Different Signaling Mechanisms Are Involved in the Norepinephrine-Stimulated TORC1 and TORC2 Nuclear Translocation in Rat Pinealocytes

J. McTague,* N. Amyotte,* R. Kanyo,* M. Ferguson, C. L. Chik, and A. K. Ho

Departments of Physiology (J.M., N.A., R.K., M.F., A.K.H.) and Medicine (C.L.C), Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

The distribution of transducers of regulated cAMP-response element-binding protein activity (TORC) between the cytoplasm and the nucleus is tightly regulated and represents one of the main mechanisms whereby the cAMP response element activation activities of TORC are controlled. Whereas both cAMP and Ca^{2+} pathways can cause translocation of TORC, the relative importance of these two pathways in regulating different TORC within the same cell is unclear. In this study, we determined the mechanism that regulated TORC1 translocation and compared it with that of TORC2 in rat pinealocytes. Stimulation of pinealocytes with norepinephrine (NE), although having no effect on *Torc1* transcription, caused rapid dephosphorylation of TORC1. Although NE also caused rapid dephosphorylation of TORC2, pharmacological studies revealed that TORC1 dephosphorylation could be induced by both β-adrenoceptor/cAMP and α-adrenoceptor/intracellular Ca^{2+} pathways contrasting with TORC2 dephosphorylation being induced mainly through the β-adrenoceptor/cAMP pathway. PhosTag gel indicated a different pattern of TORC1 desphosphorylation resulting from the selective activation of α- or β-adrenoceptors. Interestingly, only the α-adrenoceptor/intracellular Ca^{2+}-mediated dephosphorylation could translocate TORC1 to the nucleus, whereas the β-adrenoceptor/cAMP-mediated dephosphorylation of TORC1 was ineffective. In comparison, translocation of TORC2 was induced predominantly by the β-adrenoceptor/cAMP pathway. Studies with different protein phosphatase (PP) inhibitors indicated that the NE-mediated translocation of TORC1 was blocked by cyclosporine A, a PP2B inhibitor, but that of TORC2 was blocked by okadaic acid, a PP2A inhibitor. Together these results highlight different intracellular signaling pathways that are involved in the NE-stimulated dephosphorylation and translocation of TORC1 and TORC2 in rat pinealocytes. **(*Endocrinology* 153: 3839–3849, 2012)**

The binding of the coactivator cAMP response element-binding protein (CREB)-binding protein/p300 to the phosphorylated Ser-133 of CREB (pCREB) is key to the regulation of CREB-mediated transcription (1–4). However, phosphorylation of Ser-142 of CREB as well as interaction with the basic leucine zipper (bZIP) domain of CREB can also regulate CREB-mediated transcription (5–7). One established mechanism involves members of a family of CREB coactivators, transducer of regulated CREB activity (TORC) (8–10). TORC facilitate CREB-dependent gene expression by binding to the bZIP domain of CREB and enhance the association of CREB with TAFII130, a component of the general transcription factor complex needed to recruit RNA polymerase II (8). The importance of TORC in glucose homeostasis (9–12), synaptic plasticity (13), and the synthesis of CRH has been

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2012-1315 Received March 20, 2012. Accepted May 14, 2012.
First Published Online June 8, 2012

* J.M., N.A., and R.K. contributed equally to this manuscript.

Abbreviations: AA-NAT, Arylalkylamine-*N*-acetyltransferase; AP, alkaline phosphatase; bZIP, basic leucine zipper; Caly A, calyculin A; CREB, cAMP response element binding protein; CsA, cyclosporine A; DBcAMP, dibutyryl cAMP; DBcGMP, dibutyryl cGMP; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; ISO, isoproterenol; NE, norepinephrine; Oka, okadaic acid; pCREB, phosphorylated Ser-133 of CREB; PE, phenylephrine; PKA, protein kinase A; PMA, 4β-phorbol-12-myristate-13-acetate; PP, phosphoprotein phosphatase; PRAZ, prazosin; PROP, propranolol; SIK, salt-inducible kinase; Tau, tautomycin; TORC, transducer of regulated CREB activity coactivators.

established (14). Among the members of the TORC family, the expression of TORC2 is ubiquitous, whereas TORC1 is detected primarily in the brain (8).

Regulation of the coactivation activities of TORC revolve around controlling their intracellular localization (15). In the resting state, TORC are highly phosphorylated, presumably by salt-inducible kinase (SIK), and are sequestered in the cytoplasm via phosphorylation-dependent association with 14-3-3 proteins (15, 16). Upon stimulation that elevates intracellular cAMP and/or Ca^{2+}, TORC are dephosphorylated, released from 14-3-3 proteins, and translocated to the nucleus (15, 16). Because of their sensitivities toward both cAMP and Ca^{2+}, it has been suggested that TORC could serve as coincidence detectors for the cAMP and Ca^{2+} signals by converting these signals into a transcriptional response as demonstrated for TORC1 in neurons and TORC2 in insulinoma cells (13, 17). Additional studies indicate that Ca^{2+} causes activation of TORC through calcineurin-mediated dephosphorylation, whereas cAMP/cAMP-dependent protein kinase (PKA) contributes to TORC activation by inhibiting SIK, thereby preventing phosphorylation and inactivation of TORC (15, 16). However, earlier studies centered mainly on the regulation of either TORC1 or TORC2 separately and important differences may exist between the activation mechanisms of these two TORC (9–13, 15, 17). The relative contributions of the cAMP and Ca^{2+} signals, two main intracellular pathways controlling TORC1 and TORC2 activation, within the same cell has not been examined in detail. This issue merits investigation because simultaneous activation of both the cAMP and Ca^{2+} signaling pathways is a common transduction mechanism (13, 15, 17), and one example of interest is the norepinephrine (NE) stimulation of rat pinealocytes (18–21).

The main function of the rat pineal gland is to synthesize melatonin at night (22). With the onset of darkness, NE released from the sympathetic neurons causes elevations of both intracellular cAMP and Ca^{2+} levels through activation of β- and α-adrenergic receptors, respectively (18–21). Increase in cAMP leads to the activation of PKA and the phosphorylation of CREB (21, 23). The binding of pCREB to the cAMP response element of CREB target genes turns on their transcription (24, 25). This cAMP/pCREB-dependent mechanism is thought to be responsible for the 100-fold increase in the mRNA of the melatonin rhythm generating enzyme, arylalkylamine-*N*-acetyltransferase (AA-NAT) (26, 27). However, recent studies suggest that TORC may also play a role in the induction of *Aanat* in the rat pineal gland. This is based on the demonstration that overexpression of TORC2 can prolong the NE-stimulated *Aanat* expression (28). In addition, NE can induce the expression of *Sik1*, the kinase that phosphorylates and negatively regulates TORC, and that SIK1 can act as a transcription repressor of *Aanat* in rat pinealocytes (29). However, the regulation of TORC1 and the possible difference in the mechanisms involved in the activation of TORC1 and TORC2 in rat pinealocytes remain unclear.

Considering that NE stimulation can elevate both intracellular cAMP and Ca^{2+} in the pinealocyte, this provides an opportunity to compare the relative importance of these two signaling pathways in regulating TORC1 and TORC2 in the same cell. In the present study, we focus on characterizing the pathways involved in the intracellular distribution of TORC1 and TORC2 under basal and NE-stimulated conditions.

