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Norepinephrine Induction of Mitogen-Activated Protein Kinase Phosphatase-1 Expression in Rat Pinealocytes: Distinct Roles of α- and β-Adrenergic Receptors

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In this study, we investigated the mechanisms through which norepinephrine (NE) regulates MAPK phosphatase-1 (*MKP-1*) expression in rat pinealocytes. Stimulation with NE (a mixed α- and β-adrenergic agonist) caused a rapid increase in *MKP-1* mRNA and protein that peaked around 1 h post stimulation, and the response was sustained for at least 4 h. Selective activation of β-adrenergic receptors with isoproterenol for 1 h caused a similar increase in *MKP-1* mRNA and protein as observed with NE, but at 3 h, the isoproterenol response was much lower relative to NE. In contrast, selective activation of α-adrenergic receptors caused only small increases in *MKP-1* mRNA and protein and appeared to function primarily in prolonging the β-adrenergic-stimulated responses. In NE-stimulated pinealocytes, blockade of β-adrenergic receptors caused a rapid reduction in *MKP-1* mRNA, but it had a minimal effect on MKP-1 protein. In contrast, blockade of α-adrenergic receptors specifically reduced NE-induced MKP-1 protein but not mRNA. At the postreceptor level, treatment with dibutyryl cAMP caused parallel increases in *MKP-1* mRNA and protein. However, treatment with a protein kinase C activator caused a significant increase in MKP-1 protein but had little effect on *MKP-1* mRNA. Together, these results suggest that, in rat pinealocytes, NE activates the β-adrenergic receptor → protein kinase A pathway to induce transcription and translation of MKP-1 expression and the α-adrenergic receptor → protein kinase C pathway to prolong the stimulated responses through increased stability of the MKP-1 protein. (*Endocrinology* 145: 5723–5733, 2004)

THE BIOSYNTHESIS OF melatonin in the rat pineal gland is tightly regulated by the nightly release of norepinephrine (NE) from the sympathetic nerve terminals (1). By simultaneously stimulating both α- and β-adrenergic receptors, NE causes large increases in cAMP and cyclic GMP (cGMP) accumulation (2). These large increases in cyclic nucleotide accumulation are mediated through a cross-talk mechanism, whereby the α-adrenoceptor-mediated activation of protein kinase C (PKC) (3, 4) and elevation of intracellular Ca^{2+} (5, 6) potentiate the β-adrenergic-stimulated cAMP and cGMP responses (2). The main function of cAMP is to increase the activity of the rate-controlling enzyme for melatonin biosynthesis, arylalkylamine-*N*-acetyltransferase (AA-NAT), through transcriptional and posttranslational mechanisms (7–9).

Apart from induction of AA-NAT activity, NE increases the expression of transcription factors such as Fra-2 and inducible cAMP early repressor (10, 11), clock genes (12, 13), and enzymes, such as tryptophan hydroxylase and type II iodothyronine deiodinase (14, 15). Both inducible cAMP early repressor and Fra-2 have been suggested to be part of the control mechanism that regulates the daily rhythm of AA-NAT activity (16–18). NE, in addition to inducing protein synthesis, also activates other signaling pathways via phosphorylation cascades; one example relevant to the present study is MAPK (19, 20). MAPKs comprise a large family of serine/threonine protein kinases that are activated by diverse stimuli including cytokines, growth factors, neurotransmitters, hormones, and cellular stresses (21–24).

The importance of MAPK signaling in the regulation of pineal function in rats has recently been recognized (25–28). Nocturnal activation of p42/44MAPK and its downstream kinase, the 90-kDa ribosomal S6 kinase, has been found in the rat pineal gland (25, 26). Pineal cell culture studies indicate that both p42/44MAPK and p38MAPK are activated by NE (20, 28), and inhibition of either p42/44MAPK or p38MAPK activity in pinealocytes modulates the NE-mediated induction of AA-NAT activity (26, 28).

The magnitude and duration of signaling through MAPKs reflects a balance between activating kinases and inhibitory phosphatases (24, 29–31). Microarray analysis of day *vs.* night gene expression in rat pineal glands reveals a nocturnal increase in pineal transcription of MAPK phosphatase-1 (*MKP-1*) (32), raising the possibility that MKP-1, through interactions with p42/44MAPK and/or p38MAPK proteins, plays an important role in the regulation of AA-NAT activity. In this study, we characterized the adrenergic receptor-mediated mechanisms controlling *MKP-1* expression in the rat pineal gland and compared the mechanisms involved in the adrenergic induction of *MKP-1* and *AA-NAT* expression.

Materials and Methods

Materials

Dibutyryl cAMP (DBcAMP), dibutyryl cGMP (DBcGMP), isoproterenol (ISO), NE, phenylephrine (PE), 4 β-phorbol 12-myristate 13-acetate (PMA), prazosin (Praz), and propranolol (Prop) were obtained from

Abbreviations: AA-NAT, Arylalkylamine-*N*-acetyltransferase; cGMP, cyclic GMP; DBcAMP, dibutyryl cAMP; DBcGMP, dibutyryl cGMP; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; ISO, isoproterenol; MKP-1, MAPK phosphatase-1; NE, norepinephrine; PE, phenylephrine; PKC, protein kinase C; PMA, 4 β-phorbol 12-myristate 13-acetate; Praz, prazosin; Prop, propranolol.

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Sigma Aldrich Co. (St. Louis, MO). Ionomycin was obtained from Calbiochem Corp. (San Diego, CA). Polyclonal antibodies against MKP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was obtained from Ambion Inc. (Austin, TX). Polyclonal antibodies against AA-NAT (AB3314) were a gift from Dr. D. C. Klein (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD). All other chemicals were of the purest grades available commercially.

Preparation of cultured pinealocytes and drug treatment

All procedures were reviewed and approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta (Edmonton, Alberta, Canada). Sprague Dawley rats (male; weighing 150 g) were obtained from the University of Alberta animal unit. Animals were housed under a lighting regimen providing 12 h of light every 24 h, with lights on at 0600 h. For pinealocyte cell culture preparation, animals were killed 3 h after the onset of light by decapitation, and pineal glands were removed and stored in ice-cold PBS until trypsinization. Pinealocytes were prepared from freshly dissected rat pineal glands by trypsinization as described previously (33). The cells were suspended in DMEM containing 10% fetal calf serum and maintained at 37 C for 24 h in a gas mixture of 95% O_2-5% CO_2 before experiments. Aliquots of pinealocytes (0.8×10^5 cells/0.3 ml) were treated with drugs that had been prepared in concentrated solutions in water or dimethylsulfoxide for the duration indicated. Treated cells were collected by centrifugation (2 min, 12,000 $\times$ *g*).

Western blot analysis

Samples for Western blot analysis were solubilized in 1× sample buffer by boiling for 5 min and stored at −20 C until electrophoresis. The homogenization buffer contained 20 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride (pH 7.5). SDS-PAGE was performed according to the procedure of Laemmli (34), as described in our previous studies (19, 20, 26), using 10% acrylamide (Mini-Protein II gel system; Bio-Rad, Hercules, CA).

