0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(8):3344–3350

doi: 10.1210/en.2003-0215

Regulation of 90-Kilodalton Ribosomal S6 Kinase Phosphorylation in the Rat Pineal Gland

A. K. HO, M. MACKOVA, C. CHO, AND C. L. CHIK

Departments of Physiology and Medicine, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

In this study we investigated diurnal changes in the activation state of the 90-kDa ribosomal S6 kinase (p90RSK) in the rat pineal gland. In animals housed under a lighting regimen with 12 h of light, we found an increase in phosphorylated p90RSK during the dark phase, and this increase was abolished by treatment with propranolol or continuous exposure to light. To determine the intracellular mechanism involved, rat pinealocytes were treated with norepinephrine. Norepinephrine caused a parallel increase in phosphorylated p42/44 MAPK (p42/44^{MAPK}) and p90RSK that was reduced by prazosin or propranolol, indicating involvement of both α_1- and β-adrenergic receptors. Treatment with dibutyryl cGMP, 4β-phorbol 12-myristate 13-acetate, or ionomycin mimicked norepinephrine-stimulated p90RSK phosphorylation, whereas dibutyryl cAMP caused a decrease in p90RSK phosphorylation. Inhibition of p42/44^{MAPK} activation by UO126 was effective in reducing norepinephrine-stimulated p90RSK phosphorylation. Moreover, UO126 had an inhibitory effect on norepinephrine-stimulated arylalkyl-*N*-acetyltransferase activity. These results indicate that the adrenergically regulated nocturnal increase in p90RSK phosphorylation is mainly mediated through a cGMP→p42/44^{MAPK}-dependent mechanism. (*Endocrinology* 144: 3344–3350, 2003)

THE RAT PINEAL gland is stimulated by the nightly release of norepinephrine (NE) from the sympathetic nerve terminals. NE, by stimulating both α_1- and β-adrenergic receptors, causes a 100-fold increase in cAMP and cGMP accumulation (1). These large increases in cyclic nucleotide responses are the results of the potentiation of β-adrenergically stimulated cyclic nucleotide synthesis by the α_1-adrenergic receptor-mediated activation of protein kinase C (PKC) and elevation of intracellular Ca^{2+} (2–5). The primary function of cAMP in the rat pineal gland is to initiate a series of cellular events that leads to the synthesis of arylalkyl-*N*-acetyltransferase (AA-NAT), the rate-limiting enzyme in the synthesis of the pineal hormone, melatonin (6). The physiological importance of NE-stimulated cGMP accumulation in the pineal gland is unclear. However, we have shown that cGMP is involved in regulation of the L-type Ca^{2+} channel (7) as well as NE activation of p42/44 mitogen-activated protein kinase (p42/44^{MAPK}) in rat pinealocytes (8).

MAPKs, a large family of serine/threonine protein kinases, function as mediators of signal transduction and are activated by diverse stimuli, such as cytokines, growth factors, neurotransmitters, hormones, and cellular stresses (9, 10). Three main groups of MAPKs have been identified: p42/44^{MAPK} (11, 12), p38^{MAPK} (13, 14), and c-Jun N-terminal kinase (15, 16). The best studied member of MAPKs is p42/44^{MAPK}. Upon activation, p42/44^{MAPK} can regulate gene expression either directly by phosphorylating transcription factors (9, 17–19) or indirectly through phosphorylation of protein kinases such as the 90-kDa ribosomal S6 kinase (p90RSK) (20). One of the cellular functions of p90RSK that has been identified is regulation of gene expression by phosphorylating transcription factors, including cAMP response element-binding protein (CREB) (21–23). Considering the importance of CREB phosphorylation in the circadian activation of AA-NAT (24), NE activation of p90RSK may represent an important signaling mechanism that modulates the induction of AA-NAT at night. Therefore, the objectives of the present study are 3-fold. First, we will determine whether there is a day/night variation in the activation state of p90RSK in the rat pineal gland. Second, if there is a diurnal difference in the activation of p90RSK, we will investigate whether this is mediated by NE and will characterize the receptor and signaling pathways involved in the regulation of p90RSK. Third, we will determine the effect of inhibition of p42/44^{MAPK}/p90RSK on NE-stimulated AA-NAT activity.

Abbreviations: AA-NAT, Arylalkylamine *N*-acetyltransferase; CREB, cAMP response element-binding protein; MEK, MAPK kinase; NE, norepinephrine; *p-*, phosphorylated; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PMA, 4β-phorbol 12-myristate 13-acetate; p90RSK, 90-kDa ribosomal S6 kinase; ZT, zeitgeber time.

Materials and Methods

Materials

Acetyl-coenzyme A, dibutyryl cAMP, dibutyryl cGMP, isoproterenol, NE, phenylephrine, 4β-phorbol 12-myristate 13-acetate (PMA), prazosin, propranolol, and tryptamine were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Calphostin C, H89, ionomycin, KT5823, KN93, SB202474, SB203580, UO124, and UO126 were obtained from Calbiochem (San Diego, CA). Antibodies against p90RSK, p42/44^{MAPK}, and phosphorylated p42/44^{MAPK} were purchased from Sigma-Aldrich. An antibody against phosphorylated p90RSK was obtained from Cell Signaling Technology (Beverly, MA). [^{3}H]Acetyl-coenzyme A was purchased from NEN Life Science Products (Boston, MA). All other chemicals were of the purest grade available commercially.