Materials and Methods

Drugs

Dibutyryl cAMP (DBcAMP), dibutyryl cGMP (DBcGMP), isoproterenol (ISO), NE, phenylephrine (PE), 4β-phorbol-12-myristate-13-acetate (PMA), prazosin (PRAZ), and propranolol (PROP) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Calyculin A (Caly A), cyclosporine A (CsA), okadaic acid (Oka), and tautomycin (Tau) were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Antibodies used included the following: polyclonal anti-TORC2 serum was obtained from Calbiochem-Novabiochem; rabbit monoclonal antibodies against TORC1, CREB, and pCREB (Ser133) were obtained from Cell Signaling Technology (Beverly, MA); monoclonal anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody was obtained from Ambion Inc. (Austin, TX); and polyclonal anti-AA-NAT (AB3314) serum was a gift from Dr. D. C. Klein (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD).

Animal handling, isolation of pineal gland, and preparation of pinealocytes

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), obtained from the University of Alberta animal unit, were housed under a lighting regimen providing 12 h of light every 24 h with lights on at 0600 h. For the preparation of pinealocyte cell culture, 12–15 animals were killed at 0900 h and their pineal glands removed and placed in ice-cold PBS until enzymatic digestion. Pinealocytes were prepared by papain dissociation using a system from Worthington Biochemical Corp. (Lakewood, NJ). To determine the nocturnal expression of TORC1 and TORC2 in the pineal gland, groups of animals (n = 3) were conditioned for 1 wk under 14 h of light every 24 h and killed at various time points as indicated. Pineal glands were collected, flash frozen on dry ice, and stored at −75 C until preparation for RNA extraction or Western blot analysis. A dim red light was used when animals were killed during the dark period.

Drug treatments and sample preparation

Pinealocytes were suspended in DMEM containing 10% fetal calf serum and maintained at 37 C for 18 h in a mixture of 95% air and 5% CO_2 before the experiment. Aliquots of pinealocytes 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, 6000 × *g*). For Western blot analysis, cell pellets were lysed in 1× Laemmli buffer (30) by boiling for 5 min and placed at 4 C until electrophoresis. In some experiments, cell lysates were treated with calf intestinal alkaline phosphatase (AP; 1 U per 10 μl of lysate; New England Biolabs, Ipswich, MA) for 30 min at 37 C before mixing with 2× Laemmli buffer and boiled. Pinealocyte total RNA was isolated using Trizol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. For the nuclear and cytosolic fractionation, a commercial kit from BioVision Inc. (Mountain View, CA) was used.

Western blotting

SDS-PAGE (using 10% acrylamide and 1 mg/ml sodium dodecyl sulfate) and Western blotting were performed as described in our previous studies (31). After the transfer of proteins onto polyvinylidene difluoride membranes, the blots were blocked [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 and incubated overnight at 4 C with diluted specific antisera as indicated. After washing, the blots were incubated with diluted horseradish peroxidase-conjugated second antibodies (Bio-Rad Laboratories Inc., Hercules, CA) for 1 h at room temperature, washed extensively, and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL). For the Phos-Tag gel, the following modifications of the procedure were made. In the running gel, 20 μM of PhosTag-acrylamide (Wako Chemical, Richmond, VA) and 40 μM of $MnCl_2$ were included. Before blotting, the gels were first soaked in transfer buffer containing 8 mM EDTA for 15 min to remove the $MnCl_2$, and the duration of blotting was extended to 3 h.

RT-PCR

First-strand cDNA was synthesized from the isolated RNA using an Omniscript reverse transcriptase kit (QIAGEN Inc., Valencia, CA) with a random primer. Real-time PCR was run with StepOne Real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR-Green FAST reagent mixture as described previously (28). Primers used are as follows: *Torc1* forward primer, 5′-GCA GAA GTC GCA GTA TCT CCA-3′; reverse primer, 5′-AGG CCT GAG GAC TGA AAG G-3′ (corresponding to bp 135–266). Sequences of the *Aanat*, *Gapdh*, and *Torc2* primers used were previously described (28, 32, 33).

Results and statistical analysis

Western blots analysis was repeated a minimum of three times with pinealocytes from different cell preparations or with rat pineal glands from three independent experiments. Images were analyzed by Kodak 1D imaging software (Eastman Kodak Co., Rochester, NY) and normalized as indicated in the figure legend. Real-time PCR was used to determine mRNA levels, 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. Statistical significance was set at $P < 0.05$.

Results

Effect of NE stimulation on TORC1 protein in rat pinealocytes

Western blot analysis showed that treatment of pinealocytes with NE (3 μM) resulted in a rapid downward shift of the TORC1 protein band within 30 min of stimulation and a gradual increase in its intensity (Fig. 1A). Whereas the increase in band intensity persisted for 8 h, the shifting of the band lasted for less than 4 h after NE stimulation. In comparison, although the shifting of the TORC2 band showed a similar time profile as that of TORC1, the intensity of the TORC2 band showed a gradual decline over the 8 h of NE treatment. The NE-induced shifting of the TORC1 and TORC2 bands had a similar time of onset as the NE-stimulated pCREB but preceded the appearance of the NE-induced AA-NAT protein (Fig. 1A). To determine whether the increase in TORC1 protein was due to an increase in synthesis, we measured the effect of NE treatment on *Torc1* mRNA levels. Treatment of pinealocytes with NE (3 μM), although causing an expected increase in the mRNA levels of *Aanat* that peaked around 4 h, had no effect on the levels of *Torc1* mRNA (Fig. 1B), indicating that NE has no effect on *Torc1* transcription. A similar lack of effect of NE stimulation on the *Torc2* transcription has been reported previously (28).

To determine whether the onset of darkness had similar effects on the protein levels of TORC1 and TORC2 in the rat pineal gland, pineal glands were collected from rats housed under a lighting regimen of 14 h of light every 24 h at different times. As shown in Fig. 1C, Western blot analysis of TORC1 protein showed a downward shift of the TORC1 band within 1 h after the onset of darkness. This downward mobility shift of TORC1 was observed during the early part of the dark period, and the intensity of the band increased gradually toward the end of the dark period that persisted after the onset of light. In comparison, the TORC2 band appeared as a doublet, with the slower migrating upper band being more dominant in the daytime samples (Fig. 1C). The onset of darkness caused an increase in the intensity of the lower band and a decrease in the upper band that was observed within 1 h. This shift in the intensities of the TORC2 doublet persisted throughout the dark period, and the intensities of both TORC2 bands declined gradually as the dark period progressed. With the onset of light, the relative intensities of the TORC2 bands reverted to the daytime pattern within 1 h. The nighttime-induced shifting of TORC1 and TORC2 bands occurred