RT-PCR analysis

Pinealocyte total RNAs were isolated and purified using an RNeasy kit (Qiagen Inc., Valencia, CA). First-strand cDNA was synthesized from the isolated RNA using an Omniscript reverse-transcriptase kit (Qiagen) with an oligo-dT primer. Three microliters of a 1:10 dilution of cDNA were used as template for PCRs. PCR was performed in a 29.3-μl reaction mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each deoxynucleotide triphosphate, 1.25 U Taq polymerase (Cetus; PerkinElmer, Wellesley, MA), and 1 μM each of the two primers. All PCRs were performed as follows: denaturing for 1 min at 94 C, annealing for 1 min at 63 C, and extension for 1 min at 72 C. Initial denaturing and final extension were both 5 min in duration. Cycle numbers varied slightly between cell preparations, but in general, 22 cycles were used to amplify *GAPDH*, and 25 cycles were used to amplify both *MKP-1* and *AA-NAT*. All reaction sets included water blanks as negative controls. Amplified products were separated on ethidium bromide-stained 1.5% agarose gels. In all cases, *GAPDH* mRNA levels were also measured from the samples to demonstrate uniformity of sample preparation and loading. The following primers were used: *MKP-1*: left primer, 5′-CTG CTT TGA TCA ACG TCT CG-3′; right primer, 5′-AAG CTG AAG TTG GGG GAG AT-3′; and *AA-NAT*: left primer, 5′-GGT TCA CTT TGG GAC AAG GA-3′; right primer, 5′-GTG GCA CCG TAA GGA ACA TT-3′. Sequences of the GAPDH primers used were previously described (35).

Real time RT-PCR based on SYBR green fluorescence (Molecular Probes, Inc., Eugene, OR) was used to provide a relative quantification of *MKP-1* and *AA-NAT* (36). This was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Template cDNA samples were diluted 10-fold, and serial 4-fold dilutions (from 1:4 to 1:65,536) were analyzed on a 384-well plate. cDNA template samples were standardized by their OD_{260} readings, and incorporation curves for *MKP-1* and *AA-NAT* were normalized to a standard curve constructed for amplification of *GAPDH* for each sample.

Statistical analysis

For quantitation of RT-PCR analyses, gel images were acquired with Kodak 1-D software on a Kodak 2000R imaging station (Eastman Kodak Co., Rochester, NY). For analyses of Western blots, exposed films were scanned, and band densitometry of acquired images was performed with Kodak 1-D software. Densitometric values were normalized to percent maximal and presented as the mean ± SEM from at least three independent experiments. Statistical analysis involved either a paired *t* test or ANOVA with the Newman-Keuls test. Statistical significance was set at $P < 0.05$.

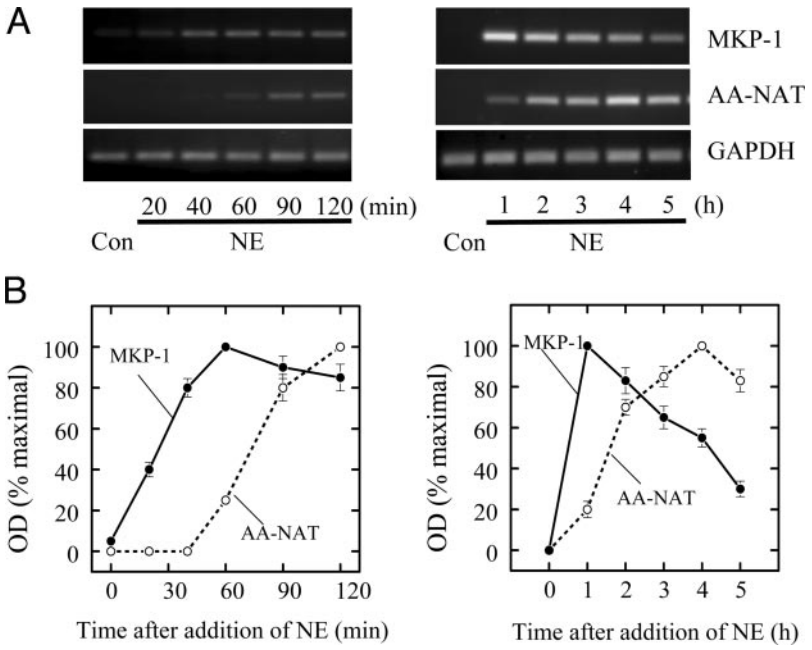

FIG. 1. Time profile of NE-induced *MKP-1* mRNA in cultured pinealocytes. Pinealocytes (0.8×10^5 cells/0.3 ml) were cultured for 24 h and treated with NE (3 μM) for different time periods. Cells were collected by centrifugation and prepared for RT-PCR as described in *Materials and Methods*. Con, Control. A, Representative ethidium bromide-stained gels showing early (*left*) and late (*right*) time profiles of *MKP-1* and *AA-NAT* mRNA expression by RT-PCR in NE-treated pinealocytes; *GAPDH* was included to demonstrate loading consistency. B, Densitometric measurements of *MKP-1* and *AA-NAT* mRNAs are presented as percentage of maximal OD value. Values represent the mean ± SEM (n = 3).

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Results

Time profile of NE induction of MKP-1 expression in rat pinealocytes

Treatment of pinealocytes with NE (a mixed α- and β-adrenergic agonist; 3 μM) caused a rapid induction of *MKP-1* mRNA that was detectable within 20 min post treatment ($P < 0.05$), peaked at 1 h, and declined gradually over the next 4 h (Fig. 1A). This differed from the time profile of NE-induced AA-NAT expression; there was a gradual increase in *AA-NAT* mRNA at 60 min ($P < 0.05$), which peaked after 4 h (Fig. 1A). The changes in NE-induced MKP-1 and AA-NAT proteins paralleled the corresponding increases in mRNA levels (Fig. 2, A and B). Induction of MKP-1 protein by NE occurred more rapidly than AA-NAT, with the peak response observed around 1 h after stimulation (Fig. 2, A and B).

Time profiles of α- and β-adrenergic activation of MKP-1 expression

To determine the relative contributions of α- and β-adrenergic receptors to the NE-stimulated *MKP-1* expression in rat pinealocytes, β-adrenergic receptors were selectively activated by using ISO (3 μM; in the presence of an α-adrenergic antagonist, Praz, 3 μM), and α-adrenergic receptors were selectively activated by PE (3 μM; in the presence of a β-adrenergic antagonist, Prop, 3 μM) as in previous studies (3–6, 37). Cells stimulated with NE for 4 h were included for comparison. Treatment with ISO caused a large increase in pineal *MKP-1* mRNA at 1 h, which was followed by a rapid decline (Fig. 3A), and at 4 h, the *MKP-1* mRNA level was lower than that induced by NE. Treatment with PE caused a small but sustained increase in *MKP-1* mRNA level