Animals

All procedures were reviewed and approved by the health sciences animal and welfare committee of the University of Alberta (Edmonton, Alberta). Sprague Dawley rats (male; weighing 150 g) were obtained from the University of Alberta animal unit. Animals were housed under a lighting regimen that provides 12 h of light every 24 h with lights on at 0600 h [zeitgeber time zero (ZT 0)]. For the preparation of pinealocyte cell culture, animals were killed 3 h after the onset of light by decapi-

tation, and the pineal glands were rapidly removed and stored in ice-cold PBS until trypsinization. For the circadian study, groups of animals were killed at various time points as indicated. The pineal glands were collected, washed in ice-cold PBS, and frozen in dry ice immediately until preparation for Western blot analysis or AA-NAT determination. When the animals were killed during the dark period, only a dim red light was used, except in the experiment when the animals were deliberately exposed to light during darkness.

Preparation of cultured pinealocytes and drug treatment

Pinealocytes were prepared from freshly dissected rat pineal glands by trypsinization as described previously (25). The cells were suspended in DMEM containing 10% fetal calf serum and were maintained at 37 C for 24 h in a gas mixture of 95% air and 5% CO_2 before the experiments. For the determination of the activation states of $p42/44^{MAPK}$ and p90RSK, aliquots of pinealocytes (5×10^5 cells/0.5 ml) were treated with drugs that were added to the medium at 200× concentrated solutions in water or dimethylsulfoxide for the duration indicated. Treated cells were collected by centrifugation (2 min, 12,000 × *g*). Samples for Western blot analysis were solubilized in 1× sample buffer by boiling for 5 min and stored until analysis. For the determination of AA-NAT activity, treated cells were collected by centrifugation (2 min, 12,000 × *g*), and cell pellets were immediately frozen in dry ice and stored at −75 C.

Preparation of pineal glands for Western blot analysis

Frozen pineal glands were homogenized in a buffer (20 mM Tris-HCl; 2 mM EDTA; 0.5 mM EGTA; 2 mM phenylmethylsulfonylfluoride; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM sodium vanadate; and 1 mM sodium fluoride, pH 7.5) by sonication. Sodium vanadate and sodium fluoride were included to inhibit phosphatase activity. An aliquot of the homogenate was used for protein determination (26). The sample buffer that contained an identical amount of protease inhibitors, EGTA and EDTA, was used as a blank. The sample, after adjusting for protein content, was mixed with 2× sample buffer, boiled for 5 min, and stored until electrophoresis.

Western blot

SDS-PAGE was performed according to the procedure described by Laemmli (27), using 10% acrylamide in the presence of 1 mg/ml sodium dodecyl sulfate (Mini-Protein II gel system, Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, gels were equilibrated for 20 min in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). Proteins were transferred onto polyvinylidene difluoride membranes (1 h, 100 V), which were then incubated with a blocking solution [5% dried skim milk in 100 mM Tris (pH 7.5) with 140 mM NaCl and 0.01% Tween 20] for a minimum of 1 h. The blots were incubated overnight at 4 C with diluted specific antisera as indicated. After washing twice with the blocking solution, the blots were incubated with diluted horseradish peroxidase-conjugated second antibodies (Bio-Rad Laboratories, Inc.) for 1 h at room temperature. The blots were washed extensively and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

AA-NAT assay

AA-NAT activity was determined as described previously (28). Briefly, the collected pineal glands or cell pellets were homogenized in a reaction mixture of 0.1 M phosphate buffer (pH 6.8) containing 30 nmol [^{3}H]acetyl coenzyme A (specific activity, 1 mCi/mmol) and 1 μmol tryptamine hydrochloride in a final volume of 60 μl. The reaction mixture was incubated at 37 C for 1 h. At the end of the incubation period, the reaction was stopped by the addition of 1 ml methylene chloride. After vortexing, the aqueous phase was removed, and the organic phase was washed three times with 0.1 M phosphate buffer (pH 6.8). The organic phase was transferred to a scintillation vial and evaporated to dryness. The radioactive acetylated product was determined, and NAT activity was expressed as picomoles per microgram of protein per hour or nanomoles per hour per 10^5 cells.

Result presentation and statistical analysis

For the Western blots, a typical blot from at least three similar experiments is shown. Selected results were quantified using densitometric measurements, analyzed by Sigmagel (Jandel Scientific, San Rafael, CA), normalized to the control level, and presented as the mean ± SEM from at least three separate experiments. AA-NAT activity was presented as the mean ± SEM of three independent observations performed in duplicate. Statistical comparisons were analyzed by either unpaired *t* test or one-way ANOVA, followed by Tukey's *post hoc* test. Statistical significance was set at $P < 0.05$.

Results

Diurnal variation in p90RSK activation in the rat pineal gland

To investigate whether there is a diurnal difference in p90RSK activation in the rat pineal gland, the level of phosphorylated p90RSK in pineal glands collected at different time points from rats housed under a 12-h light, 12-dark cycle was determined using Western blot analysis. As shown in Fig. 1, pineal glands collected during the subjective night (ZT 14, 18, and 22; lights on at ZT 0; lights off at ZT 12) were found to have 3-fold higher levels of phosphorylated p90RSK than tissues collected during the subjective day (ZT 3 or 9; Fig. 1B). However, there was no significant difference in the level of total p90RSK (both phosphorylated and unphosphorylated) protein between any time point regardless of the light-dark cycle.

Effect of continuous light exposure or propranolol treatment on the nighttime level of phosphorylated p90RSK in the rat pineal gland

To establish that the nocturnal increase in phosphorylated p90RSK is regulated by adrenergic input to the rat pineal gland, animals were either injected with propranolol (1 mg/kg, ip) 1 h before the onset of darkness or subjected to continuous exposure to light after the onset of darkness. Both treatments were effective in significantly reducing the level of phosphorylated p90RSK when measured at ZT 18 (6 h after the onset of darkness; Fig. 2). Exposure to light for 30 or 60 min at ZT 17 during the dark period caused a significant decline in the level of phosphorylated p90RSK (Fig. 3A). In comparison, acute light exposure abolished the increase in AA-NAT activity that occurred at night (Fig. 3B), as reported previously (29).