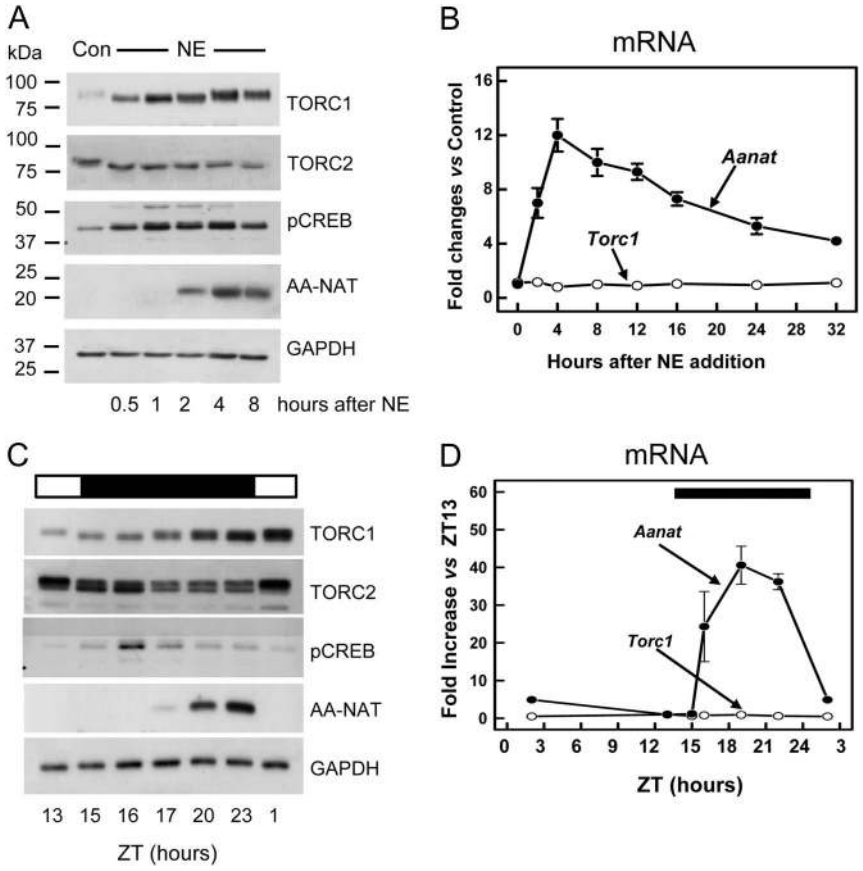

FIG. 1. *Torc1 and Torc2* mRNA and protein levels after NE stimulation in rat pinealocytes and their day/night variations in the rat pineal gland. A and B, Pinealocytes (1×10^5 cells per 0.3 ml) were stimulated with NE (3 μM) for the indicated time period. C and D, Pineal glands were collected from rats, housed under a 14-h light, 10-h dark cycle, at the time points indicated. ZT, Zeitgeber time. *Dark bar* represents the period of darkness. Pinealocytes or pineal glands were prepared for real-time PCR or Western blot analysis as described in *Materials and Methods*. A and C, TORC1 and TORC2 proteins as measured by Western blot analysis. The protein levels of pCREB, AA-NAT, and GAPDH were determined for comparison. The blots presented are representative from three separate experiments with similar results. B and D, *Torc1* and *Aanat* mRNA levels as measured by real-time PCR, normalized to *Gapdh* mRNA and expressed as fold increase against control or against ZT13. Each value indicates mean ± SEM (n = 3).

more rapidly than the appearance of pCREB or the induction of AA-NAT protein in the pineal gland (Fig. 1C). Although the nocturnal induction of the *Aanat* mRNA levels was readily detectable in the rat pineal gland, no diurnal difference in the *Torc1* mRNA was evident (Fig. 1D). A lack of a diurnal difference in the *Torc2* mRNA has been reported previously (28).

NE stimulation causes rapid dephosphorylation of TORC1 protein in the rat pinealocyte

To verify the identity of the two TORC1 bands on the Western blot, protein samples were subjected to AP treatment before SDS-PAGE analysis. As shown in Fig. 2A, AP treatment resulted in the disappearance of the upper band and the appearance of the lower band in the control sample. AP treatment had no effect on the lower TORC1 band in the NE-stimulated sample but abolished the NE-stimulated pCREB signal that was included as a positive control. A similar effect of AP treatment on the migration of TORC2 protein has been reported previously (28). Together these results indicate that the slower migrating upper band represents the phosphorylated form of TORC1. To confirm this observation, we compared the banding patterns of TORC1 and TORC2 proteins separated on PhosTag gels between control and NE-stimulated pinealocytes. The presence of PhosTag in the sodium dodecyl sulfate gel led to retardation of phosphorylated proteins in comparison with the native nonphosphorylated forms. As shown in Fig. 2B, NE stimulation caused a downward shift of TORC1 and TORC2 bands on the PhosTag gel within 15 min, indicating a rapid dephosphorylation of TORC1 and TORC2 proteins. The shifting in the band pattern of TORC1 persisted for at least 2 h and gradually returned to a pattern comparable with control after 4 h. In comparison, the NE-induced downward shift in the TORC2 bands persisted for more than 8 h (Fig. 2B).

To determine whether the onset of darkness had a similar effect on the TORC1 and TORC2 proteins, pineal glands collected at different times from rats housed under a lighting regimen of 14 h of light every 24 h were analyzed by PhosTag gels. As shown in Fig. 2C, the faster migrating TORC1 bands appeared within 1 h after the onset of darkness, persisted for 2 h before returning to the slower migrating pattern. Interestingly, a gradual increase in the intensity of the TORC1 banding pattern occurred toward the latter part of the dark period and persisted after the onset of light. In comparison, a gradual downward shifting of the TORC2 bands occurred 1 h after the onset of darkness that became better delineated after 2 h and persisted through the dark period before reverting to the daytime pattern within 1 h after the onset of light (Fig. 2C). Unlike the TORC1 and TORC2 proteins, AA-NAT protein appeared only during the latter part of the night as three separate bands when analyzed by the PhosTag gel, a finding consistent with the previously reported double-phosphorylation sites on the

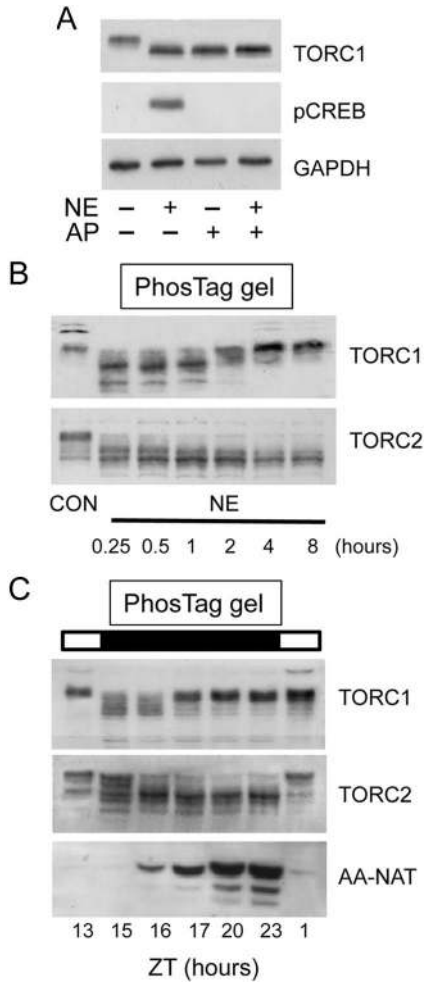

FIG. 2. Identification of the faster migrating bands of TORC that appear after NE stimulation in rat pinealocytes or after onset of darkness in the rat pineal gland. To determine the identity of the faster migrating TORC1 band, pinealocytes (1×10^5 cells per 0.3 ml) were treated as indicated. A, Pinealocytes were stimulated with or without NE (3 μM) for 15 min and collected. One set of control (CON) and NE-stimulated cells were subjected to treatment with AP before Western blot analysis for TORC1. The protein levels of pCREB and GAPDH were determined to monitor the effectiveness of the AP treatment and loading. B, Pinealocytes were treated for the indicated period with NE (3 μM). Whole-cell lysates were prepared and separated on the PhosTag gel before Western blot analysis for TORC1 and TORC2 proteins. C, Pineal glands were collected from rats, housed under a 14-h light, 10-h dark cycle, at the time points indicated. Whole-gland lysates were prepared and separated on the PhosTag gel before Western blot analysis for TORC1 and TORC2 proteins. The protein levels of AA-NAT were determined to monitor the effectiveness of the PhosTag gel to separate phosphorylated proteins. The blots are representative from at least three separate experiments with similar results.

