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Norepinephrine Stimulation of Pineal Cyclic AMP Response Element-Binding Protein Phosphorylation: Primary Role of a β-Adrenergic Receptor/Cyclic AMP Mechanism

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SUMMARY

Norepinephrine (NE) regulates melatonin production and many other aspects of pineal function through actions involving cAMP. In the present study the effects of NE on the phosphorylation of the cAMP response element-binding protein (CREB) were studied to determine whether CREB phosphorylation might be involved in cAMP signal transduction in this tissue. CREB was detected using gel mobility-shift analysis with the radiolabeled Ca^{2+}/cAMP response element of the c-*fos* promoter. CREB phosphorylation was estimated in the gel mobility-shift assay using an antiserum specific for phosphorylated CREB. This antiserum generates a supershifted CREB signal with protein extracts obtained from glands treated with NE ($EC_{50} \cong 10$ nM) in organ culture, demonstrating that NE stimulates CREB phosphorylation. CREB phosphorylation peaks 30–45 min after NE treatment is initiated and then gradually returns to base-line values. Pharmacological studies show that NE-stimulated CREB phosphorylation is mediated primarily through β_1-adrenergic receptor-stimulated increases in cAMP. Activation of α_1-adrenergic receptors, which is known to elevate the intracellular free Ca^{2+} concentration, does not cause CREB phosphorylation. However, it is possible to produce CREB phosphorylation with certain pharmacological agents that elevate the intracellular free Ca^{2+} concentration. *In vivo* studies show that CREB phosphorylation can be induced by treatment with isoproterenol (1 mg/kg), demonstrating that phosphorylation of pineal CREB occurs in intact animals. These studies indicate that cAMP-dependent CREB phosphorylation could play a role in the adrenergic regulation of gene expression in pinealocytes.

The nocturnal increase in melatonin production in the pineal gland is regulated by the circadian release of NE from sympathetic nerves terminating in the gland (1). This rhythmic release is driven by signals generated by the circadian oscillator system in the suprachiasmatic nucleus and transmitted to the pineal gland via a neural circuit that includes both central and peripheral structures (2). NE acts on pinealocytes through postsynaptic α_1- and β_1-adrenergic receptors (3). Second messengers of NE in the pineal gland include cAMP, cGMP, and Ca^{2+}. cAMP and cGMP levels are regulated by "AND" gates, through which β_1-adrenergic activation is potentiated by α_1-adrenergic elevation of $[Ca^{2+}]_i$. Activation of α_1-adrenergic receptors alone has little or no influence on pineal cyclic nucleotides (4).

cAMP has been shown to participate in the adrenergic stimulation of three pineal enzymes, i.e., NAT (EC 2.3.1.87), which is the rate-controlling enzyme in the pathway that converts serotonin to melatonin (1), tryptophan hydroxylase (5), which converts tryptophan to 5-hydroxytryptophan, and thyroxine type II 5′-deiodinase (6), which converts thyroxine to 3,5,3′-triiodothyronine. New gene transcription and protein synthesis are required for the cAMP-dependent stimulation of these enzymes (1, 5, 7). In addition, cAMP has also been implicated in the adrenergically induced increases in the early response genes c-*fos* (8) and ICER in the pineal gland (9).

Downstream effects of cAMP on gene expression are thought to be mediated through PKA phosphorylation of

ABBREVIATIONS: NE, norepinephrine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; NAT, arylalkylamine *N*-acetyltransferase; ICER, inducible cAMP early repressor; PKA, cAMP-dependent protein kinase; CREB, cAMP response element-binding protein; Rp-8-CPT-cAMPS, Rp-8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate; AEBSF, 4-(2-aminoethyl)benzylsulfonyl fluoride; PCREB, cAMP response element-binding protein phosphorylated on Ser-133; CREBtide, synthetic peptide corresponding to cAMP response element-binding protein residues 123–136; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CaCRE, Ca^{2+}/cAMP response element; CRE, cAMP response element; PCREBtide, synthetic peptide corresponding to cAMP response element-binding protein residues 123–136 and phosphorylated on Ser-133; AP-1, activator protein-1; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. AP-2, activator protein-2; GRE, glucocorticoid response element; TFIID, transcription factor IID; Oct1, octomer transcription factor 1; SP1, Simian Virus 40 promoter factor 1; NF-κB, nuclear factor-κB; CREM, cAMP response element modulator.

CREB at Ser-133 (10). This is known to activate transcription of several genes through interaction with appropriate response elements in their promoter regions (11). Therefore, it was of interest to determine whether this mechanism operates in the pineal gland, where it could participate in adrenergic receptor/cAMP regulation of gene expression. In the study presented here, we determined that CREB is present in the pineal gland and that phosphorylation of CREB appears to be primarily regulated by a β_1-adrenergic receptor/cAMP mechanism.

Experimental Procedures

Materials

Pineal glands used in organ culture and *in vitro* phosphorylation studies were obtained from 200-g, female, Sprague-Dawley rats (Taconic Farms, Germantown, NY). Rats used for *in vivo* studies were 200–250-g, male, Sprague-Dawley rats (Taconic Farms).

The following reagents were used: A23187, catalytic subunit of PKA, N^6,2′-*O*-dibutyryl-cAMP, ionomycin, isoproterenol, NE, ouabain, and propranolol (Sigma Chemical Co., St. Louis, MO); bovine serum albumin (Intergen Co., Purchase, NY); phenylephrine (Research Biochemicals, Natick, MA); Rp-8-CPT-cAMPS (Biolog Life Science Institute, La Jolla, CA); Immobilon P membranes (Millipore, Bedford, MA); ATP (Boehringer Mannheim Biochemicals, Indianapolis, IN); bestatin, cholera toxin, forskolin, and microcystin-LR (Calbiochem, San Diego, CA); leupeptin (Bachem California, Torrance, CA); poly(dI·dC)/poly(dI·dC) and spermidine trihydrochloride (United States Biochemicals, Cleveland, OH); AEBSF, aprotinin, dithiothreitol, and pepstatin A (ICN Biochemicals, Aurora, OH); consensus transcription factor oligonucleotides (Promega Biotec, Madison, WI); goat anti-rabbit IgG conjugated to horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD); T4 polynucleotide kinase (New England Biolabs, Beverly, MA); [γ-^{32}P]ATP (5000 Ci/mmol) and enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL); Teflon pellet pestle (Kontes, Vineland, NJ); and Microsep 10K centrifugal concentrator (Filtron Technology, Northborough, MA).

Prazosin was a gift from Pfizer (Groton, CT). Oligonucleotide probes were synthesized on a model 380B DNA synthesizer from Applied Biosystems (Foster City, CA). Antibodies that either recognized CREB regardless of phosphorylation state (anti-CREB) or detected the phosphorylated form of CREB (anti-PCREB) were obtained from Upstate Biotechnology (Lake Placid, NY). In addition, anti-PCREB was also provided by D. D. Ginty and M. E. Greenberg (Harvard Medical School, Cambridge, MA). An additional antibody (Ab240), which was produced against a synthetic peptide corresponding to CREB residues 89–101 plus Gly-Tyr at the carboxyl terminus (12) and which detects CREB regardless of its phosphorylation state, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CREBtide (13) was synthesized by the University of Notre Dame Bioscience Core Facility (Notre Dame, IN). All other reagents were purchased from commercial sources and were of the highest purity available. Sonication was performed with a Biosonik IV sonicator from VWR Scientific (San Francisco, CA). Gel mobility-shift images were obtained from a PhosphorImager operated with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Methods

Organ culture technique. Pineal glands were placed in organ culture as described previously (14). Briefly, pineal glands were incubated on a 6-mm nylon mesh disk at 37° (95% O_2/5% CO_2), in 200 μl of Biggers, Gwatkin, Judah medium with Fitton-Jackson modification (BGJ_b), in a 24-well tissue culture plate (two glands/well). The glands were transferred to fresh medium after 24 and 36 hr. After 48 hr they were placed into fresh medium in a table-top incubator for 2 hr before transfer to medium for treatment. At the end of treatment, glands were placed on solid CO_2 and stored at −80° until use.

NAT assay. NAT activity was assayed as described previously (15), with the exception that [^{3}H]acetyl-CoA (0.5 mM; final specific activity, 4 μCi/μmol) was used.

