University of Alberta

Regulation of Transducer of Regulated CREB 1 (TORC1) in the Rat Pineal Gland

by

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Abstract

The transducers of regulated cAMP-response element-binding protein (CREB)(TORC) are a family of co-activators that can enhance CREB-mediated gene transcription. Existing literature remains elusive of a detailed regulatory mechanism for specific TORC isoforms, and suggests dephosphorylation and nuclear accumulation are mediated through the same signalling pathways for all isoforms. Through the use of immunoblot analysis and nuclear/cytosolic fractionation we examined the norepinephrine stimulated signalling mechanism that mediates the dephosphorylation and nuclear accumulation of TORC1 in the rat pineal gland. This study reveals that the dephosphorylation and subsequent nuclear accumulation of TORC1 leading to its activation, is regulated through the α_1-adrenergic receptor causing an elevation of intracellular Ca^{2+} and the activation of protein phosphatase 2B. Once in the nucleus, TORC1 requires constant α_1- and β-adrenergic receptor activation to maintain the dephosphorylation and nuclear retention. Furthermore, we demonstrate that salt-inducible kinase 1 (SIK1) is not responsible for regulating the cellular distribution of TORC1.

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List of Abbreviations

5-HT, 5-hydroxtryptamine

AA-NAT, arylalkylamine-N-acetyltransferase

Ala, Alanine

bZIP, basic leucine zipper domain

CaMKII, calmodulin-dependent protein kinase

cAMP, cyclic adenosine monophosphate

CBP, CREB binding protein

cGMP, cyclic guanosine monophosphate

CIP, calf intestinal alkaline phosphatase

CRE, cAMP response element

CREB, cAMP response element binding protein

DAG, diacylglycerol

DBcAMP, dibutyryl cAMP

DBcGMP, dibutyryl cGMP

DMEM, Dulbecco's modified Eagle's medium

DMSO, dimethylsulfoxide

DTT, dithiothretol

EBSS, Earle's balanced salt solution

FCS, fetal calf serum

GABA, γ-amino butyric acid

GAM, goat anti-mouse antibody

GAR, goat anti-rabbit antibody

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

h, hour(s)

HIOMT, hydroxyindole-O-methyltransferase

HRP, horseradish peroxidise

IP_3, inositol triphosphate

ISO, isoproternol

KID, kinase inducible domain

min, minutes

NAS, N-acetylserotonin

NE, norepinephrine

PBS, phosphate buffered saline

PE, phenylephrine

PKA, cAMP dependent protein kinase

PKC, protein kinase C

PLC, phospholipase C

PMA, 4-beta-phorbol-12-myristate-13-acetate

PP2A, protein phosphatase 2A

PP2B, protein phosphatase 2B

PRAZ, prazosine

PROP, propanolol

PSF, penicillin-streptomycin-amphotericin B

PVN, paraventricular nucleus

RHT, retinohypothalamic tract

rpm, revolutions per minute

SCG, superior cervical ganglia

SCN, suprachiasmatic nucleus

SDS, sodium dodecyl sulfate

Ser, Serine

SIK, salt inducible kinase

TBS, Tris-Cl buffered saline

TORC, transducer of regulated CREB

TTBS, Tris-Cl buffered saline with Tween-20

wk, week

1. Introduction

1.1 Pineal gland biology

The pineal gland, a neuroendocrine organ is responsible for the production and secretion of the hormone melatonin. The main function of the rat pineal gland is to transform the circadian rhythm generated in the suprachiasmatic nucleus (SCN) into a rhythmic production of melatonin in the circulation (Klein 1985; Korf et al. 1998; Ganguly *et al.,* 2002). The tightly regulated daily rhythm of circulating melatonin closely reflects the environmental lighting condition with an increased production at night. Melatonin has been shown to play an important role in several aspects of circadian physiology (Simonneaux and Ribelayga, 2003)

By acting on both central and peripheral tissues, melatonin can have a variety of physiological effects (Simonneaux, 2011). The circulating melatonin rhythm is involved in defining seasonal information, which is of particular importance in the reproductive physiology of seasonal breeding animals (Lincoln & Hazlerigg, 2010; Korf *et al.*, 1998). In humans, melatonin has been shown to be involved in the treatment of jet lag and insomnia (Arendt *et al.*, 1995; Brown *et al.*, 2009; Zisapel*,* 2007). In addition, melatonin is currently under investigation for its involvement in many other disorders, including cancer (Bartsch & Bartsch, 2006; Blask *et al*., 2002; Jung & Ahmad, 2006).

The nocturnal release of norepinephrine (NE) from the sympathetic neurons that innervate the pineal gland is responsible for driving the nightly increase in melatonin synthesis (Klein *et al.,* 1991). The master clock located in the SCN of the hypothalamus is responsible for controlling the nightly release of NE. The release of NE

stimulates the pinealocytes to produce arylalkylamine-N-acetyltransferase (AA-NAT), which is the rhythm generating enzyme in the synthesis of melatonin.

A multi-synaptic pathway innervating the pineal gland involving the retina, SCN, paraventricular nucleus (PVN), and superior cervical ganglion (SCG) is responsible for transforming environmental lighting conditions into the controlled release of NE (FIG. 1.1)(Klein *et al.,* 1983, Moore & Klein*,* 1974). The circadian rhythm of melatonin production and release are blocked with bilateral lesions to the SCN, suggesting the importance of the SCN as the generator of the daily melatonin rhythm (Kalsbeek *et al.*, 2000). In addition, it has been shown that the genetically driven clock present in the neurons of the SCN can be reset by light stimuli (Welsh *et al.,* 1995). Environmental light stimuli received by the retina is transmitted as a neuronal signal to the SCN through the retinohypthalamic tract (RHT). Input from the RHT results in synchronization of the 24 h clock of the SCN with the environmental lighting conditions (Arendt *et al*., 1995; Wehr, 1991). The synchronization of the pineal gland with the daily light and dark cycles is important for specific diurnal gene expression and hormone output.

The circadian rhythm generated in the SCN is transmitted to the PVN, intermediolateral nucleus (IML), and ultimately leads to the release of NE from the sympathetic neurons of the SCG. A neurotransmission cascade ensures the release of NE is only occurring at the desired time. Neurons of the PVN, receive both glutamatergic (stimulatory) and GABAergic (inhibitory) inputs from the SCN (Buigs *et al.,* 2003, Teclemariam-Mesbah *et al.*, 1999). Under normal physiological circumstances,

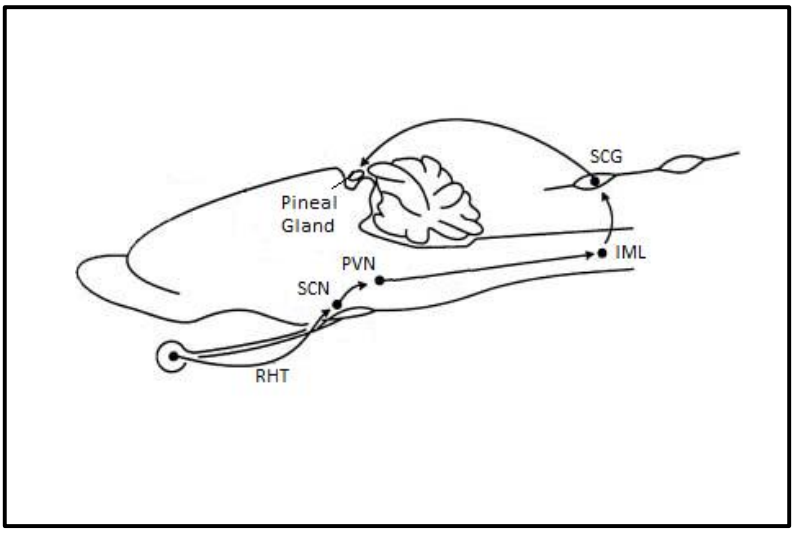

FIG. 1.1. Neuronal circuitry involved in regulating pineal gland function. The daily melatonin production from the pineal gland is modulated through changes in environmental lighting conditions. Environmental light stimuli received by the retina is transmitted as a neuronal stimulus through the retinohypothalmic tract (RHT) to the suprachiasmatic nucleus (SCN). Circadian signals from the SCN are transmitted to the paraventricular nuclei (PVN), then to the preganglionic sympathetic intermediolateral nucleus of the spinal cord, and lastly to the norephinephrine releasing neurons of the superior cervical ganglion (SCG). Figure modified from Ganguly *et al.,* 2002.

the pineal gland does not produce melatonin during the daytime. In the presence of light, the SCN releases both glutamate and GABA onto the neurons of the PVN. The inhibitory action from GABA on the PVN is able to compensate for the stimulatory action of glutamate, preventing the stimulation of the preganglionic neurons of the IML; resulting in the suppression of melatonin production (Kannan *et al.*, 1989; Kalsbeek *et al.*, 2000). At night or in the absence of light, the release of GABA from the SCN is prevented. This allows for the glutamate being released from the SCN to stimulate the PVN, which in turn initiates the release of NE from the SCG, stimulating the synthesis of melatonin in the pineal gland. Together, this series of neuronal interactions and signalling, results in the daily rhythm of melatonin production and secretion from the pineal gland.

1.2 Melatonin synthesis in the pineal gland

In the pineal gland, the nightly release of melatonin is regulated by the enzyme AA-NAT (Klein *et al.*, 1970; Klein, 1985). AA-NAT is the rate-limiting enzyme in the daily production of melatonin and is responsible for driving the daily melatonin rhythm. At night, NE stimulation leads to the induction of *Aa-nat* gene expression. This induction results in elevated levels of AA-NAT protein and activity. AA-NAT causes the acetylation of 5-hydroxytryptamine (5-HT or serotonin), resulting in an increase in the melatonin precursor, N-acetylserotonin (NAS)(FIG. 1.2)(Ganguly *et al.*, 2002; Klein & Weller, 1970). NAS is methylated by the enzyme hydroxyindole-O-methyltransferase (HIOMT) producing melatonin. There is limited variation in HIOMT activity throughout the day,

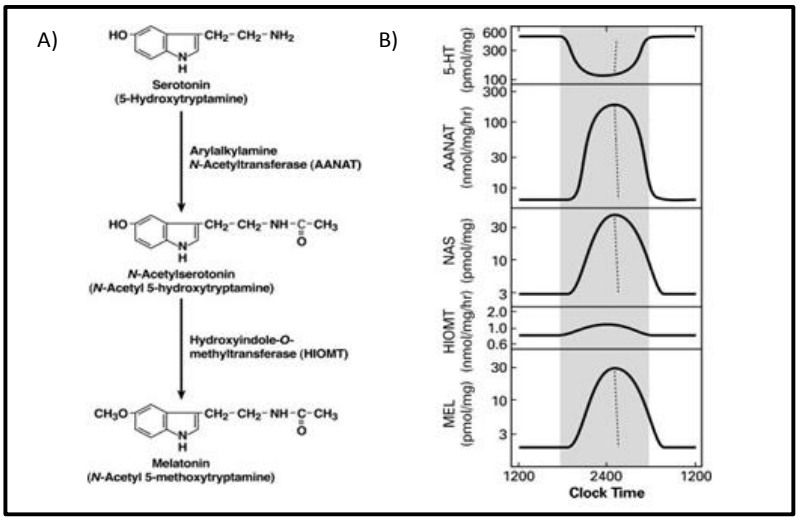

FIG. 1.2. Melatonin production pathway in the pineal gland. Arylalkylamine-N-acetyltransferase (AANAT) is the rate-limiting enzyme in the daily production of melatonin and is responsible for driving the daily melatonin rhythm. A) AA-NAT causes the acetylation of serotonin (5-hydroxtryptamine, 5-HT) into the melatonin precursor, N-acetylserotonin (NAS), which is then methylated by hydroxindole-O-methyltransferase (HIOMT) to produce melatonin. B) AA-NAT activity shows a significant daily rhythm, with a nocturnal increase in AA-NAT activity, NAS quantity, and melatonin production. HIOMT activity does not display any significant daily rhythm. Figure modified from Ganguly *et al.*, 2002.

suggesting it is constitutively active; therefore, melatonin production is regulated by AA-NAT activity and the availability of NAS to HIOMT (Klein & Lines, 1969, Klein *et al.,* 1997).

1.3 Signal transduction in the pineal gland

At night, NE released from the SCG causes the activation of α_1- and β-adrenergic receptors present in the pineal gland (Chik & Ho, 1989; Ganguly *et al.*, 2002)(FIG. 1.3). Activation of these G-protein coupled receptors leads to the initiation of two distinct signalling cascades. Initiation of these signalling pathways causes the elevation of intracellular second messengers, which leads to the activation of downstream proteins.

The binding of NE to the β-adrenergic receptor leads to the activation of the Gs signalling cascade. GTP becomes bound to the Gα-subunit of the G-protein, allowing the Gα-subunit to dissociate from the remaining Gβγ-protein dimer and the β-adrenergic receptor (Klein, 1985). The dissociated Gα-subunit is able to interact with the membrane bound protein, adenylyl cyclase, leading to its activation. Activated adenylyl cyclase catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The stimulation of the β-adrenergic receptor with NE, leads to a substantial increase in intracellular cAMP levels, due to adenylyl cyclase activity (Klein, 1985).

The interaction of NE with the α_1-adrenergic receptor leads to an elevation in intracellular Ca^{2+} and the activation of the Gq signalling cascade (Chik & Ho, 1989). Activated Gq-protein can directly activate phospholipase C, a membrane bound enzyme that cleaves phosphatidylinositol 4,5-bisphosphate to produce inositol triphosphate (IP_3) and diacylglycerol (DAG)(Ho *et al.*, 1988a; 1988b). Intracellular Ca^{2+} levels are elevated through the binding of IP_3 to the ligand-gated Ca^{2+} channels present on the endoplasmic

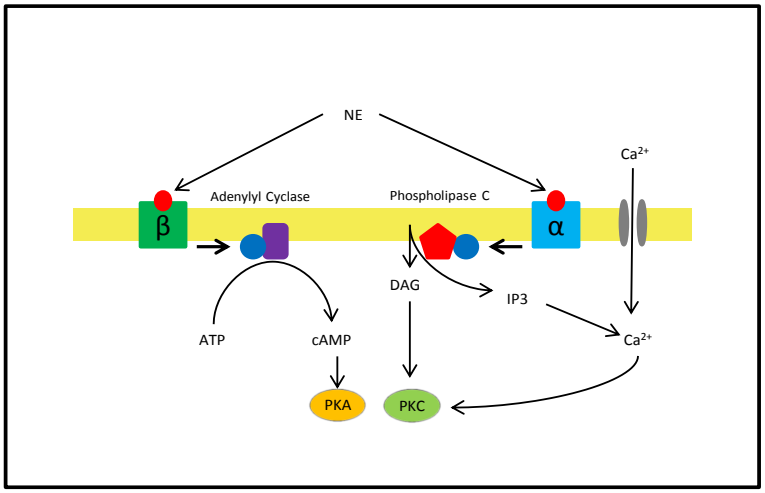

FIG. 1.3. Adrenergic signal transduction in the pineal gland. Nocturnal release of NE from the SCG causes the activation of α_1- and β-adrenergic G-protein coupled receptors present in the pineal gland. NE binding to the β-adrenergic receptor activates the Gs-protein signalling cascade, leading to the activation of adenylyl cyclase and resultant elevation in cAMP. cAMP can then lead to the activation of protein kinase A (PKA). NE binding to the α-adrenergic receptor activates the Gq-protein signalling cascade, leading to the activation of phospholipase C and resultant elevation of diacylglycerol (DAG) and inositol triphoshphate (IP_3). In addition, intracellular Ca^{2+} is elevated through the opening of Ca^{2+} channels and the depletion of intracellular Ca^{2+} stores. The elevation in DAG and intracellular Ca^{2+} lead to the activation of protein kinase C (PKC).

reticulum (ER). This interaction, results in the release of Ca^{2+} from the intracellular Ca^{2+} stores of the ER leading to the activation of Ca^{2+} channels located on the plasma membrane (Sugden, 1987; Korf, 1997). The elevation in intracellular Ca^{2+} and DAG lead to the activation of protein kinase C (PKC)(Chik & Ho, 1989; Sugden 1987)

Although stimulation of the α_1-adrenergic receptor alone is not sufficient to activate adenylyl cyclase, the downstream activation of PKC can potentiate the cAMP response (Klein 1985; Sugden & Klein, 1988; Vanecek *et al.*, 1985). PKC phosphorylates both adenylyl cyclase and the Gsα-subunit. GTPase converts the bound GTP to GDP, causing a conformational change in Gsα-subunit leading to its dissociation from adenylyl cyclase. Phosphorylation of the G-protein by PKC reduces the inherent GTPase catalytic activity, allowing the Gsα-subunit to maintain its interaction with adenylyl cyclase and sustain the production of cAMP (FIG. 1.4). Also, the direct phosphorylation of adenylyl cyclase by PKC will enhance the duration of adenylyl cyclase activity. Together these two phosphorylation events by PKC potentiate the cAMP signal initiated by the β-adrenergic receptor.