AA-NAT protein and their phosphorylation status at night (34).

Signaling mechanisms involved in the NE-induced dephosphorylation of TORC1 and TORC2 proteins

Results from the studies above indicate that whereas NE can stimulate the dephosphorylation of TORC1 and TORC2, the difference in the time profiles of responses suggests that the postreceptor mechanism involved may be different. To determine the subtype of adrenergic receptors involved in regulating the dephosphorylation of TORC1 and TORC2, selective adrenergic agonists and antagonists at concentrations established previously were used (20, 21, 29, 31, 35). PhosTag gels were used to monitor the dephosphorylation of TORC1 and TORC2 proteins. At 30 min after treatment, selective activation of α-adrenergic receptors by PE (1 μM in the presence of 3 μM of PROP) or NE (1 μM) in the presence of PROP (3 μM) caused an increase in the intensity and the appearance of the faster migrating TORC1 bands (Fig. 3A). Activation of β-adrenergic receptors alone by ISO (1 μM in the presence

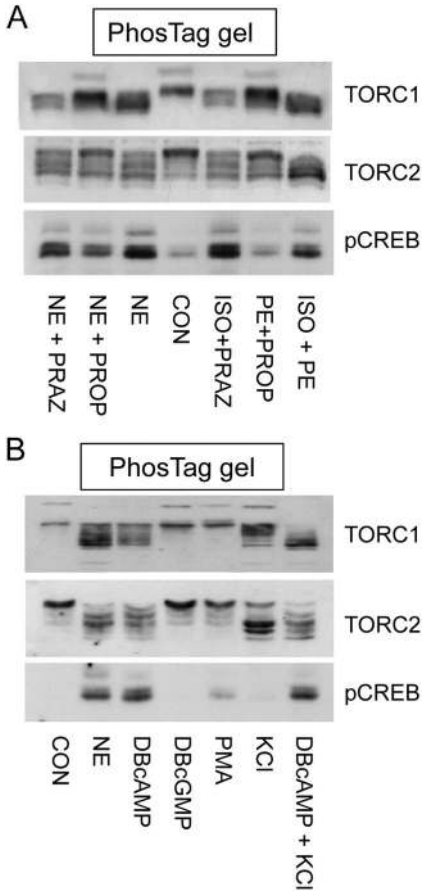

FIG. 3. Adrenergic receptors and postreceptor mechanisms involved in the NE-stimulated dephosphorylation of TORC1 and TORC2 proteins. To determine the adrenergic receptors and the postreceptor mechanisms involved, pinealocytes (1×10^5 cells per 0.3 ml) were treated for 30 min with different drugs as indicated. A, Receptor characterization. NE (1 μM) alone or in the presence of PROP (3 μM) or PRAZ (3 μM), ISO (1 μM) + PRAZ (3 μM), PE (1 μM) + PROP (3 μM), or ISO (1 μM) + PE (3 μM). CON, Control. B, Postreceptor mechanisms. NE (1 μM), DBcAMP (0.5 mM), DBcGMP (0.5 mM), PMA (0.1 μM), or KCl (30 mM). Whole-cell lysates were prepared and separated on the PhosTag gel before Western blot analysis for TORC1 and TORC2. The blots were probed for pCREB as stimulation control. The blots presented are representative from at least three separate experiments with similar results.

of 3 μM of PRAZ) or NE (1 μM) in the presence of PRAZ (3 μM) also caused the appearance of the faster migrating TORC1 bands without any apparent changes in band intensity compared with control (Fig. 3A). Activation of neither the α- nor the β-adrenergic receptors alone was able to fully mimic the NE-induced changes in the banding pattern of TORC1. To mimic the NE-stimulated pattern, simultaneous activation of both the α- and β-adrenergic receptors was required. In contrast, activation of the β-adrenergic receptors alone (ISO+PRAZ or NE+PRAZ) could mimic the effect of NE-induced changes in the banding pattern of TORC2 (Fig. 3A). However, selective activation of α-adrenergic receptors (PE+PROP or NE+PROP) had only a modest effect on the TORC2 bands. These results indicate that whereas the NE-induced TORC1 dephosphorylation is mediated through both the α- and β-adrenergic receptors, the NE effect on TORC2 dephosphorylation is mediated predominantly through the β-adrenergic receptors.

To characterize the intracellular signaling pathways involved, the effects of different protein kinase activators were tested. Treatment of pinealocytes for 30 min with the membrane-permeable cGMP analog DBcGMP (0.5 mM) or the activator of protein kinase C PMA (0.1 μM) had no effect on the TORC1 and TORC2 proteins (Fig. 3B). In comparison, treatment with DBcAMP (0.5 mM), a membrane-permeable cAMP analog, or a depolarizing concentration of KCl (30 mM), which elevates intracellular Ca^{2+} through the voltage-dependent Ca^{2+} channels, were both effective in causing the appearance of the faster migrating TORC1 bands in the PhosTag gel, indicating dephosphorylation of the TORC1 protein (Fig. 3B). However, neither agent alone was able to produce the same TORC1 banding pattern as that induced by NE (Fig. 3B). Combined stimulation with DBcAMP and KCl resulted in a predominance of the faster migrating TORC1 bands that was even more pronounced than that induced by NE (Fig. 3B). This indicates that both cAMP and Ca^{2+} pathways are involved in mediating the effect of NE on TORC1. In the case of TORC2, although both DBcAMP (0.5 mM) and KCl (30 mM) could induce the appearance of the faster migrating TORC2 bands, the band pattern induced by KCl was different from that stimulated by NE. Moreover, stimulation with DBcAMP alone was sufficient to produce the effect of NE on the TORC2 migration pattern (Fig. 3B). These results suggest that only the PKA pathway is involved in the NE-stimulated dephosphorylation of TORC2.

To gain insight into the specific phosphatase involved in dephosphorylating TORC1 after NE stimulation, established protein phosphatase inhibitors were tested (36, 37). As shown in Fig. 4A, the NE-induced shifting of the

