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Adrenergic Regulation and Diurnal Rhythm of p38 Mitogen-Activated Protein Kinase Phosphorylation in the Rat Pineal Gland

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In this study, we investigated adrenergic and photoneural regulation of p38MAPK phosphorylation in the rat pineal gland. Norepinephrine (NE), the endogenous neurotransmitter, dose-dependently increased the levels of phosphorylated MAPK kinase 3/6 (MKK3/6) and p38MAPK in rat pinealocytes. Time-course studies showed a gradual increase in MKK3/6 and p38MAPK phosphorylation that peaked between 1 and 2 h and persisted for 4 h post NE stimulation. In cells treated with NE for 2 and 4 h, the inclusion of prazosin or propranolol reduced NE-induced MKK3/6 and p38MAPK phosphorylation, indicating involvement of both α- and β-adrenergic receptors for the sustained response. Whereas treatment with dibutyryl cAMP or ionomycin mimicked the NE-induced MKK3/6 and p38MAPK phosphorylation, neither dibutyryl cGMP nor 4β-phorbol 12-myristate 13-acetate had an effect. The NE-induced increase in MKK3/6 and p38MAPK phosphorylation was blocked by KT5720 (a protein kinase A inhibitor) and KN93 (a Ca^{2+}/calmodulin-dependent kinase inhibitor), but not by KT5823 (a protein kinase G inhibitor) or calphostin C (a protein kinase C inhibitor). In animals housed under a lighting regimen with 12 h of light, MKK3/6 and p38MAPK phosphorylation increased in the rat pineal gland at zeitgeber time 18. The nocturnal increase in p38MAPK phosphorylation was blocked by exposing the animal to constant light and reduced by treatment with propranolol, a β-adrenergic blocker. Together, our results indicate that activation of p38MAPK is under photoneural control in the rat pineal gland and that protein kinase A and intracellular Ca^{2+} signaling pathways are involved in NE regulation of p38MAPK. (*Endocrinology* 145: 5194–5201, 2004)

THE RAT PINEAL gland is stimulated by the release of norepinephrine (NE) from the sympathetic nerves at night (1, 2), which activates both α- and β-adrenergic receptors (1, 3). Stimulation of β-adrenergic receptors alone produces a 7- to 10-fold increase in cAMP and a 2- to 4-fold increase in cGMP accumulation (1). Stimulation of α-adrenergic receptors, which activates protein kinase C (PKC) (4, 5) and elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (6, 7), potentiates the β-adrenergic stimulated cyclic nucleotide responses and results in a 50-fold increase in cAMP and a 100-fold increase in cGMP accumulation (1, 3). The nocturnal release of NE and elevation of cAMP level in the rat pineal gland result in the induction of arylalkyl-N-acetyltransferase (AA-NAT), the rate-controlling enzyme in melatonin synthesis at night (1, 2).

MAPK signaling pathways are involved in regulation of cellular processes including growth, differentiation, secretion, and metabolism (8–10). MAPKs comprise a family of proline-directed, serine/threonine protein kinases, with three major members: p42/44MAPK, p38MAPK, and c-Jun amino-terminal kinase (JNK) (8–10). The enzymes are evolutionary conserved and are activated by a common mechanism that involves a protein kinase cascade (11). Activation of p42/44MAPK involves phosphorylation of the enzymes by MAPK kinase (MEK or MKK)1 and MEK2 (12, 13), p38MAPK are phosphorylated by MKK3, MKK4, and MKK6 (14, 15), and JNK by MKK7 (16, 17). Whereas the p42/44MAPK signaling pathway is activated by growth factors, the JNK and p38MAPK signaling pathways are activated by cytokines or cellular stresses (19, 20). MAPKs are also activated by signaling pathways coupled to G proteins through mechanisms that are cell type and receptor specific (21–23).

The importance of the MAPK signaling pathway in the regulation of pineal function in rats has recently been recognized (24–29). Pineal culture studies indicate that NE, in addition to inducing AA-NAT, also activates p42/44MAPK (24, 25) and its downstream kinase p90 ribosomal S6 kinase (p90RSK) (26). Nocturnal increases in the activation states of MEK1/2, p42/44MAPK, and p90RSK have been demonstrated in the rat pineal gland (26, 27). Moreover, inhibition of p42/44MAPK activation modulates NE-stimulated AA-NAT activity (26). In the case of p38MAPK, NE increases the activation state of this kinase and inhibition of p38MAPK activity enhances NE-stimulated AA-NAT activity and melatonin production (29). These results suggest that members of the MAPK family are regulated in a complex manner in the rat pineal gland and play an important role in regulating melatonin synthesis. However, the signaling mechanisms that mediate the adrenergic activation of p38MAPK remain unknown. In this study, we determined the adrenergic-mediated sig-

Abbreviations: AA-NAT, Arylalkyl-N-acetyltransferase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Ca^{2+}/CMK, Ca^{2+}/calmodulin-dependent kinase; Con, control; DB, dibutyryl; ION, ionomycin, ISO, isoproterenol; JNK, c-Jun amino-terminal kinase; MEK, MAPK kinase; NE, norepinephrine; *p*-, phosphorylated; PE, phenylephrine; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PMA, 4β phorbol 12-myristate 13-acetate; Praz, prazosin; Prop, propranolol; p90RSK, p90 ribosomal S6 kinase; ZT, zeitgeber time.

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naling mechanisms involved in regulating p38MAPK activation and the diurnal changes in p38MAPK activation in the rat pineal gland.