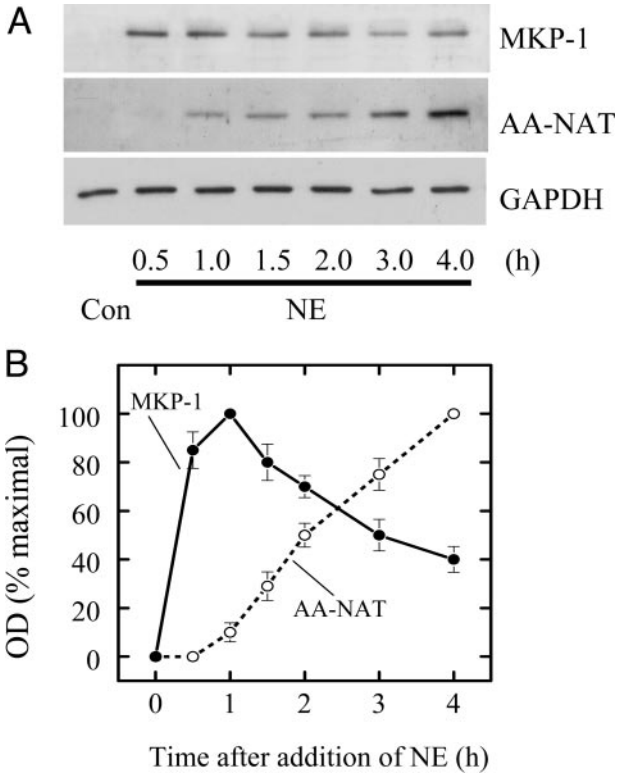

FIG. 2. Time profile of NE-induced MKP-1 protein in cultured pinealocytes. Pinealocytes (0.8×10^5 cells/0.3 ml) were treated with NE (3 μM) for different time periods. Cells were collected by centrifugation and prepared for immunoblot analysis as described in *Materials and Methods*. Con, Control. A, Representative immunoblots showing MKP-1 and AA-NAT proteins in NE-treated pinealocytes; GAPDH was included to validate loading. B, Densitometric measurements of MKP-1 and AA-NAT proteins from three independent experiments are presented as percentage of maximal OD value. Values represent the mean ± SEM (n = 3).

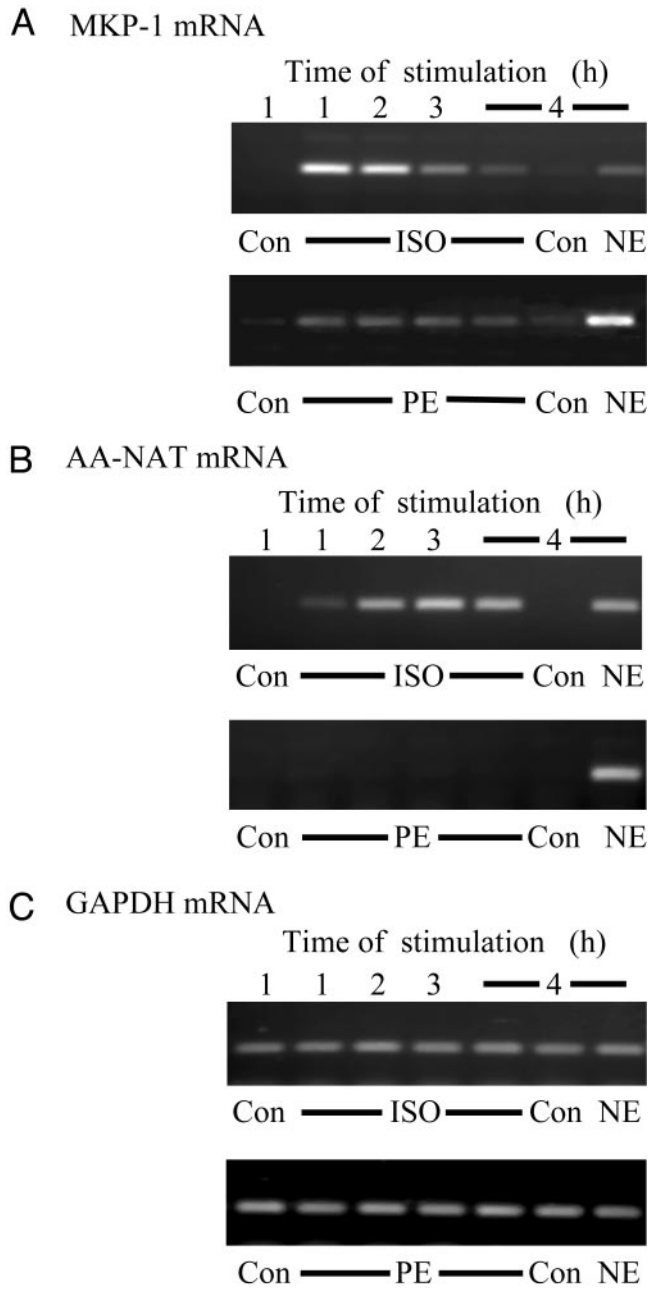

FIG. 3. Time profiles of α- and β-adrenergic receptor agonist-induced *MKP-1* transcription. Pinealocytes (0.8×10^5 cells/0.3 ml) were treated with ISO (3 μM; with Praz, 3 μM) or PE (3 μM; with Prop, 3 μM) for different time periods and NE (3 μM) for 4 h. Con, Control. Representative ethidium bromide-stained gels from three separate experiments are presented showing (A) *MKP-1*, (B) *AA-NAT*, and (C) *GAPDH* mRNAs.

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(Fig. 3A). In comparison, the ISO-stimulated *AA-NAT* mRNA induction was gradual, with a small increase at 1 h, and peaked between 3 and 4 h, whereas PE had no effect on *AA-NAT* mRNA (Fig 3B).

Interaction of α- and β-adrenergic receptors in regulating MKP-1 expression

To determine the nature of the interaction between α- and β-adrenergic receptors in the NE-stimulated *MKP-1* transcription, pinealocytes were stimulated with NE (3 μM), ISO (3 μM; with 3 μM of Praz), PE (3 μM; with 3 μM of Prop), or ISO + PE (both 3 μM) for 1 and 3 h. These time points were chosen to represent the peak and the decline phase of the NE-stimulated response, and real-time PCR was used to determine the relative changes in mRNA levels. As shown in Fig. 4A, after 1 h of drug treatment, selective activation of β-adrenergic receptors with ISO was as effective as activation of both α- and β-adrenergic receptors by NE or ISO + PE in elevating *MKP-1* mRNA levels, each resulting in a 10- to 12-fold induction of *MKP-1* mRNA. In contrast, activation of α-adrenergic receptors alone by PE resulted only in a 3-fold induction of *MKP-1* mRNA at 1 h. *AA-NAT* mRNA determinations from the same samples showed a 22-fold increase by NE at 1 h. Whereas the ISO-stimulated *AA-NAT* mRNA response at 1 h was 80% of the NE or ISO + PE response, the PE-stimulated response was minimal.

When the treatment period was extended to 3 h, the NE- and ISO + PE-stimulated *MKP-1* mRNA responses remained at approximately 10-fold above basal level (Fig. 4B). In comparison, the ISO-stimulated *MKP-1* mRNA response at 3 h dropped to 2.5-fold (from 12-fold at 1 h), whereas the corresponding decline in the PE-stimulated response was less substantial. *AA-NAT* mRNA determinations from the same cells showed NE causing a further increase to 35-fold at 3 h (from 22-fold at 1 h), whereas the relative potencies of ISO, PE, and ISO + PE to NE-stimulated AA-NAT responses remained identical to those observed at 1 h (Fig. 4B).