NE activates p90RSK in rat pinealocytes

To determine the intracellular mechanism involved in the regulation of p90RSK activation, cultured pinealocytes were used, and the level of phosphorylated $p42/44^{MAPK}$ was monitored in the same sample for comparison. To confirm that p90RSK activation is under adrenergic control in cultured pinealocytes, cells were treated with NE. Treatment with NE (10 μM) caused a time-dependent increase in the level of phosphorylated p90RSK that started within 5 min, and the increase was sustained for at least 10 min. After 15 min, there was a decline in the level of phosphorylated p90RSK that returned toward the basal level by 60 min (Fig. 4A). The time profile of NE-stimulated $p42/44^{MAPK}$ activation followed closely the time profile of NE activation of p90RSK (Fig. 4A). At 15 min, the effects of NE on phosphorylated p90RSK and

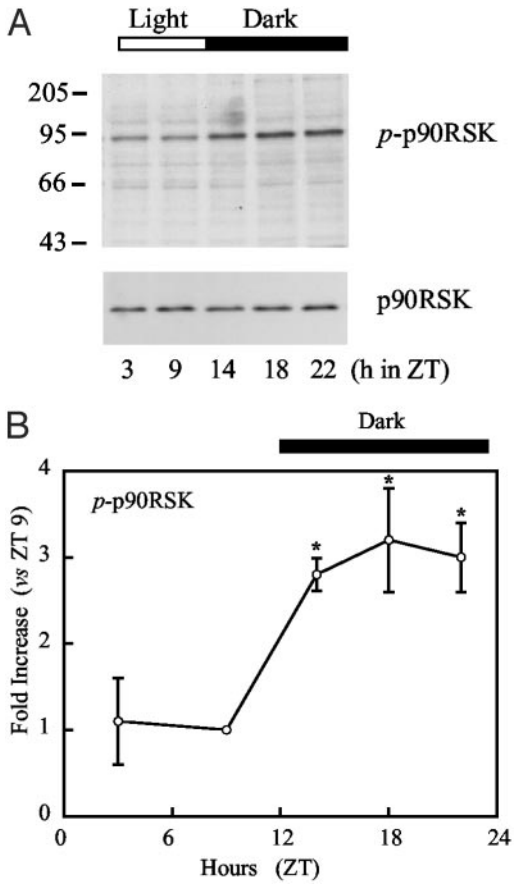

FIG. 1. Day/night variation in p90RSK activation in the rat pineal gland. Pineal glands were collected at the time points indicated from rats housed under a 12-h light, 12-h dark cycle. The glands were homogenized, mixed with 2× sample buffer, and analyzed by Western blotting using a polyclonal antibody against phosphorylated p90RSK (*p*-p90RSK) and a monoclonal antibody against p90RSK. Each lane contains 25 μg protein. See *Materials and Methods* for details. A, A representative blot from four separate experiments; B, densitometric measurements of *p*-p90RSK from the four experiments, presented as the fold increase over the value at ZT 9. *, $P < 0.05$, significantly different from ZT 9.

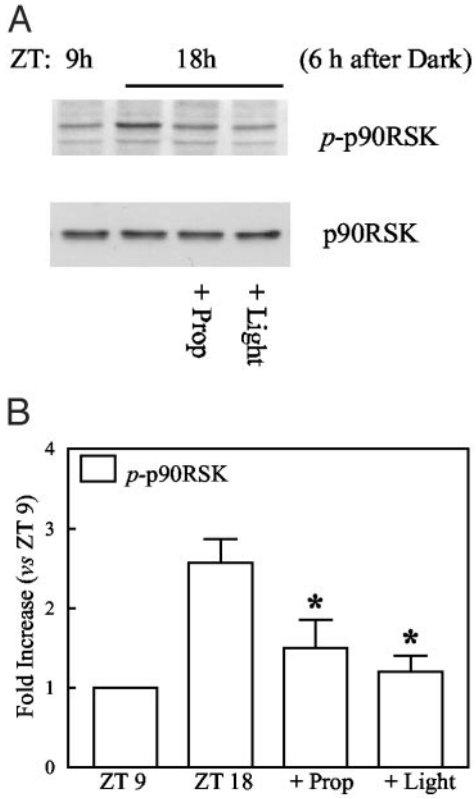

FIG. 2. Effect of continuous light exposure or propranolol treatment on the nighttime phosphorylated p90RSK level in the rat pineal gland. Rats were housed under a 12-h light, 12-h dark cycle, with lights off at ZT 12. Pineal glands were collected at ZT 9 (3 h before dark), at ZT 18 (6 h after dark), at ZT 18 with light remaining on (+ Light), or at ZT 18 with lights off but mice were injected with propranolol (1 mg/kg, ip; 1 h before dark; +Prop). The glands were homogenized, mixed with 2× sample buffer, and analyzed by Western blotting using a polyclonal antibody against *p*-p90RSK and a monoclonal antibody against p90RSK. Each lane contains 25 μg protein. See *Materials and Methods* for details. A, Representative blot from five separate experiments; B, densitometric measurements of *p*-p90RSK from five experiments, presented as the fold increase over the value at ZT 9. *, $P < 0.05$, significantly different from ZT 18.

$p42/44^{MAPK}$ levels were dependent on concentration (Fig. 4B).