Materials and Methods

Materials

Dibutyryl cAMP (DBcAMP), dibutyryl cGMP (DBcGMP), isoproterenol (ISO), NE, phenylephrine (PE), 4β phorbol 12-myristate 13-acetate (PMA), prazosin (Praz), propranolol (Prop) and antibodies against p38MAPK, phosphorylated p38MAPK (*p*-p38MAPK) and phosphorylated p42/44MAPK (*p*-p42/44MAPK) were obtained from Sigma Chemical Co. (St. Louis, MO). KT5720, KT5823, KN93, calphostin C and ionomycin (ION) were obtained from Calbiochem Corp. (San Diego, CA). Antibodies against phosphorylated MKK3/6 (*p*-MKK3/6) was from Cell Signaling Technology, Inc. (Beverly, MA). All other chemicals were of the purest grades available commercially.

Animal handling and pineal gland isolation

This study was 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 [zeitgeber time zero (ZT 0)]. For pinealocyte cell culture preparation, animals were killed 3 h after the onset of light, and pineal glands were removed and stored in ice-cold PBS until trypsinization. For analysis of the pineal glands, groups of animals were killed at various time points as indicated. Animals subjected to constant light were kept in a lighted environment for an additional indicated period after the onset of darkness. To determine the effect of acute light exposure on the nocturnal increase in p38MAPK activation, animals were exposed to light at ZT 17 (5 h after darkness) and pineal glands collected at various time points as indicated. Propranolol-treated animals were injected ip with aqueous propranolol solution (1 mg/kg body weight) 1 h before the onset of darkness. Pineal glands were cleaned in ice-cold PBS, flash-frozen on dry ice and stored at −75 C until preparation for Western blot analysis. A dim red light was used when animals were killed during the dark period.

Preparation of cultured pinealocytes and drug treatment

Pinealocytes were prepared from freshly dissected rat pineal glands by trypsinization as described previously (30). Cells were suspended in DMEM containing 10% fetal calf serum and maintained at 37 C for 24 h in a gas mixture of 95% air and 5% CO_2 before experiments. Aliquots of pinealocytes (5×10^5 cells/0.5 ml) were treated with drugs which had been prepared in 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 at −20 C until electrophoresis.

Western blot

SDS-PAGE was performed according to the procedure of Laemmli (31) using 10% acrylamide in the presence of 1 mg/ml sodium dodecyl sulfate (Mini-Protein II gel system, Bio-Rad, Hercules, CA). After electrophoresis, gels were equilibrated for 15 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. Blots were then incubated overnight at 4 C with diluted specific antisera as indicated. After washing twice with the blocking solution, blots were incubated with diluted horseradish peroxidase-conjugated second antibodies (Bio-Rad, Hercules, CA) for 1 h at room temperature. Blots were then washed extensively and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

Results presentation and statistical analysis

A representative immunoblot is shown. Results were quantified using densitometric measurements and analyzed by Kodak 1-D imaging software (Eastman Kodak, Rochester, NY). Densitometric values were normalized as indicated 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.

Results

Activation of MKK3/6 and p38MAPK by NE

Treatment of pinealocytes with NE (10 μM) caused a gradual increase in the levels of *p*-MKK3/6 and *p*-p38MAPK that was detectable at 15 min, peaked between 60 and 120 min, and the increase persisted for 240 min post treatment (Fig. 1, A and B). This differed from the time-profile of NE-induced increase in *p*-p42/44MAPK that peaked between 15 and 30 min and declined gradually after 60 min as reported previously (25). At 120 min post treatment, NE increased the levels of *p*-p38MAPK in a dose-dependent manner (Fig. 1, A and B). The time- and dose-dependent increase in *p*-p38MAPK level occurred in the absence of changes in total p38MAPK (both phosphorylated and unphosphorylated) protein (Fig. 1, A and B).

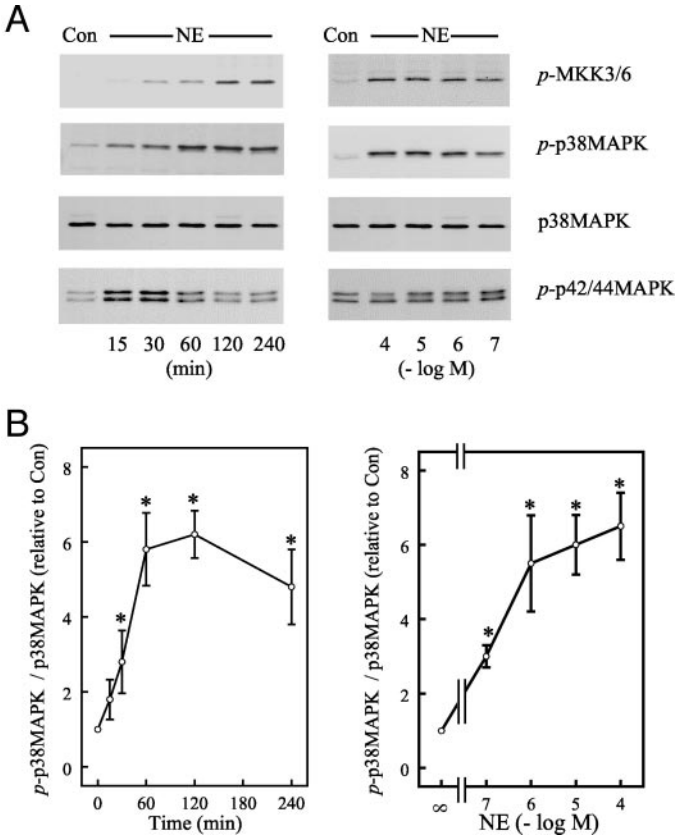

FIG. 1. Effect of NE on p38MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with NE (10 μM) for different time periods or NE (0.1–100 μM) for 2 h as indicated. Cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK and *p*-p42/44MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK as described in *Materials and Methods*. Representative immunoblots (A) and normalized densitometric measurements (B) of *p*-p38MAPK / p38MAPK presented as fold increase relative to control (Con) from three independent experiments. *, $P < 0.05$, significantly different from Con.