Oligonucleotide labeling. Single-stranded CaCRE oligonucleotide (5′-GAGCCCGTGACGTTTACACTCATTC-3′) (16) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The CaCRE is underlined. The labeled oligonucleotide was then hybridized to its reverse complement and the double-stranded oligonucleotide was purified on an 8% polyacrylamide gel.

Gel mobility-shift assay. Whole-cell extracts of pineal tissue were prepared for gel mobility-shift assays essentially as described for the suprachiasmatic nucleus (13). For each binding reaction two pineal glands were sonicated in 50 μl of 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, and the protease inhibitors AEBSF (0.5 mM), aprotinin (1.0 μg/ml), bestatin (40 μg/ml), leupeptin (0.5 μg/ml), and pepstatin A (0.7 μg/ml). Additionally, 1 mM NaF and 5 μM microcystin-LR were included as phosphatase inhibitors.

Binding reactions were performed with 10 μl of supernatant from a 10-min centrifugation at 16,000 × *g* (at 4°), 1 μg of poly(dI·dC)/poly(dI·dC), 4 μl of buffer (50 mM Tris·HCl, pH 7.5 at 25°, 20% glycerol, 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 5 mM spermidine trihydrochloride), and 30,000–40,000 dpm of labeled oligonucleotide, in a final reaction volume of 20 μl. After a 15-min incubation at room temperature, protein-DNA complexes were resolved by native PAGE (17). Bands in the dehydrated gel were visualized and quantitated by exposure to a PhosphorImager. Relative intensity was calculated by expressing the signal for each band as a percentage of the total shifted signal. The percentage decrease in the shifted signal due to competitors or antisera was approximated from a single determination.

Where indicated, anti-PCREB antibody (0.3 μg) was added immediately before the labeled probe. For reactions that contained unlabeled competitor oligonucleotides or antisera (274A or anti-CREB), pineal cell extracts were incubated (at 4°, for 15 min) with these reagents before addition of the labeled probe. The polyclonal antiserum 274A specifically detects the isoforms of the 14–3-3 proteins and has been described previously (18). The 14–3-3 proteins (19) are unrelated to CREB, and this antiserum was used as a negative control to demonstrate the specificity of the effects of the anti-CREB antiserum.

Nuclear extract preparation. Twenty-eight pineal glands were homogenized in 300 μl of 10 mM Tris·HCl, pH 7.5 at 25°, 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Nonidet P-40, 5 mM dithiothreitol, using a type B Dounce homogenizer (15 strokes). After a 10-min incubation at 4°, pinealocytes were lysed with a type A Dounce homogenizer (15 strokes). Nuclei were isolated and protein was extracted in the presence of 0.7 M KCl according to a published procedure (20). The nuclear extract was desalted using a Microsep 10K centrifugal concentrator, by addition of 19 volumes of 20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM dithiothreitol, and concentration to the original volume. Leupeptin (20 μg/ml), aprotinin (20 μg/ml), AEBSF (0.5 mM), NaF (1 mM), and microcystin-LR (5 μM) were included in all buffers.

SDS-PAGE, electroblotting, and immunodetection. Nuclear proteins (5 μg/lane) were resolved by SDS-PAGE on a 10% polyacrylamide gel (21); two identical sets of samples were run on the gel. To ensure equal loading, the protein concentrations were measured by a dye-binding method (22). After transfer to an Immobilon P membrane (23), the blot was blocked for 4 hr with 0.3% Tween. To detect PCREB a section of blot corresponding to one set of samples was probed with anti-PCREB (0.2 μg/ml), and to detect total CREB protein the other section of the blot was probed with Ab240 (1/500). After an 18-hr incubation at 4°, the two sections of the blot were washed with 20 mM Tris·HCl, pH 7.5 at 25°, containing 500 mM NaCl and 0.05% Tween-20, and were then incubated with goat anti-rabbit

IgG conjugated to horseradish peroxidase (1/10,000); immunoreactivity was visualized using the enhanced chemiluminescence detection system.

Phosphorylation of CREB in cell-free preparations with the catalytic subunit of PKA. Immediately after rats were sacrificed, pineal glands were removed and placed on solid CO_2. Glands were then sonicated (10 glands/250 μl of sonication buffer) as described above, and the supernatant from a 10-min centrifugation at 16,000 × *g* was incubated for 20 min at 37° with 1 mM ATP and 10 mM $MgCl_2$, in the presence or absence of the catalytic subunit of PKA (0.3 unit/μl). The reactions were stopped by placing the tubes on solid CO_2. Gel mobility-shift assays were then performed as described above.

Preparation of PCREBtide. CREBtide (18 μg) was phosphorylated with 0.25 unit of the catalytic subunit of PKA in 20 mM PIPES, pH 7.2, in the presence of 10 mM $MgCl_2$ and 100 μM ATP, for 30 min at 30°, in a final volume of 50 μl. The reaction was stopped by boiling for 5 min. Successful phosphorylation of the peptide was confirmed by including 1 μCi of [γ-^{32}P]ATP in a parallel reaction and monitoring for the presence of trichloroacetic acid-precipitable radioactivity.

***In vivo* study.** Male rats were housed under the control lighting conditions (light:dark 14:10; lights on 0500h) or in constant light for 7 days. At 12:00 p.m. on the day of the experiment, rats were given subcutaneous injections of 1 mg/kg isoproterenol (dissolved in 0.9% saline solution) or vehicle alone. Thirty minutes later rats were sacrificed and pineal glands were removed, placed on solid CO_2, and stored at −80° until use.

Results

CREB protein is present in the pineal gland. Gel mobility-shift assays performed on pineal extracts using a radiolabeled oligonucleotide probe that contained the CaCRE of the c-*fos* promoter (^{32}P-CaCRE) revealed the presence of four bands of retarded mobility (Fig. 1A). These signals appear to represent highly specific interactions, because all were eliminated by the addition of a 200-fold molar excess of unlabeled CaCRE (Fig. 1A). Incubation with a 200-fold molar excess of the closely related CRE (Table 1) prevented the appearance of bands 1, 2, and 4. The signal for band 3 was decreased by approximately 70%; a similar band that is not eliminated in the presence of excess CRE has been seen in other tissues under similar conditions (13). Band 3 is not present when gel mobility-shift assays are performed with pineal nuclear extracts[1] and therefore most likely represents an interaction between the CaCRE probe and a cytosolic protein or a complex containing a cytoplasmic protein and a CaCRE-binding protein.

The specificity of CaCRE binding was evident from the failure of several other transcription factor consensus oligonucleotides (Table 1) to compete with the CaCRE for protein

[1] P. H. Roseboom and D. C. Klein, unpublished observations.