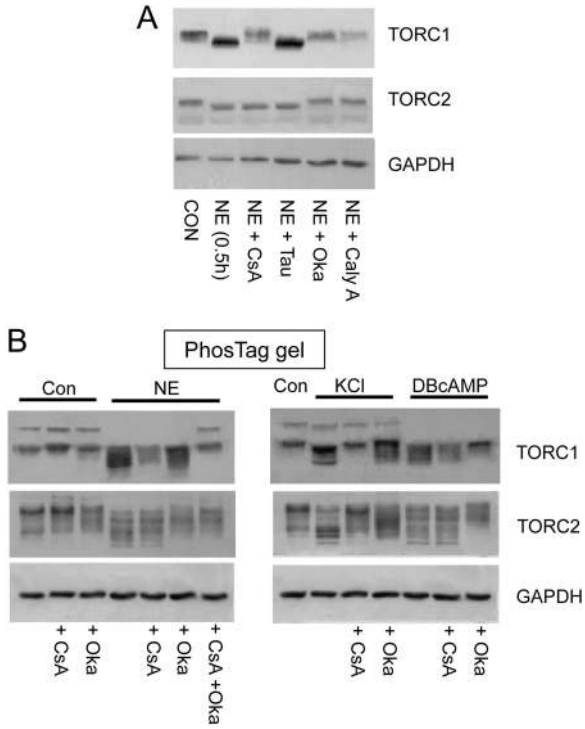

FIG. 4. Identification of phosphoprotein phosphatases involved in NE-stimulated dephosphorylation of TORC1 and TORC2 in rat pinealocytes. To determine the identity of the phosphoprotein phosphatases involved, pinealocytes (1×10^5 cells per 0.3 ml) were treated as indicated. A, Pinealocytes were treated with NE (3 μM) for 30 min in the presence and absence of CsA (1 μM), Tau (1 μM), Oka (1 μM), or Caly A (10 nM) as indicated. The inhibitors, when present, were added 15 min before NE. Whole-cell lysates were prepared and subjected to Western blot analysis for TORC1 and TORC2, with GAPDH included as loading control (CON). B, Pinealocytes were treated with NE (3 μM), KCl (30 mM), or DBcAMP (0.5 mM) for 30 min in the presence and absence of CsA (1 μM) and Oka (1 μM) alone or in combination, with the inhibitors added 15 min before other treatments. Whole-cell lysates were prepared and subjected to the PhosTag gel before Western blot analysis for TORC1 and TORC2. The blots are representative from at least three separate experiments with similar results.

TORC1 band was blocked by CsA, Oka, and Caly A and that of TORC2 was blocked by Oka and Caly A but not by CsA. Treatment of pinealocytes with Tau had no effect on either the TORC1 or TORC2 protein. Based on the pharmacology of the phosphatase inhibitors used (36), these results suggest that TORC1 can be dephosphorylated by both protein phosphatase 2 (PP2)-A and PP2B, and TORC2 can be dephosphorylated only by PP2A, whereas PP1 does not appear to be involved in the dephosphorylation of either TORC protein. The dephosphorylation of TORC1 and TORC2 was further investigated by using the PhosTag gel. As shown in Fig. 4B, the pattern of the NE-stimulated shifting of the TORC1 band was only partially reversed by treatment with either CsA or Oka alone but completely reversed by the combined treatment with both phosphatase inhibitors. When the ef-

fects of CsA and Oka were tested against the shifting of the TORC1 bands stimulated by KCl or DBcAMP, CsA but not Oka could block the effect of KCl, whereas Oka but not CsA could block the effect of DBcAMP (Fig. 4C). These results are consistent with the interpretation that the individual phosphorylation site on TORC1 probably has selective sensitivity toward specific phosphatase and the site that is sensitive to one is insensitive to another phosphatase. In the case of TORC2, the PhosTag gel revealed that Oka treatment was effective in abolishing the appearance of some of the faster migrating TORC2 bands stimulated by NE, DBcAMP or KCl (Fig. 4B). In comparison, treatment of pinealocytes with CsA had no effect on the TORC2 bands under basal, DBcAMP- and NE-stimulated conditions. This lack of effect of CsA was not due to a suboptimal concentration of the inhibitor because the same treatment was effective in reversing the effect of KCl. Together this indicates that whereas pharmacological activation of PP2B can dephosphosphorylate TORC2, the NE-stimulated dephosphophorylation is mediated mainly through PP2A.

NE-stimulated nuclear translocation of TORC1 and TORC2 in rat pinealocytes

The difference between the mechanism through which NE-induced dephosphorylation of TORC1 and TORC2 led us to compare the localization and distribution of TORC1 and TORC2 in rat pinealocytes under basal and NE-stimulated conditions. Separation of pinealocytes into nuclear and cytosolic fractions showed that most of the TORC1 and TORC2 proteins, which were in their phosphorylated forms under basal condition, were found in the cytosolic fraction of pinealocytes (Fig. 5A). NE stimulation, which induced the dephosphorylation of TORC1 and TORC2 proteins, led to the appearance of both proteins in the nucleus. However, receptor characterization studies showed that the translocation of TORC2 was stimulated by ISO, a β-adrenergic agonist, and the translocation of TORC1 was stimulated by PE, an α-adrenergic agonist. Moreover, the effect of NE on TORC2 was blocked by PROP (a β-adrenergic antagonist) and not by PRAZ (an α-adrenergic antagonist), whereas the NE effect on TORC1 was blocked by PRAZ and not by PROP (Fig. 5, A and B). Thus, the translocation of TORC2 is mediated almost exclusively through the activation of β-adrenergic receptors. However, in the case of TORC1, even though both α- and β-adrenergic stimulation can cause its dephosphorylation, its translocation is mediated mainly through activation of α-adrenergic receptors, suggesting that not all dephosphorylation can lead to nuclear translocation.

To determine and compare the signaling pathways involved in the nuclear translocation of TORC1 and TORC2, pharmacological agents that simulate different second messengers were used. As shown in Fig. 5, C and D, treatment with KCl (30 mM) was most effective in sim-