Receptor characterization of the adrenergic regulation of p38MAPK activation

Selective adrenergic agonists and antagonists were used to determine the subtypes of adrenergic receptors involved in the activation of p38MAPK. The concentrations of the agonists and antagonists chosen were based on preliminary studies that examined their potencies in stimulating or blocking adrenergic-stimulated AA-NAT activity, synthesis of cyclic nucleotides, and activation of p90RSK and p42/44MAPK. The concentrations used were also in agreement with previously published studies (3–6, 32). Selective activation of β-adrenergic receptors by ISO (1 μM in the presence of 1 μM of Praz) or α-adrenergic receptors by PE (1 μM in the presence of 1 μM of Prop) caused an increase in the levels of *p*-MKK3/6 and *p*-p38MAPK at 60 min post treatment (Fig. 2, A and B). Simultaneous activation of both α- and β-adrenergic receptors by treatment with ISO plus PE or NE (1 μM) did not cause an additional increase in *p*-MKK3/6 and *p*-p38MAPK and addition of either Praz (1 μM), an α-adrenergic blocker, or Prop (1 μM), a β-adrenergic blocker, had no effect on NE-stimulated MKK3/6 or p38MAPK phosphorylation (Fig. 2, A and B). In contrast, when the treatment period was extended to 120 or 240 min, activation of either β- or α-adrenergic receptors alone had a minimal effect on *p*-MKK3/6 or *p*-p38MAPK (Fig. 2, A and B). However, combined treatment with ISO and PE for 120 or 240 min caused a large increase in *p*-MKK3/6 and *p*-p38MAPK to levels achieved with NE stimulation. Moreover, the presence of Praz or Prop caused a reduction in NE-induced MKK3/6 and p38MAPK phosphorylation (Fig. 2, A and B).

Post-receptor mechanisms involved in p38MAPK activation

To establish the signaling pathways involved in NE-stimulated p38MAPK phosphorylation, the effects of protein kinase activators and inhibitors were tested. Treatment with DBcAMP (0.5 mM), a membrane permeable cAMP analog, mimicked the effects of NE on *p*-MKK3/6 and *p*-p38MAPK. Peak increases in *p*-MKK3/6 and *p*-p38MAPK level were observed between 120 and 240 min after DBcAMP treatment (Fig. 3, A and B). The time-dependent increase in *p*-p38MAPK levels occurred in the absence of changes in total p38MAPK protein (Fig. 3A). In contrast to its effect on *p*-MKK3/6 and *p*-p38MAPK, DBcAMP caused a reduction in p42/44MAPK activation throughout the test period (Fig. 3A) as reported previously (25).

Activation of α-adrenergic receptors elevates $[Ca^{2+}]_i$ in rat pinealocytes (6, 7). To determine the involvement of elevation of $[Ca^{2+}]_i$ on p38MAPK activation, cells were stimulated with

