Activation of \( \alpha_1 \) adrenergic receptors not only stimulates smooth muscle contraction but also modifies gene expression. We wondered if \( \alpha_1 \) adrenergic receptors could activate transcription of genes regulated by the cAMP response element-binding protein (CREB). Using Rat1 cells stably transfected with each of the three cloned human \( \alpha_1 \) adrenergic receptor subtypes, norepinephrine strongly stimulated CREB phosphorylation in \( \alpha_{1A} \) and \( \alpha_{1B} \) but more weakly in \( \alpha_{1D} \)-transfected cells. Norepinephrine increased the activity of a somatostatin cAMP-regulated enhancer-chloramphenicol acetyltransferase reporter in these cells. \( \alpha_1 \) adrenergic receptors are known to activate protein kinase C (PKC) and CREB phosphorylation was not mediated via the mitogen-dependent pathway.

These results demonstrate that \( \alpha_1 \) adrenergic receptors have more prolonged effects on cell function that involve regulation of gene expression. Many of these actions likely involve additional signal transduction pathways utilized by these receptors. Substantial evidence demonstrates that \( \alpha_1 \) adrenergic receptors in cardiomyocytes induce expression of genes involved in cardiac hypertrophy by activation of MAPK1 kinase pathways (5). Also, in vascular smooth muscle cells, \( \alpha_1 \) adrenergic receptors promote cell growth and activation of proto-oncogenes (6, 7).

There is increasing evidence that \( \alpha_1 \) adrenergic receptors activate the Ras/MAPK pathway and stimulate DNA synthesis in vascular smooth muscle cells (8). Multiple enhancer elements regulate gene expression in response to extracellular signals. A major signal transduction pathway for receptors that activate adenyl cyclase is stimulation of cAMP-dependent protein kinase (PKA), which modulates gene expression through the cAMP response element (CRE) in the promoter region of target genes (9–11). CREB is a transcription factor that binds to CREs; it is a member of the basic leucine zipper superfamily of proteins. Extracellular agonists that stimulate a rise in intracellular cAMP promote dissociation of the subunits of PKA; the released active catalytic subunits enter the nucleus and phosphorylate CREB at serine-133 (12). Phosphorylation of CREB at this site allows a conformational change that recruits the coactivator CREB-binding protein and results in activation of CRE-dependent transcription (13, 14).

CREB can be activated through phosphorylation by other kinases besides PKA. Recent studies have shown that the Ca\(^{2+}\) response elements of the \( c-fos \) and proenkephalin genes are indistinguishable from CREs (15). Furthermore, Ca\(^{2+}\) influx leads to phosphorylation of CREB at serine-133; CaMKs have been found to phosphorylate CREB at serine-133 in vitro (15, 16). Activation of the Ras/MAPK pathway by growth factors can also lead to CREB serine-133 phosphorylation. Ribosomal S6 kinase 2 (RSK2), a downstream substrate of MAPK, has...
been identified as a kinase that catalyzes CREB phosphorylation after stimulation of growth factor receptors (17). Phosphorylation of CREB at serine-133 has been shown to be an important regulatory step in growth factor induction of c-fos transcription (18).

It is widely recognized that catecholamines regulate the CREB/CRE-signaling pathway by stimulating β adrenergic receptors leading to increased cAMP accumulation and activation of PKA. CREB phosphorylation and activation of CRE-dependent transcription potentially can be regulated by signal pathways known to be activated by α1 adrenergic receptors, i.e. Ca^{2+} and MAPK pathways. This raises the interesting possibility of cross-talk between α1 and β adrenergic receptor signaling pathways at the level of gene transcription. We have asked whether α1 adrenergic receptors stimulate CREB phosphorylation and what mechanism might mediate this response.

The results demonstrate that norepinephrine stimulation of Rat1 cells expressing α1 adrenergic receptors induces CREB serine-133 phosphorylation and CRE-dependent transcription. However, PKC, Ca^{2+}, and MAPK/RSK2 signal pathways do not seem to be involved in α1 adrenergic receptor activation of CREB. Instead, α1 adrenergic receptor activation of CRE-dependent transcription surprisingly requires activation of PKA and serine-133 phosphorylation of CREB.