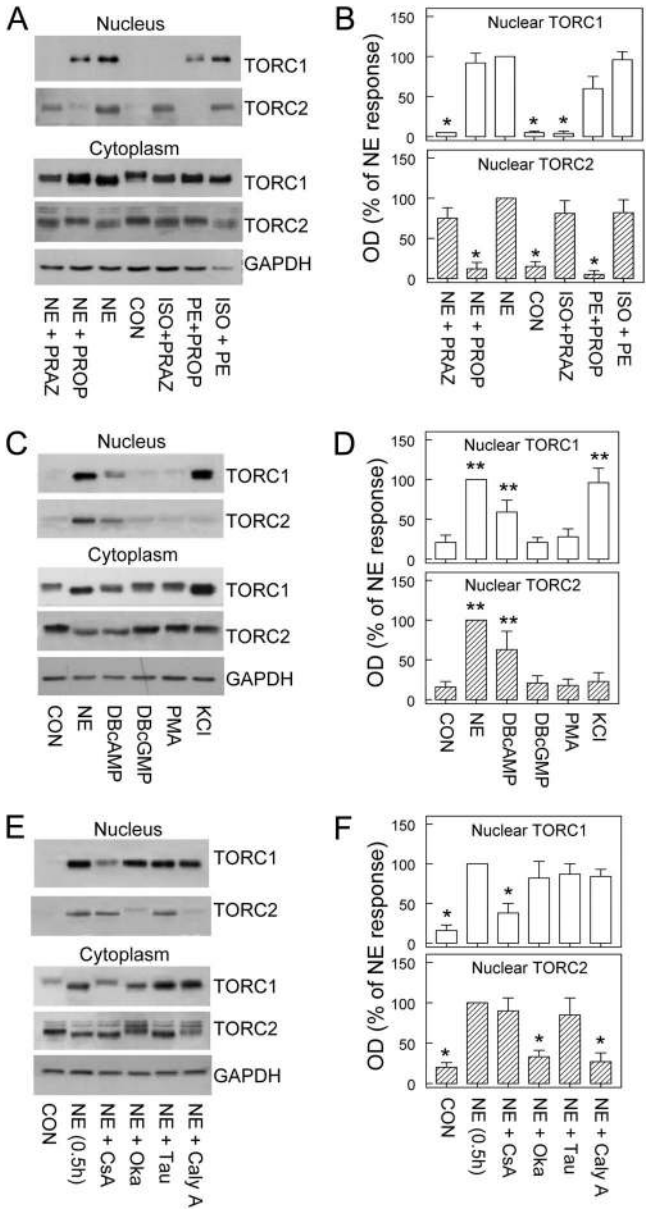

FIG. 5. Nuclear translocation of TORC1 and TORC2 in rat pinealocytes. To determine the cellular redistribution of TORC1 and TORC2 proteins after adrenergic stimulation and the mechanism involved, pinealocytes (1×10^5 cells per 0.3 ml) were treated for 30 min with NE (1 μM) alone or in the presence of PROP (3 μM) or PRAZ (3 μM), ISO (1 μM) + PRAZ (3 μM), PE (1 μM) + PROP (3 μM), or ISO (1 μM) + PE (3 μM) (A); NE (1 μM), DBcAMP (0.5 mM), DBcGMP (0.5 mM), PMA (0.1 μM), or KCl (30 mM) (C); or NE (1 μM) alone or in the presence of CsA (1 μM), Oka (1 μM), Tau (1 μM), or Caly A (10 nM), the phosphatase inhibitors added 15 min before NE (E) before separation into the cytoplasmic and nuclear fractions. The proteins in the fractions were separated by SDS-PAGE and subjected to Western blot analysis for TORC1 and TORC2. CON, Control. B, D, and F, Histograms of the densitometric measurement of the nuclear TORC1 (top panel) and TORC2 (bottom panel) bands from A, C, and E presented as percentage of the NE response. Each value indicates the mean ± SEM (n = 3). *, Significantly different from the NE-stimulated response; **, significantly different from CON.

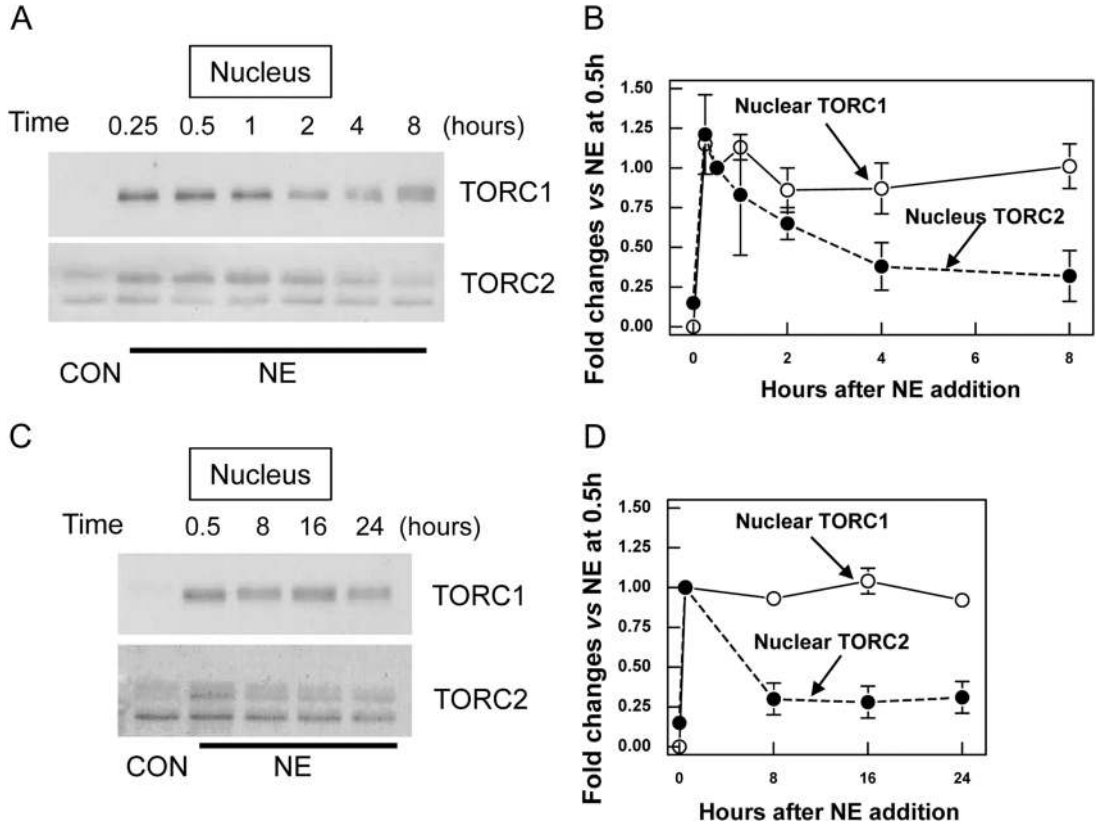

FIG. 6. Time course of NE-stimulated nuclear localization of TORC1 and TORC2 in rat pinealocytes. To determine the time course of nuclear localization of TORC1 and TORC2 proteins after NE stimulation, pinealocytes (1×10^5 cells per 0.3 ml) were stimulated with NE (3 μM) for the indicated period of time. The cells were then collected, fractionated into cytoplasmic and nuclear fractions, and the proteins in the fractions separated by SDS-PAGE and subjected to Western blot analysis for TORC1 and TORC2. Short (0–8 h) (A) and long (0–24 h) (C) time-course studies of nuclear contents of TORC1 and TORC2 after NE stimulation are shown. B and D, Histograms of the densitometric measurement of the nuclear TORC1 and TORC2 bands from A and C presented as fold changes against the NE response at 0.5 h. Each value indicates the mean ± SEM (n = 3).

ulating the NE-stimulated TORC1 nuclear translocation and, to a lesser degree, treatment with an activator of PKA (DBcAMP). In comparison, activator of protein kinase G (DBcGMP) or protein kinase C (PMA) was ineffective. In contrast, only DBcAMP was effective in causing the nuclear translocation of TORC2. Together these results indicate that NE stimulation used elevation of intracellular Ca^{2+} as the intracellular mechanism for nuclear translocation of TORC1 and elevation of cAMP for TORC2.

To establish the phosphatase involved in the translocation of TORC1 and TORC2, different protein phosphatase inhibitors were tested. As shown in Fig. 5, E and F, the NE-stimulated TORC1 translocation was blocked by pretreatment with CsA but not by Oka, Tau, or Caly A. In comparison, the translocation of TORC2 was blocked by Oka and Caly A but not by CsA or Tau. These results suggest that although PP2B is responsible for the dephosphorylation and nuclear translocation of TORC1, PP2A performs the same function for TORC2.

In view of the difference in the intracellular mechanism involved in translocating TORC1 and TORC2, we compared the time profiles of NE-stimulated nuclear localization of TORC1 and TOCR2. Figure 6 indicates that NE stimulation induces rapid nuclear localization of TORC1 and TORC2, with peak levels observed within 15 min of stimulation. Whereas the NE-stimulated TORC1 response persisted for 24 h, the NE-stimulated TORC2 response showed a substantial decline within 4 h after stimulation.

Discussion

The importance of transcription coactivators TORC1 and TORC2 in regulating the expression of inducible genes through binding of the bZIP domain on CREB is well recognized (8, 13). The regulation of coactivation activities of TORC revolves around controlling the intracellular localization of TORC, specifically, dephosphorylation of TORC in the cytoplasm and translocation to the nucleus (15). In the case of the CREB target gene *Aanat* in the rat pineal gland, we have previously shown that NE stimulation causes nuclear translocation of TORC2 by dephosphorylating the protein through a β-adrenoceptor/cAMP

mechanism and that overexpressing TORC2 can enhance the NE-induced *Aanat* transcription (28).