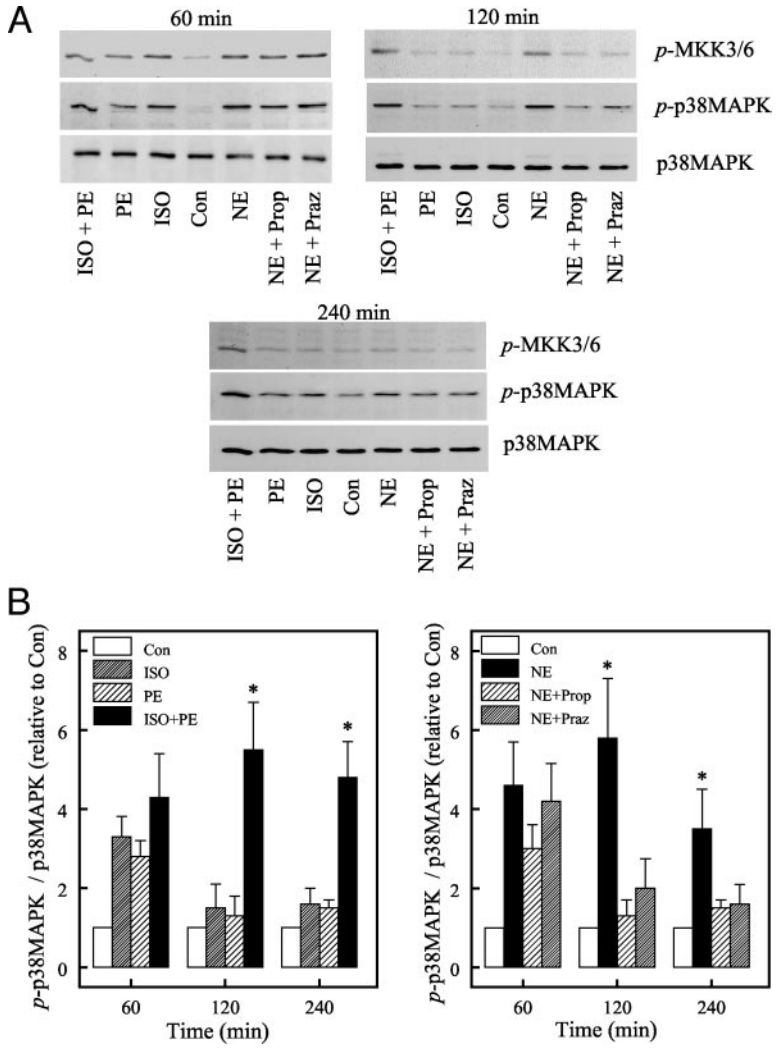

FIG. 2. Receptor characterization of NE-mediated MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with ISO (1 μM) + PE (1 μM), PE [1 μM with Prop (1 μM)], ISO [1 μM with Praz (1 μM)], NE (1 μM) alone or in the presence of Prop (1 μM) or Praz (1 μM) for 60, 120, or 240 min. Cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK and polyclonal antibodies against *p*-MKK3/6 and p38MAPK as described in *Materials and Methods*. Representative immunoblots (A) and histograms (B) of normalized densitometric measurements of *p*-p38MAPK/p38MAPK presented as fold increase relative to Con from three independent experiments. *Left panel*: *, $P < 0.05$, significantly different from ISO or PE. *Right panel*: *, $P < 0.05$, significantly different from NE + Praz or NE + Prop.

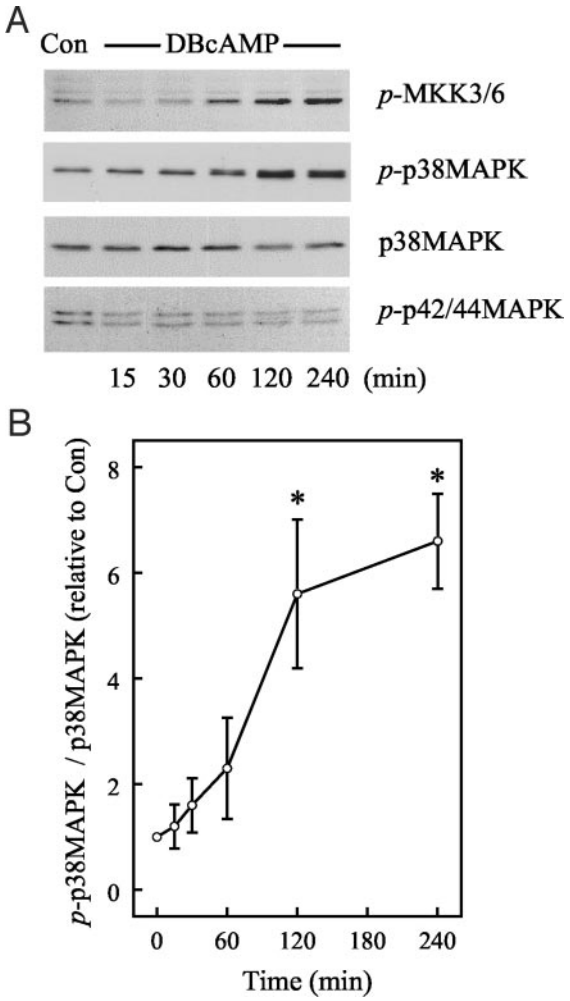

FIG. 3. Effect of DBcAMP on p38MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with DBcAMP (0.5 mM) for different time periods as indicated. Cells were collected by centrifugation, dissolved in 1× sample buffer and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK and *p*-p42/44MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK as described in *Materials and Methods*. Representative immunoblots (A) and normalized densitometric measurements (B) of *p*-p38MAPK/p38MAPK presented as fold increase relative to Con from three independent experiments. *, $P < 0.05$, significantly different from Con.

ION, a calcium ionophore. ION (1 μM) caused a gradual increase in *p*-MKK3/6 and *p*-p38 MAPK that peaked between 60 and 120 min followed by a decline at 240 min post treatment (Fig. 4, A and B). This differed from the time-profile of ION-stimulated increase in the level of *p*-p42/44MAPK, which peaked at 15 min and declined to basal level within 240 min (Fig. 4A) as reported previously (25). Treatment with DBcGMP (0.5 mM) or PMA (0.1 μM) for 120 min had no effect on MKK3/6 or p38MAPK phosphorylation (Fig. 5, A and B).

In the presence of different protein kinase inhibitors, NE (3 μM) stimulated MKK3/6 and p38MAPK phosphorylation was reduced by cotreatment with a protein kinase A (PKA) inhibitor KT 5720 (3 μM) or a Ca^{2+}/calmodulin-dependent protein kinase (Ca^{2+}/CMK) inhibitor KN93 (10 μM) but not by cotreatment with a protein kinase G (PKG) inhibitor KT5823 (3 μM) or a PKC inhibitor calphostin C (1 μM) (Fig. 5, A and C).