Time profiles of α- and β-adrenergic-stimulated MKP-1 protein expression

The time profiles of ISO- and PE-stimulated MKP-1 protein levels (Fig. 5A) followed closely their effects on *MKP-1*

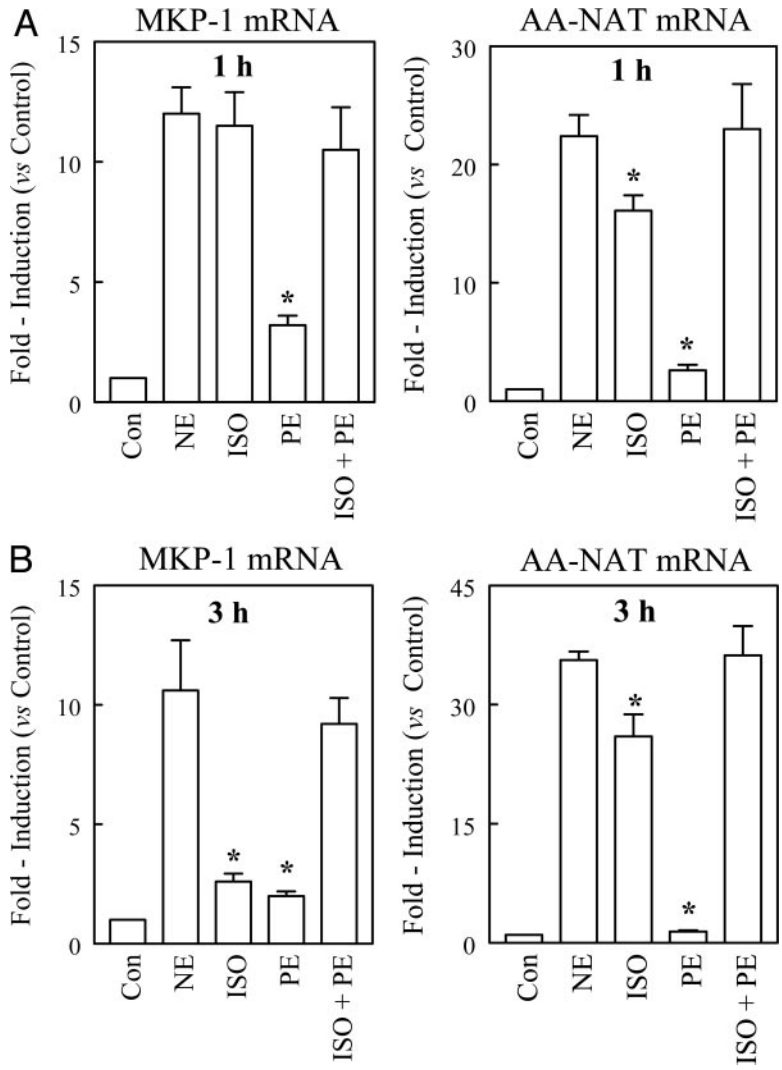

FIG. 4. Real-time RT-PCR analysis of α- and β-adrenergic receptor agonist-induced *MKP-1* transcription. Pinealocytes (0.8×10^5 cells/0.3 ml) were treated with NE (3 μM), ISO (3 μM; with Praz, 3 μM), PE (3 μM; with Prop, 3 μM), or ISO (3 μM) + PE (3 μM) for 1 and 3 h. Cells were collected by centrifugation and prepared for real-time RT-PCR as described in *Materials and Methods*. Con, Control. Histograms of *MKP-1* mRNA (*left*) and *AA-NAT* mRNA (*right*), normalized to *GAPDH* mRNA, are expressed as fold induction *vs.* untreated controls after 1 h (A) and 3 h (B) of treatment with adrenergic agonists. Values represent the mean ± SEM (n = 3, each run in duplicate). *, Significantly different from NE treatment.

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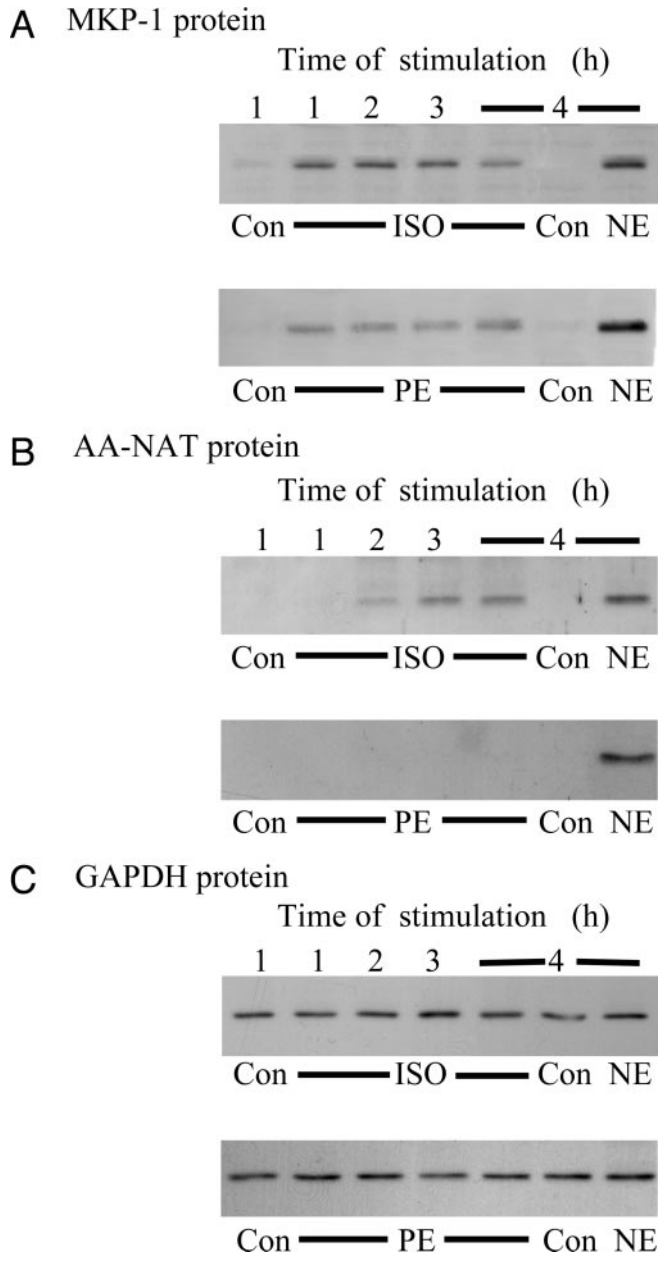

FIG. 5. Time profiles of α- and β-adrenergic receptor agonist-induced MKP-1 protein. Pinealocytes (0.8×10^5 cells/0.3 ml) were treated with ISO (3 μM; with Praz, 3 μM) or PE (3 μM; with Prop, 3 μM) for different time periods and NE (3 μM) for 4 h. Representative immunoblots from three separate experiments are presented showing (A) MKP-1, (B) AA-NAT, and (C) GAPDH proteins. Con, Control.

mRNA (Fig. 4, A and B). In cells stimulated with ISO, there was a peak increase in MKP-1 protein level between 1 and 2 h, followed by a gradual decline (Fig. 5A). In contrast, although PE was less effective in inducing MKP-1 protein than NE at 4 h, its effect was sustained between 1 and 4 h post treatment. The time profiles of ISO and PE on MKP-1 protein levels differed from their effects on AA-NAT protein levels. Whereas ISO caused a gradual increase in AA-NAT protein, PE had no effect (Fig. 5B). These changes in AA-NAT protein levels paralleled the changes in *AA-NAT* mRNA levels.