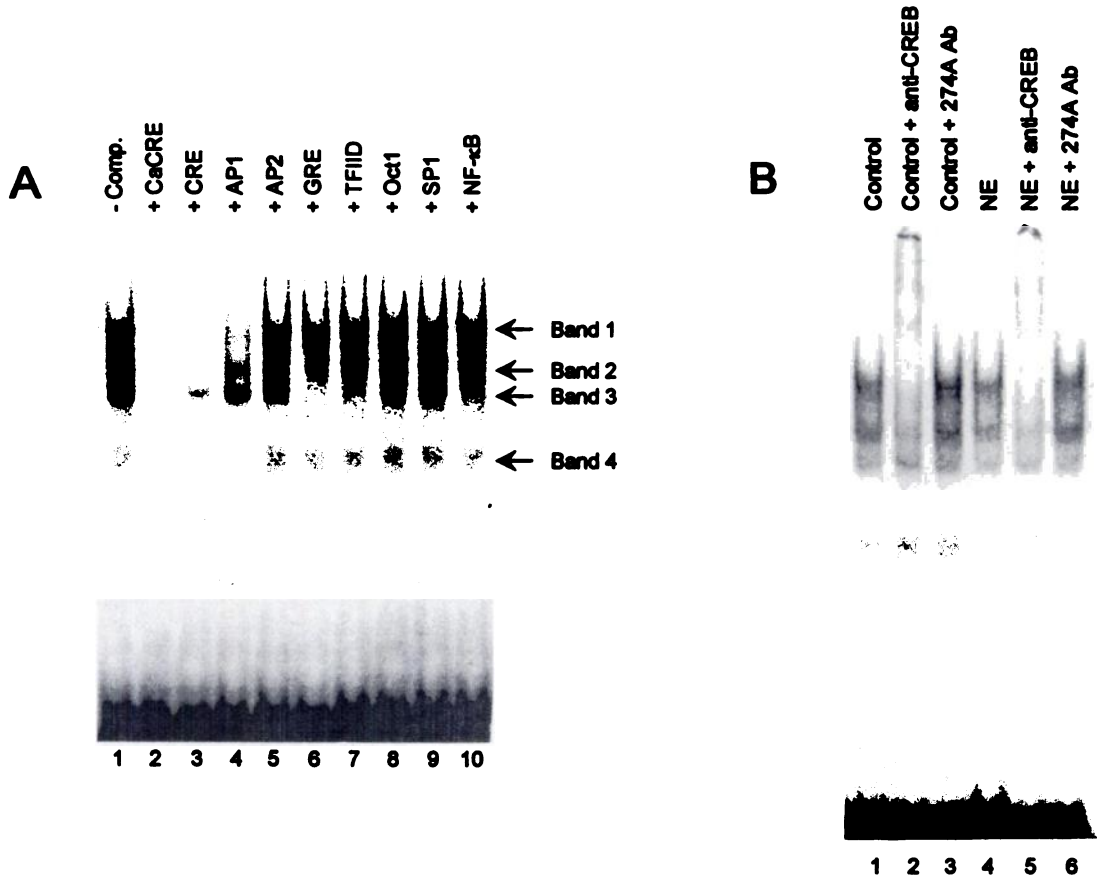

Fig. 1. Gel mobility-shift assay measuring protein binding to a CaCRE oligonucleotide in rat pineal homogenates. Pineal glands were incubated under control conditions, as described in Experimental Procedures, and then transferred to control medium or medium containing 1 μM NE for 30 min. Whole-cell pineal extracts were prepared and the gel mobility-shift assay was performed as described in Experimental Procedures. A, Extracts were incubated in the presence or absence of a 200-fold molar excess of the indicated unlabeled competitors (Table 1) for 15 min at 4° before addition of ^{32}P-CaCRE. Unlabeled CaCRE, CRE, and to a limited extent AP-1 oligonucleotides could compete with the binding, whereas several other transcription factor consensus oligonucleotides did not compete. B, Extracts were incubated, as in A, with anti-CREB or 274A antiserum (final dilution, 1/100). Incubation with antiserum that specifically recognizes CREB (anti-CREB) decreased the amount of protein binding to the CaCRE, whereas antiserum raised against the unrelated 14–3-3 proteins (274A) failed to decrease the binding.

TABLE 1

Sequences of consensus oligonucleotides used as competitors

To demonstrate the specificity of the binding to the CaCRE probe used in the gel mobility-shift assay, double-stranded oligonucleotides containing consensus binding sites for a variety of transcription factors were used as competitors (Fig. 1). The sequences from one strand are shown in the 5′ to 3′ direction, and the core consensus sequences are underlined.

Transcription factor binding site	Oligonucleotide sequence
CaCRE	GAGCCCGTGACGTTTACACTCATTC
CRE	AGAGATTGCCTGACGTCAGAGAGCTAG
AP-1	CGCTTGATGAGTCAGCCGGAA
AP-2	GATCGAACTGACCGCCCGCGGCCCGT
GRE	TCGACTGTACAGGATGTTCTAG
TFIID	GCAGAGCATATAAGGTGAGGTAGGA
Oct1	TGTCGAATGCAAATCACTAGAA
SP1	ATTCGATCGGGGCGGGGCGAGC
NF-κB	AGTTGAGGGGACTTTCCCAGGC

binding (Fig. 1A); specifically, AP-2, Oct1, and SP1 did not compete. Other oligonucleotides, however, exhibited only partial competition. There was a significant decrease (60–70%) in the signal for band 3 in the presence of the GRE (Fig. 1A, *lane 6*), TFIID (Fig. 1A, *lane 7*), and NF-κB (Fig. 1A, *lane 10*) consensus oligonucleotides; the basis and significance of this are not clear. The AP-1 oligonucleotide partially prevented the formation of the protein-CaCRE complexes (Fig. 1A, *lane 4*); band 1 was reduced by 75%, band 2 by 60%, band 3 by 30%, and band 4 by 50%. The AP-1 consensus sequence varies from the CRE consensus sequence by only a single base deletion (Table 1), and the observation that the AP-1 oligonucleotide partially competes with the CaCRE probably reflects the affinity of CREB for the AP-1 sequence (24). It is unlikely that the labeled CaCRE probe binds to AP-1 protein, because we have found that addition of an antiserum that detects the c-*fos* component of the AP-1 complex (anti-c-$fos_{129–153}$) (25) does not significantly affect the shifted signal (data not shown).

To determine whether CREB was present in the protein-CaCRE complexes, an antiserum that recognizes CREB regardless of phosphorylation state was used (13). Incubation of pineal extracts with this anti-CREB antiserum significantly altered the gel mobility-shift pattern (Fig. 1B, *lanes 1* and *2*). The intensity of band 1 was decreased by approximately 75%, and band 2 was decreased by approximately 40%; bands 3 and 4 were not significantly affected by the antiserum. Binding to the CaCRE probe was not significantly altered in the presence of another unrelated rabbit antiserum (274A) (Fig. 1B, *lanes 1* and *3*), indicating that the effects of the anti-CREB antiserum were specific. In addition, bands 1 and 2 were the only bands abolished by another CREB antiserum (Ab244) (data not shown), which was raised against a synthetic peptide corresponding to CREB residues 128-162 (12). Taken together, these results, and the observation that the anti-CREB antiserum used in Fig. 1B does not cross-react with the other CRE-binding factors, CREM and activating transcription factor 1 (13), indicate that the majority of the protein-DNA complexes being detected in bands 1 and 2 in this assay contain CREB. The identity of the protein binding that remains in the presence of anti-CREB is unknown but may represent binding to CREM or activating transcription factor 1, because the anti-CREB antiserum does not recognize these proteins and therefore should not interfere with their binding to the CaCRE probe.

NE does not alter CaCRE binding activity. The amount and pattern of CaCRE binding were essentially the same in extracts of control and NE-treated pineal glands (Fig. 1B, *lanes 1* and *4*). In addition, NE treatment did not consistently alter the amount of CaCRE binding activity that was blocked by incubation with anti-CREB antiserum (Fig. 1B, *lanes 2* and *5*). These observations indicate that a 30-min treatment with 1 μM NE does not alter CaCRE binding activity and therefore does not affect the total amount of pineal CREB protein.

NE treatment results in phosphorylation of CREB. To determine whether CREB phosphorylation could be produced by adrenergic stimulation of pineal glands maintained in organ culture, anti-PCREB antiserum was used in the gel mobility-shift assay. In the presence of this antiserum, a supershifted band was detectable in extracts of NE-treated (1 μM, 30 min) pineal glands (Fig. 2A, *lane 9*). This band was absent if the antiserum was absent (Fig. 2A, *lane 7*), if a 200-fold excess of unlabeled CRE was present (Fig. 2A, *lane 10*), or if the antiserum had been preadsorbed with PCREBtide (Fig. 2A, *lane 11*). However, it was present if the antiserum had been preadsorbed with the unphosphorylated CREBtide (Fig. 2A, *lane 12*). Identical results were obtained with another anti-PCREB antiserum (Ab5322) (data not shown), which was produced against a phosphorylated synthetic peptide corresponding to CREB residues 128-141 (26). These findings indicate that CREB phosphorylation is increased by NE treatment of pineal glands.

NE-induced pineal CREB phosphorylation was also observed using Western blot technology. Anti-PCREB antiserum detected a band of immunoreactivity with a molecular mass of 45 kDa (Fig. 2B) on Western blots of pineal nuclear protein, which corresponds approximately to the previously reported molecular mass of 43 kDa for rat CREB (27). The intensity of this band was increased in extracts obtained from glands that had been treated with 1 μM NE for 30 min (Fig. 2B, *lanes 3* and *4*); however, NE treatment did not alter the total amount of CREB protein present in the pineal nuclear extracts, as detected with the affinity-purified, CREB-specific antibody Ab240 (Fig. 2B, *lanes 1* and *2*). These results indicate that a 30-min treatment with 1 μM NE induces pineal CREB phosphorylation without altering the total amount of CREB protein present in the nucleus. In addition to the major band at 45 kDa, two minor bands at 39 kDa and 41 kDa were detected with both anti-PCREB and Ab240 antisera. These unidentified proteins are probably related to CREB, because they were detected by both antibodies. Additionally, the intensity of the anti-PCREB immunoreactivity corresponding to the two smaller bands increased with NE treatment (Fig. 2B, *lanes 3* and *4*), suggesting that these proteins may also be phosphorylated in response to NE treatment.