Activation of the α_1- and β-adrenergic receptor signalling cascades have been shown to produce a 100-fold increase in cAMP levels, which can cause the activation of protein kinase A (PKA)(Klein, 1985). PKA in combination with other mediators is responsible for initiating the transcription of *Aa-nat* (Baler *et al.*, 1997; Klein & Berg 1970; Klein *et al.*, 1970; Roseboom *et al.*, 1995; Roseboom *et al.* 1996).

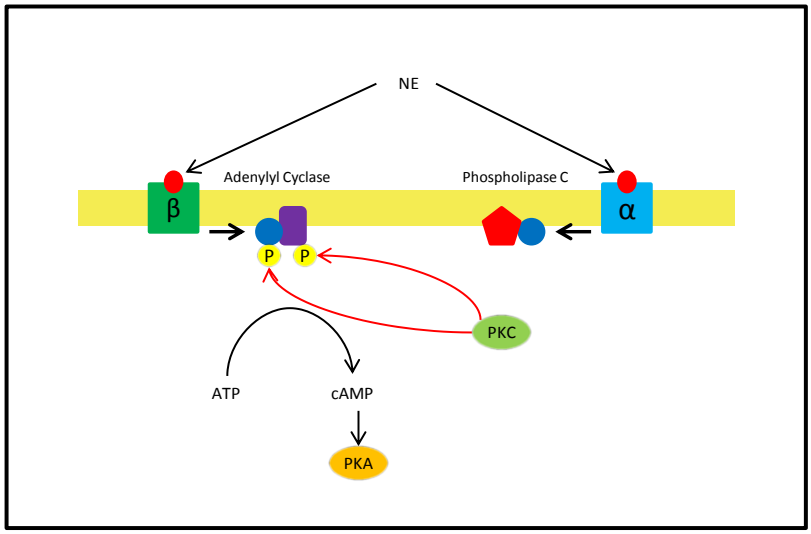

FIG. 1.4. Potentiation of cAMP production by PKC. PKC is activated through the interaction of NE with the α_1-adrenergic receptor. PKC activity potentiates the production of cAMP, through phosphorylating adenylyl cyclase and the Gsα-protein. This phosphorylation maintains the activity of adenylyl cyclase, resulting in a potentiation of cAMP production. The elevated levels of cAMP, lead to cAMP interacting with the regulatory subunit of PKA. This interaction leads to a conformational change, allowing the liberation of the active catalytic subunit from the regulatory subunit.

1.4 CREB-mediated gene transcription

Elevated levels of cAMP caused by the nightly release of NE from sympathetic nerves leads to the activation of PKA (Gonzalez & Montminy, 1989). PKA is a heterotetrameric protein composed of two regulatory and two catalytic subunits. Under basal conditions, the heterotetramer remains intact in the cytoplasm and catalytically inactive. Upon elevation in cAMP levels, cAMP is able to bind to the two corresponding binding sites on the regulatory subunits; resulting in a conformational change and the dissociation of the catalytic subunits. Released catalytic subunits are able to migrate to the nucleus and phosphorylate the cAMP response element-binding protein (CREB) on Ser-133, initiating the transcription of *Aa-nat* (Gonzalez & Montiminy, 1989; Roseboom & Klein, 1995). The nuclear entry of the catalytic PKA subunit appears to be the rate-limiting step in CREB phosphorylation (Hagiwara *et al.* 1993). The activation of CREB-mediated gene transcription leads to a >100-fold increase in *Aa-nat* mRNA, while also leading to the induction of over 600 other genes in the rat pineal gland (Bailey *et al*., 2009; Roseboom *et al.,* 1996).

Phosphorylated CREB is able to activate target gene transcription by binding as a dimer to the conserved cAMP response element (CRE)(TGACGTCA) in the target gene promoter(Comb *et al*., 1986*;* Montminy *et al.*, 1986) Once bound, phosphorylated CREB is able to recruit the binding of the key transcription factor, CREB binding protein (CBP) (Chrivia *et al.,* 1993; Kwok *et al.,*1994; Arias *et al.,*1994)(FIG. 1.5). CBP contains inherent histone acetyltransferases properties. Negatively charged DNA remains tightly bound around positively charged histone proteins. When DNA is bound to histones, transcription factors cannot access the DNA. CBP transfers an acetyl group to the

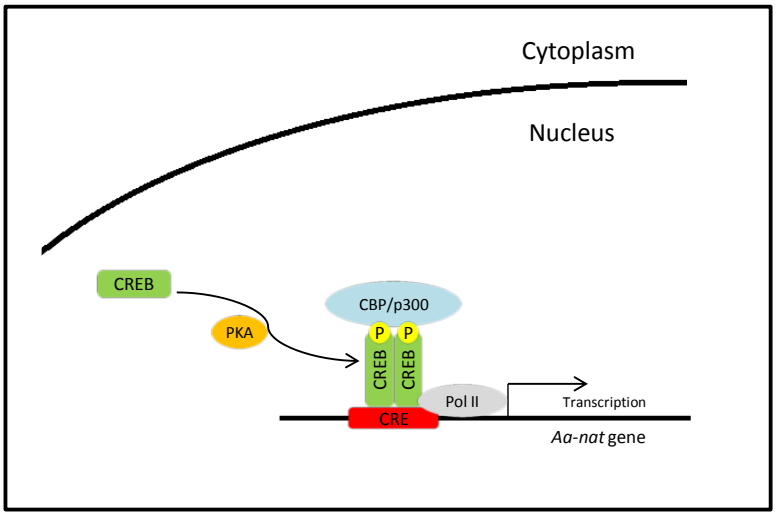

FIG. 1.5. cAMP response element binding protein (CREB) mediated gene transcription. The catalytic subunit of PKA translocates into the nucleus, where it can phosphorylate CREB on S133. Phosphorylated CREB binds to the cAMP-response element (CRE) region of the *Aa-nat* promotor, leading to the recruitment of CREB binding protein (CBP). The transcription factor CBP can also cause the recruitment of other transcription factors, such as, RNA polymerase II; leading to an active transcriptional complex.

positively charged histones leading to a neutralization of the positive charge, reducing the affinity between the histone and DNA. Once acetylated, the DNA becomes loosely bound, allowing transcription factors to access the DNA and initiate gene transcription (Brownell *et al*., 1996).

1.5 Regulation of CREB-mediated gene transcription

The ubiquitously expressed CREB family of proteins is composed of CREB, CRE modulator (CREM), and activating transcription factor 1(ATF-1). CREB and its family share similar domain organization. The following CREB functional domains have been shown to be important in CREB's transcriptional activity: the kinase-inducible domain (KID), the glutamine-rich consistitutively active Q2 domain, and the basic leucine zipper domain (bZIP) (Brindle, 1993; Mayr & Montminy, 2001; Quinn, 1993).

The constitutively active Q2 domain is able to enhance target gene transcription through an interaction with components of the basal transcription factor IID (TAFIID)(Ferreri *et al.* 1994; Nakajima *et al.,* 1997). Interaction with TAFIID is important in stabilizing the basic transcriptional machinery. Studies confirming the functional role of the Q2 domain showed that cAMP agonists were not able to activate CREB activity in cells containing a knockdown of a component of TAFIID (Mengus *et al.,* 1998). Furthermore, removal of the Q2 domain results in CREB acting as a repressor. The Q2 domain has been shown to be essential in the activation of CREB-mediated transcription by promoting assembly of the transcriptional complex (Felinski, 2001).

In contrast to the Q2 domain, the activity of the KID domain is phosphorylation-dependent. Activation of the KID domain occurs through the phosphorylation of Ser-133 by PKA, which is currently accepted as the key event in the regulation of CREB

activity (Gonzalez & Montminy, 1989; Hagiwara *et al.*, 1993; Mayr & Montminy, 2001). In the PC12 cell line that is deficient in PKA, CREB phosphorylation through elevation in cAMP is abolished (Gonzalez & Montminy, 1989). The phosphorylation of Ser-133 in the KID domain leads to the recruitment of CBP (Chrivia *et al*., 1993; Parker *et al.*, 1996). Mutagenesis studies show that replacement of Ser-133 with Ala eliminates the transcriptional activation ability of CREB (Gonzalez & Montminy, 1989). CBP activity has been shown to facilitate the activation of the RNA-polymerase II complex (Kee et al., 1996; Kim & Maniatis, 1998). Together, the functional role of the Q2 domain leads to the assembly of the transcriptional machinery, with the phosphorylation of the KID domain facilitating the activation of the RNA-polymerase II complex.

Phosphorylation of CREB at Ser-133 by non-cAMP signals does not lead to the recruitment of CBP, resulting in no induction of CREB activity (Mayr *et al.*, 2001; Ravnskjaer,*et al.* 2007; Wagner *et al*., 2000). CBP recruitment is able to decipher between cAMP-induced and non-cAMP-induced CREB phosphorylation (Mayr, 2001). PKC-mediated Ser-133 phoshphorylation through the use of the specific PKC activator, phorbol 12-myristate 13-acetate (PMA), did not promote CBP recruitment or initiate CREB activity. It is thought that further accessory phosphorylation sites present in the KID domain are responsible for modulating CREB activity with different signal inputs (Altarejos & Montminy, 2011). Studies in support of this idea have shown that phosphorylation of Ser-142 of the KID domain by calmodulin-dependent kinase II (CaMKII) can block the CREB-CBP interaction, preventing the expression of CREB-meditated gene transcription (Sun *et al.*, 1994). The tight regulation involved in the activation of CREB ensures that CREB-mediated gene transcription only occurs with the specific signal activation. In regard to *Aa-nat*, this control mechanism ensures that

expression of *Aa-nat* occurs only during the night in response to elevation of cAMP caused by the release of NE from the sympathetic nerve terminals.

In addition to the constitutively active Q2 domain and the inducible KID domain, the bZIP domain of CREB provides an additional level of control in the regulation of CREB-mediated gene transcription (Carlezon *et al.*, 2005; Mayr & Montminy, 2001). The bZIP domain promotes dimerization and binding of the CRE promoter (Schumacher *et al.*, 2000). In addition, the transducers of regulated CREB (TORC) are a family of CREB specific co-activators that are able to interact with the bZIP domain of CREB, resulting in the potentiation of CREB-activity (Conkright *et al.,* 2003).

1.6 Transducer of regulated CREB

The TORC family of proteins, consisting of three members (TORC1, TORC2, and TORC3), have been shown to function as specific CREB transcriptional co-activators (Conkright *et al.,* 2003). The use of high-throughput screens for proteins capable of potentiating a CRE-luciferase reporter, led to the discovery of TORC (Conkright *et al.,* 2003; Iourgenko *et al.,* 2003). TORC proteins are evolutionary conserved, with TORC homologues identified in *Drosophila melanogaster* and *Caenorhabditis elegans* (Wang *et al.,*2008; Mair *et al.,* 2011). The TORC isoforms show distinct expression in different tissues. The expression of TORC2 is ubiquitous, whereas TORC1 is detected mainly in the brain (Conkright *et al.,* 2003). Studies investigating the physiological significance of TORC have shown their involvement in glucose homeostasis and hippocampal long-term synaptic plasticity (Dentin *et al.*, 2007; Dentin *et al.*, 2008; Koo *et al.*, 2005; Kovacs *et al.*, Liu *et al.*, 2008; Screaton *et al.*, 2004; Zhou *et al.*, 2006).

All TORC family members share a similar structural organization (Altarejos & Montminy, 2011). TORC proteins contain a conserved coiled-coil N-terminal domain, which interacts with the bZIP domain of CREB (Conkright *et al*., 2003). A gene reporter assay study involving TORC segments fused to the GAL4 DNA-binding region, were tested for their induction of a minimal promoter linked to GAL4 binding sites (Iourgenko *et al*., 2003). This study determined that TORC possesses a C-terminal transactivation domain. Lastly, TORC contains a central regulatory domain, which is the region where post-translational modification may occur.

In the nucleus, TORC can interact with the bZIP domain of CREB, independent of Ser-133 phosphorylation (Takemori & Okamoto, 2008). This interaction results in enhanced association of TAFII with CREB (Conkright *et al*., 2003). In addition, TORC is able to interact directly with CBP, leading to the stabilization of CBP with the CRE promoter (FIG. 1.6)(Ravnskjaer *et al.*, 2007; Xu *et al*., 2007) Together these result in TORC potentiating CREB-mediated transcription.

1.7 Transducer of regulated CREB regulation

Under basal conditions, TORC is thought to remain sequestered in the cytoplasm, through the phosphorylation-dependent interaction with cytoplasmic scaffolding protein, 14-3-3 (Bittinger *et al.,* 2004; Screaton *et al.,* 2004). Upon activation, TORC becomes dephosphorylated causing its dissociation from 14-3-3, which leads to its nuclear translocation. The nuclear localization of TORC is required for TORC to interact with the bZIP domain of CREB and perform its co-activator function.

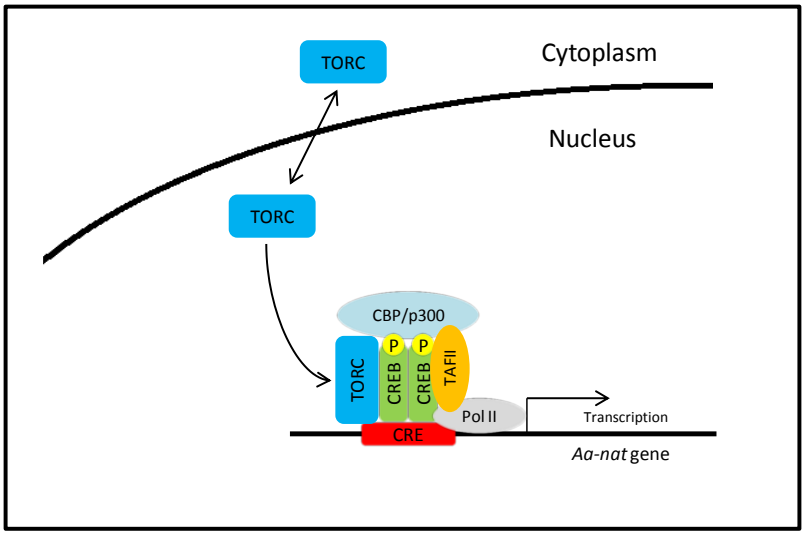

FIG. 1.6. Transducer of regulated CREB role in CREB-mediated gene transcription. Under basal conditions TORC1 resides in the cytoplasm. Upon activation, TORC1 is able to enter the nucleus and bind to the basic leucine zipper (bZIP) domain of CREB. This interaction enhances the interaction between CREB and other transcription factors, in particular CBP. Together, this results in TORC1 potentiating CREB-mediated gene transcription.

Salt inducible kinase(SIK), a member of the sucrose non-fermenting (SNF1)/adenosine monophosphate activated protein kinase (AMPK) family, has been shown to be important in the regulation of metabolism under energy stress (Wang *et al.*, 1999; Hardie, 2007). SIK1, which was originally isolated from the adrenal glands of rats feed a high salt diet (Wang *et al.*, 1999), has been shown to be nocturnally activated and induced by cAMP in the rat pineal gland (Bailey *et al.,* 2009; Kanyo *et al.,* 2009). SIK1 is thought to be the key mediator controlling the regulation of TORC (Takemori *et al*., 2007; Katoh *et al*., 2006).

It has been assumed that different TORC isoforms share a similar regulatory mechanism (Takemori *et al*., 2007; Takemori & Okamoto, 2008). Under basal conditions, TORC is phosphorylated by SIK. Upon stimulation, resulting in the elevation of either cAMP or intracellular Ca^{2+}, TORC becomes dephosphorylated. The sensitivity of TORC towards cAMP and intracellular Ca^{2+} signals suggest that TORC may serve as a coincident detector for these two signals (Kovacs *et al*., 2007; Screaton*, et al*., 2004). Activation of PKA through the elevation of cAMP contributes to TORC activation by the inhibition of SIK1 through the PKA-mediated phosphorylation of SIK1 at Ser-577 (Katoh *et al.,* 2004), whereas elevated intracellular Ca^{2+} causes activation of TORC through calcineurin mediated dephosphorylation, allowing its dissociation from 14-3-3 and migration into the nucleus (Bittinger, *et al.*, 2004). Once in the nucleus, it can interact with the bZIP domain of CREB, and potentiate target gene transcription.