**EXPERIMENTAL PROCEDURES**

**Materials**—Myelin basic protein and PMA were purchased from Sigma. GP109203X was purchased from LC Laboratories (Woburn, MA). H-89 was purchased from CalBiochem. Myelin basic protein and PMA were purchased from Sigma.

**Cell Culture**—Rat1 fibroblasts stably transfected with α1 adrenergic receptors were gifts from Dr. G. Johnson of Pfizer Laboratories (19) and were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 5% fetal bovine serum and 500 units/ml of penicillin.

**Cell lysates** were prepared, and equal amounts of protein were suspended in loading buffer, boiled, and separated by gel electrophoresis. Cell lysates were incubated with indicated antibodies, anti-phosphorylated CREB antibody that recognizes serine-133-phosphorylated CREB species (New England Biolabs, MA), or anti-phosphorylated MAPK antibody that recognizes tyrosine-phosphorylated ERK1 and ERK2 (New England Biolabs). The blots were visualized with the ECL detection system (Amersham Pharmacia Biotech; all other reagents of molecular biology grade were obtained from standard commercial sources.

**RESULTS**

**Activation of α1 Adrenergic Receptors Induces CREB Phosphorylation**—To study the potential capacity of each of the α1 adrenergic receptor subtypes to induce CREB phosphorylation, Rat1 cells were stably transfected with α1A, α1B, or α1D adrenergic receptor genes; clones were selected having comparable levels of receptor expression. Norepinephrine strongly stimulated CREB phosphorylation, cells were first treated with timolol to block β adrenergic responses and then stimulated with norepinephrine for 30 min. Norepinephrine strongly stimulated phosphorylation of CREB proteins in cells expressing α1A or α1B receptors with a relatively weaker response in cells expressing α1D receptors, as detected on Western blots probed with an antibody that only recognizes CREB species phosphorylated on serine-133 (Fig. 1A). The stimulation of CREB phosphorylation was not due to differences in protein loading because the same membrane, when stripped and blotted with a general CREB antibody (detects both phosphorylated and nonphosphorylated forms), showed similar levels of CREB protein in all lanes (Fig. 1B).

**CREB Serine-133 Phosphorylation Induced by α1A Adrenergic Receptor Activates Transcription via CRE**—To investigate the mechanism of α1 adrenergic receptor-mediated CREB phosphorylation, further experiments were conducted utilizing the α1A Rat1 cells. To determine whether α1 adrenergic receptor-induced CREB phosphorylation leads to transcriptional activation, α1A Rat1 cells were transiently transfected with a CRE-CAT reporter construct (12), and CAT activity was measured following stimulation and described under “Experimental Procedures.” We found that norepinephrine strongly stimulated CRE-CAT expression in α1A Rat1 cells. Indeed, these responses were similar to those induced by forskolin, a well known activator of adenyl cyclase (Fig. 2A). The extent of α1A adrenergic receptor-induced CREB phosphorylation was also similar to that induced by forskolin (data not shown).

**References**

1. M. E. Greenberg. Boston, MA) and the GAL4-luciferase reporter plasmid (Promega, Madison, WI) was also cotransfected to control for differences in transfection efficiency. 48 h after transfection, the cells were made quiescent in serum-free medium then treated with agonists for 5 h. The cell lysates were first measured for firefly luciferase activity using a luminometer (Promega). The reaction was then stopped; the Renilla luciferase substrate was added to the same sample, and the luciferase activity was again measured by a luminometer. The results are expressed as a ratio of firefly/ Renilla luciferase activity. In vitro Kinase Assays—After treatment, cells were rinsed with ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate plasma. Cells were incubated with lysis buffer (1% Triton X-100, 25 mM Heps, pH 7.5, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 10 mM okadic acid, 0.11 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µM/mL of aprotinin and leupeptin) for 15 min on ice. Insoluble material was removed by centrifugation. The lysate was incubated with antibody against ERK1 or RSK2 (Santa Cruz Biotechnology, 2 µg/mg protein) for 2 h and then with 20 µL of protein A/G plus-agarose (Santa Cruz Biotechnology) for 1 h. The beads containing the immunoprecipitates were washed 4 times with lysis buffer and once with kinase reaction buffer and then subjected to kinase activity assays. Assay of MAPK activity was performed following a method described previously (8). The washed immunocomplexes were resuspended in 40 µL of kinase buffer with 1 mM dithiothreitol, 40 µM ATP, 1 µCi of [γ-32P]ATP, and 25 µg of myelin basic protein. The reaction mixture was incubated for 10 min at 30 °C. The reaction was stopped by adding loading buffer, boiled, and separated by gel electrophoresis, and results were visualized by autoradiography.