Interaction of α- and β-adrenergic receptors in regulating MKP-1 protein level

In parallel with their effects on *MKP-1* mRNA, activation of β-adrenergic receptors alone by ISO caused a similar induction in MKP-1 protein level as did NE at 1 h (Fig. 6A). In contrast, activation of α-adrenergic receptors alone by PE produced a small increase (18% of the NE response) in MKP-1 protein at 1 h (Fig. 6A), whereas AA-NAT protein measured from the same cells showed no induction by these treatments (data not shown). At 3 h, stimulation by ISO or PE increased MKP-1 protein levels that were 45 and 23% of the NE-stimulated response, respectively (Fig. 6B). However, combined stimulation of α- and β-adrenergic receptors by NE or ISO + PE resulted in an increase in MKP-1 protein level larger than the summation of the individual stimulation (Fig. 6B). Similar potentiation of the β-adrenergic-stimulated response by α-adrenergic agonist was observed with AA-NAT protein (Fig. 6B).

Contributions of α- and β-adrenergic receptors in the maintenance of NE-induced MKP-1 expression

To address the roles of α- and β-adrenergic receptors in the maintenance of NE-induced *MKP-1* expression, pinealocytes were treated with NE (3 μM) for 3 h to induce *MKP-1* expression before the addition of Prop or Praz. Blocking β-adrenergic receptors with Prop (3 μM) caused a significant reduction in NE-induced *MKP-1* mRNA within 30 min (Fig. 7, A and B). In contrast, blocking α-adrenergic receptors with Praz (3 μM) had little effect on NE-induced *MKP-1* mRNA (Fig. 7, A and B). Neither Prop nor Praz had a significant effect on NE-stimulated *AA-NAT* mRNA (Fig. 7, A and B).

In contrast to their effects on *MKP-1* mRNA, Prop (3 μM) had no effect on the level of NE-induced MKP-1 protein, whereas Praz (3 μM) caused a 50% reduction within 1 h (Fig. 8, A and B). In comparison, Prop (3 μM) caused a pronounced reduction in NE-stimulated AA-NAT protein level within 1 h, whereas Praz treatment had no effect (Fig. 8, A and C).

Roles of adrenergic-activated signaling pathways in induction of MKP-1 mRNA and protein levels

NE, through activation of β-adrenergic receptors, elevates cAMP and cGMP accumulation (1, 2) and, through activation of α-adrenergic receptors, causes activation of PKC and elevation of intracellular Ca^{2+} (3–6). To determine the relative roles of these signaling pathways in regulating *MKP-1* induction in rat pinealocytes, cells were treated with DBcAMP, DBcGMP, PMA (a PKC activator), and ionomycin (a Ca^{2+} ionophore) for 2.5 h. The effect of NE (3 μM) on *MKP-1* expression was included for comparison. DBcAMP (1 mM) and ionomycin (1 μM) increased *MKP-1* mRNA levels but with different potencies. Whereas DBcAMP induced *MKP-1* mRNA to a level similar to that of NE, ionomycin was less effective, and the effect of PMA was just significant (Fig. 9, A and B). In contrast, DBcGMP (1 mM) caused no increase in the *MKP-1* mRNA level (Fig. 9, A and B). When *AA-NAT* mRNA levels were determined from the same cells, only NE and DBcAMP were effective (Fig. 9, A and C).

At the protein level, NE and DBcAMP caused a significant

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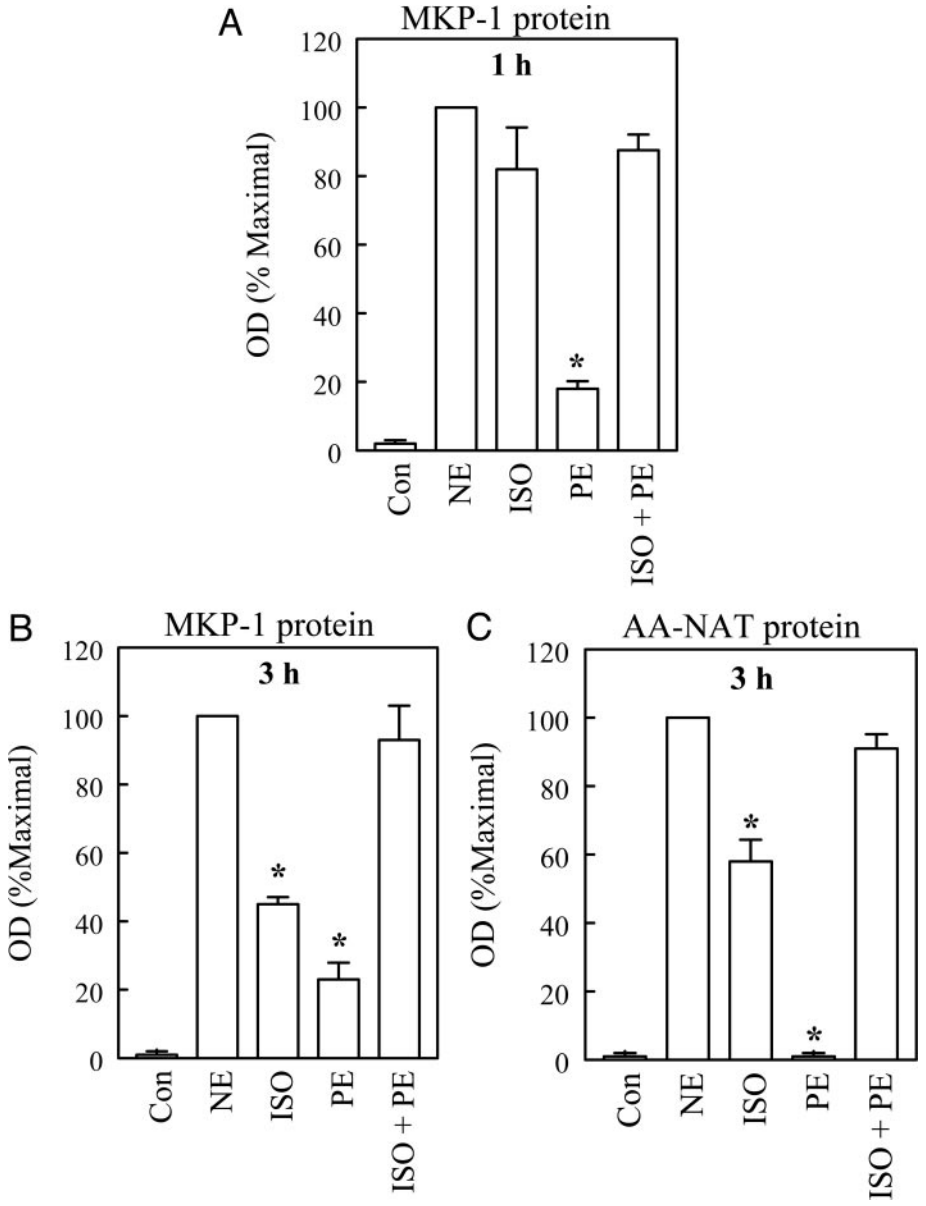

FIG. 6. Densitometric measurements of α- and β-adrenergic receptor agonist-induced MKP-1 protein. Pinealocytes (0.8×10^5 cells/0.3 ml) were treated with NE (3 μM), ISO (3 μM; with Praz, 3 μM), PE (3 μM; with Prop, 3 μM), or ISO (3 μM) + PE (3 μM) for 1 and 3 h. Con, Control. Histograms showing densitometric measurements of MKP-1 protein after 1 h (A) and 3 h (B) and AA-NAT protein after 3 h (C) of treatment with adrenergic agonists are presented as percentage of maximal OD value. Values represent the mean ± SEM (n = 3). *, Significantly different from NE treatment.

induction of MKP-1, whereas ionomycin was less effective, which parallels their effects on *MKP-1* mRNA levels (Fig. 9, A and B). Interestingly, PMA treatment caused an increase in MKP-1 protein level (63% of the DBcAMP response), even though it only had a small inducing effect on *MKP-1* mRNA (Fig. 9, A and B). In parallel with their effects on *AA-NAT* mRNA, only treatment with NE or DBcAMP caused an increase in AA-NAT protein (Fig. 9, A and C).