In the present study, we further demonstrate that NE stimulation has no effect on *Torc1* mRNA and TORC1 protein levels in the rat pineal gland as in the case of TORC2 (28). However, there is a rapid appearance of the faster migrating TORC1 protein bands in the rat pineal gland after the onset of darkness and in cultured pinealocytes after NE stimulation. Moreover, by using the PhosTag gel and AP treatment, we show that these faster migrating TORC1 bands represent the dephosphorylated form of TORC1. Therefore, these results imply that, just like the TORC2 protein, most of the TORC1 protein is phosphorylated under basal condition and that NE stimulation induces rapid TORC1 dephosphorylation in the rat pineal gland.

Although NE stimulation induces similar dephosphorylation and nuclear localization of both TORC1 and TORC2 in the rat pineal gland, there are distinct differences in the receptors and signaling mechanisms involved in these effects of NE within the same cell. By using selective adrenergic agonists and antagonists and separation of proteins by the PhosTag gel, we establish that activation of both α- and β-adrenergic receptors are involved in mediating the NE-stimulated TORC1 dephosphorylation. In comparison, activation of β-adrenergic receptors alone is sufficient to simulate the effect of NE stimulation on TORC2.

At the second messenger level, our results show that both DBcAMP and elevating intracellular Ca^{2+} through depolarization with K^+ can cause dephosphorylation of TORC1, but the pattern of the dephosphorylated proteins on the PhosTag gel is different. More importantly, neither treatment of pinealocytes with DBcAMP and KCl alone can produce the NE-stimulated TORC1 dephosphorylation pattern. To produce the NE-stimulated TORC1 dephosphorylation pattern, simultaneous activation of both pathways by DBcAMP and KCl is probably required. In the case of TORC2, both DBcAMP and intracellular Ca^{2+} elevation can cause dephosphorylation of TORC2. In contrast to TORC1, treatment with DBcAMP alone is sufficient to simulate the pattern of rapid TORC2 dephosphorylation induced by NE. These findings are consistent with the results from the receptor characterization studies because the synthesis of cAMP in rat pinealocytes is regulated through the β-adrenergic receptor (22), whereas elevation of intracellular Ca^{2+} is controlled by the α-adrenergic receptor (20). Together these results indicate that although NE can activate multiple signaling pathways in the pinealocyte, the dephosphorylation of TORC1 in this tissue is regulated through both the cAMP and intracellular Ca^{2+} signaling pathways, but that of TORC2 is primarily regulated through the cAMP signaling pathway.

Investigation into the phosphatase involved by comparing the effects of different phosphoprotein phosphatase inhibitors also reveals a major difference between the dephosphorylation of TORC1 and TORC2 in the rat pinealocyte. Whereas inhibitors of PP2A (CalyA and Oka) and PP2B (CsA) appear to be effective in reversing the NE-stimulated TORC1 dephosphorylation as observed in SDS-PAGE, the PhosTag gels reveal that inhibition of either PP2A or PP2B alone can only partially reverse the NE-stimulated TORC1 dephosphorylation. To block the NE-stimulated effect completely, inhibition of both phosphatases are required. In comparison, inhibition of PP2A (CalyA and Oka) alone but not inhibition of PP1 (Tau) or PP2B (CsA) is effective in blocking the NE-stimulated dephosphorylation of TORC2. Together these results indicate that TORC1 can be dephosphorylated by both PP2A and PP2B, but the NE-stimulated TORC2 dephosphorylation is mediated mainly through PP2A. Another finding of interest is that whereas both PP2A and PP2B can dephosphorylate TORC1 after NE stimulation, they appear to act on different sites on TORC1. This is based on the observation that inhibition of these two phosphatases produce very different TORC1 protein migration patterns on the PhosTag gel.

In regard to TORC2, our results show that elevation of intracellular Ca^{2+} through activation of voltage-dependent Ca^{2+} channels can cause dephosphorylation but elevation of intracellular Ca^{2+} through stimulation of α-adrenergic receptors by NE has no effect. This may reflect the difference in the concentration of intracellular Ca^{2+} attained through the two intracellular Ca^{2+} elevating mechanisms and hence the level of PP2B activation. However, we cannot exclude the contribution of other pathways that regulate Ca^{2+} entry in rat pinealocytes such as the acetylcholine-mediated depolarization of L-type Ca^{2+} channels through activation of nicotinic receptors (38). In any case, our results indicate that compared with TORC1, TORC2 is less sensitive to PP2B dephosphorylation in the rat pinealocyte.

The physiological significance of dephosphorylating TORC1 and TORC2 proteins is their translocation to the nucleus whereby they can function as coactivators in CREB-regulated transcription. By separating the pinealocytes into cytoplasmic and nuclear fractions, we demonstrate that the NE-stimulated dephosphorylation of TORC1 and TORC2 leads to similar nuclear translocation of TORC1 and TORC2 in the rat pinealocyte. Whereas only the dephosphorylated forms of TORC1 and TORC2 are found in the nucleus, both phosphorylated and native forms are present in the cytoplasmic fraction

after NE stimulation. This suggests that only a portion of the dephosphorylated TORC1 or TORC2 is translocated and implies that besides dephosphorylation, other factors may limit the nuclear localization of both TORC1 and TORC2.

Besides the above similarities, our study also reveals important differences in the pathways involved in TORC1 and TORC2 nuclear translocation in rat pinealocytes. For the translocation of TORC1, NE uses predominantly the α-adrenergic receptors/intracellular Ca^{2+} signaling pathway and the phosphatase PP2B in mediating the response. In contrast, NE uses predominantly the β-adrenergic receptor/cAMP signaling pathway and PP2A as the phosphatase in inducing the nuclear translocation of TORC2. Together our results indicate that the signaling pathways that cause translocation of TORC1 and TORC2 into the nucleus are not identical. This difference in the regulatory mechanism between TORC1 and TORC2 translocation in rat pinealocytes may account for the distinct time profiles of NE-stimulated nuclear localization of TORC1 and TORC2 with a more sustained response of TORC1. The duration of nuclear localization of TORC1 and TORC2 in turn may have an impact on their transcriptional activities on the large number of CREB target genes induced by NE in the rat pineal gland (25). In the case of *Aanat*, our working model is that although *Aanat* expression is upregulated through the NE/cAMP mechanism, to shape the time profile of induction, NE, through activation of α- and β-adrenergic receptors, also induces the translocation of the transcriptional coactivators, TORC1 and TORC2, to the nucleus to prolong the transcription of *Aanat* initiated by pCREB. These results also extend the previously demonstrated potentiating effect of α-adrenoceptor-mediated Ca^{2+} elevation on the β-adrenoceptor /cAMP stimulation of AA-NAT in the rat pineal gland (39, 40).

Earlier studies have suggested that both TORC1 and TORC2 can function as a Ca^{2+} and cAMP sensitive coincident detector (13, 17). In agreement with these studies, an increase in intracellular Ca^{2+} or cAMP can induce dephosphorylation of TORC1 and TORC2 in the rat pineal gland. However, studies on the nuclear localization of TORC1 and TORC2 indicate that the translocation of TORC1 can only be stimulated by Ca^{2+} elevation but not by cAMP; and the translocation of TORC2 can be stimulated only by cAMP but not by Ca^{2+} elevation. These results indicate that in rat pinealocytes, although the dephosphorylation of TORC may still serve as a Ca^{2+} and cAMP coincident detector, the translocation of TORC1 and of TORC2 to the nucleus is controlled by separate and independent signaling mechanisms. Equally important, our results also demonstrate that not all stimulated dephosphorylation of TORC1 or TORC2 can lead to nuclear localization and that the phosphorylation status alone may not reflect accurately the activation status of TORC. This raises the issue of whether TORC1 and TORC2 can function as a Ca^{2+}- and cAMP-sensitive coincident detector in all cell types. It also highlights the necessity of investigating the role of individual phosphorylation site on the TORC proteins, specifically one in which dephosphorylation is coupled with nuclear translocation and allows TORC to function as a transcription coactivator. In view of the importance of 14-3-3 proteins in the dynamics of dephosphorylation and nuclear translocation of TORC proteins (15, 16), the precise role 14-3-3 proteins play in this process merits further investigation.