Assay of RSK2 was performed using the washed immunocomplexes resuspended in 40 µL of kinase buffer with 1 mM dithiothreitol, 40 µM ATP, 1 µCi of [γ-32P]ATP, and 10 µg of RSK peptide substrate (Santa Cruz Biotechnology). The reaction mixture was incubated for 10 min at 30 °C. The reaction was stopped by spotting an aliquot of the reaction mixture on phosphocellulose paper. Following extensive washing, radioactivity was quantified by a scintillation detector.
FIG. 1. Western blotting analysis of CREB phosphorylation induced by α₁ adrenergic receptor subtypes. Rat1 cells stably transfected with α₁A, α₁B, or α₁D adrenergic receptors were preincubated with 1 μM timolol for 1 h; the cells were then either treated with 1 μM norepinephrine (NE) for 30 min or left untreated (CT). Equal amounts of protein (100 μg) from each sample were separated by SDS-polyacrylamide gel electrophoresis, and an antibody that recognizes phosphorylated CREB at serine-133 (also cross-reacts with phosphorylated ATF1) was used to blot the membrane (top panel). The same membrane was then stripped and rebotted with a CREB antibody (recognizes both phosphorylated and nonphosphorylated forms), which confirmed equal loading of CREB protein (bottom panel). Experiments were repeated at least three times with similar results.

To determine whether α₁ adrenergic receptor activation of CRE-dependent transcription is mediated by serine-133 phosphorylation of CREB, we cotransfected α₁A Rat1 cells with a GAL4-luciferase reporter construct and an expression plasmid encoding either the wild-type or a phosphorylation-defective GAL4-CREB expression plasmid mutated at the CREB serine-133 site. A similar system has been used previously to demonstrate that RSK2 activation of CRE-dependent transcription requires CREB serine-133 phosphorylation (17). In cells transfected with the wild-type GAL4-CREB, luciferase activity in cells treated with an α₁ adrenergic receptor agonist, phenylephrine, was at least 10-fold greater than in untreated cells (Fig. 2B). This result confirms our hypothesis that α₁A adrenergic receptor activation of CRE-dependent transcription is mediated by CREB. More importantly, we also found, following stimulation of the cells with phenylephrine, that the luciferase activity in cells transfected with the serine-133 phosphorylation-defective GAL4-CREB mutant was much lower than in cells transfected with wild-type GAL4-CREB (Fig. 2B). This result suggests that α₁A adrenergic receptor-stimulated CREB phosphorylation at serine-133 plays a major role in its activation of CRE-dependent gene expression. As expected, forskolin-induced CRE-dependent transcription is also reduced when cells are transfected with the mutant GAL4-CREB. However, it is interesting to note that CRE-dependent transcription induced by α₁A receptor is significantly greater than that induced by forskolin.

Mechanisms of α₁A Adrenergic Receptor-induced CREB Phosphorylation—Norepinephrine stimulation of CREB phosphorylation was reduced by a specific α₁ antagonist doxazosin (Fig. 3A). It is well known that α₁ adrenergic receptors can activate PKC and raise intracellular Ca²⁺ concentrations; both pathways could potentially lead to CREB phosphorylation at serine-133. CREB can be phosphorylated by PKC in vitro or at least three different residues, leading to enhanced DNA binding (20). However, the PKC inhibitor GF109203X did not inhibit norepinephrine-stimulated CREB phosphorylation at serine-133 (Fig. 3A). As expected, GF109203X did inhibit CREB phosphorylation induced by phorbol ester (PMA, 0.1 μM) (data not shown). Also, after PKC was down-regulated by A 24-h treatment with 0.1 μM PMA, norepinephrine-induced activation of CREB remained intact (Fig. 3B). In contrast, PKC down-regulation inhibited PMA-induced CREB phosphorylation. These
results suggest that PKC is not involved in the α1A adrenergic receptor-mediated induction of CREB phosphorylation.