Both α_1- and β-adrenergic receptors are involved in NE-stimulated p90RSK activation in rat pinealocytes

To determine the subtype of adrenergic receptors involved in the activation of p90RSK, selective adrenergic agonists and antagonists were used. Selective activation of β-adrenergic receptors by isoproterenol (1 μM) in the presence of prazosin (1 μM) had no effect on p90RSK activation at 15 min (Fig. 5). Activation of α_1-adrenerigc receptors by phenylephrine (1 μM) in the presence of propranolol (1 μM) caused a small increase in phosphorylated p90RSK (Fig. 5). Combined treatment with isoproterenol (1 μM) and phenylephrine (1 μM) caused a 3-fold increase in the phosphorylated 90RSK level, an effect similar to that of NE (10 μM; Fig. 5). Furthermore, the presence of either prazosin (1 μM) or propranolol (1 μM) caused a reduction in NE-mediated p90RSK activation (Fig. 5). These results suggest that both α_1- and β-adrenergic receptors are involved in the NE activation of p90RSK. The requirement for both α_1- and β-adrenoceptors was similar to that seen in the regulation of $p42/44^{MAPK}$ by NE as reported previously (30) and shown in Fig. 5 for comparison.

Signaling pathways involved in the activation of p90RSK by NE in rat pinealocytes

NE, through activation of both α_1- and β-adrenergic receptors, causes activation of protein kinase A (PKA), protein kinase G (PKG), and PKC as well as elevation of intracellular Ca^{2+} (2–5). To investigate the relative contributions of NE-activated signaling pathways in NE activation of p90RSK, pharmacological agents were used. Phosphorylated levels of $p42/44^{MAPK}$ were measured for comparison. As shown in Fig. 6, activation of PKG by dibutyryl cGMP (1 mM) and of PKC by PMA (0.1 μM) as well as elevation of intracellular Ca^{2+} by ionomycin (1 μM) were effective in increasing the phosphorylated levels of p90RSK. In comparison, treatment with dibutyryl cAMP (1 mM) caused a reduction in the phos-

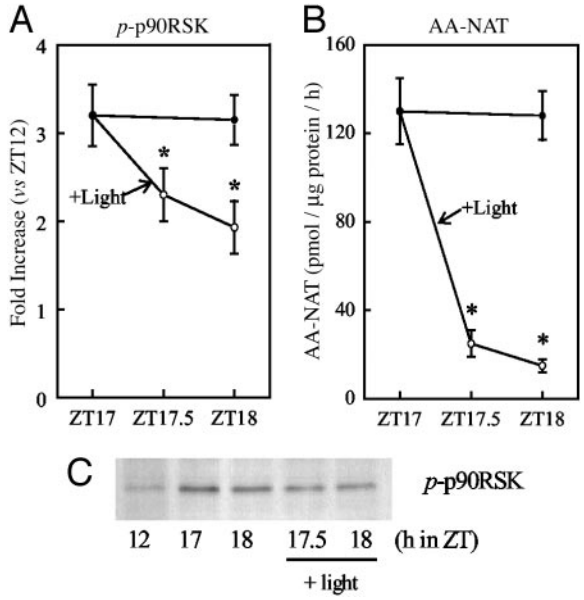

FIG. 3. Effect of acute light exposure on the nighttime phosphorylated p90RSK level and AA-NAT activity in the rat pineal gland. Rats were housed under a 12-h light, 12-h dark cycle, with lights off at ZT 12. Pineal glands were collected at the time points indicated. ●, Animals remaining in the dark; ○, animals subjected to light from ZT 17. The glands were homogenized, mixed with 2× sample buffer, and analyzed by Western blotting using a polyclonal antibody against *p*-p90RSK or were assayed for AA-NAT activity. See *Materials and Methods* for details. A, Densitometric measurements of *p*-p90RSK from four experiments presented as the fold increase over the value at ZT 12; B, AA-NAT activity from three independent experiments. AA-NAT activity from glands obtained at ZT 12 was not detectable. C, A representative blot of *p*-p90RSK from four separate experiments. *, $P < 0.05$, significantly different from ZT 17.

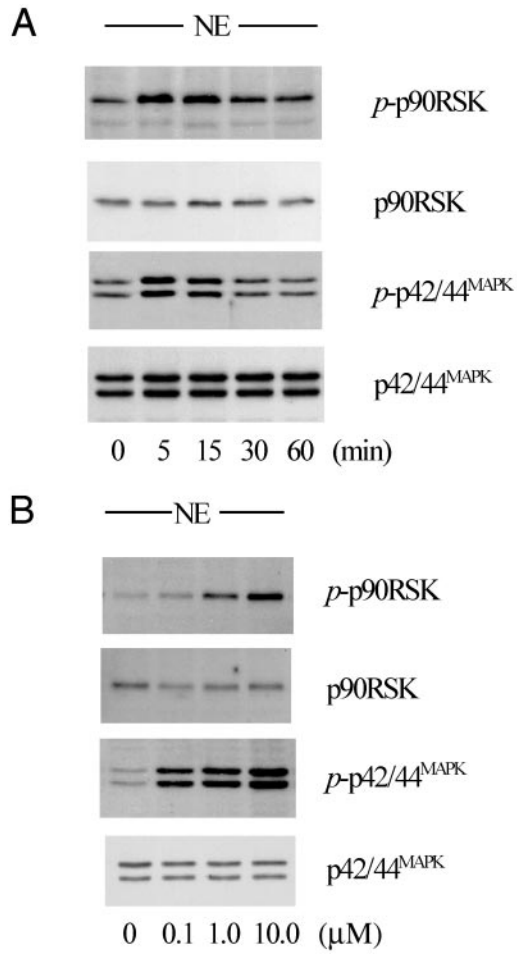

FIG. 4. Effect of NE on p90RSK activation in rat pinealocytes. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with NE (10 μM) for different time periods as indicated (A) and NE (0.1–10 μM) for 15 min (B). The cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a polyclonal antibody against *p*-p90RSK and monoclonal antibodies against p90RSK and phosphorylated p42/44^{MAPK} (*p*-p42/44^{MAPK}) as described in *Materials and Methods*. The blot presented is representative of three separate experiments.

phorylated p90RSK level. Thus, it appears that whereas pharmacological activation of PKG and PKC as well as elevation of intracellular Ca^{2+} enhance the phosphorylation of p90RSK, activation of PKA has the opposite effect. The pharmacological profiles of these agents in activating p90RSK were similar to their profiles in activating p42/44^{MAPK} (Fig. 6A).