Acknowledgments

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

This work was supported by a grant from the Canadian Institutes of Health Research.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH** 1993 Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855–859
2. **Gonzalez GA, Montminy MR** 1989 Cyclic AMP stimulates somatostatin gene transcription by phoshorylation of CREB at serine 133. Cell 59:675–680
3. **Mayr B, Montminy M** 2001 Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599–609
4. **Vo N, Goodman RH** 2001 CREB binding protein and p300 in transcriptional regulation. J Biol Chem 276:13505–13508
5. **Gau D, Lemberger T, von Gall C, Kretz O, Le Minh N, Gass P, Schmid W, Schibler U, Korf HW, Schütz G** 2002 Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock. Neuron 34:245–253
6. **Dwarki VJ, Montminy M, Verma IM** 1990 Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J 9:225–232
7. **Schumacher MA, Goodman RH, Brennan RG** 2000 The structure of a CREB bZIP-somatostatin CRE complex reveals the basis for selective dimerization and divalent cation-enhanced DNA binding. J Biol Chem 275:35242–35247
8. **Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M** 2003 TORCs: transducers of regulated CREB activity. Mol Cell 12:413–423
9. **Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M** 2005 The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 437:1109–1111
10. **Dentin R, Liu Y, Koo SH, Hedrick S, Vargas T, Heredia J, Yates 3rd**

J, Montminy M 2007 Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. Nature 449:366–369
11. **Altarejos JY, Montminy M** 2011 CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol 12:141–151
12. **Wang B, Goode J, Best J, Meltzer J, Schilman PE, Chen J, Garza D, Thomas JB, Montminy M** 2008 The insulin-regulated CREB coactivator TORC promotes stress resistance. Cell Metab 7:434–444
13. **Kovács KA, Steullet P, Steinmann M, Do KQ, Magistretti PJ, Halfon O, Cardinaux JR** 2007 TORC1 is a calcium- and cAMP-sensitive coincidence detector involved in hippocampal long-term synaptic plasticity. Proc Natl Acad Sci USA 104:4700–4705
14. **Liu Y, Coello AG, Grinevich V, Aguilera G** 2010 Involvement of transducer of regulated cAMP response element-binding protein activity on corticotrophin releasing hormone transcription. Endocrinology 151:1109–1118
15. **Bittinger MA, McWhinnie E, Meltzer J, Iourgenko V, Latario B, Liu X, Chen CH, Song C, Garza D, Labow M** 2004 Activation of cAMP response element-mediated gene expression by regulated nuclear transport of TORC proteins. Curr Biol 14:2156–2161
16. **Takemori H, Kajimura J, Okamoto M** 2007 TORC-SIK cascade regulates CREB activity through the basic leucine zipper domain. FEBS J 274:3202–3209
17. **Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S, Yates 3rd JR, Takemori H, Okamoto M, Montminy M** 2004 The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. Cell 119:61–74
18. **Klein DC, Berg GR, Weller J** 1970 Melatonin synthesis: adenosine 3′5′-monophosphate and norepinephrine stimulate *N*-acetyltransferase. Science 168:979–980
19. **Chik CL, Ho AK** 1989 Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. Prog Biophys Mol Biol 53:197–203
20. **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
21. **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
22. **Klein DC** 1985 Photoneural regulation of the mammalian pineal gland. Photoperiodism, melatonin and the pineal. Ciba Found Symp 117:38–56
23. **Maronde E, Schomerus C, Stehle JH, Korf HW** 1997 Control of CREB phosphorylation and its role for induction of melatonin synthesis in rat pinealocytes. Biol Cell 89:505–511
24. **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
25. **Bailey MJ, Coon SL, Carter DA, Humphries A, Kim JS, Shi Q, Gaildrat P, Morin F, Ganguly S, Hogenesch JB, Weller JL, Rath MF, Møller M, Baler R, Sugden D, Rangel ZG, Munson PJ, Klein DC** 2009 Night/day changes in pineal expression of >600 genes: Central role of adrenergic/cAMP signaling. J Biol Chem 284:7606–7622
26. **Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC** 1996 Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. Endocrinology 137:3033–3045
27. **Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Bégay V, Falcón 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 52:307–358
28. **Kanyo R, Amyotte N, McTague J, Chik CL, Ho AK** 2011 Adrenergic regulation of the distribution of transducer of regulated cAMP-response element-binding protein (TORC2) in rat pinealocytes. Endocrinology 152:3440–3450
29. **Kanyo R, Price DM, Chik CL, Ho AK** 2009 Salt-inducible kinase 1 in the rat pinealocyte: adrenergic regulation and role in arylalkylamine *N*-acetyltransferase gene transcription. Endocrinology 150: 4221–4230
30. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
31. **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
32. **Chik CL, Liu QY, Li B, Klein DC, Zylka M, Kim DS, Chin H, Karpinski E, Ho AK** 1997 α_{1D} L-type Ca^{2+}-channel currents: inhibition by a β-adrenergic agonist and pituitary adenylate cyclase-activating polypeptide (PACAP) in rat pinealocytes. J Neurochem 68:1078–1087
33. **Chik CL, Wloka MT, Price DM, Ho AK** 2007 The role of repressor proteins in the adrenergic induction of type II iodothyronine deiodinase in rat pinealocytes. Endocrinology 148:3523–3531
34. **Ganguly S, Weller JL, Ho A, Chemineau P, Malpaux B, Klein DC** 2005 Melatonin synthesis: 14-3-3-dependent activation and inhibition of arylalkylamine *N*-acetyltransferase mediated by phosphoserine-205. Proc Natl Acad Sci USA 102:1222–1227
35. **Price DM, Chik CL, Ho AK** 2004 Norepinephrine induction of mitogen-activated protein kinase phosphatase-1 expression in rat pinealocytes: distinct roles of α- and β-adrenergic receptors. Endocrinology 145:5723–5733
36. **Oliver CJ, Shenolikar S** 1998 Physiologic importance of protein phosphatase inhibitors. Front Biosci 3:D961–D972
37. **Ho AK, Chik CL** 1995 Phosphatase inhibitors potentiate adrenergic-stimulated cAMP and cGMP production in rat pinealocytes. Am J Physiol 268:E458–E466
38. **Letz B, Schomerus C, Maronde E, Korf HW, Korbmacher C** 1997 Stimulation of a nicotinic ACh receptor causes depolarization and activation of L-type $Ca2^+$ channels in rat pinealocytes. J Physiol 499:329–340
39. **Klein DC, Sugden D, Weller JL** 1983 Postsynaptic α-adrenergic receptors potentiate the β-adrenergic stimulation of pineal serotonin *N*-acetyltransferase. Proc Natl Acad Sci USA 80:599–603
40. **Yu L, Schaad NC, Klein DC** 1993 Calcium potentiates cyclic AMP stimulation of pineal arylalkylamine *N*-acetyltransferase. J Neurochem 60:1436–1443