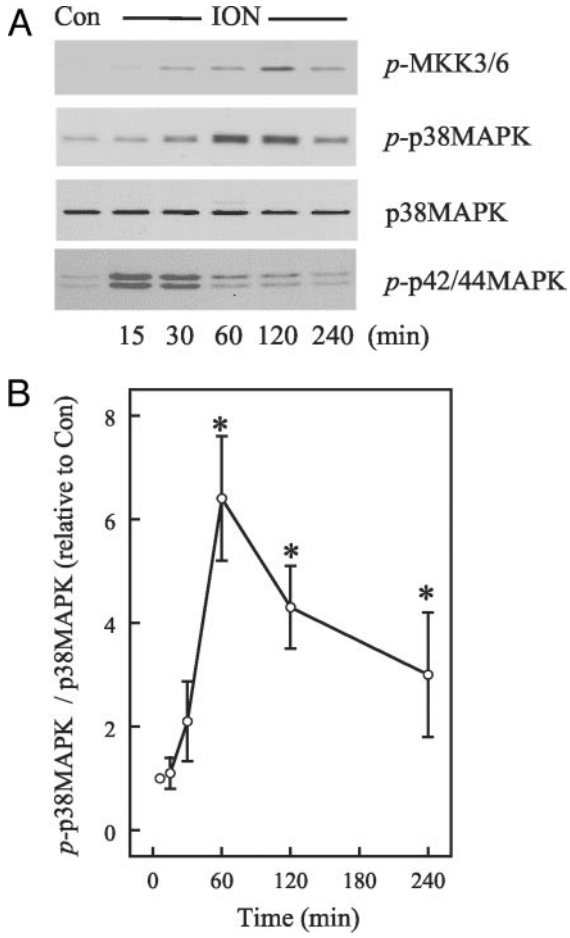

FIG. 4. Effect of ION on p38MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated with ION (1 μM) for different time periods as indicated. Cells were collected by centrifugation, dissolved in 1× sample buffer and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK and *p*-p42/44MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK as described in *Materials and Methods*. Representative immunoblots (A) and normalized densitometric measurements (B) of *p*-p38MAPK/p38MAPK presented as fold increase relative to Con from three independent experiments. *, $P < 0.05$, significantly different from Con.

Diurnal variation in MKK3/6 and p38MAPK activation in the rat pineal gland

To investigate whether there is a diurnal difference in MKK3/6 and p38MAPK activation in the rat pineal gland, the levels of *p*-MKK3/6 and *p*-p38MAPK in pineal glands collected at different time points from rats housed under a 12-h light, 12-h dark cycle was determined using Western blot analysis. As shown in Fig. 6, pineal glands collected during the mid to late subjective night (ZT 18 and 22; lights on at ZT 0; lights off at ZT 12) were found to have higher levels of *p*-MKK3/6 and *p*-p38MAPK when compared with tissues collected during either the subjective day (ZT 3 or 9) or the early subjective night (ZT 14). Compared with ZT 9, the levels of *p*-MKK3/6 and *p*-p38MAPK at ZT 18 were 6-fold higher (Fig. 6, B and C). However, there was no significant difference in the level of total p38MAPK protein between any time points regardless of the light-dark cycle (Fig. 6A). Unlike cultured pinealocytes, a nonspecific band of slightly

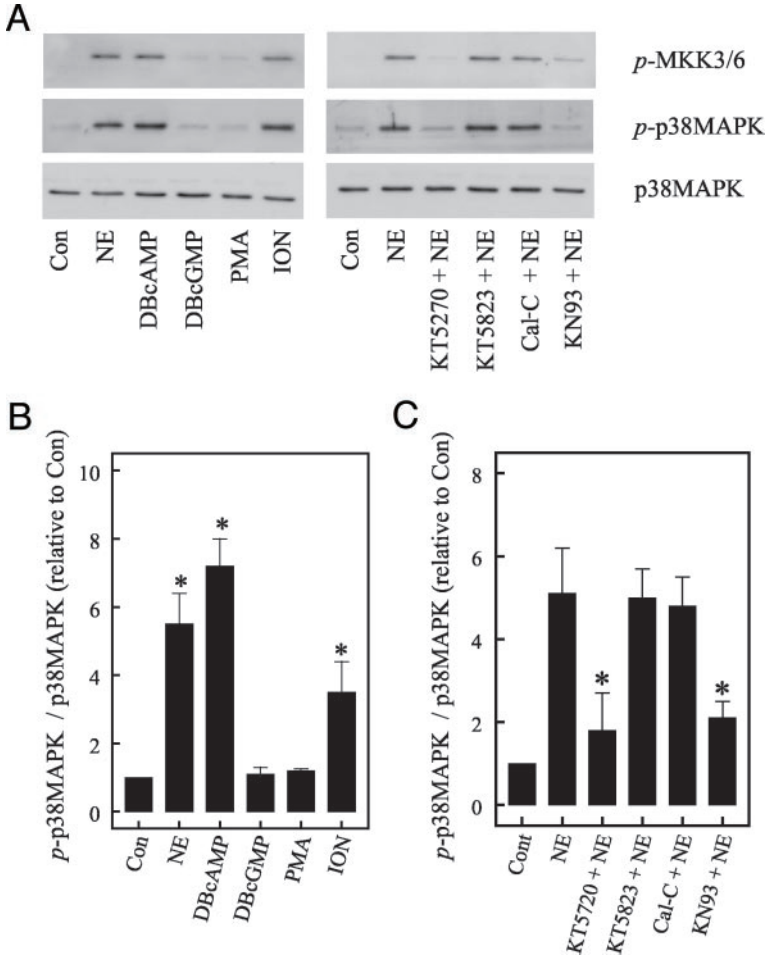

FIG. 5. Effects of protein kinase activators and inhibitors on NE-stimulated p38MAPK activation. Pinealocytes (5×10^5 cells/0.5 ml) were cultured for 24 h and treated for 2 h with NE (3 μM), DBcAMP (0.5 mM), DBcGMP (0.5 mM), PMA (0.1 μM), ION (1 μM); or NE (3 μM) in the absence or presence of KT5720(3 μM), KT5823 (3 μM), calphostin C (Cal-C, 1 μM), or KN93 (10 μM). Cells were collected by centrifugation, dissolved in 1× sample buffer, and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK as described in *Materials and Methods*. Representative immunoblots (A) and histograms of normalized densitometric measurements (B) of *p*-p38MAPK/p38MAPK presented as fold increase relative to Con from three independent experiments. *Left panel*: *, $P < 0.05$, significantly different from Con. *Right panel*: *, $P < 0.05$, significantly different from NE.

lower molecular weight was recognized by the monoclonal antibody against *p*-p38MAPK in most studies that involved homogenates of intact pineal glands (Fig. 6A).