1.8 Aim of the study

As indicated in the background, the pineal gland is an excellent model to study adrenergic regulated rhythmic gene transcription. The amplitude and duration of gene expression can be important in regulating the rhythm of specific hormones in the body. In the case of the rhythm generating enzyme *Aa-nat*, its transcription is predominantly stimulated through the elevation of cAMP by stimulation of the β-adrenergic receptor (Roseboom & Klein, 1995). However, other signalling pathways that are activated through NE also participate in modulating the amplitude and duration of *Aa-nat* expression, through their effects on CREB transcription factors and co-activators (Ho & Chik, 2010).

One specific CREB co-activator of interest is TORC, which can enhance the rate and amplitude of CREB-mediated gene transcription. Investigation of TORC2 in the rat pineal gland showed that the phosphorylation status and cellular distribution of TORC2 was regulated by NE (Kanyo *et al.*, 2011). In pinealocyte culture, the activation of the β-adrenergic receptor/cAMP pathway, leads to the dephosphorylation and subsequent nuclear accumulation of TORC2. Dephosphorylation and nuclear translocation were seen with the elevation of cAMP; however, activation of the α_1-adrenergic receptor leading to an elevation in intracellular Ca^{2+} or elevation in intracellular Ca^{2+} with a depolarizing concentration of KCl did not lead to the dephosphorylation and nuclear translocation of TORC2. In addition, protein phosphatase 2 A (PP2A) was shown to be the key phosphatase involved in the NE-mediated dephosphorylation of TORC2. These results contrast with the current regulatory dogma, since only elevation in cAMP, and not intracellular Ca^{2+}, was responsible for the activation of TORC2. Moreover, in view of

the observation that overexpression of TORC2 led to a notable elevation in the NE-mediated *Aa-nat* transcription, whether TORC1 is involved in enhancing the CREB mediated transcription of *Aa-nat,* merits investigation (Kanyo *et al.,* 2011).

Existing literature defining the regulation of TORC1 remains elusive of a detailed regulatory mechanism. TORC1 is the main isoform expressed in the brain. Therefore, the specific aim of this study is to determine if TORC1 is present in the rat pineal and resolve the mechanism involved in regulating TORC1. The multiple interacting signal cascades initiated by NE in the rat pineal gland have been well established. Through the use of various pharmacological tools, this study provides insight into the detailed regulatory mechanism of TORC1 in the rat pineal gland.

2. Materials and Methods

2.1 Materials

Monoclonal antiglyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody was obtained from Ambion, INC. (Austin, TX). Calyculin A (CALY A), cyclosporine A (CSA), okadaic acid (OKA), polyclonal anti-TORC1 antibody, and tautomycin (TAU) were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Monoclonal anti-CREB and phosphor-CREB (Ser 133) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal anti-AA-$NAT_{25\text{-}200}$ (AB3314) antibody was a gift from Dr. D.C. Klein (National Institute of Child Health and Human Development, NIH, Bethesda, MD). Horse radish peroxidise (HRP)-linked goat anti-rabbit (GAR) and HRP-linked goat anti-mouse (GAM) antibodies were obtained from Chemicon Corp. (Temecula, CA). Adeno-X rapid titer kit was obtained from Clontech (Mountain View, CA). Entry vectors and destination vectors were obtained from Invitrogen (Burlington, ON). DYEnamic ET Terminator Cycle Sequencing Kit was obtained from GE Healthcare (Buckinghamshire, UK). Qiagen Quantitec reverse transcriptase kit obtained from Qiagen Inc. (Valencia, CA). Polyclonal anti-MKP-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dibutyryl cAMP (DBcAMP), dibutyryl cGMP (DBcGMP), GenElute Plasmid Miniprep Kit, isoproterenol (ISO), norepinephrine (NE), phenylephrine (PE), 4β phorbol 12-myristate 13-acetate (PMA), prazosin (PZ), propranolol (PRO) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Papain dissociation kit was obtained from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals used were of the purest grades commercially available.

2.2 Animal handling and pineal gland isolation

Procedures were reviewed and approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Sprague Dawley Rats (male, weighing 150 g) were obtained from the University of Alberta animal unit. For pinealocyte culture, animals were housed under a lighting regimen consisting of 12 h of light every 24 h; 12 animals were sacrificed 3 h after the onset of light. The pineal glands were removed and placed in ice-cold Dulbecco's modified Eagle's medium (DMEM) with Ca^{2+}/Mg^{2+} until enzymatic digestion. Pinealocyte culture yield was approximately 7 x 10^5 cells per gland. For whole gland studies to determine the nocturnal expression of TORC1, rats were conditioned for 1 wk under a lighting regimen of 14 h light every 24 h. After conditioning, groups of animals were sacrificed at various times throughout the day. Under a dim red light, pineal glands were collected and extra tissue was removed in ice-cold PBS (phosphate buffered saline). The glands were flash frozen on dry ice, and stored at -80 ^{0}C until preparation for RNA extraction or Western blot analysis.

2.3 Preparation of cultured pinealocytes and drug treatment

Cultured pinealocytes were prepared by papain dissociation of freshly dissected rat pineal glands. The glands were cleaned in ice-cold DMEM with Ca^{2+}/Mg^{2+} to remove excess tissue, and then washed several times with ice-cold PBS, pH 7.2. The glands were treated with 0.005% DNase and 20units/mL papain (Worthington Biochemical Corp., Lakewood, NJ) in Earle's balanced salt solution (EBSS) and incubated at 37 ^{o}C for 40 min under 95% air and 5% CO_2. After the digestion, fetal calf serum (FCS) was added to

terminate the reaction and glands were collected by centrifugation (10 min, 1300 x g). Glands were further dissociated by resuspension and pipetting in pineal media (DMEM + 10 % FCS + 0.01 % ascorbic acid + 1% penicilin, streptomycin, and amphotericin (PSF)) containing 0.005% DNase. The digested glands were pelleted (10 min, 1300 x g), then resuspended in EBSS containing albumin ovomucoid inhibitor (Worthington Biochemical Corp) and 0.005% DNase. Tissue contaminants were removed by layering the suspension over an albumin ovomuciod inhibitor solution. The layered gradient was centrifuged (8 min, 500 x g) to collect the pinealocytes. Cells were washed thoroughly with pineal media and counted with a hemocytometer. Cells were then resuspended in pineal media and maintained at 37 ^{o}C for 18 h under 95% air and 5% CO_2 prior to use.

Cell aliquots were treated for the times indicated with drugs prepared in concentrated solutions of water or dimethylsulfoxide (DMSO). Treated cells were collected by centrifugation (2 min, 6000 x g). Samples for Western blot analysis were boiled for 8 min in 1X sample buffer solution and stored at 4 ^{o}C until analysis. Samples for total RNA isolation were homogenized in Trizol.

Cells subjected to adenoviral transfection, were transduced with titered adenoviral constructs encoding shRNA or full-length *Sik1* according to a previously developed protocol for rat pinealocytes (Ho, 2007). Adenovirus was added directly to pinealocytes suspended in pineal medium. Samples were incubated at 37 ^{o}C for 24 h under 95% air and 5% CO_2, and then washed with fresh pineal media. Samples were incubated overnight prior to being treated with drugs as indicated.

2.4 Design and construction of *Sik1* full-length overexpression adenovirus

The full-length *Sik1* cDNA sequence was obtained from the National Center for Biotechnology Information website (*Sik1*; accession no. NM-021693). Cloning primers flanking the *Sik1* coding region were designed using Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA; http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The *Sik1* forward cloning primer: 5' – CAT GGT GAT CAT GTC GGA GT –3'; and *Sik1* reverse cloning primer: 5' – TTG CTT GGA AGA GTC CAT – 3' CC were used to amplify full-length *Sik1* from a cDNA collection prepared from NE-treated pinealocytes. For PCR amplification, the cycling regimen was as follows: denaturing at 93 ^{o}C for 30 sec, annealing at 58 ^{o}C for 15 sec, and extension at 72 ^{o}C for 3 min. The polymerases utilized for the reaction was a mixture of *Thermus aquaticus* (*Taq*) and *Pyrococcus furiosis* (*Pfu*) enzymes at a ratio of 10:1. The amplification took place for 2 sets of 12 cycles with fresh enzyme added between sets. The initial denaturing took place for 3.5 min and the final extension occurred for 8 min. PCR amplified *Sik1* was subjected to electrophoresis separation on an agarose ethidium bromide gel. A band corresponding to *Sik1*'s expected mobility was removed from the gel. The excised fragment was gel-purified using the Qiaex II gel purification kit (Qiagen). The purified product was verified for size on an agarose ethidium bromide gel.

The purified *Sik1* product was ligated into the pCR8/GW/TOPO vector as per the manufacturer's instructions, and the resultant vector was used to transform DH5α *Escherichia coli* (E. coli) cells. The transformed cells were grown overnight at 37 ^{o}C on agar plates containing 100 μg/mL spectinomycin. Positive colonies were selected and placed in luria broth (LB) containing spectinomycin (100 μg/mL), and were shaken at 225

rpm for 18 h at 37 ^{o}C. GenElute Plasmid Miniprep Kit (Sigma) was utilized to harvest the plasmid from the transformed cells. Purified plasmid was quantified with a spectrophotometer, then sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare). Inserts containing the correct sequence were recombined into the pAd/CMV/V5-DEST vector using GATEWAY LR Clonase (Invitrogen), then transformed into DH5α cells and grown overnight at 37 ^{o}C on agar plates containing 100 μg/mL ampicillin. Colonies were selected and shaken at 225 rpm for 18 h at 37 ^{0}C. The resulting plasmids were harvested using GenElute Plasmid Miniprep Kit and quantified. The linear adenoviral genomic fraction was liberated from the vector using PacI (New England Biolabs, Ipswich, MA) digestion. This digested product was confirmed on an agarose ethidium bromide gel. Lipofectamine 2000 (Invitrogen) was combined with the linear adenoviral fraction in Opti-MEM I medium (Gibco) and allowed to incubate for 20 min at room temperature. The mixture was added directly to 293A human embryonic kidney (HEK) cells incubating in HEK media (DMEM + 10 % FCS + 1 % non-essential amino acids (NEAA) + 10 mM sodium pyruvate) without PSF, and allowed to incubate overnight at 37 ^{o}C under 95% air and 5% CO_2. The media was changed to HEK media containing PSF, and allowed to incubate at 37 ^{o}C under 95% air and 5% CO_2 until adenovirus was ready for harvest. Adenovirus was titered using Adeno-X^{TM} Rapid Titer Kit (Clontech, Mountain View, CA) and stored at -80 ^{o}C .

2.5 Design and construction of *Sik1* short hairpin RNA (shRNA) - expressing adenovirus

Small interfering RNA (siRNA) targets were designed using an online shRNA (short hairpin) design utility site (Invitrogen), by submitting the full-length rat *Sik1* cDNA

sequence (*Sik1*; accession no. NM-021693). The *Sik1* shRNA target used for this study was 5' – GGA TAC GTC TCT CAC TCA AGG – 3' (1726–1747). Single-stranded shRNA DNA oligonucleotide sequences were synthesized by Sigma's oligonucleotide synthesis service. The oligonucleotides were annealed to form a double stranded segment. The double stranded oligonucleotide was combined with pENTR/U6 RNAi vector (Invitrogen) and T4 DNA ligase; the ligation reaction was incubated for 60 min at room temperature. The remainder of the shRNA generation took place as previously described for the full-length; with the following modifications incorporated in the full-length adenovirus generation procedure. Bacterial transformations using the entry vector were grown on agar plates containing 50 µg/mL kanamycin and the pAd/BLOCK-iT DEST (Invitrogen) destination vector was used.

2.6 Western Blotting

Cell pellets were lysed in 1X sample buffer (2X Sample Buffer (Sigma) and Buffer A, containing 20 mM Tris-HCl, 2 mM EDTA, 0.5mM EGTA, 2 mM phenylmethylsulfonylfluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM sodium vanadate, and 1 mM sodium fluoride, pH 7.5). Samples were then boiled for 8 min, and stored at 4 ^{o}C until analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the procedure of Laemmli (Laemmli, 1970; Price *et al*., 2004) using 9% acrylamide and 1mg/mL sodium dodecyl sulphate (Mini-Protein II gel systems, Bio-Rad Laboratories, Inc., Hercules, CA). Electrophersis was performed using 170 V for 1 h in electrophoresis buffer (25 mM Tris-HCl, 190 mM glycine, 3.5 mM SDS). Then, gels were equilibrated for 15 min in Towbin's buffer (25

mM Tris-HCL, 190 mM glycine, and 20 % methanol). Proteins were transferred onto polyvinylidene difluoride membranes (1.5 h, 50 V), using the mini vertical gel system from E-C Apparatus Corporation (St. Petersburg, FL). Membranes were then incubated for a minimum of 1 h in blocking solution containing 5% dried skim milk in TTBS (20mM Tris-HCl, 0.5 M NaCl, 0.05% Tween-20). Blots were incubated overnight at 4 ^{o}C in primary antibody diluted in blocking solution, to a final concentration of: AA-NAT(1:8,000), CREB, phosphorylated S133 CREB (1:20,000), GAPDH (1:250,000), TORC1(1:6,700). After extensive washing in TTBS, blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were thoroughly washed in TTBS, then placed in TBS (20mM Tris-HCl, 0.5M NaCl) prior to developing with enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL) and exposing to X-ray film (Fuji Film Co, Mississauga, Ontario).

For samples subjected to PhosTag gel, the following alterations were made. 10 µM PhosTag-acrylamide (Wako Chemical, Rechmond, VA) and 20 µM $MnCl_2$ were included in the gel. Gels were run at 150 V, and prior to transfer, gels were incubated in Towbin's buffer containing 8 mM EDTA for 15 min to remove $MnCl_2$, and then equilibrated in Towbin's buffer containing 0.005% sodium dodecyl sulphate (SDS) for 10 min. Blotting took place for 2.5 h at 100V in Towbin's buffer containing 0.005% SDS using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont.)

2.7 Cellular fractionation

The nuclear extract was separated from the cytoplasmic fraction of cultured pinealocytes using Biovision's nuclear/cytosol fractionation kit (Biovision, Milpitas, CA). Treated cells were pelleted by centrifugation (3 min, 2500 x g). Cell pellets were resuspended in hypotonic cytosolic extraction buffer-A (CEBA) containing protease inhibitor and dithiothreitol (DTT), then incubated for 10 min at 0 ^{o}C. Next, the cells were lysed with 2% cytosolic extraction buffer B (CEBB) detergent for 1 min. Pinealocytes transfected with adenovirus required 5% CEBB detergent. The nuclear fraction was pelleted by centrifugation (1 min, 3000 x g), and the cytoplasmic fraction was removed by aspiration. Nuclear pellets were lysed in 1X sample buffer (2X sample buffer and buffer A) and cytoplasmic fractions were solubilized with the addition of 3X sample buffer. Samples were then boiled for 8 min, and stored at 4 ^{o}C until analysis.

2.8 Calf intestinal alkaline phosphatase treatment

Phosphate groups were removed through the use of a calf intestinal alkaline phosphatase (CIP) kit from New England Biolabs (Pickering, ON). Cells were collected by centrifugation (2 min, 6000 x g) and the supernatant was removed. Pellets were suspended in resuspension buffer (NEB3, EGTA, leupeptin, aprotinin, protease inhibitor p-8340, and protease inhibitor p-2714), and immediately placed on dry ice. Samples were lysed by subjection to multiple freeze/thaw cycles. 1U of CIP was added to each sample, and allowed to incubate at 37 ^{o}C for 20 min. 2X sample buffer was added to the samples. Samples were then boiled for 8 min, and stored at 4 ^{o}C until analysis.