To investigate whether calcium-dependent pathways are involved in CREB phosphorylation induced by α1 adrenergic receptors, BAPTA-AM was used to chelate intracellular Ca2+. α1A Rat1 cells were treated with 10 μM BAPTA-AM for 1 h before stimulation with norepinephrine. Pretreatment with the Ca2+ chelator did not block norepinephrine-induced CREB phosphorylation (Fig. 3A). In contrast, BAPTA-AM significantly reduced α1A induction of c-fos mRNA expression (Fig. 3B). In separate experiments utilizing the indicator dye Fura-2, we confirmed that BAPTA-AM had the expected result of blocking α1A adrenergic receptor-induced rises in intracellular Ca2+ concentrations (data not shown).

Activation of α1A Adrenergic Receptors in Rat1 Cells Does Not Activate MAPK and RSK2—Other investigators have reported that activation of MAPK (ERK1 and ERK2) by growth factors such as epidermal growth factor can lead to CREB phosphorylation and stimulation of gene expression via CRE elements (17). MAPK activates RSK2, which also phosphorylates CREB at serine-133. Our laboratory reported previously that activation of α1 adrenergic receptors in vascular smooth muscle cells stimulates MAPK activity (8). To determine whether norepinephrine activates MAPK or RSK2 in α1A Rat1 cells, MAPK and RSK2 activity were measured with immunocomplex kinase assays. Surprisingly, norepinephrine did not stimulate MAPK activity but instead inhibited PDGF activation of MAPK in these cells (Fig. 5A). Norepinephrine also did not stimulate tyrosine phosphorylation of ERK1 or ERK2; indeed, it further decreased the low basal values of tyrosine-phosphorylated ERK species (Fig. 5B). Similarly, norepinephrine did not stimulate RSK2 activity but also inhibited PDGF activation of RSK2 (Fig. 5C). These results indicate that α1A adrenergic receptor activation of CREB is not via the MAPK pathway in Rat1 cells.

α1 Adrenergic Receptor Induction of CRE-CAT Activity Is PKA-dependent—Since the results suggested that norepinephrine-induced CREB phosphorylation was not dependent on PKC, Ca2+, or MAPK/RSK2 signal pathways, we tested whether the classic pathway associated with CREB phosphorylation, namely mediated by PKA, was activated by α1 adrenergic receptors. There is evidence that α1 adrenergic receptors can stimulate cAMP accumulation in some cells (3, 21, 22). Also, it has been reported that M1 muscarinic receptors, which are also coupled to Gq, increase cAMP accumulation in Rat1 cells (23).

cAMP values were measured following norepinephrine and forskolin stimulation, utilizing a cAMP immunosay kit (Biokit EIA, Amersham Pharmacia Biotech). Both norepinephrine (4.2 pmol cAMP/60-mm dish/3 min) and forskolin (6.4 pmol cAMP/60-mm dish/3 min) increased cAMP concentrations above basal values (2.1 pmol cAMP/60 mm dish) (averages of two experiments done in duplicate). To determine whether α1A adrenergic receptor activation of the CRE reporter gene involved PKA, we utilized a plasmid that expresses the heat-stable PKI, which selectively inhibits the catalytic subunit of PKA (24). In transient transfection experiments, this PKI expression vector was cotransfected with CRE-CAT in α1A Rat1 cells. PKI blunted the norepinephrine-induced induction of CAT activity (Fig. 6). PKI also suppressed forskolin-induced stimulation of CRE-CAT expression (data not shown). To further test the importance of PKA in this response, we used an expression plasmid (mREVab) that encodes a dominant negative mutant of the PKA regulatory subunit. (25). The mutant protein does not bind cAMP and therefore cannot be released from the PKA catalytic subunits, preventing activation of PKA by cAMP. Expression of mREVab inhibited norepinephrine stimulation of CRE-CAT expression (Fig. 6). We also found that α1A adrenergic receptor-induced CREB phosphorylation was inhibited by pretreatment of cells with a PKA inhibitor, H-89 (data not shown), consistent with our results using PKI and the dominant negative regulatory subunit of PKA to block the PKA signaling pathway.

