W. F., Benfield, P., Carini, D. J., Lee, R. J., Wester, R. R., Saye, J. A. M., and Smith, R. D. (1993) Pharmacol. Rev. 45, 205–251.
38. Hordijk, P. L., Verlaan, J., van Corven, E. J., and Moolenaar, W. H. (1994) J. Biol. Chem. 269, 333–342.
39. Pfeifer-Ohlsson, S., Watt, R., Funderud, S., Godal, T., and Ohlsson, R. (1989) Mol. Pharmacol. 36, 577–588.
40. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) J. Biol. Chem. 270, 13191–13195.
41. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) J. Biol. Chem. 270, 13191–13195.
42. Wolf, G., Killen, P. D., and Neilson, E. G. (1991) Cell Regul. 2, 219–227.
43. Wolf, G., Zahnier, G., Monfort, U., Schoeppe, W., and Stahl, R. A. K. (1993) Nephron. Dial. Transplant. 8, 128–133.
44. Seckl, M., and Rozengurt, E. (1993) Circ. Res. 73, 424–438.
45. Zachary, I. G., Bock, U., and Vetter, H. (1992) J. Biol. Chem. 264, 651–655.