Effect of continuous light exposure or propranolol treatment on the increase in p-MKK3/6 and p-p38MAPK in the rat pineal gland

To establish that the nocturnal activation of the p38MAPK pathway 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 levels of *p*-MKK3/6 and *p*-p38MAPK when measured at ZT 18 (6 h after the onset of darkness) (Fig. 7). Exposure to light for 30 or 60 min at ZT 17 during the dark period caused a gradual but significant decline in the levels of *p*-MKK3/6 and *p*-p38MAPK (Fig. 8). After 1 h of light exposure during the dark period, the levels of *p*-MKK3/6 and *p*-p38MAPK declined by about 50% (Fig. 8, B and C).

Discussion

In rat pinealocytes, NE increases the activation state of p38MAPK and inhibition of p38MAPK activity enhances NE-stimulated melatonin synthesis (29). In this study, we focus on the regulation of p38MAPK activation and the intracellular mechanisms involved. Our results indicate that NE induces p38MAPK phosphorylation in a time- and dose-dependent manner with involvement of both α- and β-adrenergic receptors in cultured rat pinealocytes. Moreover, pathways activated by PKA and to a lesser extent Ca^{2+}/CMK contribute to the NE activation of p38MAPK. Furthermore, activation of p38MAPK is under photoneural control with increased *p*-MKK3/6 and *p*-p38MAPK during the dark phase in the rat pineal gland.

Activation of p38MAPK requires phosphorylation of both threonine and tyrosine residue within the regulatory site of the enzyme (18). By monitoring the phosphorylation states of p38MAPK and its upstream kinase, MKK3/6, we showed in the present study that NE treatment, although having no effect on p38MAPK protein, increases the levels of *p*-MKK3/6 and *p*-p38MAPK. These results indicate that, similar to p42/44MAPK (25), the p38MAPK signaling pathway is a downstream target of adrenergic stimulation in the rat pinealocyte. A parallel increase in *p*-MKK3/6 further suggests that NE interacts with a site upstream of MKK3/6 activation.

Receptor characterization experiments show synergistic interaction between both α- and β-adrenergic receptors to NE activation of p38MAPK, similar to the NE regulation of cyclic nucleotide accumulation and AA-NAT activity (1, 2, 32). By activating both receptors, the duration of p38MAPK activation is extended beyond that achieved by activating the individual

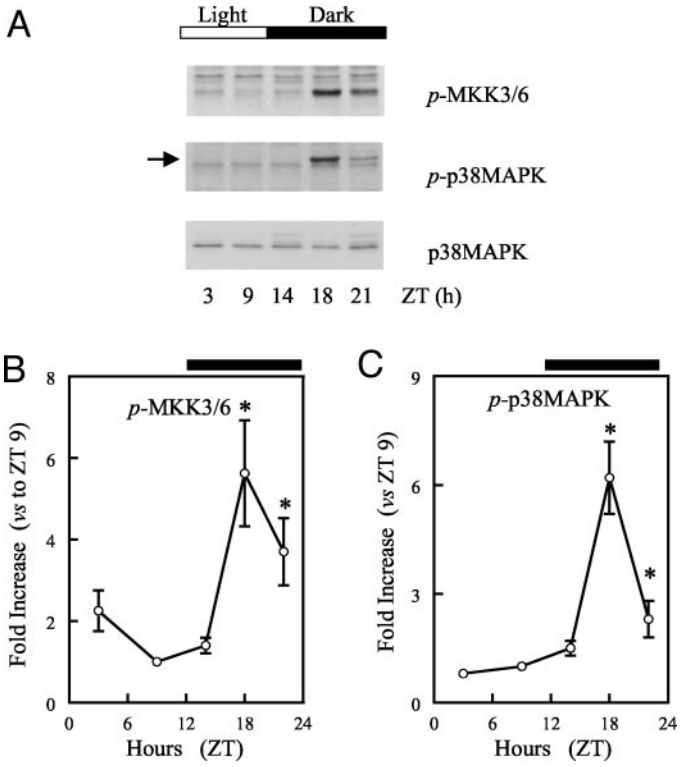

FIG. 6. Day/night variation in p38MAPK activation in the rat pineal gland. Pineal glands were collected from rats, housed under a 12-h light, 12-h dark cycle, at the time points indicated. The glands were homogenized, mixed with 2× sample buffer, and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK. Each lane contains 25 μg of protein. See *Materials and Methods* for details. Representative immunoblots (A) and relative densitometric measurements (B) of *p*-MKK3/6 and (C) *p*-p38MAPK from four independent experiments presented as fold increase against the value at ZT 9. *, $P < 0.05$, significantly different from ZT 9; →, specific band for *p*-p38MAPK.