2.9 Reverse transcription – polymerase chain reaction

Total RNA was isolated from samples using Trizol (Invitrogen Co., Carlsbad, CA). Samples were homogenized with the addition of Trizol, and left to incubate for 5 min at room temperature. Chloroform was then added, and samples were mixed vigorously prior to centrifugation (15 min, 12000 x g, 4 ^{o}C). Glycogen was added to the aqueous phase and the RNA was precipitated with isopropyl alcohol. RNA was left to precipitate at room temperature for 10 min, prior to centrifugation (10 min, 12000 x g, 4 ^{o}C). Pellets were gently washed with 70% ethanol, then centrifuged (5 min, 7500 x g, 4 ^{o}C). The ethanol was removed by aspiration and pellets were dried. Pellets were dissolved in RNase-free water, and incubated for 10 min at 55 ^{o}C.

cDNA was synthesized from isolated RNA using Qiagen Quantitec reverse transcriptase kit (Qiagen). Genomic removal buffer was added and allowed to incubate for 2 min at 42 ^{o}C . A master mix was prepared containing 5X reverse transcriptase buffer, reverse transcriptase primers, and Omniscript enzyme. The master mix was added to the samples and allowed to incubate for 30 min at 42 ^{o}C. The reaction was terminated through heat inactivation for 3 min at 95 ^{o}C. Distilled water was used to dilute each sample and samples were stored at -20 ^{o}C until analysis.

Real-time PCR was performed on cDNA samples using StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). SYBR-Green fast reagent was used for a PCR reaction volume of 10 µL with a forward and reverse primer concentration of 300 nM. PCR cycling conditions were as follows: 3 sec at 95 ^{o}C for denaturing, 30 sec at 60 ^{o}C for annealing and extension for 40 cycles. StepOne Software version 2.0.1 (Applied Biosystems) was used to determine the relative quantity of PCR product; expressed as a

fold difference relative to an internal control containing a fixed amount of pooled cDNA. The relative quantity was normalized to *Gapdh* to correct for sample loading. Primer sequences were designed from rat gene cDNA obtained from the National Center for Biotechnology Information website. Primers were selected from 3' regions using the internet based primer design program, *Primer3* (*http://frodo.wi.mit.edu/*). The primers used were as follows: *Aa-nat*: forward primer, 5' –TCC CTG CCA GTG AGT TCC -3'; reverse primer, 5' – AGC TCT GGA CAC AGG GTG AG – 3'; *Gapdh*: forward primer, 5' – GTCGGTGTGAACGGATTTG-3'; reverse primer, 5' – CTT GCC GTG GGT AGA GTC AT – 3'; *Torc1*: forward primer, 5' –GCA GAA GTC GCA GTA TCT CCA-3'; reverse primer, 5' – AGG CCT GAG GAC TGA AAG G– 3'.

2.10 Results and statistical analysis

For analysis of real time-PCR, obtained values were normalized to *Gapdh* and presented as the mean ± SEM from three independent experiments.

3. Results

3.1 Regulation of *Torc1* transcription

To determine if *Torc1* transcription is regulated in the rat pineal gland, pineal glands were collected at various times from rats housed under a lighting regimen of 14 h light every 24 h, as described in *Methods and Materials.* Real-time PCR was used to determine *Aa-nat* and *Torc1* mRNA levels. FIG. 3.1 shows there is a dramatic increase in mRNA levels of *Aa-nat* with the onset of darkness; however, no significant changes in *Torc1* mRNA levels occurred throughout the day.

To confirm the result from the whole animal study, cultured pinealocytes were used for *in-vitro* study to determine if activation of various NE-induced post-receptor signalling pathways would affect *Torc1* mRNA levels. Pinealocytes were treated with NE (3 µM), dibutyryl cyclic adenosine monophosphate (DBcAMP)(1 mM) a membrane permeable cyclic adenosine monophosphate (cAMP) analog, dibutyryl cyclic guanosine monophosphate (DBcGMP)(1 mM) a membrane permeable cyclic guanosine monophosphate (cGMP) analog, phorbol-12-myristate 13-acetate (PMA)(0.1 µM) a protein kinase C activator, or a depolarizing concentration of KCl (30 mM) leading to elevation in intracellular Ca^{2+}. Real-time PCR was used to determine the mRNA levels of *Aa-nat* and *Torc1*. NE and DBcAMP led to an elevation in *Aa-nat* mRNA levels, while no treatment caused any significant changes in *TORC1* mRNA levels (FIG. 3.2).

These results suggest that *Torc1* transcription is not induced and shows no diurnal regulation. Furthermore, they demonstrate that *Torc1* transcription is not adrenergically regulated.

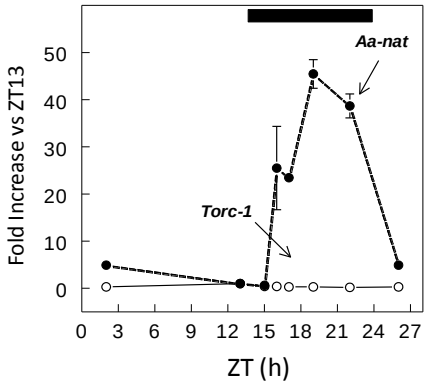

FIG. 3.1 Day/Night variation in *Torc1* mRNA levels in the rat pineal gland. Pineal glands were collected at the indicated zeitgeber time (ZT) from rats housed under a lighting regiment of 14 h light every 24 h. mRNA extraction and real-time PCR, as described in *Materials and Methods*, was utilized to measure the *Torc1* and *Aa-nat* mRNA levels, which were normalized to *Gapdh*. Data is expressed as fold change vs ZT13.

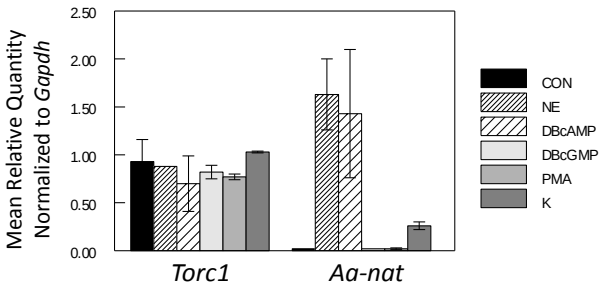

FIG. 3.2 Effects of post-receptor signalling mechanism on *Torc1* mRNA levels. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to 3 h treatment with the following: NE (3 µM), DBcAMP (1 mM), DBcGMP (1 mM), PMA (0.1 µM), and KCl (30 mM). mRNA extraction and real-time PCR, as described in *Materials and Methods*, was used to measure the *Torc1* and *Aa-nat* mRNA levels. Data was expressed as the mean relative quantity of mRNA, normalized to *Gapdh*.

3.2 Regulation of TORC1 through phosphorylation

The previous result indicated that *Torc1* transcription is not under adrenergic control; suggesting, regulation may occur through post-translational modification. To determine if there is a variation in TORC1 protein throughout the day, pineal glands were collected at various times from rats housed under a lighting regimen of 14 h light every 24 h, as described in *Methods and Materials*. Western blot analysis was used to determine the TORC1 protein levels. FIG. 3.3A shows that during daytime TORC1 produced a single band at approximately 78 kDa; however, with the onset of darkness TORC1 shifted to a faster migrating band. The lower band of TORC1 was present throughout the period of darkness, but its intensity gradually decreased over this period. After 2 h of darkness, there was a gradual formation of a doublet TORC1 band. TORC1 did not shift back to the high molecular weight band until the onset of light. Phosphorylated CREB at Ser-133 (pCREB) and AA-NAT protein were monitored as a positive control for the NE stimulation. TORC1 band shifting occurred prior to the appearance of pCREB and AA-NAT. GAPDH was measured to determine loading consistency.

The addition of PhosTag to normal sodium dodecyl sulphate (SDS) gel causes the retardation of phosphorylated proteins. PhosTag gel was used to determine if changes in the phosphorylation status of TORC1 was responsible for the shifting of the TORC1 band in the whole pineal gland samples. During daylight TORC1 remains as a higher molecular weight band, but with the onset of darkness there is a shift to a lower molecular weight band (FIG. 3.3B). After 2 h of darkness, there is a partial shift back to a higher molecular weight band, but TORC1 does not again form a band at the control

weight regions until the period of darkness has ceased. This suggests that *in-vivo,* TORC1 of the rat pineal gland becomes dephosphorylated during night, and is again rephosphorylated with the onset of daylight.

To confirm this dephosphorylation event *in-vitro*, NE stimulated pinealocytes were subjected to calf intestinal alkaline phosphatase (CIP) treatment prior to Western blot analysis. NE stimulation of pinealocytes caused a shifting of TORC1 to a faster migrating band. CIP treatment resulted in the elimination of the slower migrating band of TORC1, and produced a control TORC1 band that migrated the same distance as the stimulated TORC1 band. CIP treatment did not lead to any further changes in the stimulated TORC1 band (FIG. 3.4). This suggests that NE stimulation of pinealocytes leads to the rapid dephosphorylation of TORC1.

Together, these results confirm that TORC1 is post-translationally modified through phosphorylation. Under basal conditions TORC1 remains phosphorylated, while the onset of darkness in whole animal pineal gland samples or NE stimulation in pinealocyte culture leads to the dephosphorylation of TORC1.

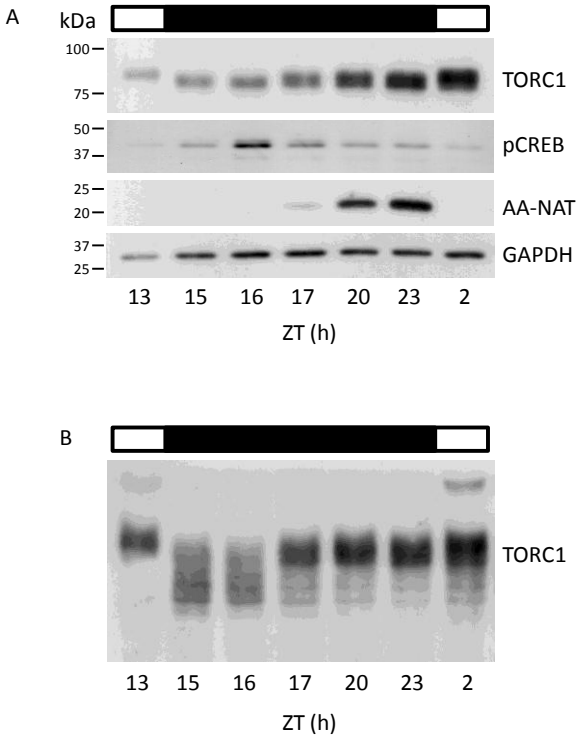

FIG. 3.3 Day/Night variation in TORC1 protein levels in the rat pineal gland. Pineal glands were collected at the zeitgeber time (ZT) indicated from rats housed under a lighting regiment of 14 h light every 24 h. A) Western blot analysis, as described in *Materials and Methods*, was used to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, AA-NAT, and GAPDH. GAPDH demonstrates loading consistency. B) Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was used to determine TORC1 protein phosphorylation. Immunoblots were probed for TORC1.

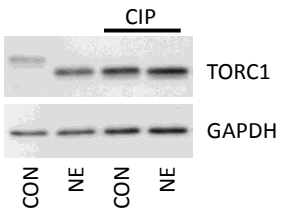

FIG. 3.4 Confirmation of NE-induced faster migrating TORC1 dephosphorylated band. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to 0.5 h NE (3 μM) treatment. Samples were subjected to CIP prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, and GAPDH. GAPDH demonstrates loading consistency.

3.3 The α-adrenergic receptor is responsible for the NE-mediated dephosphorylation and nuclear translocation of TORC1 protein

To characterize what adrenergic receptor subtype was involved in the NE-mediated dephosphorylation of TORC1, selective adrenergic agonists and antagonists were utilized. The concentrations used were previously established (Ho *et al.*, 2000; Price *et al*., 2004). Selective activation of the α_1-adrenergic receptor (1 μM phenylephrine (PE) in the presence of 3 μM propanolol (PROP)) or the β-adrenergic receptor (1 μM isoproternol (ISO) in the presence of 3 μM prazosin (PRAZ)), led to an enhancement in the intensity of the lower TORC1 band (FIG 3.5A). pCREB was measured as a control for the stimulation, and was reduced in the samples containing propanolol. NE stimulation in the presence of PRAZ or PROP, also led to shifting of TORC1 to the lower band. This suggests that activation of either adrenergic receptor subtype can lead to the dephosphorylation of TORC1.

To determine if the dephosphorylation of TORC1 caused by activation of either adrenergic receptor subtype was identical, samples were run on a PhosTag gel and the band pattern of TORC1 was compared. Selective activation of the α_1-adrenergic receptor (1 μM PE in the presence of 3 μM PROP) or β-adrenergic receptor (1 μM ISO in the presence of 3 μM PRAZ), both led to the dephosphorylation of TORC1; however, they produced distinct band patterns (FIG. 3.5B). Activation of the α_1-adrenergic receptor led to a TORC1 band pattern similar to what was seen with NE, while activation of the β-adrenergic receptor produced a band pattern dissimilar to NE. NE stimulation in the presence of PROP, and not PRAZ was capable of producing a TORC1 band pattern similar to that of NE. Although both adrenergic receptor subtypes can lead to the

dephosphorylation of TORC1, the α_1-adrenergic receptor is responsible for the NE-mediated dephosphorylation of TORC1.

To investigate the cellular distribution of TORC1, samples were fractionated into nuclear and cytosolic fractions, prior to Western blot analysis. Under basal conditions TORC1 resided in the cytoplasm, but upon stimulation with NE (1 μM) the quantity of TORC1 in the nucleus was elevated (FIG. 3.6). NE stimulation in the presence of either PRAZ (3 μM) or PROP (3 μM) caused the dephopshorylation of TORC1 in the cytoplasm; however, treatment with PRAZ abolished any TORC1 nuclear translocation. To control for fractionation, pCREB, representative of nuclear proteins, was monitored in the nuclear fraction, while GAPDH, representative of cytosolic proteins, was measured in the cytoplasm.

Together, these results suggest that activation of either adrenergic receptor subtype is capable of leading to the dephosphorylation of TORC1, but only the α_1-adrenergic receptor leads to the NE-mediated dephosphorylation of TORC1. Furthermore, activation of the α_1-adrenergic receptor is responsible for the NE-mediated nuclear translocation of TORC1.

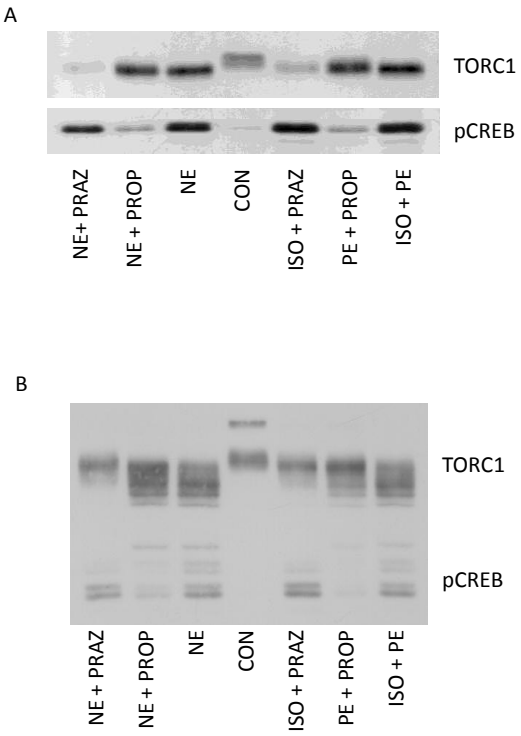

FIG. 3.5 Adrenergic receptor characterization of NE-mediated TORC1 dephosphorylation. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to treatment for 0.5 h with NE (1 µM), ISO (1 µM), or PE (1 µM) in the presence or absence of PROP (3 µM) or PRAZ (3 µM). A) Western blot analysis, as described in *Materials and Methods*, was utilized to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, and GAPDH. GAPDH demonstrates loading consistency. B) Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was utilized to determine TORC1 protein phosphorylation. Immunoblots were probed for TORC1 and pCREB.

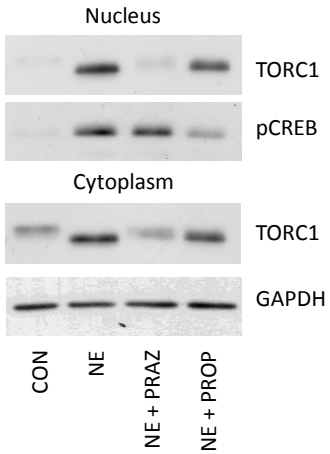

FIG. 3.6 Adrenergic receptor responsible for NE-mediated TORC1 translocation. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to treatment for 0.5 h with NE (1 µM) in the presence or absence of PROP (3 µM) or PRAZ (3 µM). Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods*, to determine TORC1 protein levels. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

3.4 Post-receptor signalling mechanism involved in the NE-mediated dephosphorylation and nuclear localization of TORC1

To characterize which post-receptor signalling mechanism is responsible for the NE-mediated dephosphorylation of TORC1, several post-receptor signalling pathway activators were employed. Separation on PhosTag gel allowed for the comparison of the TORC1 band pattern. NE (3 μM) produced a TORC1 band pattern with the majority of TORC1 concentrated in the lower molecular weight bands (FIG. 3.7). Either DBcAMP (1 mM) or KCl (30 mM) led to the dephosphorylation of TORC1; however, both of these produced a band pattern distinct from that of NE treatment. The band pattern from DBcAMP had TORC1 uniformly distributed between the upper and lower weight bands, while KCl produced a band pattern with the bulk of TORC1 concentrated in higher molecular weight bands. Interestingly, the combination of DBcAMP and KCl was required to produce a band pattern similar to NE, where TORC1 is concentrated at the lower molecular weight bands. This result suggests that the combination cAMP and elevation of intracellular Ca^{2+} is required for the NE-mediated dephosphorylation of TORC1.