To confirm the role the individual signaling pathways play in NE activation of p90RSK, the effects of different protein kinase inhibitors on the NE-stimulated p90RSK response were determined (Fig. 6B). Whereas H89 (1 μM), a selective PKA-selective inhibitor with a Ki less than 0.05 μM, was effective in enhancing the NE activation of p90RSK, the effect of KT5823 (1 μM), a selective PKG inhibitor with a Ki less than 0.3 μM, was inhibitory (Fig. 6B). Calphostin C (1 μM), a selective PKC inhibitor with a Ki less than 0.05 μM, and KN93 (1 μM), a selective Ca^{2+}/calmodulin-dependent protein kinase inhibitor, had little effect (Fig. 6B). The effects of these kinase inhibitors on NE-stimulated p90RSK activation were similar to their effects on NE-stimulated p42/44^{MAPK} activation (Fig. 6B).

p42/44^{MAPK} mediates the effect of NE on p90RSK on rat pinealocytes

To confirm that p42/44^{MAPK} mediates the effect of NE on p90RSK, inhibitors against MAPK kinase (MEK1/2) and p38^{MAPK} were used. Pretreatment of pinealocytes with a MEK1/2 inhibitor, UO126 (1 μM), caused a significant reduction in NE activation of p90RSK and p42/44^{MAPK} (Fig. 7 and Table 1). In comparison, UO124 (1 μM), the inactive analog, had no effect. Inhibition of p38^{MAPK} by SB203580 (10 μM), a treatment that we have previously shown to enhance NE-stimulated p42/44^{MAPK} activation (31), caused a further increase in NE-stimulated p90RSK activation. The inactive analog, SB202474 (10 μM), had no effect on the NE-mediated increase in phosphorylated p90RSK (Fig. 7). Together these results suggest that NE increases phosphorylated p90RSK through activation of the p42/44^{MAPK} pathway.

Effect of an MEK1/2 inhibitor on NE stimulation AA-NAT activity in rat pinealocytes

To determine whether activation of the p42/p44^{MAPK}/p90RSK pathway has an effect on pineal function, we inves-

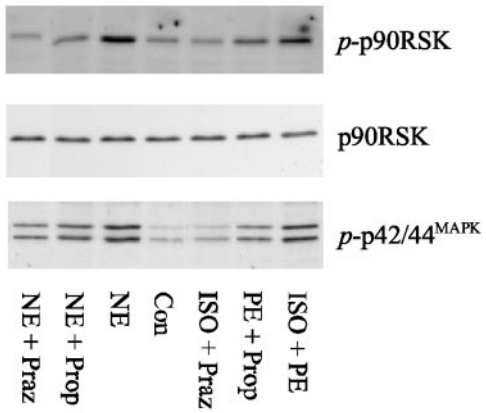

FIG. 5. Receptor characterization of NE-mediated p90RSK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated for 15 min with NE (10 μM) with prazosin (Praz; 1 μM) or propranolol (Prop; 1 μM), NE (10 μM) alone, isoproterenol (ISO; 1 μM) with Praz (1 μM), phenylephrine (PE; 1 μM) with Prop (1 μM), or ISO (1 μM). Cont, Control. The cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a polyclonal antibody against *p*-p90RSK and monoclonal antibodies against p90RSK and *p*-p42/44^{MAPK} as described in *Materials and Methods*. The blot presented is representative of three separate experiments.

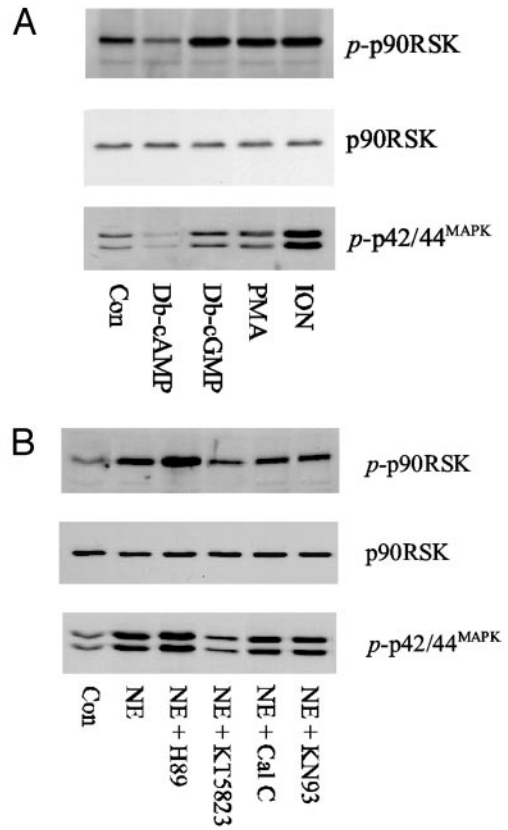

FIG. 6. Effects of protein kinase activators on p90RSK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated for 15 min with A) dibutyryl cAMP (Db-cAMP; 1 mM), dibutyryl cGMP (Db-cGMP; 1 mM), PMA (0.1 μM), or ionomycin (ION; 1 μM); and B) NE (10 μM) in the absence or presence of H89 (1 μM), KT5823 (1 μM), calphostin C (Cal C; 1 μM), or KN93 (1 μM). Cont, Control. The cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a polyclonal antibody against *p*-p90RSK and monoclonal antibodies against p90RSK and *p*-p42/44^{MAPK} as described in *Materials and Methods*. The blot presented is representative of three separate experiments.

tigated the effects of UO126 on NE-stimulated AA-NAT activity in cultured pinealocytes. Treatment with NE (1 μM) for 6 h caused a significant increase in AA-NAT activity (Table 1). The presence of UO126 (1 μM) caused a 40% reduction in NE-stimulated AA-NAT activity (Table 1). In contrast, UO124 (1 μM) had no effect on NE-stimulated AA-NAT activity (Table 1).