**DISCUSSION**

Compelling evidence points toward an important role for regulation of gene expression by catecholamines such as epinephrine and norepinephrine, especially in cardiomyocytes and
vascular smooth muscle cells. The results of the current study demonstrate that stimulation of \( \alpha_1 \) adrenergic receptors leads, via a cAMP-PKA-dependent mechanism, to phosphorylation of CREB at serine-133 and activation of CRE-mediated gene expression. Although \( \alpha_1 \) adrenergic receptors can potentially activate several protein kinases, including PKC, Ca\(^{2+}\)-dependent protein kinases, MAPK, and RSK2 kinases, none of these pathways appeared to be involved in inducing CREB phosphorylation.

Although previous observations indicate that catecholamines can increase cAMP accumulation in some target tissues by activating \( \alpha_1 \) adrenergic receptors (22, 26–27), this is, to our knowledge, the first report of \( \alpha_1 \) adrenergic receptor-induced CREB phosphorylation and CRE-dependent gene transcription. This result suggests possible physiologic relevance for activation of this signaling pathway by \( \alpha_1 \) adrenergic receptors involving regulation of transcription of genes containing CRE enhancer elements.

PKI is a highly specific peptide inhibitor derived from the heat-stable protein kinase inhibitor in rabbit skeletal muscle. PKI potently inhibits the catalytic subunit of PKA. Grove et al. (24) developed a PKI expression plasmid system that inhibited PKA activation of a CRE reporter gene in JEG-3 cells (24). In addition, overexpression of PKA R1 regulatory subunit mutated at both cAMP binding sites, mtREVab, prevents binding of cAMP to the regulatory subunit, leading to dominant inhibition of the catalytic subunit (25). Transfection of cells with either PKI or mtREVab inhibited \( \alpha_1 \) adrenergic receptor-stimulated CRE-CAT reporter activity, strongly suggesting that CRE-dependent gene transcription induced by \( \alpha_1 \) adrenergic receptors in Rat1 fibroblasts is mediated by PKA-induced phosphorylation of CREB. Using a phosphorylation-defective CREB mutant, we further demonstrated that \( \alpha_1 \) adrenergic receptor activation of CRE-mediated gene expression is at least partially dependent on Ser-133 phosphorylation of CREB. Although the mutant GAL4-CREB construct was partially inducible by \( \alpha_1 \) adrenergic receptor, this probably reflects heterodimerization of endogenous CREB phosphorylated at Ser-133 with the mutant GAL4-CREB via the leucine zipper dimerization motif. At the same time, our result does not rule out the possibility that there may be an additional site that is phosphorylated in response to stimulation of \( \alpha_1 \) adrenergic receptors that contributes to activation of CREB. It has been shown that Ser-129 is a secondary phosphorylation site required for activation of CREB and that phosphorylation at this site requires an initial phosphorylation at Ser-133 (28).

Although there are multiple potential mechanisms by which \( \alpha_1 \) adrenergic receptors may enhance cAMP accumulation, we do not know the mechanism responsible in the transfected Rat1 cells. \( \beta_\gamma \) subunits released from G proteins can activate some adenylyl cyclase isoforms such as type II in the presence of G\(_\alpha\)bg (29). It is thought that \( \beta_\gamma \) subunits from G proteins such as G\(_i\) or G\(_o\) are responsible for this stimulation. Potentially, \( \beta_\gamma \) subunits released from \( \alpha_1 \) adrenergic receptors coupled to certain G proteins could be responsible for activating adenylyl cyclase. Also, \( \alpha_1 \) adrenergic receptors may activate G\(_i\) (21). Cellular effects elicited by \( \beta_\gamma \) subunits can be inhibited by expression of transducin (\( \alpha_i \)) or the \( \beta_\gamma \) adrenergic receptor kinase fragment to sequester free \( \beta_\gamma \) subunits (30), and interestingly, M1 receptor-induced cAMP accumulation was found to be partially inhibited by expression of \( \alpha_i \) or \( \beta_\gamma \) adrenergic receptor kinase fragment (31). Future studies will investigate whether \( \alpha_1 \) adrenergic receptor-induced CREB activation and cAMP accumulation are mediated by G protein \( \beta_\gamma \) subunits and/or G proteins that are pertussis toxin-sensitive.