Discussion

Induction of *MKP-1*, an immediate early gene product, functions as a transcriptional mechanism targeting inactivation of MAPKs in many physiological processes (29, 30). In the rat pineal gland, microarray studies showed an induction of *MKP-1* mRNA at night (32). In the present study, we characterized the contribution of specific adrenergic receptor subtypes and the signaling mechanisms involved in the transcriptional and translational control of *MKP-1* expression. By selectively activating α- or β-adrenergic receptors by pharmacological agents, our results indicate that each receptor subtype has a distinct role in NE-stimulated *MKP-1* expression. During the early phase of stimulation, activation of β-adrenergic receptors alone is sufficient to induce *MKP-1* expression of a similar magnitude as seen with NE, and simultaneous activation of α-adrenergic receptors has no additional effect on β-adrenergic-stimulated *MKP-1* expression. However, compared with NE, β-adrenergic-stimulated *MKP-1* expression is less sustained, with a decline in *MKP-1* mRNA after 1 h and a decline in MKP-1 protein after 2 h.

In contrast to the results with selective activation of β-adrenergic receptors, selective activation of α-adrenergic receptors only has a minimal effect on *MKP-1* mRNA and protein levels (<20% of the NE response). However, activation of this pathway accounts for the sustained effect of NE

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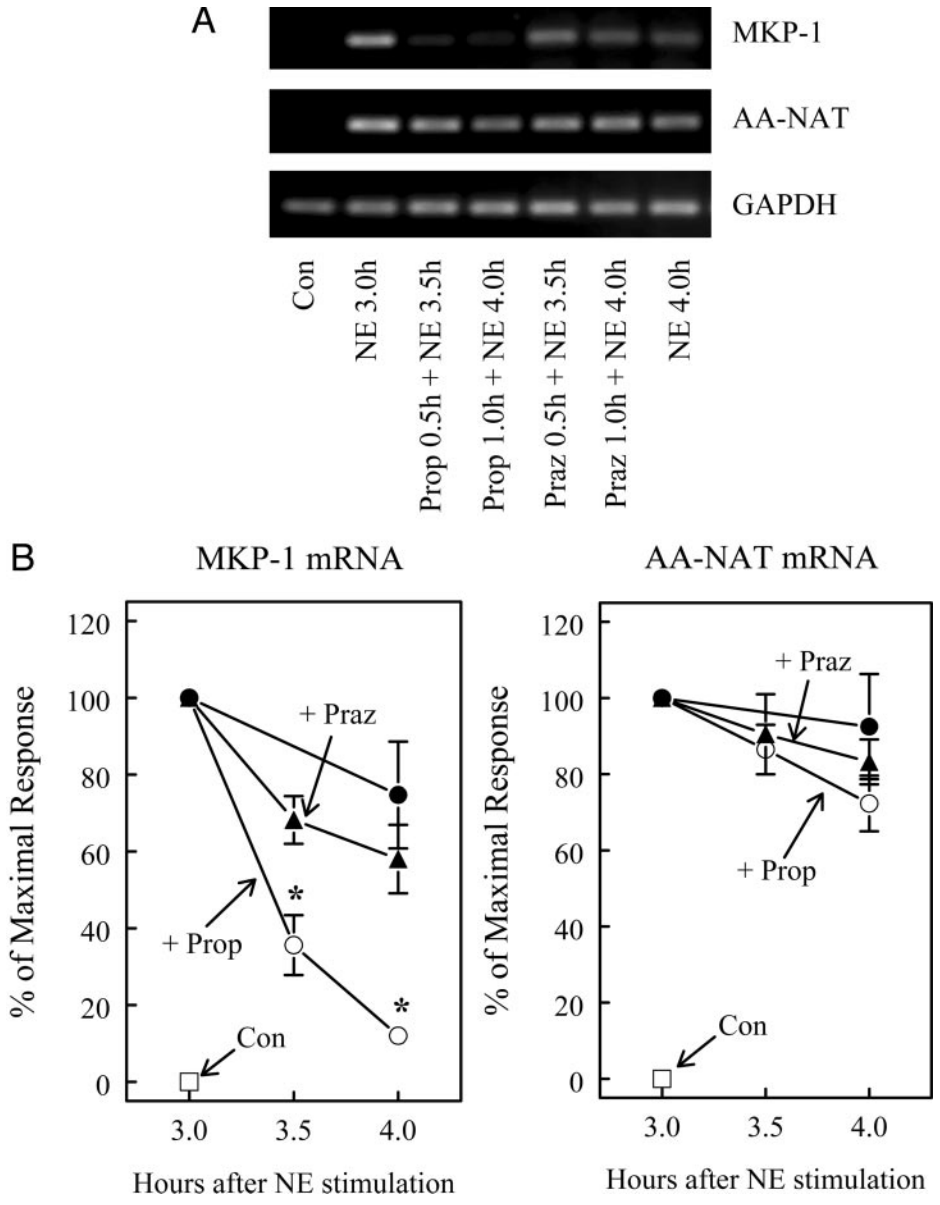

FIG. 7. Blockade of β-adrenergic receptors reduces NE-induced *MKP-1* mRNA. Pinealocytes (0.8×10^5 cells/0.3 ml) were stimulated with NE (3 μM) (●) for 3 h before treatment with Prop (3 μM) (○) or Praz (3 μM) (▲) for 30 and 60 min. Con, Control (□). A, Representative ethidium bromide-stained gels from three separate experiments are presented showing *MKP-1*, *AA-NAT*, and *GAPDH* mRNAs. B, Densitometric measurements of *MKP-1* (*left*) and *AA-NAT* (*right*) mRNAs are presented as percentage of maximal response. Values represent the mean ± SEM (n = 3). *, Significantly different from NE treatment at 3 h.

on *MKP-1* expression by prolonging the duration of the β-adrenergic-stimulated responses. This is achieved, not by raising the maximal β-adrenergic-stimulated responses during the early phase of stimulation, but by potentiating and prolonging the β-adrenergic-stimulated responses during the decline phase. With this potentiation, *MKP-1* mRNA and protein levels are maintained at levels achieved by NE at 4 h.

Another important difference between the effects of α- and β-adrenergic activation on *MKP-1* mRNA and protein levels is highlighted by the experiment in which α- or β-adrenergic receptor antagonists are added after *MKP-1* expression is induced by NE for 3 h. Our results show that, in the presence of NE induction, blockade of β-adrenergic receptors results in a rapid reduction of *MKP-1* mRNA but not of MKP-1 protein. In contrast, blockade of α-adrenergic receptors has no effect on *MKP-1* mRNA but causes a significant reduction in MKP-1 protein. These results indicate that continuous stimulation of both receptor subtypes is required for the maintenance of *MKP-1* expression.