Discussion

We have shown that in rat pinealocytes, NE treatment increases the activation state of p42/44^{MAPK} (8, 30), a pathway known to stimulate CREB phosphorylation (18, 19). Moreover, p90RSK, the downstream component of p42/44^{MAPK}, has also been shown to enhance CREB phosphorylation (21, 22), suggesting that the p42/44^{MAPK} pathway may be involved in adrenergically stimulated AA-NAT induction at the transcriptional level. In the present study we focused on the regulation of p90RSK and the intracellular mechanism involved. Our results showed that the activation state of p90RSK not only is increased by NE in cultured pinealocytes, but is also regulated by the light/dark cycle, with increased phosphorylation during the dark phase in the rat pineal gland. Because the increase in phosphorylated p90RSK during the dark phase occurs without any increase in the protein level of p90RSK, the nocturnal increase in the activation state of p90RSK is related to enhanced phosphorylation of existing protein.

In support of nocturnal activation of p90RSK, subjecting the animals to continuous light from the onset of darkness is effective in blocking the nocturnal increase in p90RSK phosphorylation. Moreover, treatment with propranolol reduces the nocturnal increase in p90RSK phosphorylation, an observation indicating that activation of p90RSK is under adrenergic regulation. Considering that the pineal gland is stimulated by the release of NE from the sympathetic nerve at night (1, 32), these results suggest that adrenergically stimulated p90RSK phosphorylation in the pineal gland may be driven by the endogenous circadian clock in the suprachiasmatic nucleus (33, 34), similar to other cellular processes that have a circadian rhythm.

Our result also showed that the nocturnal increase in p90RSK phosphorylation is inhibited by acute light exposure. Within 30 min of light exposure, there is a decline in the increase in phosphorylated p90RSK during the dark phase. Although the decline in phosphorylated p90RSK is fairly rapid after light exposure, this appears slower than the decline in AA-NAT activity after similar light exposure. These results indicate that similar to the regulation of AA-NAT, a sustained stimulation is required for the nighttime activation of p90RSK. However, the mechanism involved in the inactivation of p90RSK may not be identical to that of AA-NAT.

In cultured pinealocytes we also found that both α_1- and β-adrenergic receptors are involved in NE-stimulated p90RSK activation. Furthermore, potentiation at the postreceptor level also constitutes part of the intracellular mechanism through which NE regulates p90RSK activation. This

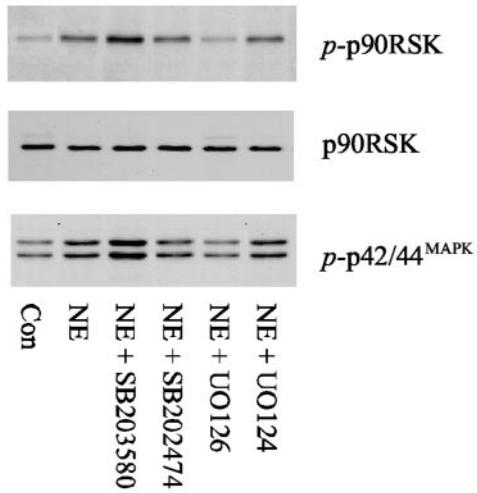

FIG. 7. Effects of SB203580, SB202474, UO126, and UO124 on NE-mediated p90RSK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated for 15 min with NE (10 μM) in the absence or presence of SB203580 (10 μM), SB202474 (10 μM), UO126 (1 μM), or UO124 (1 μM). Con, Control. The cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a polyclonal antibody against *p*-p90RSK and monoclonal antibodies against p90RSK and *p*-p42/44^{MAPK} as described in *Materials and Methods*. The blot presented is representative of four separate experiments.

TABLE 1. Effect of UO126 on NE-stimulated AA-NAT activity and phosphorylated p90RSK in rat pinealocytes

Treatment	AA-NAT activity (nmol/h·100,000 cells)	Phosphorylated p90RSK (fold increase)
Control	0.07 ± 0.02	1
UO126	0.09 ± 0.03	0.98 ± 0.03
UO124	0.08 ± 0.02	1.01 ± 0.04
NE (1 μM)	1.52 ± 0.11[a]	2.95 ± 0.15[a]
+ UO 126 (1 μM)	0.93 ± 0.20[b]	1.25 ± 0.13[b]
+ UO 124 (1 μM)	1.35 ± 0.24	2.67 ± 0.33

Pinealocytes (5×10^4 cells/0.5 ml) in DMEM with 10% fetal bovine serum were stimulated by different drugs as indicated for 6 h. Cells were then collected by centrifugation, and AA-NAT activity was determined as described in *Materials and Methods*. Each value represents the mean ± SEM of determinations done in duplicate from four independent experiments.

[a] Significantly different from control.

[b] Significantly different from NE-treated cells. Densitometric measurements of *p*-p90RSK from the four experiments from Fig. 7 (presented as fold increase against control) were included for comparison.

is based on observations that NE-mediated p90RSK phosphorylation can be reduced by treatment with either an α_1- or a β-adrenergic receptor antagonist. Moreover, treatment with an α_1-adrenergic receptor agonist causes a small increase in phosphorylated p90RSK, whereas treatment with a β-adrenergic receptor has no effect. However, combined treatment with both an α_1- and a β-adrenergic receptor agonist results in a large increase in the level of phosphorylated p90RSK.