NE-induced CREB phosphorylation is time and dose dependent. Time course analysis indicated that CREB phosphorylation was first detectable at 1 min, reached a peak at 30–45 min, and did not show a significant decrease until 2 hr of NE stimulation (Fig. 3). This parallels the known time course for NE stimulation of cAMP accumulation in the pineal gland (28). There is a trend for the intensity of band 4 (lowest band) to increase with NE treatment at the later time points (2 hr and greater); this trend was seen with time courses determined on three sets of glands. Additionally,

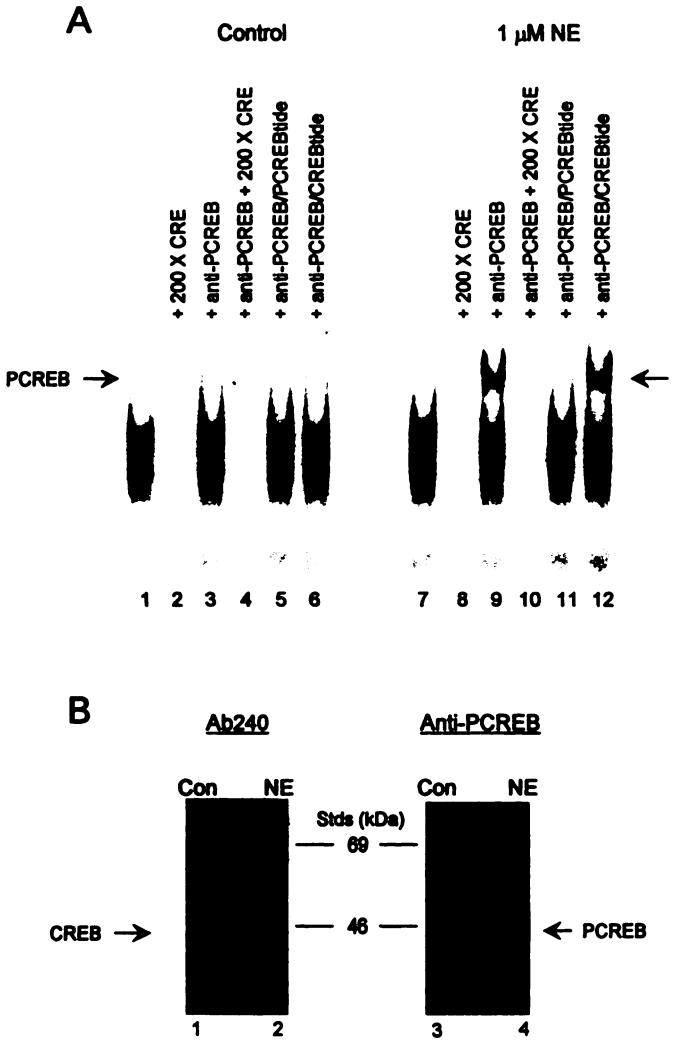

Fig. 2. NE-induced CREB phosphorylation in the pineal gland in organ culture. Pineal glands were incubated in control medium or medium containing 1 μM NE for 30 min. A, Gel mobility-shift assay reactions contained pineal gland extract and probe alone (*lanes 1* and *7*) or also included 3.5 pmol of consensus CRE oligonucleotide (*lanes 2* and *8*), anti-PCREB antibody (0.3 μg) (*lanes 3* and *9*), anti-PCREB antibody and 3.5 pmol of consensus CRE oligonucleotide (*lanes 4* and *10*), anti-PCREB antibody that had been preadsorbed with PCREBtide (0.3 μg) (*lanes 5* and *11*), or anti-PCREB antibody that has been preadsorbed with CREBtide (0.3 μg) (*lanes 6* and *12*). Antisera (0.15 μg/μl) were preadsorbed with peptide (0.3 μg/μl) by incubation for 20 hr at 4°, using an end-over-end rotator. *Arrows*, location of the supershifted band. The lower half of the gel containing the unbound probe is not included in the image. The supershifted bands were seen only in extracts obtained from NE-treated pineal glands, and the appearance was blocked in the presence of excess unlabeled CRE or when the anti-PCREB antibody was preadsorbed with PCREBtide. B, Immunodetection of NE-induced CREB phosphorylation in pineal nuclear extracts is shown. Pineal nuclear proteins were extracted, resolved by SDS-PAGE, electroblotted, and immunodetected as described in Experimental Procedures. To detect CREB one half of the blot was probed with Ab240 (1/500), and to detect PCREB the other half of the blot was probed with anti-PCREB (0.2 μg/ml). Treatment with 1 μM NE for 30 min increased anti-PCREB immunoreactivity in rat pineal nuclear extracts but did not alter the amount of total CREB protein. Similar results were obtained from two separate nuclear extract preparations. *Con*, control.

studies measuring ^{32}P-CaCRE binding activity in pineal extracts obtained from rats sacrificed at various times of day revealed an increase in the intensity of band 4 at night and a return to control values during the day (data not shown). The identity of the ^{32}P-CaCRE-binding protein in band 4 is a matter of speculation. One possibility is that it is ICER, a small CRE-binding protein that is up-regulated in the pineal gland at night, by an adrenergic mechanism (9). However, it is clear that the identity of this band is unknown and needs to be more rigorously established.

Dose-dependence analysis of the effect of NE on CREB phosphorylation indicated that an effect could be seen at 20 min with a concentration of 10 nM NE (Fig. 4, *lane 6*), with a maximum response being produced with a concentration of 100 nM NE ($EC_{50} \cong 10$ nM) (Fig. 4, *lane 8*). This is similar to or more potent than the previously reported concentration-effect curves for NE stimulation of cAMP accumulation in dispersed pinealocytes (29, 30) and of NAT activity in cultured pineal glands (31). Additionally, NE is more potent at stimulating CREB phosphorylation ($EC_{50} \cong 10$ nM) than at stimulating MEKA (phosducin) phosphorylation ($EC_{50} = 110$ nM) (30).

Effects of NE primarily involve β-adrenergic receptors. The relatively selective β-adrenergic agonist isoproterenol was approximately as effective as NE in increasing CREB phosphorylation (Fig. 5A, *lanes 2-5* and *11*). In contrast, the α_1-adrenergic receptor-selective agonist phenylephrine was approximately 10,000-fold less potent than isoproterenol (Fig. 5A, *lane 10*) and the maximum CREB phosphorylation appeared to be approximately 60% of the maximal signal seen with NE or isoproterenol. Additionally, attempts to potentiate the effects of isoproterenol by treatment with phenylephrine were unsuccessful (data not shown).

The effects of the β-adrenergic receptor-selective antagonist propranolol and the α_1-adrenergic receptor-selective antagonist prazosin on the 100 nM NE-induced CREB phosphorylation were assessed. Incubation of pineal glands with increasing concentrations of the relatively selective β-adrenergic receptor antagonist propranolol blocked the 100 nM NE-induced CREB phosphorylation (Fig. 5B, *lanes 3-6*). In contrast, increasing concentrations of the relatively selective, α_1-adrenergic receptor antagonist prazosin did not significantly alter the 100 nM NE-induced CREB phosphorylation (Fig. 5B, *lanes 7-10*). In the same experiment, incubation with 10 μM propranolol or 10 μM prazosin decreased the NAT response to a 4-hr treatment with 100 nM NE by 90% or 55%, respectively (data not shown), indicating that effective concentrations of both antagonists were achieved inside the gland. These results suggest that the β_1-adrenergic receptor plays a major role in mediating the effects of NE on CREB phosphorylation.

cAMP protagonists and the catalytic subunit of PKA stimulate pineal CREB phosphorylation. CREB phosphorylation was increased by treatment with cAMP protagonists, including N^6,2′-*O*-dibutyryl-cAMP (1 mM), forskolin (10 and 100 μM), and cholera toxin (50 μg/ml) (Fig. 6A). The maximum amount of phosphorylation obtained with each of these reagents was similar to that seen with NE (Fig. 2A). In contrast, incubation with the cGMP protagonist N^6,2′-*O*-dibutyryl-cGMP (1 mM) did not produce phosphorylation of pineal CREB (data not shown).