Since multiple post-receptor signalling pathways could cause the dephosphorylation of TORC1, pinealocytes treated with several post-receptor signalling pathway activators were separated into nuclear and cytosloic fractions to determine which signalling pathway was responsible for the nuclear translocation of TORC1. FIG. 3.8 shows that the NE-mediated nuclear translocation of TORC1 is mimicked by elevation of intracellular Ca^{2+} with a depolarizing concentration of KCl (30 mM).

Treatment with DBcAMP (1 mM), DBcGMP (1 mM), and PMA (0.1 µM) did not lead to significant TORC1 nuclear translocation.

These results suggest that the NE-mediated dephosphorylation of TORC1 is mediated through an elevation of both intracellular Ca^{2+} and cAMP. Either an elevation in cAMP or elevation in intracellular Ca^{2+} can cause the dephosphorylation of TORC1; however, it is the elevation of intracellular Ca^{2+} that is responsible for the NE-mediated nuclear translocation of TORC1.

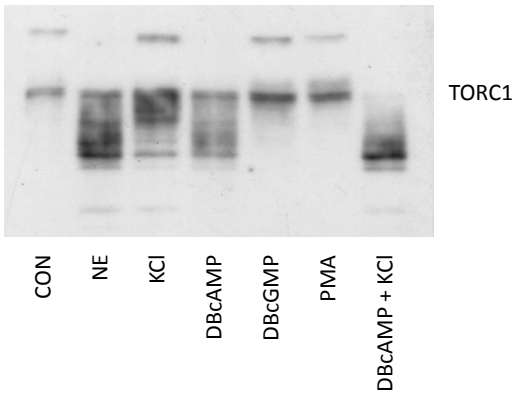

FIG. 3.7 Post-receptor signalling mechanism involved in the NE-mediated dephosphorylation of TORC1. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to 0.5 h treatment with the following: NE (3 µM), DBcAMP (1 mM), DBcGMP (1 mM), PMA (0.1 µM), and KCl (30 mM). Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was utilized to determine TORC1 protein phosphorylation. Immunoblots were probed for TORC1.

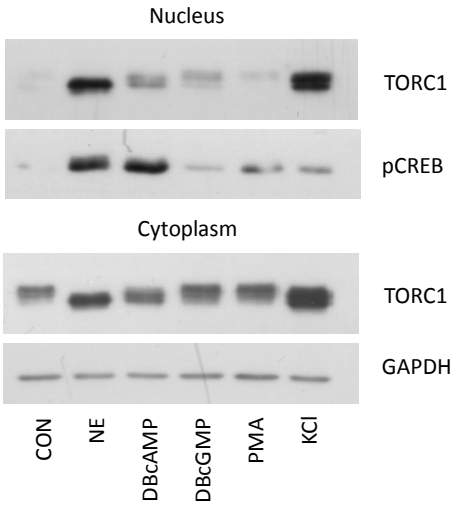

FIG. 3.8 Post-receptor signalling mechanism involved in the NE-mediated nuclear translocation of TORC1. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to 0.5 h treatment with the following: NE (3 µM), DBcAMP (1 mM), DBcGMP (1 mM), PMA (0.1 µM), and KCl (30 mM). Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods.* Immunoblots were probed for TORC1, pCREB, and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

3.5 Phosphatases involved in the NE-mediated dephosphorylation and nuclear localization of TORC1

NE-mediated dephosphorylation of TORC1 leads to the nuclear translocation of TORC1. In order to determine which phosphatase was responsible for this dephosphorylation, pinealocytes were treated with a variety of phosphatase inhibitors that target against different protein phosphatases. PhosTag gel was used to demonstrate that okadaic acid (OKA) (1 μM) a protein phosphates 2A (PP2A) inhibitor and cyclosporine A (CSA) (1 μM) a protein phosphatase 2B (PP2B/calcineurin) inhibitor were able to partially prevent the NE-mediated dephoshorylation of TORC1 (FIG. 3.9 A and B). In contrast, tautomycin a protein phosphatase 1 (PP1) inhibitor, had no effect on TORC1 dephosphorylation. Although both CSA and OKA alone could cause a reduction in the NE-mediated TORC1 dephosphorylation, neither could completely abolish the dephosphorylation. Cotreatment with both OKA and CSA was able to completely prevent the NE-mediated dephosphorlyation and produce a band that mimicked control conditions (FIG. 3.10A).

pCREB represents a positive control for stimulation and OKA function, since the dephosphorylation of CREB is facilitated through PP2A. In order to ensure that the concentration of phosphatase inhibitor was appropriate, samples containing OKA and CSA were stimulated with DBcAMP (1 mM) and KCl (30 mM). PP2B activity is initiated through elevation of intracellular Ca^{2+}, while PP2A is activated through cAMP. CSA (1 μM) was able to block dephosphorylation by KCl treatment (FIG. 3.10B). CSA or OKA (1 μM) could prevent the DBcAMP-mediated dephosphorylation. This suggests the additive effect seen with the cotreatment of CSA and OKA is consistent with each

inhibitor's effect in preventing the dephosphorylation through their individual regulatory pathway. In order to determine which phosphatase could dephosphorylate TORC1 making it a suitable candidate for nuclear translocation, pinealocytes were treated with NE in the presence of various phosphatase inhibitors. Samples were fractionated prior to Western blot analysis (FIG. 3.11). CSA(1 μM) caused a reduction in the nuclear quantity of TORC1; while OKA(1 μM), TAU(1 μM), and calyculin A (CALY A) (10 nM)(PP1 and PP2A phosphatase inhibitor) had no effect on TORC1 nuclear quantity.

Together, these results suggest that both PP2A and PP2B are used in the NE-mediated dephosphorylation of TORC1; however, it is only the dephosphorylation caused by PP2B that allows for TORC1 nuclear accumulation.

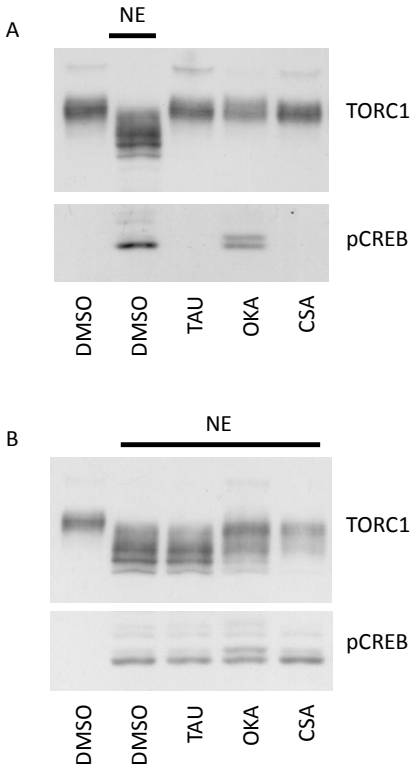

FIG. 3.9 Phosphatases responsible for the NE-mediated dephosphorylation of TORC1.
Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h, prior to pharmacological treatment. Cells were treated for 15 min with NE (3 µM), in the presence of tautomycin (TAU) (10^{-6}M), okadaic acid (OKA) (10^{-6}M), or cyclosporine A (CSA) (10^{-6}M). A) and B) Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was used to determine TORC1 protein phosphorylation. pCREB was included to control for drug activity and stimulation

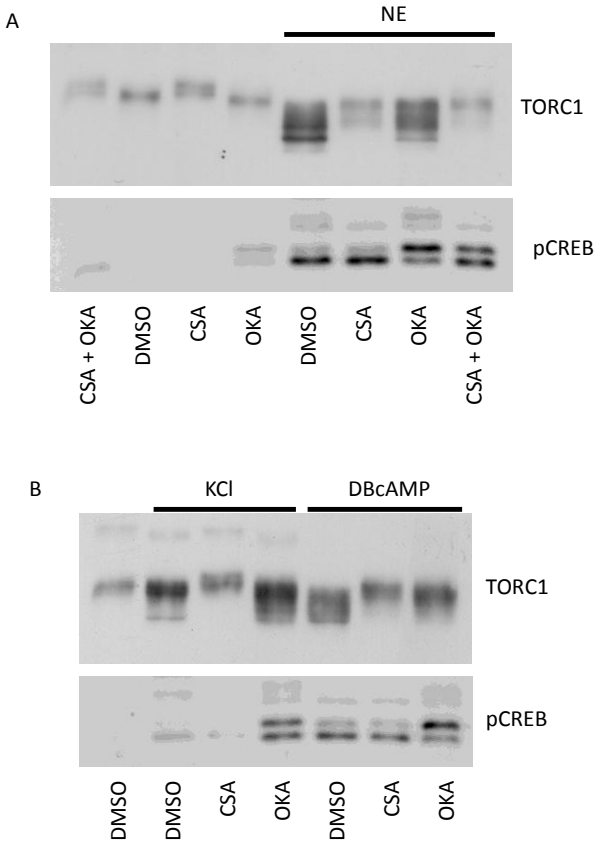

FIG. 3.10 Phosphatases responsible for the NE-mediated dephosphorylation of TORC1. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h, prior to pharmacological treatment. A) Cells were treated for 15 min with NE (3 µM), in the presence of OKA (1 µM) or (1 µM). Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was utilized to determine TORC1 protein phosphorylation. pCREB was included to control for drug activity and stimulation. B) Cells were treated for 0.25 h with NE (3 µM), DBcAMP (1 mM), or KCl (30 mM) in the presence of either OKA (1 µM) or CSA (1 µM). Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was utilized to determine TORC1 protein phosphorylation. pCREB was included to control for drug activity and stimulation.

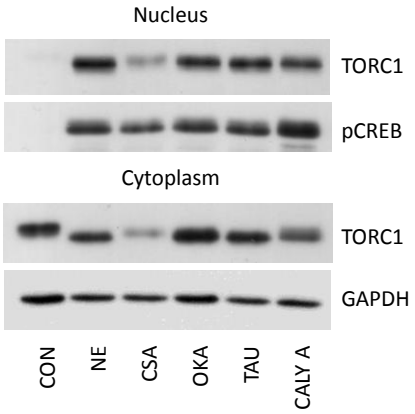

FIG. 3.11 Phosphatases responsible for the NE-mediated nuclear translocation of TORC1. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h, prior to pharmacological treatment. Cells were treated for 0.5 h with NE (3 µM), in the presence of CSA (1 µM), OKA (1 µM), TAU(10 nM), or CALY A(10^{-8}M). Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

3.6 Time course response of NE-mediated TORC1 cellular distribution

To understand the dynamics of TORC1 translocation from the cytoplasm to the nucleus the time course response was investigated. Pinealocytes were treated with NE (3 μM) for the periods indicated; then fractionated prior to Western blot analysis (FIG. 3.12 A and B). Within 0.25 h of NE stimulation TORC1 became dephosphorylated and was translocated to the nucleus. TORC1 remained dephosphorylated and present in the nucleus throughout the time-course. AA-NAT and pCREB were monitored for stimulation control.

After 4 h of NE stimulation TORC1 is partially rephosphorylated, forming a TORC1 doublet band that remains in both the cytoplasm and nucleus for the balance of the time-course (FIG. 3.13). The presence of partially phosphorylated TORC1 in the nucleus after 4 h, suggests that phosphorylation may not be the absolute determinant of TORC1 cellular distribution.

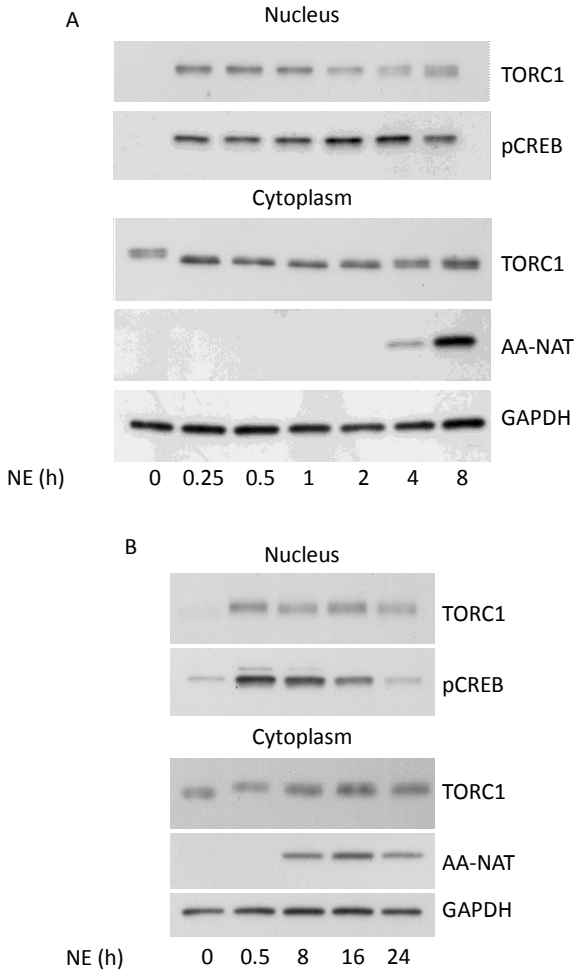

FIG. 3.12 Time course response of NE-mediated TORC1 nuclear translocation. A) and B) Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to treatment with NE (3 µM) for the times indicated. Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, AA-NAT, and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

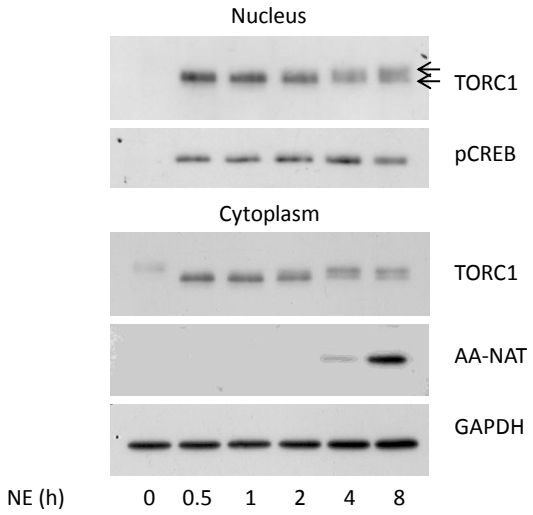

FIG. 3.13 Time course response of NE-mediated TORC1 nuclear translocation. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to treatment with NE (3 µM) for the times indicated. Samples were fractionated into nuclear and cytolslic fractions prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, AA-NAT, and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

3.7 Continuous adrenergic stimulation is necessary to maintain the NE-mediated dephosphorylation and nuclear localization of TORC1

The previous result demonstrates that the NE-mediated TORC1 dephosphorylation and nuclear translocation is maintained indefinitely in the presence of NE. To determine if TORC1 dephosphorylation and maintenance in the nucleus is a regulated process, samples were subjected to NE stimulation prior to the addition of specific adrenergic receptor antagonists. FIG. 3.14A shows that there is no change in TORC1 between 0.5 h, 1 h, or 1.5 h of NE (1 μM) stimulation; however, the addition of either PRAZ (3 μM) or PROP (3 μM) causes TORC1 to become rephosphorylated. Increased duration of stimulation produced a band pattern with no variation between 6 h and 7 h of NE stimulation, but again showed blockage of either adrenergic receptor with PRAZ and PROP led to the rephosphorylation of TORC1 (FIG. 3.14B). PhosTag gel was used to confirm that blocking either adrenergic pathway would lead to alterations in the phosphorylation status of TORC1 (FIG. 3.15). The addition of PROP was able to cause a reduction of both pCREB and AA-NAT. Treatment with either PRAZ or PROP for 1 h after 4 h of NE stimulation led to rephosphorylation of TORC1.