Each of the three cloned \( \alpha_1 \) adrenergic receptors has been shown to stimulate increases in \([\text{Ca}^{2+}]_i\) concentrations in
HEK293 cells (32). We have also found similar effects on [Ca^{2+}]_i in Rat1 cells. However, our data indicate that the capacity to increase [Ca^{2+}]_i concentrations does not appear to play a role in α_1 adrenergic receptor-induced CREB phosphorylation because chelation with BAPTA-AM completely abolished the sharp rise in intracellular [Ca^{2+}], but did not inhibit increased CREB phosphorylation. Although several groups have reported that CaMK II and IV phosphorylate CREB at serine-133 in vitro (16, 33, 34), the role of Ca^{2+}-dependent protein kinases in regulating CRE-dependent gene expression remains unclear. There is recent evidence that CaMK II activates CRE-dependent transcription using ATF1 rather than CREB (35). In our studies, chelation of intracellular Ca^{2+} significantly inhibited α_1 adrenergic receptor-induced c-fos mRNA expression. This result suggests that α_1 receptor activation of the fos gene is not mediated by CREB in Rat1 cells; however, it is still possible that α_1 adrenergic receptors may stimulate transcription of the fos gene via CRE through ATF1 rather than CREB.

We used PMA to down-regulate PKC in Rat1 cells. van Dijk et al. (36) found that Rat1 cells express PKC isofoms α, δ, and ε; these enzymes were markedly down-regulated by prolonged treatment with phorbol ester. The atypical PKC isoform, PKC-ζ, also expressed in Rat 1 cells, was not down-regulated by phorbol ester. However, these investigators found that PKC-ζ activated MAPK in Rat1 cells (36); these results suggest that α_1 adrenergic receptors are unlikely to activate PKC-ζ since these receptors inhibit rather than activate MAP kinase in these cells. Taken together, the results with PMA treatment and with the PKC inhibitor GF109203X suggest that PKC is not involved in α_1 adrenergic receptor-induced phosphorylation of CREB.

It is possible that α_1 adrenergic receptors may also activate another pathway leading to CRE-dependent transcription. This hypothesis is compatible with our data in that expression of neither the PKI nor the dominant negative PKA completely inhibited α_1 adrenergic receptor-induced CRE-dependent transcription (Fig. 6). It has been reported that p70 S6 kinase can phosphorylate CREB in vitro and activation of p70 S6 kinase can stimulate CRE-dependent gene expression (37); we have found that α_1 adrenergic receptors activate p70 S6 kinase in vascular smooth muscle cells and in Rat1 cells. This hypothesis is also compatible with our data in which the cAMP accumulation stimulated by α_1 adrenergic receptors was relatively small compared with that of forskolin in Rat1 cells, whereas the level of CREB phosphorylation and CRE-dependent transcription induced by α_1 adrenergic receptors was at least as efficacious as that induced by forskolin (Fig. 2).

Further investigation is required to understand the role of CREB phosphorylation and CRE-dependent transcription by α_1 adrenergic receptors with specific target genes. For example, it would be interesting to determine whether α_1 adrenergic receptors enhance CRE-dependent transcription of the β adrenergic receptor gene (38). Vascular smooth muscle cells express α_1 and β adrenergic receptors that have opposing effects on vascular tone; stimulation of α_1 adrenergic receptors potentially could lead to increased β receptor expression, which would tend to diminish vascular contraction.

In summary, our results demonstrate α_1 adrenergic recep-

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2 Chin, J., unpublished observations.

3 Hu, Z. W., unpublished data.