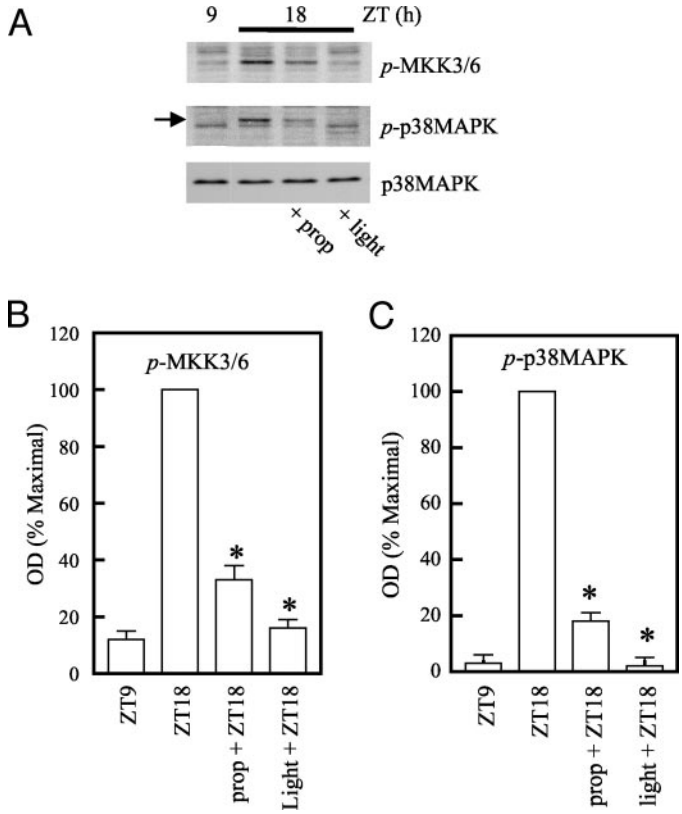

FIG. 7. Effect of continuous light exposure or propranolol treatment on the nocturnal activation of MKK3/6 and p38MAPK in the rat pineal gland. Rats were housed under a 12-h light, 12-h dark cycle with light off at ZT 12. Pineal glands were collected at ZT 9 (3 h before dark), ZT 18 (6 h after dark), ZT 18 with light remaining on (+light) or ZT 18 with light off but injected with propranolol (1 mg/kg ip, 1 h before dark) (+prop). Glands were homogenized, mixed with 2× sample buffer and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK. Each lane contains 25 μg of protein. See *Materials and Methods* for details. Representative immunoblots (A) and relative densitometric measurements (B) of *p*-MKK3/6 and (C) *p*-p38MAPK from three independent experiments presented as percentage of maximal OD value. *, $P < 0.05$, significantly different from ZT 18; →, specific band for *p*-p38MAPK

receptor. This conclusion is based on observations that, at 60 min post treatment, activation of either α- or β-adrenergic receptors alone produces similar level of p38MAPK phosphorylation as activation of both receptors, and blockade of either adrenergic receptor has no discernible effect on NE-stimulated increases in *p*-MKK3/6 and *p*-p38MAPK levels. However, at 120 and 240 min post treatment, whereas the effect of activation of either α- or β-adrenergic receptors alone on *p*-MKK3/6 and *p*-p38MAPK is minimal, simultaneous activation of both receptors by ISO + PE or NE results in a large increase in *p*-MKK3/6 and *p*-p38MAPK. The ability to extend the duration of p38MAPK activation appears to be mediated by signaling mechanism(s) activated by both receptor subtypes.

Downstream from the adrenergic receptors, in contrast to previous reports indicating the involvement of $G\alpha_{q/11}$ or $G\beta\gamma$ in G protein-coupled receptor activation of MAPK signaling pathways (33–35), our results strongly indicate the involvement of second messengers rather than G proteins in NE-stimulated p38MAPK phosphorylation. This is based on observations that DBcAMP and ION mimics the effect of NE on p38MAPK activation and KT5720, a PKA inhibitor, as well as KN93, a Ca^{2+}/CMK inhibitor, reduce NE-stimulated phopsphorylation of MKK3/6 and p38MAPK. However, the duration of p38MAPK activation by ION appears shorter than that by DBcAMP or NE treatment. Our results also indicate that PKG is not involved in p38MAPK phosphorylation in rat pinealocytes even though PKG is the main signaling mechanism that mediates NE activation of p42/44MAPK (24). Moreover, activation of PKC also fails to stimulate p38MAPK phosphorylation in rat pinealocytes despite the involvement of this pathway in p38MAPK activation in other cell types (36, 37). Together, these results suggest that although multiple signaling pathways are activated by NE, only PKA and Ca^{2+}/CMK contribute to activation of p38MAPK. Similar to our results, PKA is involved in adrenergic regulation of p38MAPK in other cell types (38, 39).

The involvement of PKA and Ca^{2+}/CMK in p38MAPK regulation also helps to explain the prolonged duration of p38MAPK activation that occurs with simultaneous activation of α- and β-adrenergic receptors. One possible explanation is that, during the initial phase of NE stimulation, activation of either the α-adrenergic receptor → Ca^{2+}/CMK