In order to determine if the duration of adrenergic blockade would affect the magnitude of TORC1 rephosphorylation caused by specific adrenergic blockade, pinealocytes were treated with PRAZ (3 μM) or PROP (3 μM) for varying durations after 4 h NE (1 μM) stimulation (FIG. 3.16 A and B). Surprisingly, the blockage of the β-adrenergic signalling pathway with the addition of PROP was able to cause a more rapid and pronounced TORC1 rephoshorylation, than blockage of the α_1-adrenergic receptor with PRAZ.

Since blockage of adrenergic stimulation led to the rephosphorylation of TORC1, the effect of this rephosphorlyation on the nuclear quantity of TORC1 was investigated. Samples were stimulated with NE (1 μM) before the addition of specific adrenergic receptor antagonists; then fractionated prior to Western blot analysis. NE led to an accumulation of TORC1 in the nucleus that remained constant from 60-70 min of treatment; however, the addition of PRAZ (3 μM) or PROP (3 μM) for 10 min caused a rapid reduction in TORC1 nuclear accumulation (FIG. 3.17A). To confirm this effect at a later time point, PRAZ and PROP were added to samples after 4 h of NE stimulation (FIG. 3.17B). Both PRAZ and PROP led to a rapid decrease in TORC1 nuclear accumulation. This suggests that both adrenergic receptor pathways must be continually stimulated to maintain the nuclear presence of TORC1.

TORC1 nuclear translocation and nuclear maintenance are differentially regulated processes. Activation of the α_1-adrenergic receptor is sufficient for TORC1 nuclear translocation, while activation of both the α_1-adrenergic receptor and the β-adrenergic receptor are required to maintain the nuclear presence of TORC1.

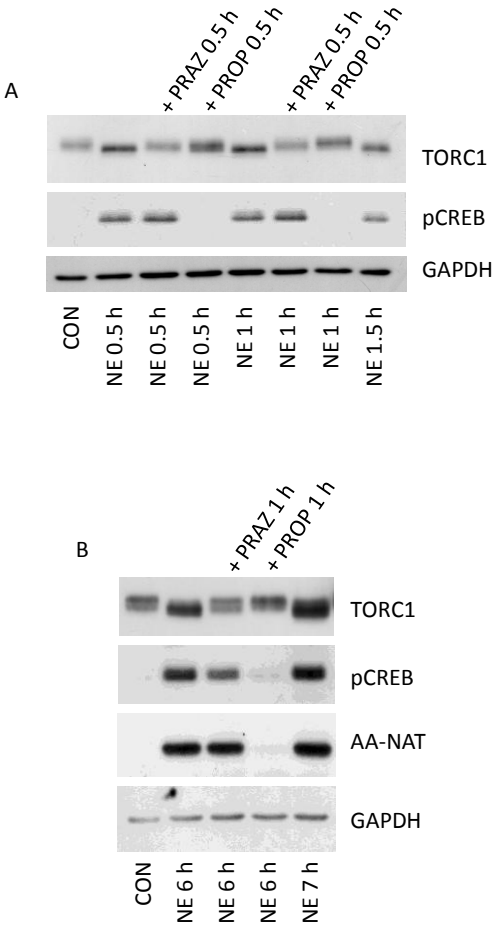

FIG. 3.14 Continuous adrenergic stimulation is required for TORC1 to remain dephosphorylated. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to pharmacological treatment. A) Samples were pretreated with NE (1 µM) for the times indicated prior to 0.5 h treatment with PRAZ (3 µM) or PROP (3 µM). Western blot analysis, as described in *Materials and Methods*, was utilized to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, and GAPDH. GAPDH demonstrates loading consistency. B) Samples were pretreated with NE (1 µM) for 6 h prior to 1h treatment with PRAZ (3 µM) or PROP (3 µM). Western blot analysis, as described in *Materials and Methods*, was utilized to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, AA-NAT, and GAPDH. GAPDH demonstrates loading consistency

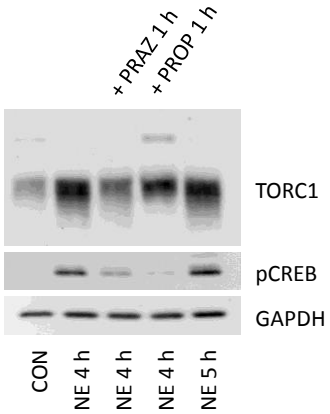

FIG. 3.15 Continuous adrenergic stimulation is required for TORC1 to remain dephosphorylated. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to pharmacological treatment. Samples were pretreated with NE (1 µM) for 4 h prior to 1 h treatment with PRAZ (3 µM) or PROP (3 µM). Western blot analysis using PhosTag gel, as described in *Materials and Methods*, was utilized to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, and GAPDH. GAPDH demonstrates loading consistency.

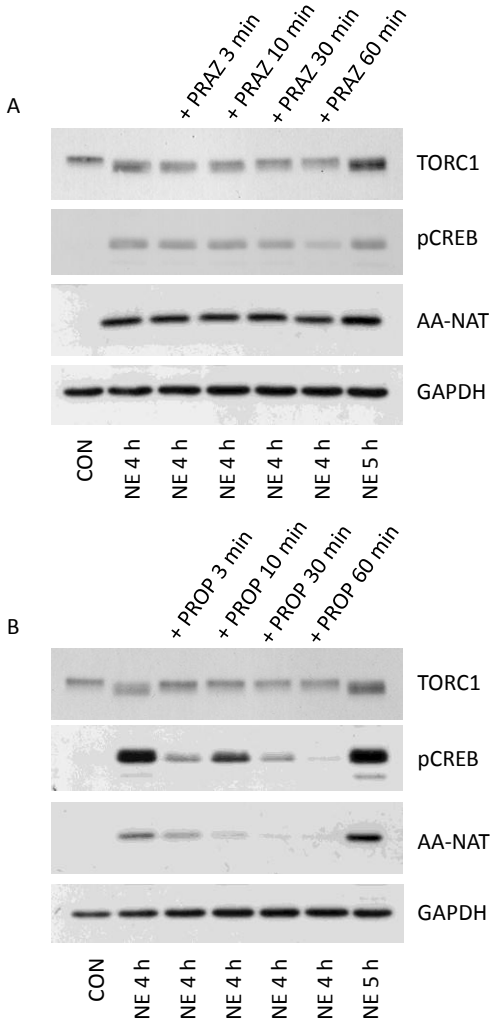

FIG. 3.16 Effect of varying duration of adrenergic blockade on TORC1 phosphorylation. A) and B) Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h prior to pharmacological treatment. Samples were pretreated with NE (1 µM) for 4 h prior to treatment with PRAZ (3 µM) or PROP (3 µM) for the times indicated. Western blot analysis, as described in *Materials and Methods*, was utilized to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, AA-NAT and GAPDH. GAPDH demonstrates loading consistency

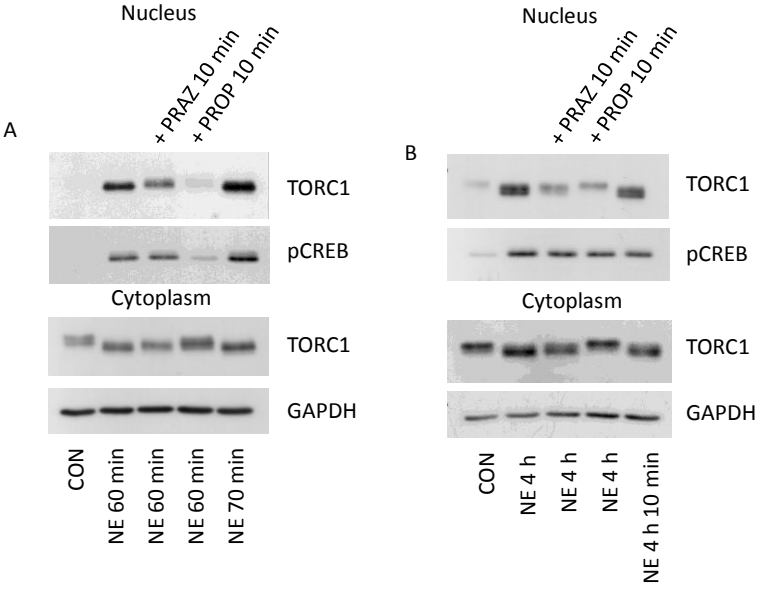

FIG. 3.17 Continuous adrenergic stimulation is required for maintenance of NE-mediated nuclear TORC1. A) and B) Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h, prior to pharmacological treatment. Samples were pretreated with NE (1 µM) for the times indicated prior to 10 m treatment with PRAZ (3 µM) or PROP (3 µM). Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

3.8 Continuous phosphatase action is necessary to maintain the NE-mediated dephosphorylation and nuclear localization of TORC1

Constant adrenergic stimulation is necessary to maintain the dephosphorylation of TORC1. In order to determine if this dephosphorylation is maintained through constant phosphatase action; NE stimulated pinealocytes were treated with specific phosphatase inhibitors. Samples were stimulated with NE (3 μM) for 1 h prior to the addition of CSA (1 μM) and OKA (1 μM). FIG. 3.18 shows that there is no variation in TORC1 between 1 h and 2 h of NE treatment; however, the addition of either CSA or OKA led to the rephosphorlyation of TORC1. To ensure the phosphatase inhibitors were working correctly, samples received 0.5 h phosphatase inhibitor pretreatment, with 0.5h of NE treatment as a positive control. Continuous phosphatase activity is necessary to maintain TORC1 in a dephosphosorylated state.

To determine if continual phosphatase activity is essential for TORC1 nuclear maintenance, stimulated pinealocytes were treated with specific phosphatase inhibitors. Samples were then fractionated prior to Western blot analysis. The nuclear quantity of TORC1 was dramatically reduced within 15 min in the presence of CSA (1 μM), and was eliminated after 30 min of CSA (FIG. 3.19). Although OKA (1 μM) led to some reduction in TORC1 nuclear quantity, its effect was minimal in comparison to CSA.

Constant PP2A and PP2B activity are required to maintain the NE-mediated TORC1 dephosphorylation. Furthermore, PP2B activity is essential for preserving the NE-mediated nuclear presence of TORC1.

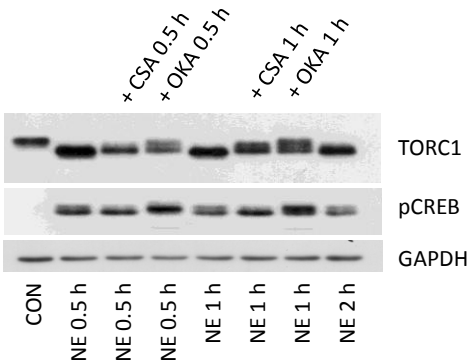

FIG. 3.18 Continuous phosphatase activity is required to maintain the NE-mediated dephosphorylation of TORC1. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h, prior to pharmacological treatment. Samples were pretreated with NE (3 µM) for the times indicated prior to treatment with CSA(1 µM) or OKA(1 µM). Western blot analysis, as described in *Materials and Methods*, was utilized to determine TORC1 protein levels. Immunoblots were probed for TORC1, pCREB, and GAPDH. GAPDH demonstrates loading consistency.

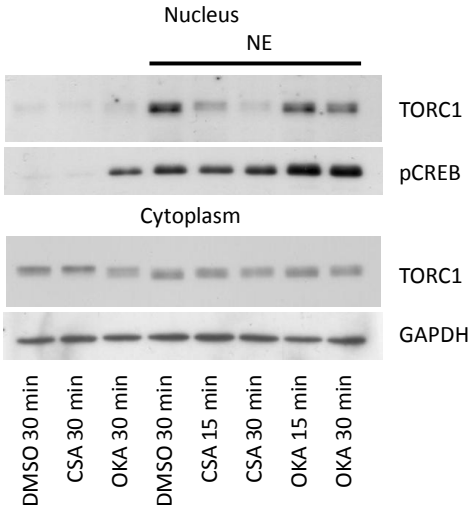

FIG. 3.19 Continuous phosphatase activity is required for maintenance of NE-mediated nuclear TORC1. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were cultured for 18 h, prior to pharmacological treatment. Samples were pretreated with NE (3 μM) for the times indictheated prior to treatment with CSA(1 μM) or OKA(1 μM). Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency.

3.9 Role of SIK1 in regulating the NE-mediated TORC1 cellular distribution

Existing literature suggests that SIK1 is responsible for phosphorylating TORC1, leading to its retention in the cytoplasm (Takemori H & Okamoto M, 2008). In order to determine if SIK1 is capable of phosphorylating TORC1, the quantity of SIK1 was manipulated through knockdown and overexpression. Pinealocytes were transfected with adenoviruses encoding *Sik1* short-hairpin (*Sik1SH*) and wild-type full-length *Sik1* (*Sik1FL*). To control for viral transfection all other samples were transfected with adenovirus encoding *LacZ*. FIG 3.20 shows that manipulation in the quantity of SIK1 does not lead to substantial changes in TORC1 under basal conditions. Upon NE (3 μM) stimulation, there is no significant difference in the phosphorlyation pattern of TORC1 between the *LacZ* and *SikSH* sample; however, the sample transfected with *Sik1FL* showed an increased intensity of the upper TORC1 band and decreased intensity of the lower band. To control for the viral treatment, the quantity of AA-NAT was monitored since SIK1 has been shown to repress *Aa-nat* transcription (Kanyo *et al.*, 2009). NE-induced AA-NAT was increased in the sample containing *Sik1SH*, while the sample containing *Sik1FL* showed a reduction in AA-NAT. This result confirms that TORC1 can be phosphorylated by SIK1.

Previous results, demonstrated that blockade of either α_1- adrenergic receptor or β- adrenergic receptor can abolish the NE-mediated dephosphorylation and lead to the rapid rephosphorylation of TORC1(FIG 3.14). To examine if SIK1 is responsible for this rapid rephosphorylation, pinealocytes were transfected with adenovirus encoding *Sik1SH*. Samples were then stimulated with NE (1 μM) for 4 h prior to the addition of specific adrenergic antagonists. FIG. 3.21 A and B show that the rephosphorylation of

TORC1, with the addition of PRAZ (3 μM) or PROP (3 μM) for 15 min, was not prevented by cells subjected to SIK1 knockdown. AA-NAT were measured as a positive control for stimulation and SIK1 knockdown. This suggests that SIK1 may not be the only kinase involved in the rapid rephosphorylation of TORC1, when adrenergic stimulation is blocked.

To examine the contribution of SIK1 in regulating the cellular distribution of TORC1, the quantity of SIK1 was manipulated through overexpression and knockdown. Pinealocytes were transfected with adenovirus encoding *Sik1SH* or *Sik1FL*. Samples were stimulated with NE (3 μM), prior to cellular fractionation and Western blot analysis (FIG. 3.22A). After 4 h of NE stimulation the nuclear intensity of TORC1 showed no significant variation between *LacZ, SikSH,* and *SikFL*. Interestingly, *Sik1FL* led to TORC1 remaining exclusively in the upper band in both the nuclear and cytoplasmic fraction. To determine if SIK1 manipulation would cause changes in TORC1 distribution at a later time-point, the experiment was repeated with a 16 h NE stimulation (FIG. 3.22B). Again, manipulating the quantity of SIK1 did not cause significant changes in TORC1 nuclear localization. AA-NAT was monitored to control for SIK1 manipulation.

Together these results suggest that SIK1 is not the main kinase involved in phosphorylating TORC1 when NE stimulation has ceased. SIK1 does not contribute significantly to the regulation of the NE-mediated TORC1 nuclear localization. Lastly, the phosphorylation of TORC1 caused by SIK1 overexpression does not cause TORC1 to be exported from the nucleus.

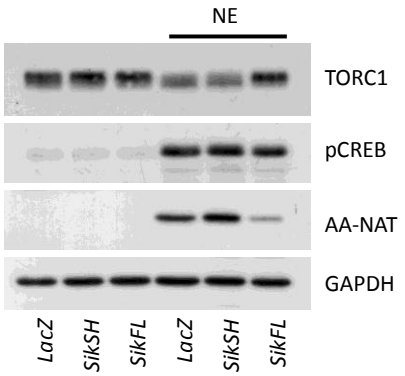

FIG. 3.20 Effect of *Sik1* manipulation on TORC1 phosphorylation. Pinealocytes (1.0 x 10^5 cells/0.3 mL) were transfected with either *LacZ*, *Sik1SH*, or *Sik1FL*, as described in *Materials and Methods* prior to pharmacological treatment. Samples were stimulated for 4 h with NE (3 µM) prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, AA-NAT and GAPDH. GAPDH demonstrates loading consistency.