Downstream from the adrenergic receptors, it has previously been shown that NE causes elevation of cAMP and cGMP, elevation of intracellular Ca^{2+}, as well as activation of PKC (2). When these signaling pathways are activated pharmacologically, treatment that either activates PKG or PKC or causes elevation of intracellular Ca^{2+} is effective in activating p90RSK. In comparison, activation of PKA reduces the level of phosphorylated p90RSK. Our results also indicate that, similar to p42/44^{MAPK} (8, 30), PKG appears to be the main pathway involved in NE activation of p90RSK phosphorylation. This is based on observations that whereas selective inhibition of PKG by KT5823 is effective in blocking the NE activation of p90RSK, inhibition of either PKC or the Ca^{2+}/calmodulin pathway has only a minimal effect on NE-stimulated p90RSK phosphorylation. Although treatment with either PMA or ionomycin causes a large increase in p90RSK phosphorylation, activation of α_1-adrenergic receptors alone only has a modest effect on p90RSK phosphorylation. This difference can be explained by the smaller NE-mediated increases in membrane-associated PKC and elevation of $[Ca^{2+}]i$ compared with those of PMA or ionomycin (35, 36).

The opposite effect of PKA and PKG on the activation state of p90RSK also provides an explanation for the requirement for activation of α_1- and β-adrenergic receptors for enhancement of p90RSK phosphorylation in rat pinealocytes. When β-adrenergic receptors are activated alone, the increase in cyclic nucleotide contents, in particular the cGMP response, is relatively small (2). Under these conditions, the inhibitory effect of PKA on p90RSK phosphorylation is adequate to counteract the stimulatory effect of PKG, hence resulting in little or no effect on p90RSK phosphorylation. In contrast, when both α_1- and β-adrenergic receptors are activated, p90RSK phosphorylation stimulated by the large increase in cGMP is sufficient to overcome the inhibitory effect of PKA. In agreement with this is the observation that the isoproterenol- or NE-mediated p90RSK activation is enhanced by a PKA inhibitor, but is reduced by a PKG inhibitor.

The similarities between the adrenergic receptors involved in the activation of p90RSK and p42/44^{MAPK} (30) as well as the predominant involvement of the PKG pathway (8, 30) suggest that NE-mediated activation of p90RSK may occur as a consequence of p42/44^{MAPK} activation. Other supportive evidence includes the observation that reducing the NE-mediated p42/44^{MAPK} phosphorylation by inhibition of MEK1/2 reduces the NE-mediated activation of p90RSK. Moreover, increasing NE-mediated p42/44^{MAPK} phosphorylation by an inhibitor of p38^{MAPK} (31) increases NE-mediated activation of p90RSK.

Previously, p42/44^{MAPK} has been shown to play an important role in pineal clock oscillation in chicks (37). In this study we found that UO126, an inhibitor of MEK1/2, has an inhibitory effect on NE-stimulated AA-NAT, hence supporting a modulating role of p42/44^{MAPK}/p90RSK in the adrenergic regulation of melatonin synthesis. The specific mechanism through which p42/44^{MAPK} modulates AA-NAT activity remains unclear. However, previous studies have shown the presence of cAMP response element in the promoter of the AA-NAT gene (38) and regulation of this gene by the binding activity of activating protein-1 (39). Therefore, p42/44^{MAPK}/p90RSK could modulate AA-NAT activity through phosphorylation of CREB and activation of activating protein-1 as shown in other cell types (22, 40). Moreover, p90RSK may be the downstream element through which PKG regulates cellular targets such as the L-type Ca^{2+} channel (7) and synaptic ribbons in the rat pineal gland (41).

Acknowledgments

Received February 18, 2003. Accepted April 14, 2003.

Address all correspondence and requests for reprints to: Dr. A. K. Ho, Department of Physiology, 7-26 Medical Sciences Building, Edmonton, Alberta, Canada T6G 2H7. E-mail: anho@ualberta.ca.

This work was supported by grants from the Canadian Institutes of Health Research and an Alberta Heritage Foundation for Medical Research summer studentship (to C.C.).