Increases in intracellular cAMP levels result in the activation of PKA. To ascertain whether the CREB present in the pineal gland can serve as a substrate for PKA, a cell-free supernatant prepared from pineal glands was incubated in

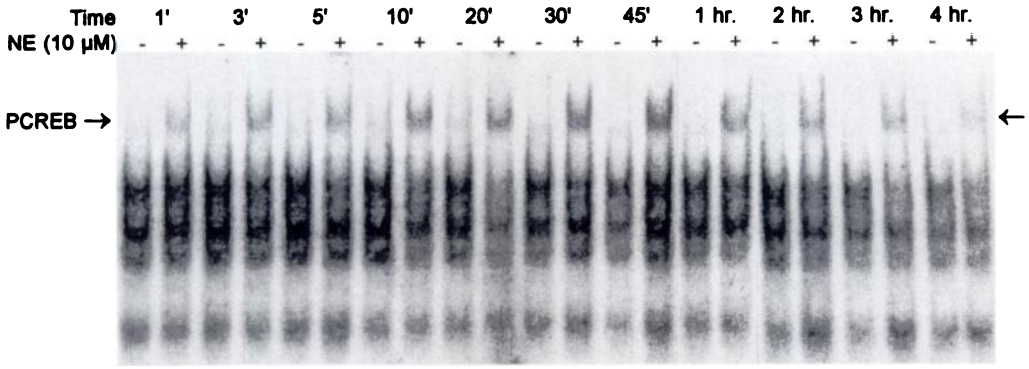

Fig. 3. Time dependence of NE-induced CREB phosphorylation in pineal glands in organ culture. Pineal glands were incubated under control conditions, as described in Experimental Procedures, and then transferred to control medium or medium containing 10 μM NE. Pineal glands were incubated for the indicated times, and the gel mobility-shift assay was performed with ^{32}P-CaCRE as described in Experimental Procedures. The supershifted band appeared 1 min after NE stimulation, reached a maximum after 30–45 min, and displayed a decrease after 2 hr. These results were confirmed in a second study.

the presence of the catalytic subunit of PKA. This resulted in a significant phosphorylation of CREB (Fig. 6B, *left* versus *right*).

Inhibition of PKA blocks NE-induced CREB phosphorylation. To determine whether the effects of NE on CREB phosphorylation resulted from activation of PKA, pineal glands were incubated with 10 nM NE in the presence of Rp-8-CPT-cAMPS. This cAMP analogue appears to antagonize the effects of cAMP through competitive binding to the regulatory subunits of PKA (32). Treatment with Rp-8-CPT-cAMPS inhibited NE-dependent CREB phosphorylation ($IC_{50} \cong 300$ μM) (Fig. 7A, *lanes 3-6*); Rp-8-CPT-cAMPS by itself had no effect on CREB phosphorylation (data not shown). Rp-8-CPT-cAMPS also inhibited with a similar potency another cAMP-dependent effect of NE in the pineal gland, i.e., NE-induced increases in NAT activity (Fig. 7B). These data suggest that PKA mediates the effects of NE on both CREB phosphorylation and NAT activity.

Elevation of $[Ca^{2+}]_i$ induces pineal CREB phosphorylation. To determine whether elevation of $[Ca^{2+}]_i$ can also produce CREB phosphorylation, several $[Ca^{2+}]_i$-elevating agents with different mechanisms of action were used. The Ca^{2+} ionophores ionomycin (10 μM) and A23187 (10 μM) did not induce significant CREB phosphorylation (Fig. 8, *lanes 2* and *4*). These concentrations of ionophores are known to mimic the effects of α_1-adrenergic receptor activation on NAT activity (15). Depolarizing treatments, i.e., KCl (45 mM) or ouabain (10 μM), stimulated CREB phosphorylation to levels similar to those produced by NE treatment (Fig. 8, *lanes 6, 9,* and *10*). These concentrations of KCl and ouabain have been previously reported to increase the $[Ca^{2+}]_i$ in pinealocytes (33). These results indicate that some, but not all, agents that elevate $[Ca^{2+}]_i$ also cause CREB phosphorylation in the pineal gland.

Isoproterenol treatment induces pineal CREB phosphorylation in rats maintained in constant light. In the pineal gland, stimulus deprivation produces an increased cAMP and NAT response to adrenergic stimulation (34, 35). This has been shown to be mediated, in part, by an increase in β-adrenergic receptors (36), as well as postreceptor mechanisms (35, 37). To determine whether stimulus deprivation would increase the PCREB response to adrenergic stimulation, rats were placed in constant light for 7 days and then given subcutaneous injections of the adrenergic agonist isoproterenol. Administration of isoproterenol to rats maintained under constant light significantly increased the amount of PCREB detected in pineal extracts, compared with saline-treated controls (Fig. 9, *lane 3* versus *lane 4*). In contrast, administration of isoproterenol to rats housed under a control lighting cycle did not significantly increase the amount of PCREB (Fig. 9, *lane 1* versus *lane 2*). These results indicate that stimulus deprivation, in the form of constant light, reveals adrenergic agonist-induced pineal CREB phosphorylation. Constant-light treatment also produced a significant decrease in the intensity of the lowest band of retarded mobility (band 4), compared with control light treatment (Fig. 9, *lanes 1* and *2* versus *lanes 3* and *4*). As mentioned previously, the identity of band 4 is unknown, but it may represent the small molecular weight transcription factor ICER.

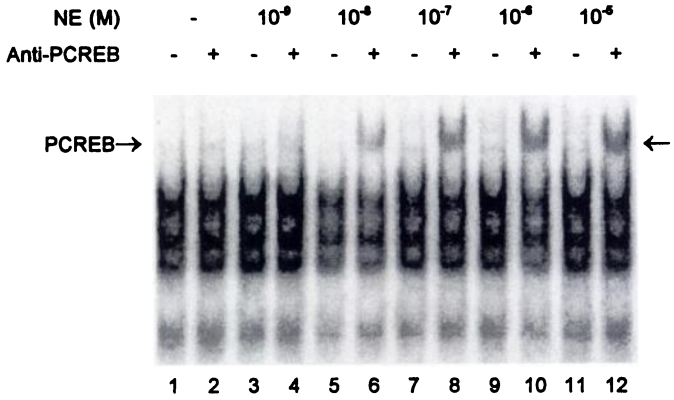

Fig. 4. Dose dependence of NE-induced CREB phosphorylation in pineal glands in organ culture. Pineal glands were incubated under control conditions, as described in Experimental Procedures, transferred to medium containing NE at the indicated concentrations, and incubated for 20 min. The gel mobility-shift assay was performed with ^{32}P-CaCRE as described in Experimental Procedures. The supershifted band can be seen at 10 nM NE, with the maximal response being obtained at 100 nM. Similar dose-response curves were obtained in four separate studies.

Discussion

In the present study, regulation of the transcription factor CREB in the rat pineal gland was investigated using gel mobility-shift assays with a radiolabeled CaCRE probe. Two