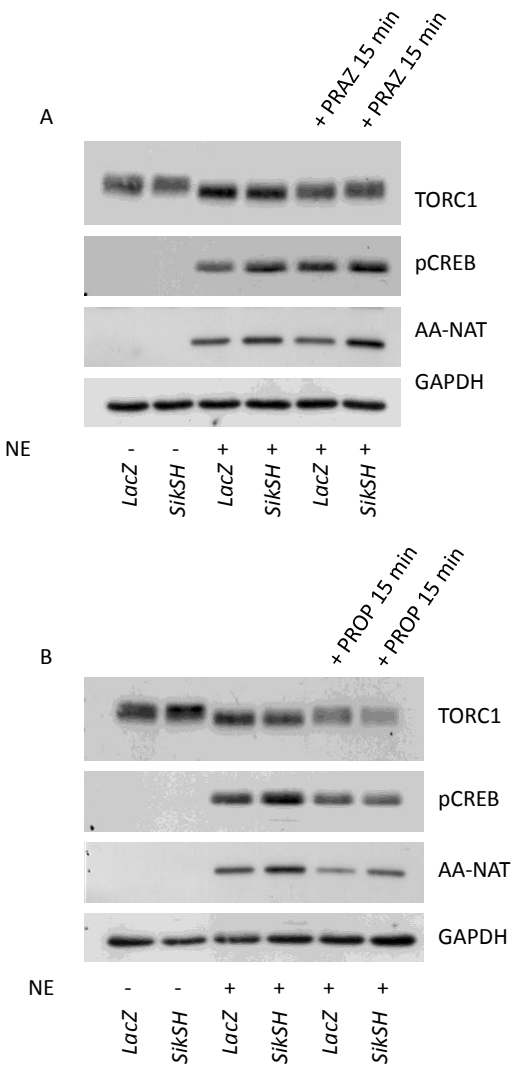

FIG. 3.21 Effect of *Sik1* knockdown on rephosphorylation of TORC1 when adrenergic stimulation is blocked. A) and B) Pinealocytes (1.0 x 10^5 cells/0.3 mL) were transfected with either *LacZ*, *Sik1SH*, or *Sik1FL*, as described in *Materials and Methods* prior to pharmacological treatment. Samples were pretreated with NE (1 µM) for 4 h prior to 15 min treatment with (3 µM) or PROP (3 µM). prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, AA-NAT and GAPDH. GAPDH demonstrates loading consistency.

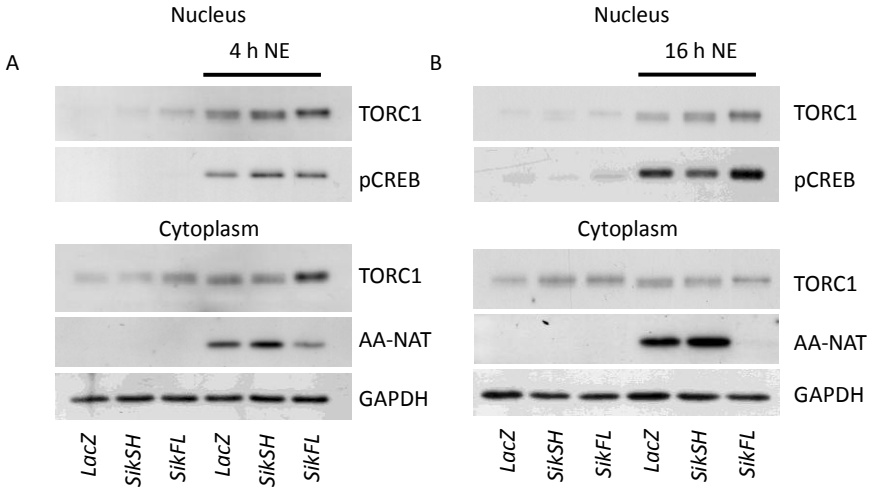

FIG. 3.22 Effect of *Sik1* manipulation on NE-mediated TORC1 nuclear accumulation. A) and B) Pinealocytes (1.0 x 10^5 cells/0.3 mL) were transfected with either *LacZ*, *Sik1SH*, or *Sik1FL*, as described in *Materials and Methods* prior to pharmacological treatment. Samples were stimulated with NE (3 µM) for the times indicated. Samples were fractionated into nuclear and cytosolic fractions prior to Western blot analysis, as described in *Materials and Methods*. Immunoblots were probed for TORC1, pCREB, AA-NAT and GAPDH. pCREB demonstrates nuclear proteins and GAPDH controls for cytoplasmic proteins and loading consistency

4. Discussion

Coactivators have been shown to play a vital role in regulating the transcription of inducible genes. In the rat pineal gland, the nocturnal release of NE from the SCG is responsible for initiating the induction of the CREB-mediated transcription of *Aa-nat* (Klein, 1985; Roseboom & Klein, 1995). The rhythmic expression of *Aa-nat* is responsible for controlling the daily rhythm of melatonin synthesis and release (Klein *et al.* 1997). Coactivators can enhance the amplitude and/or duration of expression, which shape the adrenergic regulated *Aa-nat* expression.

The nocturnal release of NE in the rat pineal gland leads to the activation of multiple signalling pathways. Initiation of these signalling pathways can cause the activation of coactivators and respressors that together are responsible for shaping the temporal-profile of *Aa-nat* expression (Ho & Chik, 2010). It is accepted that the nocturnal release of NE causes the induction of *Aa-nat* transcription through the cAMP→PKA→pCREB pathway (Roseboom *et al*., 1995; Klein *et al.*, 1997). In addition to *Aa-nat*, 600 other genes are induced through the cAMP→PKA→pCREB pathway in the pineal gland (Bailey *et al.*, 2009).

The importance of CREB activity in the induction of *Aa-nat* led to the investigation of the CREB specific coactivator TORC1 (Roseboom & Klein, 1995). In addition to the activation of CREB through the phosphorylation of Ser-133 in the KID domain, the transcriptional activity of CREB can be regulated through the bZIP domain (Carlezon *et al.*, 2005; Mayr & Montminy, 2001). The regulatory model for TORC1, suggested by current literature remains vague. In addition, an identical regulatory mechanism of SIK phosphorylation, and elevation in cAMP or intracellular Ca^{2+} leading to

the dephosphorylation and nuclear translocation, is thought to control all TORC isoforms (Takemori & Okamoto, 2008). Earlier investigation showed that the regulation of TORC2, in the rat pineal gland differed from the accepted model in the literature, with dephosphorylation and nuclear translocation occurring solely though the cAMP→PKA pathway (Kanyo *et al.*,2011). Considering that NE stimulation can elevate both intracellular Ca^{2+} and cAMP in rat pinealocytes, this provided an excellent model to study the regulatory mechanism of TORC1 and how this differed from those of TORC2 (Chik & Ho, 1989).

4.1 Regulation of TORC1 activation in the rat pineal gland

4.1.1 Adrenergic regulation of *Torc1* gene expression

Proteins that are capable of modulating the expression of specific genes, such as coactivators, must have their activity tightly regulated to ensure the expression of the target gene is at both the appropriate time and quantity (Ho & Chik, 2010). One possible regulatory mechanism is through the balance of controlled gene expression and rate of mRNA degradation. By having specific signals controlling the induction of a desired coactivator gene, and a rapid rate of degradation, the presence of this coactivator can be tightly controlled.

The whole animal time-course study reveals that *Torc1* shows no nocturnal induction in the rat pineal gland. In addition, *Torc1* shows no significant day/night variation in mRNA levels. Activation of various NE-induced post-receptor signalling pathways, that have been shown to be activated in the pineal gland, did not cause the

induction of *Torc1* transcription in cultured pineal cells(Chik & Ho, 1989; Sugden *et al*. 1987; Vanecek *et al*., 1985). Together this demonstrates that the gene expression of *Torc1* is not adrenegically regulated, suggesting that regulation may occur at the protein level.

4.1.2 NE-mediated dephosphorylation of TORC1

TORC proteins are thought to be regulated through a phosphorylation dependent mechanism, hence controlling their cellular distribution (Screaton *et al.,* 2004). Phosphorylation is thought to control the subcellular localization and stability of TORC1 by maintaining phosphorylated TORC1 in the cytoplasm through a phosphorylation dependent interaction with 14-3-3(Screaton *et al.*, 2004). The distribution of TORC1 is responsible for controlling its activity, since nuclear localization is imperative in order to fulfil its coactivator function.

Immunoblots from the whole animal time-course study reveal that TORC1 protein displays a diurnal rhythm. The onset of darkness leads to changes in TORC1 protein migration, with the appearance of a faster migrating TORC1 band. The faster migrating band of TORC1 remains throughout the period of darkness. In addition, after two hours of darkness a TORC1 doublet band is formed. The onset of light leads to a shift of TORC1 back to the slower migrating band. PhosTag polyacrylamide gel and CIP treatment demonstrate that the faster migrating band of TORC1, with the onset of darkness in whole animal samples or NE stimulation of cultured pinealocytes, is dephosphorylated TORC1. This suggests that under basal conditions TORC1 remains

phosphorylated, and the nocturnal release of NE from the SCG causes its dephosphorylation.

Selective activation of either the α_1-adrenergic receptor or β-adrenergic receptor results in the rapid dephosphorylation of TORC1. Comparison of the TORC1 band pattern produced on PhosTag gel with selective adrenergic receptor activation reveals that the NE-mediated dephosphorylation of TORC1 is mediated mainly through the activation of the α_1-adrenergic receptor. In addition, the selective adrenergic antagonist study revealed that a band pattern similar to the NE-mediated dephosphorylation of TORC1 was produced in the presence of PROP the β-adrenergic receptor antagonist, but not PRAZ, the α_1-adrenergic receptor.

Stimulation by elevation of intracellular cAMP or intracellular Ca^{2+} can lead to the dephosphorylation of TORC (Screaton, 2004; Bittinger *et al.*, 2004; Spencer and Weiser, 2010). The specific second messenger signal responsible for the NE-mediated dephosphorylation of TORC1 through the activation of adrenergic receptors was investigated. α_1-adrenergic receptor activation causes the elevation of intracellular Ca^{2+} and the activation of PKC, whereas β-adrenergic receptor activation leads to the elevation of cAMP and cGMP (Chik & Ho, 1989). My results demonstrate that either cAMP or elevation of intracellular Ca^{2+} can lead to the dephosphorylation of TORC1; however, the combination of cAMP and Ca^{2+} are involved in the NE-mediated dephosphorylation of TORC1. Elevation in cGMP or the activation of PKC cannot initiate the dephosphorylation of TORC1. These results are consistent with the receptor characterization findings, demonstrating that both the α_1-adrenergic receptor and the β-adrenergic receptor can cause the dephosphorylation of TORC1. Moreover, the NE-

mediated dephosphorylation of TORC1 requires the simultaneous activation of both pathways.

Investigation into the phosphatases responsible for the NE-mediated dephosphorylation of TORC1, indicated that both PP2A and PP2B are involved in the dephosphorylation of TORC1. The ability of different phosphatase inhibitors to block the NE-mediated dephosphorylation of TORC1 was compared. PP2A is activated through the cAMP-> PKA pathway, whereas PP2B becomes activated through elevation of intracellular Ca^{2+} and the activation of calmodulin (Oliver & Shenolikar, 1998). The NE-mediated dephosphorlyation of TORC1 is partially prevented with CSA or OKA; however CSA is able to block the dephosphorlyation to a greater extent than OKA. This suggests that PP2B is the primary phosphatase involved in the NE-mediated dephosphorylation of TORC1. In order to completely prevent the NE-mediated dephosphorylation, the combination of CSA and OKA is required. This suggests, that different phosphatases are able to dephosphorylate different Ser residues of TORC1, or multiple Ser residues. In addition, TAU has no effect on the NE-mediated dephosphorylation, demonstrating that PP1 is not involved in this process. However, CSA can block the dephosphorylation caused by cAMP or elevated intracellular Ca^{2+}, while OKA can only block the dephosphorylation initiated by cAMP. Together, this leads to the idea that the effect of PP2B may be less specific than PP2A, and further imply that the NE effect on TORC1 is mediated mainly through PP2B.

4.1.3 NE-mediated nuclear translocation of TORC1

In order for TORC1 to perform its coactivator function, by interacting with CREB and potentiating CREB-mediated transcription, TORC1 must be present in the nucleus. Elevation of cAMP or intracellular Ca^{2+} leading to the dephosphorylation of TORC1, is thought to cause the nuclear accumulation of TORC1, where it can freely associate with the bZIP domain of CREB (Screaton *et al*., 2004; Bittinger *et al*., 2004). Cellular fractionation of pinealocytes demonstrated that the NE-mediated dephosphorylation of TORC1 causes a rapid translocation of TORC1 to the nucleus. However, only a small fraction of the dephosphorylated TORC1 enters the nucleus, with the majority remaining detectable in the cytoplasmic fraction.

Surprisingly, only activation of the α_1-adrenergic receptor leads to the nuclear translocation of TORC1. NE stimulation in the presence of PRAZ can block the NE-mediated nuclear translocation of TORC1, while NE stimulation in the presence of PROP has no effect on the nuclear accumulation of TORC1. Although both the α_1-adrenergic receptor and the β-adrenergic receptor can cause the dephosphorlyation of TORC1, the NE-mediated dephosphorylation leading to the nuclear translocation of TORC1 occurs only through the α_1-adrenergic receptor.

Examination at the second messenger level demonstrates that the NE-mediated nuclear translocation of TORC1 occurs primarily through the elevation of intracellular Ca^{2+}. Other NE-induced post-receptor second messengers, such as cAMP, cGMP, and PKC have little effect on the nuclear translocation of TORC1 (Chik & Ho, 1989). This result is consistent with the NE-mediated nuclear translocation occurring through activation of the α_1-adrenergic receptor, which leads to an elevation of intracellular Ca^{2+}.

In addition, it confirms that although cAMP is capable of dephosphorylating TORC1, this dephosphorylation does not lead to significant TORC1 translocation.

The phosphorylation status of TORC1 is thought to determine its cellular localization (Screaton *et al.*, 2004). In order to determine which phosphatase acts on TORC1 and render it a suitable candidate for nuclear translocation, the blocking ability of various phosphatase inhibitors on the NE-mediated nuclear entry of TORC1 was compared. PP2B is identified as the phosphatase responsible for the NE-mediated dephosphorylation of TORC1 and its subsequent nuclear translocation. In addition, blocking PP1 or PP2A has no effect on the TORC1 nuclear translocation, suggesting these phosphatases are not involved in the NE effect.

4.1.4 Regulation of TORC1 activation

The existing model of regulation of TORC1 activation states that under basal conditions TORC1 remains phosphorylated in the cytoplasm, but upon stimulation TORC1 becomes dephosphorylated and translocates to the nucleus (Screaton, 2004; Bittinger *et al.*, 2004) Although the basic framework of this model does fit the findings of my study; current literature remains elusive of a detailed TORC1 regulatory mechanism and fails to distinguish any differences in regulation between TORC isoforms.

The results of this study provide additional insight into the regulatory mechanism that controls the activation of TORC1. Stimulation of the α_1-adrenergic receptor, leads to the activation of PP2B through the elevation of intracellular Ca^{2+}(FIG

4.1). PP2B is able to dephosphorylate TORC1 leading to its subsequent nuclear translocation. Although both PP2A and PP2B are involved in the NE-mediated dephosphorylation of TORC1, the activation of the α_1-adrenergic receptor pathway is sufficient for the NE-mediated TORC1 nuclear translocation.

PhosTag gel reveals that different signalling pathways can dephosphorylate TORC1 to varying extents, inferring that there are multiple phosphorylation sites present on TORC1. Dephosphorylation of a specific site(s) may be a requirement for the nuclear translocation of TORC1. This can possibly explain why elevation of cAMP is capable of causing the dephosphorylation of TORC1; however, this dephosphorylation does not lead to the nuclear accumulation of TORC1.

It has been assumed that all TORC isoforms share an identical regulatory mechanism. In the existing model, elevation of either cAMP or intracellular Ca^{2+} will cause the dephosphorylation and nuclear entry of all TORC isoforms (Screaton *et al*., 2004; Bittinger *et al*., 2004; Altarejos & Montminy, 2011). By having both TORC1 and TORC2 present in the pineal gland, we were able to compare the distinct mechanisms involved in regulating the activation of TORC1 and TORC2. The NE-mediated dephosphorylation and nuclear translocation of TORC2 occurs through the activation of the β-adrenergic receptor leading to an elevation in cAMP and the activation of PP2A (Kanyo *et al*., 2011). However, the regulatory mechanism controlling the activation of TORC2 is dramatically different from our findings for TORC1, demonstrating that TORC1 and TORC2 are regulated through different mechanisms in the rat pineal gland. This difference in regulation, challenges the current idea, that all TORC isoforms share an identical regulatory mechanism.