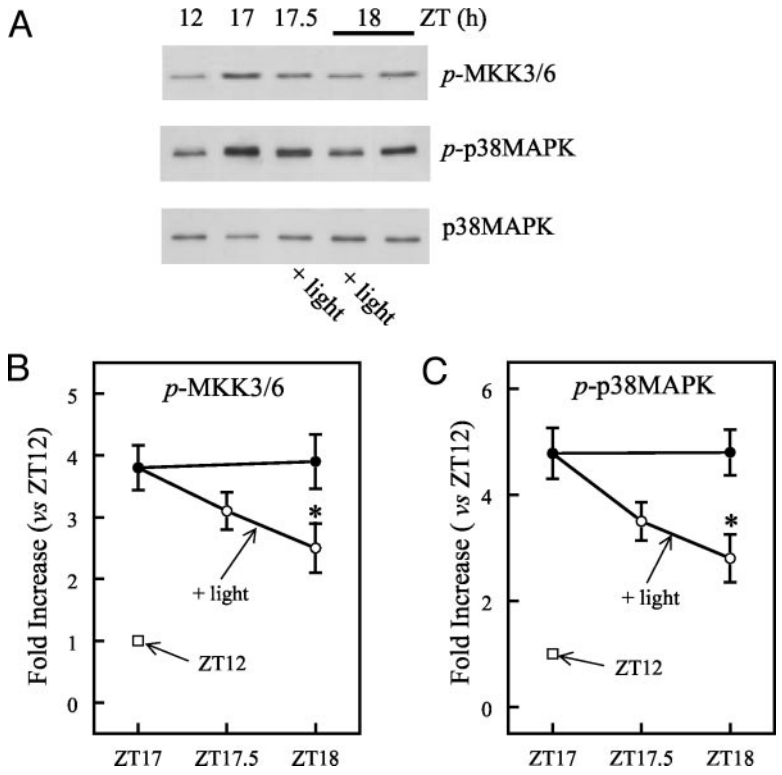

FIG. 8. Effect of acute light exposure on the nocturnal activation of MKK3/6 and p38MAPK in the rat pineal gland. Rats were housed under a 12-h light, 12-h dark cycle with light off at ZT 12. Pineal glands were collected at the time points indicated. *Closed circles* represent animals remaining in the dark, and *open circles* represent animals subjecting to light from ZT 17. Glands were homogenized, mixed with 2× sample buffer and analyzed by Western blotting using a monoclonal antibody against *p*-p38MAPK or polyclonal antibodies against *p*-MKK3/6 and p38MAPK. Each lane contains 25 μg of protein. See *Materials and Methods* for details. Representative immunoblots (A) and relative densitometric measurements (B) of *p*-MKK3/6 and (C) *p*-p38MAPK from three independent experiments presented as fold increase against the value at ZT12. *, $P < 0.05$, significantly different from ZT 17.

pathway or the β-adrenergic receptor → cAMP/PKA pathway alone is sufficient to induce a maximal p38MAPK response. However, at 120 and 240 min post stimulation, with a substantial decline in the β-adrenergic stimulated cAMP response, simultaneous activation of α-adrenergic receptors is required to potentiate the β-adrenergic stimulated cAMP response (1, 3) and maintains full activation of p38MAPK. The precise nature through which PKA and Ca^{2+}/CMK interact with the p38MAPK signaling pathway remains unclear. Nevertheless, MKK3/6 is likely downstream of PKA and Ca^{2+}/CMK interaction because of the parallel increases of *p*-MKK3/6 and *p*-p38MAPK post agonist stimulation.

The difference in time profiles of NE-induced p42/44MAPK and p38MAPK activation is of interest. Although the NE-stimulated p42/44MAPK activation in the pinealocytes is rapid in onset and transient in duration (onset within minutes and lasting less than 1 h), the NE-stimulated p38MAPK activation is slower in onset and sustained in duration. This difference in time profiles cannot be explained by involvement of different second messengers because the time-course of NE-stimulated cAMP and cGMP responses is similar (40, 41). Moreover, the delayed activation of the p38MAPK signaling pathway also cannot be attributed to the time required for elevation of cAMP or $[Ca^{2+}]_i$ because the NE-induced cAMP accumulation or elevation of $[Ca^{2+}]_i$ peaks within 15 min post treatment (7, 39–41). Furthermore, a similar delay in p38MAPK activation is also seen in cells stimulated with DBcAMP or ION.

Our results also indicate activation of the p38MAPK signaling pathway in the night pineal gland is under the control of photoneural input. The nocturnal increase in p38MAPK phosphorylation is secondary to enhanced phosphorylation of existing protein because of the parallel increase in *p*-MKK3/6 as well as the increase in *p*-p38MAPK in the absence of changes in p38MAPK protein during the dark phase. In support of a photoneural regulation of p38MAPK, subjecting the animals to continuous light from the onset of darkness blocks the nocturnal activation of MKK3/6 and p38MAPK. Moreover, blockade of the β-adrenergic input by propranolol reduces the nocturnal increase in MKK3/6 and p38MAPK phosphorylation. Considering that the pineal gland is stimulated by the release of NE from the sympathetic nerves at night (1, 2), these results suggest that the adrenergic-stimulated p38MAPK phosphorylation in the rat pineal gland is driven by the endogenous circadian clock in the suprachiasmatic nucleus (43, 44), similar to other cellular processes that have a circadian rhythm.

Our results also show a significant decline in p38MAPK activation within 60 min of acute light exposure during the dark phase, indicating that similar to the regulation of AA-NAT, a sustained stimulation is required for the night activation of the p38MAPK signaling pathway. However, the decline in phosphorylated MKK3/6 or p38MAPK levels are relatively slow after acute light exposure in comparison to the decline in AA-NAT activity after similar light exposure (27, 45). Therefore, the mechanism involved in the inactivation of MKK3/6 and p38MAPK may not be identical with that of AA-NAT.

It is of interest to note that, in the chick pineal gland, p38MAPK is involved in the regulation of the endogenous circadian clock and p38MAPK is activated during the daytime (46) in contrast to its nighttime activation in the rat pineal gland. The reason for this difference is not clear. Apart from species difference, one possible explanation is that in the chick pineal gland, the effect of NE on melatonin production is inhibitory (47) rather than stimulatory as in the rat pineal gland.

Our results indicate that activation of p38MAPK pathway is under photoneural control in the rat pineal gland and that PKA

and intracellular Ca^{2+} signaling pathways are involved in NE-regulation of p38MAPK. This adds to the complexity of the contributions by different members of the MAPK family to the regulation of pineal function. The sequential nocturnal activation of p42/44MAPK (27) and p38MAPK and the opposing effects of inhibiting p42/44MAPK and p38MAPK activation on NE-induced AA-NAT activity and melatonin production (26, 29) further suggest members of the MAPK family being an integral part of the control mechanism that frames the nocturnal increase in AA-NAT activity in the rat pineal gland.

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