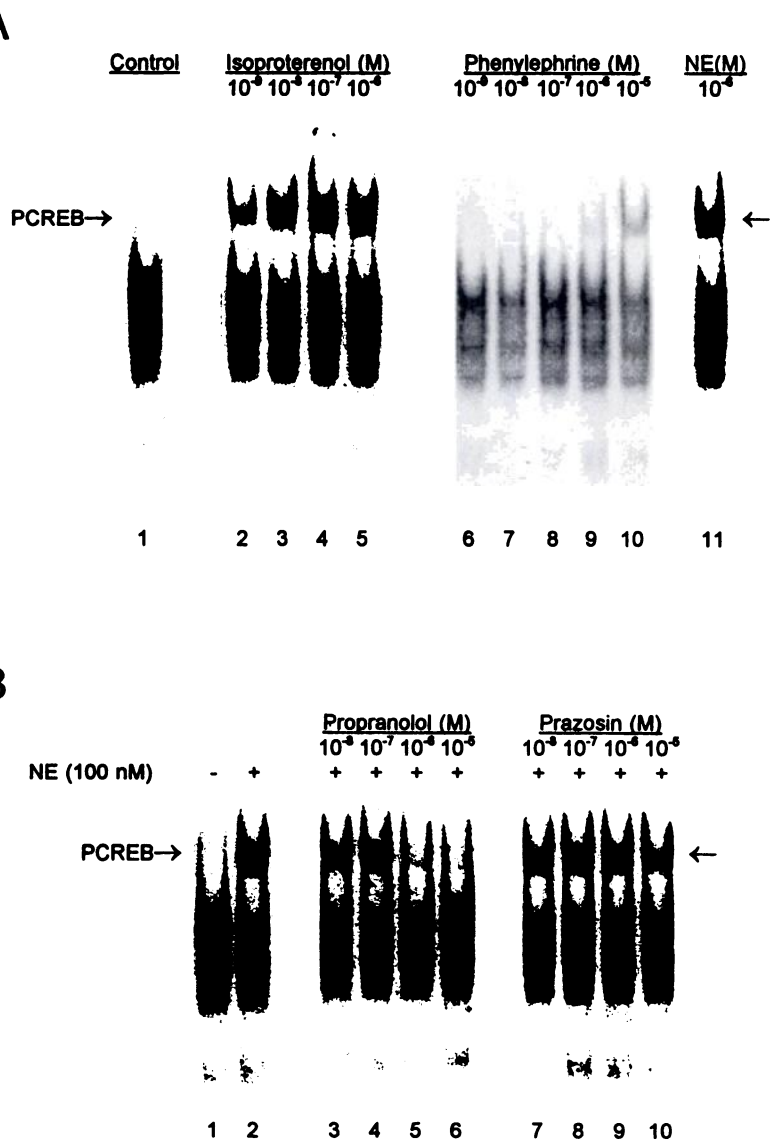

Fig. 5. Effects of α- and β-adrenergic receptor-selective ligands on pineal CREB phosphorylation. Pineal glands were incubated under control conditions, as described in Experimental Procedures, transferred to fresh medium containing the indicated concentrations of agonist, and incubated for 30 min. For experiments using antagonists, the glands were preincubated for 30 min with the indicated concentration of antagonist before transfer to medium containing antagonist and 100 nM NE and incubation for an additional 30 min. The gel mobility-shift assays were performed with ^{32}P-CaCRE as described in Experimental Procedures. A, Incubation with the β-adrenergic receptor-selective agonist isoproterenol induced significant CREB phosphorylation at concentrations as low as 1 nM, whereas incubation with the α_1-adrenergic receptor-selective agonist phenylephrine produced significant CREB phosphorylation only at 10 μM. Similar dose-response curves were obtained with three separate sets of pineal glands. B, Preincubation of glands with 1 μM or 10 μM propranolol significantly reduced the 100 nM NE-induced CREB phosphorylation; however, increasing concentrations of prazosin did not have a significant effect on the 100 nM NE-induced CREB phosphorylation. Similar results were obtained with three separate sets of pineal glands.

observations demonstrate that the majority of the protein that bound to the CaCRE represents CREB. First, only CRE-containing oligonucleotides eliminated the ^{32}P-CaCRE oligonucleotide binding to protein; second, incubation with a CREB-specific antiserum greatly decreased the protein binding to the ^{32}P-CaCRE. These results indicate that CREB protein is present in the rat pineal gland, which is consistent with a previous report of pineal CREB mRNA observed by Northern blot analysis (9).

Treatment of pineal glands with NE increased phosphorylation of CREB without significantly altering the total amount of CaCRE binding activity. This is consistent with previous reports that PKA-dependent phosphorylation of CREB does not alter DNA binding activity but that phosphorylation does enhance transcription (38). This appears to occur at the level of *trans*-activation through an allosteric mechanism (39).

Table 2 summarizes the ability of a variety of agents to induce CREB phosphorylation in cultured pineal glands. Our findings indicate that CREB is phosphorylated in the pineal gland in response to adrenergic stimulation and that this phosphorylation is mediated primarily through β_1-adrenergic receptor stimulation of cAMP accumulation. Several observations lead to this conclusion. First, the time course and dose-response relationship for NE induction of CREB phosphorylation are similar to those previously reported for cAMP accumulation (28, 29). Second, submicromolar concentrations of the β-adrenergic receptor-selective ligand isoproterenol, which stimulates cAMP production in the pineal gland, produced CREB phosphorylation comparable to that seen with NE. In contrast, similar concentrations of the α_1-adrenergic receptor-selective ligand phenylephrine, which does not elevate cAMP, did not produce CREB phosphorylation. Third, the β-adrenergic receptor-selective antagonist propranolol significantly decreased NE-induced CREB phosphorylation, whereas the α_1-adrenergic receptor-selective antagonist prazosin did not. Fourth, incubation with various cAMP protagonists or the catalytic subunit of PKA produced CREB phosphorylation. Lastly, Rp-8-CPT-cAMPS, a competitive inhibitor of PKA, blocked NE-induced pineal CREB phosphorylation. These data indicate that, whereas α_1-adrenergic receptor activation can lead to phosphorylation of CREB to a limited extent, NE-induced CREB phosphoryla-

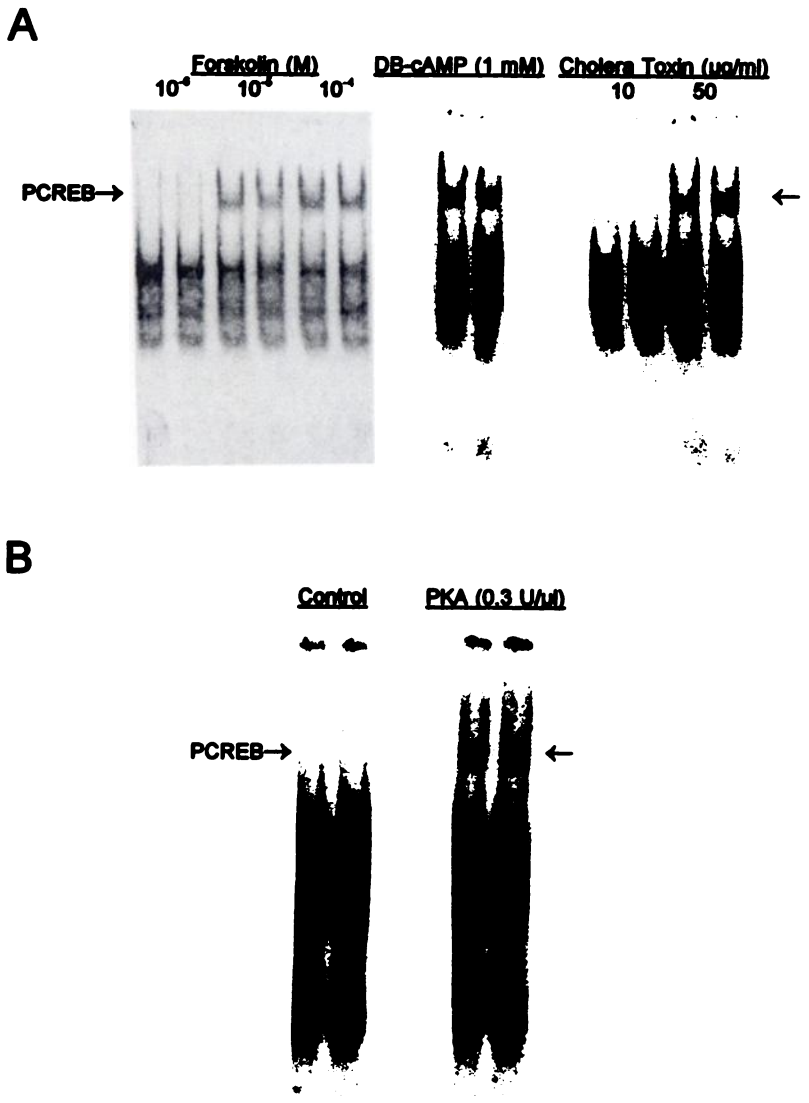

Fig. 6. Induction of pineal CREB phosphorylation by cAMP-elevating agents and the catalytic subunit of PKA. The gel mobility-shift assay was performed as described in Experimental Procedures. A, Pineal glands were incubated under control conditions, as described in Experimental Procedures, transferred to fresh medium containing the indicated drug at the various concentrations, and incubated for 30 min. For each concentration of agent, duplicate binding reactions were performed with extracts obtained from two pairs of pineal glands. Incubation with each agent resulted in CREB phosphorylation similar to that seen with NE. *DB-cAMP*, dibutyryl-cAMP. B, Pineal cytosol was prepared as described in Experimental Procedures and incubated with 1 mM ATP and 10 mM $MgCl_2$, in the presence or absence of 0.3 unit/μl PKA catalytic subunit, for 20 min at 37°. Duplicate binding reactions were performed with extracts obtained from two pairs of control or PKA-treated pineal glands. Incubation with the catalytic subunit of PKA produced significant phosphorylation of CREB.

tion is produced primarily through β_1-adrenergic receptor-mediated increases in cAMP accumulation.