References

1. **Klein DC** 1985 Photoneural regulation of the mammalian pineal gland. In: Evered D, Clark S, eds. Photoperiodism, melatonin and the pineal. Ciba Foundation Symposium. London: Pitman; vol 117:38–56
2. **Chik CL, Ho AK** 1989 Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. Prog Biophys Mol Biol 53:197–203
3. **Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB** 1985 Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 314:359–361
4. **Sugden LA, Sugden D, Klein DC** 1987 α_1-Adrenoceptor activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. J Biol Chem 262:741–745
5. **Ho AK, Chik AL, Klein DC** 1987 Protein kinase C is involved in adrenergic stimulation of pineal cGMP accumulation. J Biol Chem 262:10059–10064
6. **Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM, Baler R** 1997 The melatonin rhythm-generating enzyme: molecular regulation of serotonin *N*-acetyltransferase in the pineal gland. Recent Prog Horm Res 52:307–357
7. **Chik CL, Liu QY, Li B, Karpinski E, Ho AK** 1995 cGMP inhibits L-type Ca^{2+} channel currents through protein phosphorylation in rat pinealocytes. J Neurosci 15:3104–3109
8. **Ho AK, Hashimoto K, Chik CL** 1999 3′, 5′-cyclic guanosine monophosphate activates mitogen-activated protein kinase in rat pinealoctyes. J Neurochem 73:598–604
9. **Seger R, Krebs EG** 1995 The MAPK signaling cascade. FASEB J 9:726–735
10. **Cobb MH** 1999 MAP kinase pathways. Prog Biophys Mol Biol 71:479–500
11. **Ray LB, Sturgill TW** 1987 Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc Natl Acad Sci USA 84:1502–1506
12. **Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH** 1990 An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249:64–67
13. **Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J** 1996 Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). J Biol Chem 271:17920–17926
14. **Stein B, Yang MX, Young DB, Janknecht R, Hunter T, Murray BW, Barbosa MS** 1997 p38-2, a novel mitogen-activated protein kinase with distinct properties. J Biol Chem 272:19509–19517
15. **Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR** 1991 Phosphorylation of c-jun mediated by MAP kinases. Nature 353:670–674
16. **Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ** 1996 Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15:2760–2770
17. **Rosenberger SF, Finch JS, Gupta A, Bowden GT** 1999 Extracellular signal-regulated kinase 1/2-mediated phosphorylation of JunD and FosB is required for okadaic acid-induced activator protein 1 activation. J Biol Chem 274:1124–1130
18. **Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME** 1998 Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol 18:1946–1955
19. **Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, Gallo V, Schinelli S** 2001 cAMP-dependent protein kinase induces cAMP-response element-binding protein phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. J Biol Chem 276:11487–11495
20. **Gomez N, Cohen P** 1991 Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. Nature 353:170–173
21. **Frodin M, Gammeltoft S** 1999 Role and regulation of 90kDa ribosomal S6 kinase (RSK) in signal transduction. Mol Cell Endocrinol 151:65–77
22. **Pende M, Fisher TL, Simpson PB, Russell JT, Blenis J, Gallo V** 1997 Neurotransmitter- and growth factor-induced cAMP response element binding protein phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C, and mitogen-activated protein kinase/ribosomal S6 kinase pathway. J Neurosci 17:1291–1301
23. **Roberson ED, English JD, Adams JP, Selcher JC, Kondriatick C, Sweatt JD** 1999 The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19:4337–4348
24. **Roseboom PH, Klein DC** 1995 Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: primary role of a β-adrenergic receptor/cyclic AMP mechanism. Mol Pharmacol 47:439–449
25. **Buda M, Klein DC** 1978 A suspension culture of pinealocytes: regulation of *N*-acetyltransferase activity. Endocrinology 103:1483–1493
26. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
27. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
28. **Ho AK, Grota LJ, Brown GM** 1984 Relationship between pineal *N*-acetyltransferase activity, pineal melatonin and serum melatonin in rats under different lighting conditions. Neuroendocrinology 39:465–470
29. **Klein DC, Weller JL** 1972 Rapid light-induced decrease in pineal serotonin *N*-acetyltransferase activity. Science 177:532–533
30. **Ho AK, Chik CL** 2000 Adrenergic regulation of mitogen-activated protein kinase in rat pinealocytes: opposing effects of protein kinase A and protein kinase G. Endocrinology 141:4496–4502
31. **Mackova M, Man J R, Chik CL, Ho AK** 2000 $p38^{MAPK}$ inhibition enhances basal and norepinephrine-stimulated $p42/p44^{MAPK}$ phosphorylation in rat pinealocytes. Endocrinology 141:4202–4208
32. **Deguchi T, Axelrod J** 1972 Induction and superinduction of serotonin *N*-acetyltransferase by adrenergic drugs and denervation in rat pineal organ. Proc Natl Acad Sci USA 69:2208–2211
33. **Pevet P, Pitrosky B, Vuillez P, Jacob N, Teclemariam-Mesbah R, Kirsch R, Vivien-Roels B, Lakhdar-Ghazal N, Canguilhem B, Masson-Pevet M** 1996 The suprachiasmatic nucleus: the biological clock of all seasons. Prog Brain Res 111:369–384
34. **Reppert SM, Weaver DR** 2002 Coordination of circadian timing in mammals. Nature 418:935–941
35. **Ho AK, Ogiwara T, Chik CL** 1996 Thapsigargin modulates agonist-stimulated cyclic AMP responses through cytosolic calcium-dependent and -independent mechanisms in rat pinealocytes. Mol Pharmacol 49:1104–1112
36. **Ho AK, Thomas TP, Chik CL, Anderson WB, Klein DC** 1988 Protein kinase C: subcellular redistribution by increased Ca^{2+} influx. Evidence that Ca^{2+}-dependent subcellular redistribution of protein kinase C is involved in potentiation of β-adrenergic stimulation of pineal cAMP and cGMP by K^+ and A23187. J Biol Chem 263:9292–9297
37. **Sanada K, Hayashi Y, Harada Y, Okano Y, Fukada Y** 2000 Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. J Neurosci 20:986–991
38. **Baler R, Covington S, Klein DC** 1997 The rat arylalkylamine *N*-acetyltransferase gene promoter. cAMP activation via a cAMP-responsive element-CCAAT complex. J Biol Chem 272:6979–6985
39. **Guillaumond F, Sage D, Deprez P, Bosler O, Becquet D, Francois-Bellan AM** 2000 Circadian binding activity of AP-1, a regulator of the arylalkylamine *N*-acetyltransferase gene in the rat pineal gland, depends on circadian Fra-2, c-Jun, and Jun-D expression and is regulated by the clock's zeitgebers. J Neurochem 75:1398–1407
40. **Young MR, Nair R, Bucheimer N, Tulsian P, Brown N, Chapp C, Hsu TC, Colburn NH** 2002 Transactivation of Fra-1 and consequent activation of AP-1 occur extracellular signal-regulated kinase dependently. Mol Cell Biol 22:587–598
41. **Spessert R, Gupta BB, Seidel A, Maitra SK, Vollrath L** 1992 Involvement of cyclic guanosine monophosphate (cGMP) and cytosolic guanlyate cyclase in the regulation of synaptic ribbon numbers in rat pineal gland. Brain Res 570:231–236