Our results also provide important information regarding the regulated stability of *MKP-1* mRNA and protein. During NE stimulation, continuous activation of the β-adrenergic-mediated mechanism, but not the α-adrenergic-mediated mechanism, is required to maintain the transcription of *MKP-1* mRNA. The rapid decline in *MKP-1* mRNA level after β-adrenergic blockade, apart from demonstrating a role of β-adrenergic receptors in the NE-stimulated response at 3–4 h, also suggests a high degradation rate for *MKP-1* mRNA in rat pinealocytes. This result is in sharp contrast to the result observed with NE-regulated *AA-NAT* transcription in which blockade of either adrenergic receptor had only a small effect on *AA-NAT* mRNA level. Therefore, *AA-NAT* mRNA ap-

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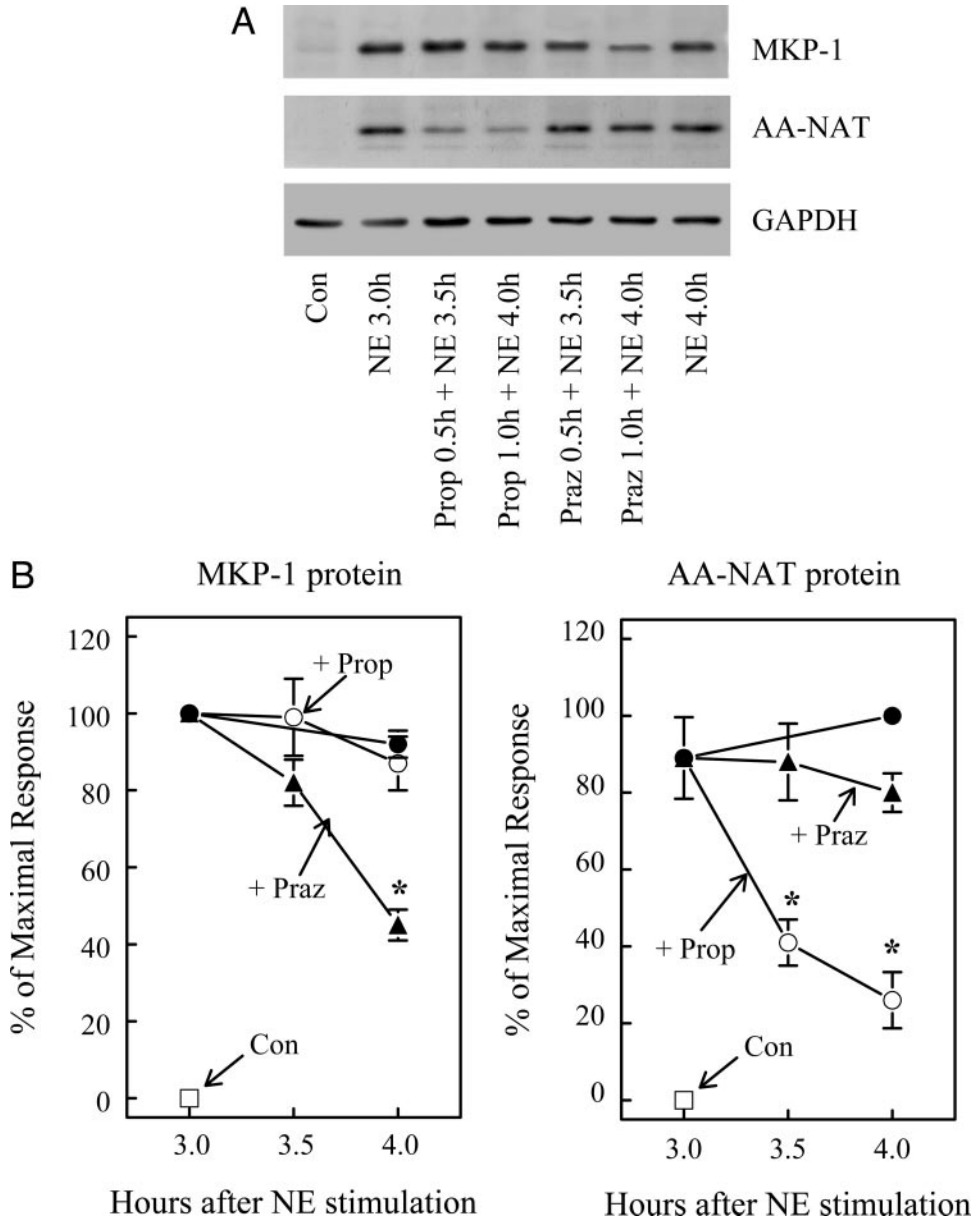

FIG. 8. Blockade of α-adrenergic receptors reduces NE-induced MKP-1 protein. Pinealocytes (0.8×10^5 cells/0.3 ml) were stimulated with NE (3 μM) (●) for 3 h before treatment with Prop (3 μM) (○) or Praz (3 μM) (▲) for 30 and 60 min. Con, Control (□). A, Representative immunoblots from three separate experiments are presented showing MKP-1, AA-NAT, and GAPDH proteins. B, Densitometric measurements of MKP-1 (*left*) and AA-NAT (*right*) proteins presented as percentage of maximal response. Values represent the mean ± SEM (n = 3). *, Significantly different from NE treatment at 3 h.

pears to have a longer half-life than *MKP-1*, and a duration of longer than 1 h is required to demonstrate a significant decline in *AA-NAT* mRNA.

A different picture emerges when the effects of adrenergic blockers are examined at the protein level. Blockade of α-adrenergic receptors, rather than the β-adrenergic receptors, is effective in reducing MKP-1 protein induced after 3 h of NE treatment. This indicates that an α-adrenergic-activated signaling mechanism appears to play a dominant role in maintaining the stability of MKP-1 protein. In comparison, blockade of β-adrenergic receptors, although only having a small effect on *AA-NAT* mRNA, causes a rapid decline in NE-stimulated AA-NAT protein, which is similar to previous reports (8, 38). This rapid decline in AA-NAT protein is due to dephosphorylation of AA-NAT protein at the protein kinase A phosphorylation sites and its subsequent dissociation from 14-3-3 protein (9). This dissociation in turn leads to a rapid degradation of the AA-NAT protein through proteosomal proteolysis (8). In the case of MKP-1 protein, it is not known whether phosphorylation of the protein itself or binding to 14-3-3 protein plays a role in protein stability. However, in other cell types, proteosomal proteolysis has been reported to play an important role in regulating the level of MKP-1 protein (39, 40).

The present study also identifies signaling mechanisms that mediate the adrenergic-induced expression of *MKP-1*. Activation of β-adrenergic receptors causes elevation of cAMP amd cGMP levels in rat pinealocytes (1, 2). Whereas treatment with DBcAMP is effective in inducing *MKP-1* expression, DBcGMP has no inductive effect. These results suggest that protein kinase A, rather than protein kinase G, is involved in the induction of *MKP-1* mRNA and protein. In agreement with our findings are the previous observations that cAMP response elements are present in the promoter