It appears that the large increase in cAMP accumulation produced by NE is not required for maximal phosphorylation of CREB in response to adrenergic stimulation. Although previous studies have reported that isoproterenol stimulates a 10-fold increase in cAMP levels in pinealocytes, compared with the approximately 100-fold increase in cAMP produced by NE (40), the present study indicates that isoproterenol and NE induced CREB phosphorylation to similar extents. Additionally, the NE-induced pineal CREB phosphorylation reached a maximum at 100 nM NE, whereas the NE-induced increase in cAMP accumulation does not reach a maximum until 1–10 μM NE (29, 30, 40). These observations suggest that submaximal increases in cAMP accumulation are sufficient to produce maximal phosphorylation of CREB.

The results of the *in vivo* study are consistent with the conclusion that pineal CREB phosphorylation is regulated in intact animals by an adrenergic receptor/cAMP mechanism, because treatment with isoproterenol, which elevated cAMP levels, also caused CREB phosphorylation. However, it should be noted that prior sensitization by stimulus deprivation (37) was required to reveal these effects. We suspect that the difficulty associated with demonstrating adrenergic stimulation of CREB phosphorylation might reflect rapid events that occur during removal of tissue and that reverse or otherwise mask phosphorylation. Additionally, it seems possible that the maximal amount of *in vivo* CREB phosphorylation detectable might be limited in some way by unidentified factors, e.g., a nuclear protein phosphatase might be present at high levels *in vivo* and at low levels after 48 hr of organ culture.

Increases in $[Ca^{2+}]_i$ have been reported to induce CREB phosphorylation in PC-12 cells (16) and primary cultures of hippocampal neurons (13); however, in the study presented here it was found that stimulation of pineal glands with phenylephrine, which increases $[Ca^{2+}]_i$ through activation of α_1-adrenergic receptors, has little or no effect on CREB phosphorylation. Similar results were obtained with two ionophores. In contrast, treatment with the depolarizing agents KCl and ouabain induces CREB phosphorylation to a great extent. This indicates that, whereas there clearly is a potential for Ca^{2+}-dependent phosphorylation of CREB, this does not appear to play a primary role in the effects of NE.

However, it is unclear why some $[Ca^{2+}]_i$-elevating agents (KCl and ouabain) cause CREB phosphorylation, whereas others (A23187, ionomycin, and phenylephrine) do not. One explanation might be that each mechanism through which

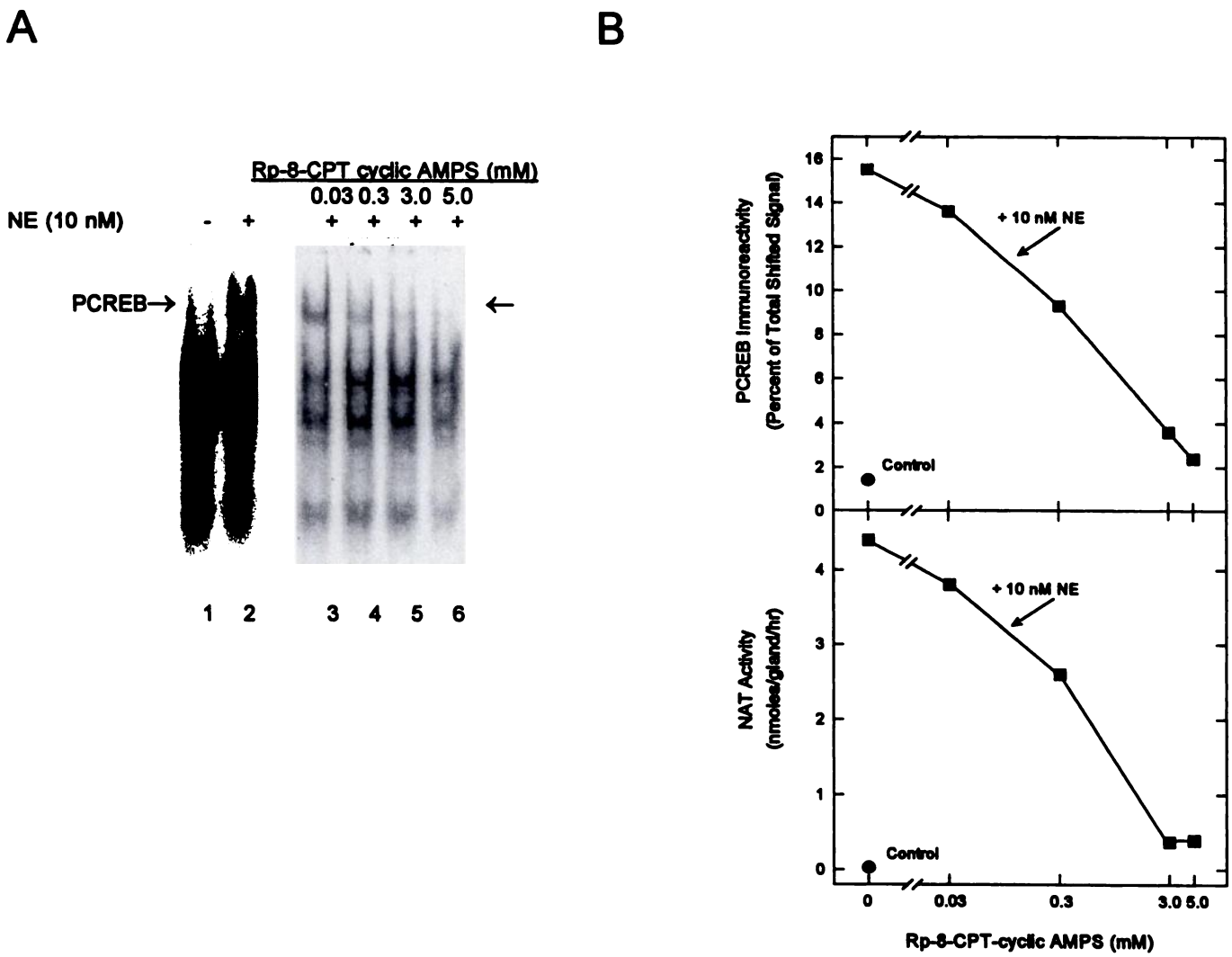

Fig. 7. Inhibition by the PKA antagonist Rp-8-CPT-cAMPS of NE stimulation of pineal CREB phosphorylation and of NAT activity. Pineal glands were incubated under control conditions as described in Experimental Procedures. After 36 hr, the glands were transferred to 40 μl of control medium or medium containing the indicated concentration of Rp-8-CPT-cAMPS, in a 96-well plate. The incubation was continued for 18 hr, and then glands were transferred to fresh medium containing the same concentrations of antagonist, in a table-top incubator. After an additional 2-hr incubation, NE was added to the medium to a final concentration of 10 nM. The incubation was continued for 30 min to measure PCREB and for 4 hr to measure NAT activity. A, The gel mobility-shift assay was performed with ^{32}P-CaCRE as described in Experimental Procedures. Similar results were obtained with a second set of pineal glands. B, The concentration-effect curves for Rp-8-CPT-cAMPS inhibition of NE-stimulated CREB phosphorylation and NAT activity are compared. The intensity of the supershifted PCREB signal is expressed relative to the intensity of the signal for all shifted bands in A; this includes the supershifted signal plus the signal for all four shifted bands, as identified in Fig. 1. NAT activity was measured as described in Experimental Procedures and is expressed as nmol of [^{3}H]acetyltryptamine produced/gland/hr. The values for NAT activity represent the average obtained from four glands in a single experiment. Rp-8-CPT-cAMPS inhibited NE stimulation of CREB phosphorylation and NAT activity with similar potencies ($IC_{50} \cong 300$ μM).