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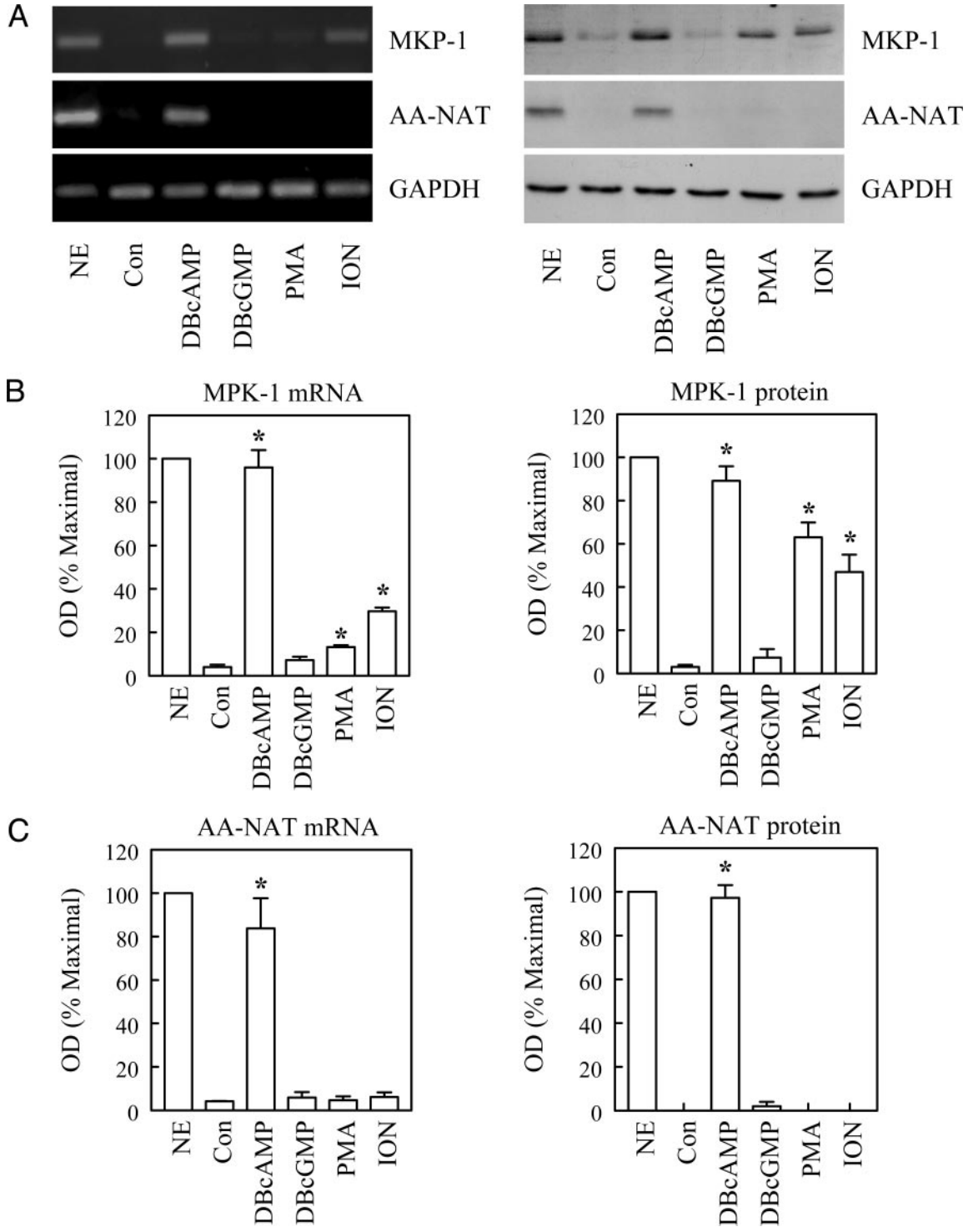

FIG. 9. Induction of *MKP-1* expression by postreceptor mechanisms. Pinealocytes (0.8×10^5 cells/0.3 ml) were treated with NE (3 μM), DBcAMP (1 mM), DBcGMP (1 mM), PMA (0.1 μM), and ionomycin (ION; 1 μM) for 2.5 h. Con, Control. A, Representative ethidium bromide-stained gels showing *MKP-1*, *AA-NAT*, and *GAPDH* mRNAs (*left*) and immunoblots showing MKP-1, AA-NAT, and GAPDH proteins (*right*). Histograms showing densitometric measurements of (B) *MKP-1* mRNA (*left*) and protein (*right*) and (C) *AA-NAT* mRNA (*left*) and protein (*right*) are presented as percentage of maximal OD value. Values represent the mean ± SEM (n = 3). *, Significantly different from untreated controls.

region of the *MKP-1* gene (41) and that *MKP-1* expression is induced by cAMP-elevating agents in other cell types (42, 43). Although induction of *MKP-1* expression by a cGMP-mediated mechanism has been reported in other cell types (44, 45), this pathway does not appear to be involved in NE-induced *MKP-1* expression in rat pinealocytes.

Activation of α-adrenergic receptors is coupled with elevation of intracellular Ca^{2+} and activation of PKC in rat

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pinealocytes (3–6). In this study, elevation of intracellular Ca^{2+} by ionomycin causes only a modest induction of *MKP-1* mRNA and protein. In contrast, activation of PKC by PMA, which has a minimal effect on *MKP-1* mRNA, causes a large increase in MKP-1 protein that approaches the effect of NE or DBcAMP. These results suggest that the contribution of PKC to *MKP-1* expression is mainly at the protein level. Hence, the effect of PMA on *MKP-1* expression is similar to that of PE, an α-adrenergic agonist, suggesting that activation of PKC may be the key mechanism through which α-adrenergic receptors act to maintain MKP-1 protein stability.

In other cell systems, activation of p42/44MAPK has been shown to induce *MKP-1* expression (24, 46). Moreover, p42/44MAPK can phosphorylate and stabilize MKP-1 protein (47). However, it is unlikely that activation of p42/44MAPK contributes to the NE-stimulated induction of *MKP-1* in rat pinealocytes. This conclusion is based on the observations that DBcGMP, which activates p42/44MAPK in rat pinealocytes (19), has no effect on the *MKP-1* level, whereas DBcAMP, which inhibits p42/44MAPK (20), stimulates *MKP-1* induction. However, the NE-mediated induction of *MKP-1* could be responsible for the rapid inactivation of p42/44MAPK observed after NE stimulation (20) despite the presence of continuous stimulation.

Because the magnitude and duration of signaling through MAPKs reflect a balance between activating kinases and inhibitory phosphatases (22, 29–31), our results suggest that NE initiates a sequential activation of upstream kinases and a downstream phosphatase in controlling the duration of MAPK activation. The time profile of MKP-1 activation may account for our observation that NE treatment increases the activation states of p42/44MAPK and p38MAPK with different time profiles (20, 28). Peak activation of p42/44MAPK occurs at 5 min after NE treatment (20) (before MKP-1 activation), whereas peak activation of p38MAPK occurs at 2 h (28), subsequent to the maximal activation of MKP-1 reported in this study. Given the known modulating effects of p42/44MAPK and p38MAPK on NE-induced AA-NAT activity (26, 28), MKP-1 probably plays an important role in shaping the darkness-induced profile of pineal AA-NAT activity and melatonin synthesis.

MKP-1 is an important regulator of MAPK activity in a wide array of physiological processes (24, 29, 30). Therefore, our observations suggesting the involvement of converging signaling mechanisms in the induction of *MKP-1* expression in the rat pineal gland is likely of general interest. Together, our results suggest that, although activation of protein kinase A and, to a lesser extent, elevation of intracellular Ca^{2+} are effective in inducing *MKP-1* mRNA/protein expression, activation of PKC appears to maintain the stability of MKP-1 protein. However, the precise nature of the interaction between different intracellular signaling pathways in regulating the transcription, translation, and protein level of MKP-1 requires further investigation. Nonetheless, it will be of interest to determine whether similar converging signaling mechanisms are involved in the control of *MKP-1* expression in other cell types.

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