Ca^{2+} enters the cell is linked to a distinctly different biochemical pathway, as may be the case in hippocampal neurons (41). The process by which phenylephrine or the endogenous ligand NE increases $[Ca^{2+}]_i$ in the pineal gland differs from the processes used by either KCl or ouabain (34). It is possible that the Ca^{2+} that enters the pinealocytes in response to KCl or ouabain treatment is available to bind calmodulin and phosphorylate CREB through activation of Ca^{2+}/calmodulin-dependent protein kinases I and II, as has been previously reported for KCl treatment of either PC-12 cells (16) or hippocampal neurons (13). However, the Ca^{2+} that enters in response to treatment with phenylephrine or NE may not be available to activate CREB. Another possibility is that KCl or ouabain has a far greater effect on the Ca^{2+} concentration in a critical pool. For example, those compounds might increase the nuclear Ca^{2+} concentration more than does phenylephrine, A23187, or ionomycin, and the nuclear Ca^{2+} concentration may be of primary importance in the regulation of CREB phosphorylation.

Our studies point to the possibility that the pharmacological effects of KCl and ouabain might involve CREB phosphorylation. For example, the adrenergic receptor/cAMP stimulation of NAT activity is completely blocked by KCl and ouabain (42), whereas phenylephrine, A23187, and ionomycin potentiate the response to isoproterenol or cAMP protagonists (15). This inhibitory effect of KCl and ouabain may be mediated by CREB phosphorylation-dependent induction of the inhibitory transcription factor ICER (9).

CREB phosphorylation results in the transcriptional activation of various genes that contain CREs in their promoter

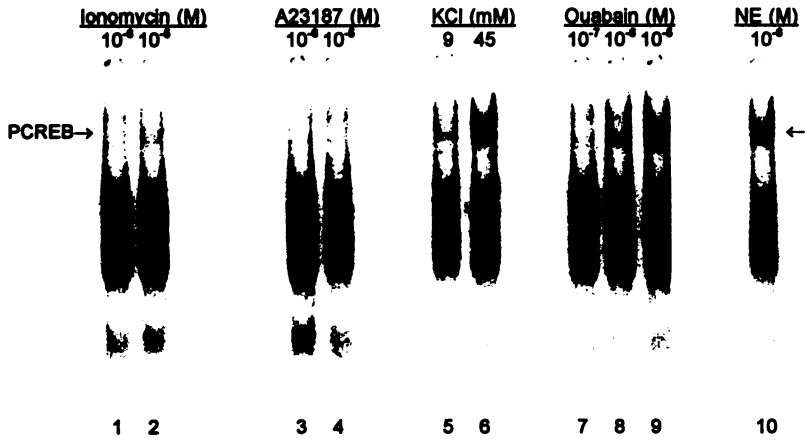

Fig. 8. Effects of the $[Ca^{2+}]_i$-elevating agents ionomycin, A23187, KCl, and ouabain on pineal CREB phosphorylation. Pineal glands were incubated under control conditions, as described in Experimental Procedures, transferred to fresh medium that contained the indicated drugs at various concentrations, and incubated for 30 min. The gel mobility-shift assay was performed with ^{32}P-CaCRE as described in Experimental Procedures. Both KCl and ouabain phosphorylated CREB to an extent similar to that seen with NE; ionomycin and A23187 produced only a slight increase in CREB phosphorylation. Similar results were obtained with two different sets of pineal glands.

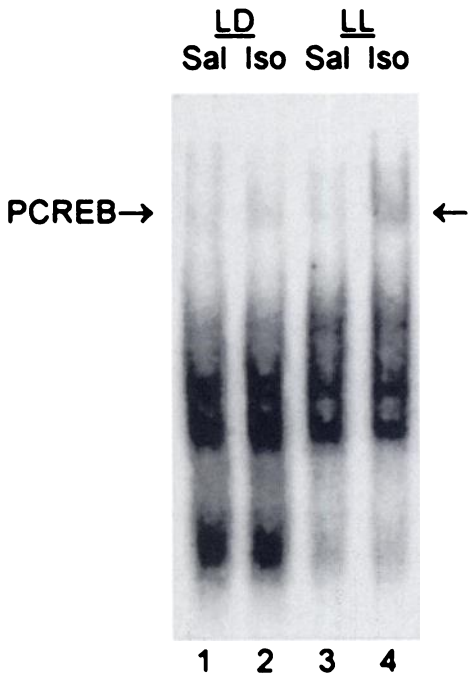

Fig. 9. Induction of pineal CREB phosphorylation by isoproterenol treatment in rats maintained in constant light. Rats were housed under control lighting conditions or in constant light for 7 days (see Experimental Procedures). On the day of the experiment, rats were given injections of 1 mg/kg isoproterenol (*Iso*) or 0.9% saline (*Sal*) at 12:00 p.m. Thirty minutes later rats were sacrificed and pineal glands were removed, placed on solid CO_2, and stored at −80° until assayed. The gel mobility-shift assay was performed with ^{32}P-CaCRE as described in Experimental Procedures. Administration of isoproterenol induced CREB phosphorylation in rats housed for 7 days in constant light (LL) but not in rats housed under control (LD) lighting conditions. Identical results were obtained with two different sets of rats treated on the same day.

TABLE 2

Relative ability of various agents to induce pineal CREB phosphorylation

Using the gel mobility-shift assay, as described in Experimental Procedures, the ability of various agents to stimulate pineal CREB phosphorylation was measured. The table summarizes data presented in Results.

Classification	Relative effectiveness		
	Strong	Weak	Undetectable
Receptor agonists	NE Isoproterenol	Phenylephrine	
Cyclic nucleotide protagonists	Forskolin Cholera toxin-Dibutyryl-cAMP		Dibutyryl-cGMP[a]
$[Ca^{2+}]_i$-elevating agents	KCl Ouabain	A23187[b] Ionomycin[b]	
Protein kinase	Catalytic subunit of PKA		

[a] Significant CREB phosphorylation was not detected with 1 mM dibutyryl-cGMP (data not shown).

[b] The Ca^{2+} ionophores A23187 and ionomycin are categorized as weak because these agents occasionally produced a weak PCREB signal in gel mobility-shift assays at the highest concentration tested (10 μM); however, this effect was not reproducible.

regions (11). Therefore, it can be predicted from the present study that elevation of pineal cAMP levels, which induces CREB phosphorylation, should result in increased transcription of various CRE-containing genes. Indeed, transcription of two CRE-containing genes, ICER and c-*fos*, is induced in the pineal gland by adrenergic stimulation (8, 9). Additionally, the promoter region of the β_1-adrenergic receptor gene contains a putative CRE sequence (43), and transcription of this gene is induced in the pineal gland in response to adrenergic stimulation (44). The observation that CREB is phosphorylated within 1 min after initiation of stimulation is temporally consistent with the putative role of CREB phosphorylation as an initiator of gene transcription for these three CRE-containing genes.

However, the NE/cAMP/PCREB cascade is not the major pathway regulating c-*fos* expression, according to pharmacological studies that indicate that NE controls c-*fos* expression primarily through an α_1-adrenergic receptor/$[Ca^{2+}]_i$ mechanism (45). This suggests that a transcription factor other than CREB might mediate the induction of c-*fos* mRNA in response to α_1-adrenergic receptor-mediated increases in $[Ca^{2+}]_i$. For example, in hippocampal neurons, entry of Ca^{2+} through the *N*-methyl-D-aspartate subtype of glutamate receptors results in the induction of c-*fos* mRNA through a mechanism that requires the presence of the serum response element but not the CaCRE in the c-*fos* promoter (41). Therefore, it is possible that α_1-adrenergic receptor-mediated increases in $[Ca^{2+}]_i$ in pinealocytes may stimulate c-*fos* transcription by phosphorylation of serum response element-binding proteins.

These studies are consistent with the hypothesis that cAMP controls pineal gene expression in part through PKA-dependent phosphorylation of CREB. The observation that Rp-8-CPT-cAMPS inhibits NE stimulation of both CREB phosphorylation and NAT activity, with similar potencies, provides indirect evidence that the cAMP/PKA/CREB cascade may play a role in

the NE stimulation of NAT activity. It will be of interest to determine whether cAMP-dependent CREB phosphorylation also mediates the increases in gene transcription that are required for NE induction of NAT, tryptophan hydroxylase, and thyroxine type II 5′-deiodinase activity.

Acknowledgments

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