The rapid dephosphorylation and nuclear accumulation of TORC1 is crucial for TORC1 to fulfil its coactivator role. My results demonstrate that TORC1 is both expressed and regulated in the pineal gland. The NE-mediated dephosphorylation and nuclear accumulation of TORC1 occurs prior to the induction of pCREB and AA-NAT. In order for TORC1 to potentiate the transcription of *Aa-nat*, TORC1 must be localized in the nucleus prior to *Aa-nat* expression. One can speculate that the nocturnal release of NE from the SCG causes the rapid dephosphorylation of TORC1 seen in the whole animal study. This dephosphorylation leads to the nuclear translocation of TORC1. TORC1 localized in the nucleus will be able to associate with CREB, resulting in the potentiation of *Aa-nat* transcription leading to an enhancement in the amplitude and duration of *Aa-nat* expression. Elevation in expression will result in increased synthesis and activity of AA-NAT protein, which leads to elevated levels and/or duration of circulating melatonin (Ganguly *et al.*, 2002).

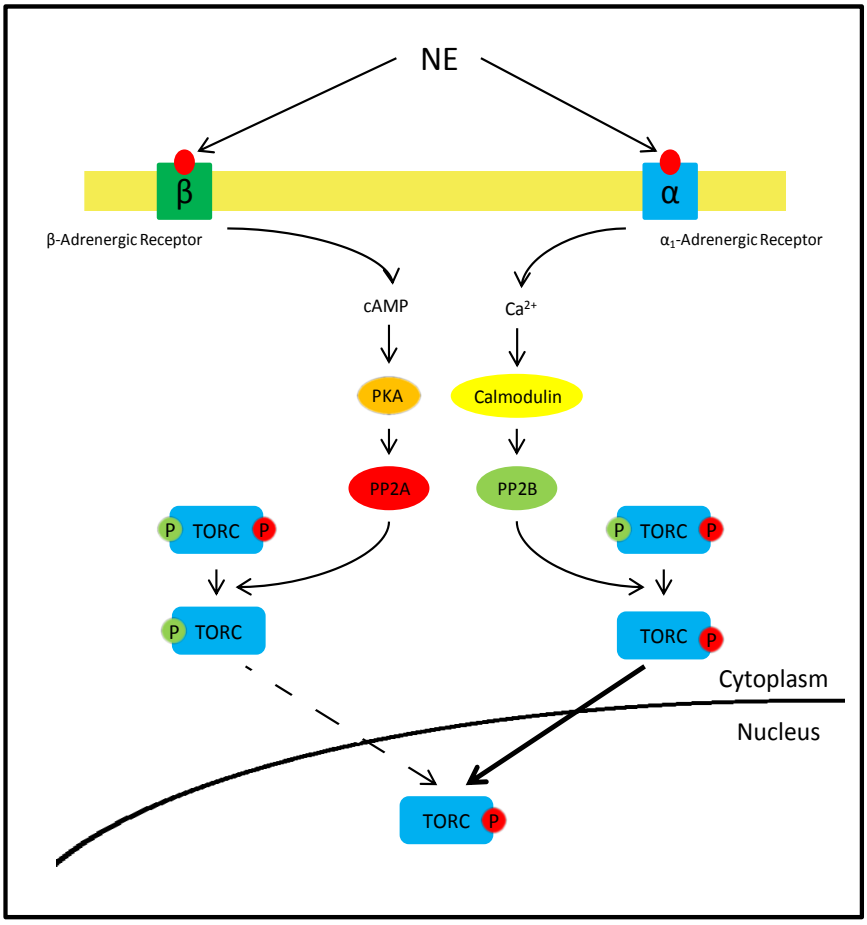

FIG. 4.1. TORC1 activation model. The interaction of NE with the α_1- and β-adrenergic receptors causes their activation, leading to an elevation in cAMP and intracellular Ca^{2+}. cAMP causes the activation of PKA, ultimately leading to the activation of protein phophatase 2A. Increased intracellular Ca^{2+}, activates calmodulin, which subsequently activates protein phosphatase 2B. Together these phosphatases cause the dephosphorylation of TORC1; however, only the dephosphorylation of TORC1 through PP2B activity leads to the significant nuclear entry of TORC1. TORC1 activation pathway is as follows: stimulation of the β – adrenergic receptor, causes the elevation of intracellular Ca^{2+}, and leads to the activation of PP2B, which can then dephosphorylate TORC1, leading to its nuclear translocation.

4.2 Regulation involved in the maintenance of TORC1 activity after NE stimulation

Current studies pertaining to the regulation of TORC are focused primarily around the initial nuclear translocation (reviewed in: Altarejos & Montminy, 2011; Takemori & Okamoto, 2008). These studies fail to investigate the time profile of stimulated TORC1 cellular distribution and the mechanism involved in maintaining TORC1 activity. It is believed that the nuclear entry of TORC1 is the main step in the regulation of TORC1, and the reverse of its activation mechanism is responsible for its inactivation. In addition, it is assumed that active SIK1 will phosphorylate TORC1, leading to its nuclear export and sequestration in the cytoplasm through the phosphorylation dependent interaction with 14-3-3 (Takemori & Okamoto, 2008). What is involved in maintaining the NE-mediated nuclear presence of TORC1 and how this process is regulated has not been previously investigated.

The NE time course study performed in fractionated pinealocytes demonstrates that TORC1 is present in the nucleus throughout the period of NE stimulation. The lower dephosphorylated TORC1 band remains throughout the period of stimulation; however, after 4 h of NE, a partially phosphorylated TORC1 band appears. The presence of the partially phosphorylated TORC1 is also seen in the whole gland study. Together, this demonstrates that TORC1 becomes rapidly dephosphorylated and translocates to the nucleus, but after a few hours of stimulation TORC1 becomes partially rephosphorylated and remains in this form for the remainder of the stimulation period. The time profile of NE-stimulated cellular distribution of TORC1 contrasts with that of TORC2, in which the nuclear accumulation of TORC2 decreases over-time, further highlighting differences in their regulation (Kanyo *et al.* 2011). In addition, partially phosphorylated TORC1 is detected in both the nucleus and cytoplasm after 4 h of

stimulation. The presence of this partially phosphorylated species of TORC1 in the nucleus, challenges the existing regulatory model of TORC1 where by phosphorylation determines TORC1 cellular distribution (Takemori & Okamoto, 2008). Partially phosphorylated TORC1 present in the nucleus implies that any phosphorylation of TORC1 may not be an absolute determinant of TORC1 cellular distribution.

This study also establishes that constant adrenergic stimulation is necessary to maintain the NE-mediated dephosphorylation of TORC1. Blockade of specific adrenergic receptor with either PRAZ or PROP, leads to the rapid rephosphorylation of TORC1. The rephosphorylation with the addition of the blockers was seen after both short and long duration of NE stimulation, suggesting that continuous adrenergic stimulation is necessary to maintain the dephosphorylation of TORC1 throughout its period of activity. Interestingly, the addition of PROP causes a more rapid and pronounced TORC1 rephopshorylation, than compared with the effect after the addition of PRAZ. The NE-mediated dephoshphorylation of TORC1 occurs through the activation of the α_1-adrenergic receptor; however, once dephosphorylated both adrenergic pathways must be continuously activated in order to maintain this dephosphorylation.

Continuous activation of both the α_1-adrenergic receptor and the β-adrenergic receptor is also required to maintain the NE-mediated nuclear presence of TORC1. Specific adrenergic blockade through the addition of PRAZ or PROP to NE stimulated pinealocytes led to the rapid export of TORC1 from the nucleus. The specific adrenergic receptor blockade after various durations of stimulation, results in the rapid rephosphorylation and nuclear export of TORC1. This demonstrates that the initial nuclear translocation and the maintenance of TORC1 in the nucleus are both regulated

processes. The results of this study reveal that activation of the α_1-adrenergic receptor is sufficient to cause the nuclear translocation of TORC1; however, once in the nucleus, activation of both the α_1-adrenergic receptor and the β-adrenergic receptor are required to maintain the nuclear localization of TORC1. This demonstrates that the cellular distribution and nuclear retention of TORC1 are regulated processes. In addition, the onset of light in the whole animal study leads to the rapid rephosphorylation of TORC1. One can speculate that this rephosphorylation will lead to the nuclear export of TORC1. Once TORC1 has been exported from the nucleus it can no longer perform its coactivator function.

The initial NE-mediated dephosphorylation and subsequent nuclear import of TORC1 occurs through the action of PP2B. The blockage of PP2A and PP2B after NE-stimulation demonstrates that continuous activity of these phosphatases is required to maintain the NE-mediated dephosphorylation of TORC1. The blockage of PP2B after NE stimulation leads to a significant reduction in TORC1 nuclear quantity, while the nuclear quantity of TORC1 is not altered with the blockage of PP2A. My results demonstrate that the activity of PP2B is responsible for both the initial dephosphorylation of TORC1 leading to its nuclear translocation, but also for maintaining TORC1 in a dephosphorylated state that can be retained in the nucleus. The need for constant α_1-adrenergic receptor stimulation to maintain the nuclear presence of TORC1 is consistent with my findings for PP2B, suggesting that the continuous stimulation of the α_1-adrenergic receptor is required in sustaining PP2B activity. Although PP2A activity is controlled through the β-adrenergic→cAMP→PKA pathway, this phosphatase is not involved in maintaining TORC1 in the nucleus, implying that the necessity of continuous

β-adrenergic receptor activation in maintaining the nuclear presence of TORC1 is not mediated through PP2A.

The duration and amplitude of *Aa-nat* expression must be tightly controlled to ensure the nocturnal increase of melatonin closely reflects the duration of night (Ganguly *et al.*, 2002). Having the coactivator function of TORC1, tightly regulated through its cellular distribution, allows for the precise modulation of the temporal profile of the CREB-mediated *Aa-nat* expression. My results demonstrate that the maintenance of the NE-mediated nuclear accumulation of TORC1, is regulated through a mechanism distinct from the one involved in the initial NE-mediated activation of TORC1. This idea challenges the current TORC1 regulatory model, which suggests that the turnoff mechanism of TORC1 is simply a reversal of its activation mechanism (Altarejos & Montminy, 2011; Takemori & Okamoto, 2008). This difference in regulation, where activation of the α_1- adrenergic receptor is sufficient to cause the initial dephosphorylation and nuclear entry of TORC1, while activation of both the α_1- and β-adrenergic receptors are required to maintain the nuclear presence of TORC1, may be due to differences in the subcellular localization of regulatory kinases or phosphatases. It is possible that dephosphorylation of a specific Ser residue of TORC1 is required for the nuclear translocation; however, once in the nucleus, phopshorylation of a different Ser residue may be necessary to cause the nuclear export of TORC1. The rapid rate of rephosphorylation when adrenergic stimulation is blocked, poses the idea that the kinases or phosphatases responsible for maintaining the dephosphorylation of TORC1, or causing the rapid rephosphorlyation are located in close proximaty to TORC1. Potential involvement of an A-kinase-anchoring protein could provide an explanation for

the close proximity, and ultimately rapid effect of the enzymes responsible for controlling the phosphorylation status of TORC1.

4.3 Role of SIK1 in the regulation of TORC1

A recent *Nature* review suggests that TORC is regulated through phosphorylation by salt-inducible kinases (SIKs) (Altarejos & Montminy, 2011). Studies involving various cell types propose that TORC activity is controlled via phosphorylation by SIK; however, evidence confirming this phenomenon is limited (Takemori *et al.,* 2007 Takemori & Okamoto, 2008; Liu *et al.*, 2012). The proposed model, in the literature of TORC1 regulation involves phosphorlyation of specific Ser residues on TORC1 by SIK1, leading to the sequestration of TORC1 in the cytoplasm through the phosphorylation dependent interaction with 14-3-3 (Altarejos & Montminy, 2011; Li *et al.*, 2009; Takemori & Okamato, 2008). Elevation in cAMP causes the activation of PKA, which results in the inhibition of SIK1 through the PKA-mediated phosphorylation of Ser-577 (Katoh *et al*, 2004). The inactivation of SIK1 leads to the dephosphorylation of TORC1, allowing it to dissociate away from 14-3-3 and translocate to the nucleus (Altarejos & Montminy, 2011; Takemori & Okamato, 2008).

Considering the significant role SIK1 is thought to play in the regulation of TORC1, transfection studies were used to investigate the relationship between TORC1 and SIK1. Overexpression of SIK1 in NE stimulated pinealocytes results in TORC1 remaining exclusively in the upper phosphorylated band. This enhanced phosphorylation seen with SIK1 overexpression confirms that SIK1 can phosphorylate TORC1. In addition, under basal conditions, TORC1 remains in the upper

phosphorylated band in the presence of SIK1 knockdown. Together these results demonstrate that SIK1 can phosphorylate TORC1, but SIK1 does not seem to be the sole kinase responsible for the basal phosphorylation of TORC1.

The cellular localization of TORC1 in pinealocytes is not controlled through SIK1 phosphorylation. Manipulation of SIK1 through adenoviral overexpression and knockdown, does not alter the quantity of TORC1 present in the nucleus after NE stimulation. SIK1 knockdown does not cause the nuclear translocation of TORC1 under basal conditions and does not lead to an enhancement in the NE-mediated nuclear accumulation of TORC1. The quantity of TORC1 translocated to the nucleus after NE stimulation is unchanged in the presence of SIK1 overexpression. Furthermore, SIK1 overexpression results in an enhancement in the upper phosphorylated TORC1 band in the nucleus, confirming that the SIK1-mediated phosphorylation of TORC1 does not lead to the nuclear export of TORC1. The same result is obtained after both short and long stimulation periods, implying the lack of SIK1 involvement at any time. These results demonstrate that the SIK1-mediated phosphorylation of TORC1 is not responsible for controlling the cellular distribution of TORC1.

The lack of SIK1 involvement in regulating the activation of TORC1, leads to the investigation of whether SIK1 is involved in the rapid rephosphorylation, when adrenergic stimulation is blocked. The rapid rephosphorylation with the addition of PRAZ or PROP was not eliminated in samples containing SIK1 knockdown. One can speculate that this rapid rephosphorylation occurring in the absence of SIK1 with the addition of PRAZ or PROP, will lead to the nuclear export of TORC1. My results suggest that SIK1 is not the sole kinase involved in the phosphorylation of TORC1. In addition,

the rephosphorylation leading to the nuclear export of TORC1 when adrenergic stimulation is blocked is not mediated through SIK1.

My results challenge the current regulatory dogma, and suggest that SIK1 is not the kinase responsible for controlling the cellular distribution of TORC1. SIK1 can phosphorylate TORC1; however, this phosphorylation does not lead to the sequestration of TORC1 in the cytoplasm. Furthermore, SIK1 is not responsible for the rephosphorylation of TORC1 when adrenergic stimulation is blocked. Together my results suggest that the enhancement in AA-NAT seen with SIK1 knockdown and the decrease in AA-NAT seen with *Sik1* overexpression is not facilitated through the regulation of TORC1 cellular distribution (Kanyo *et al.*, 2009). SIK1-mediated phosphorylation of TORC1 may still play a role in the regulation of TORC1 activity, but this may be through altering the ability of TORC1 to perform its coactivator role, such as affecting its interaction with CREB or CBP, rather than controlling its cellular distribution.

5. Future directions

1) Investigation into the role TORC1 plays in the NE induced transcription of *Aa-nat*.

My results demonstrate that, the specific CREB coactivator, TORC1 is both expressed and regulated in the pineal gland. Investigation into the ability of TORC1 to modulate the NE induced CREB-mediated transcription of *Aa-nat,* would be of great importance to further the understanding of how the transcription of *Aa-nat* is regulated in the pineal gland.

2) Identification of the specific functional role of various TORC1 phosphorylation sites.

It has been shown that there are multiple phosphorylation sites present on TORC2 (Uebi *et al.,* 2010). My results suggest that TORC1 can be phosphorylated at multiple sites. Different aspects of regulating TORC1, such as; nuclear translocation, nuclear export, and coactivator potential, may be controlled through specific phosphorylation sites present on TORC1. In order to further the understanding of how TORC1 is regulated, investigation into the functional role of each of these sites would enhance our understanding of TORC1 regulation.

3) Contribution of other SIK family members in the regulation of TORC1 cellular distribution.

Contrary to what is suggested in the existing literature, my results demonstrate that SIK1 is not the key kinase involved in the regulation of TORC1. The fact that TORC1 is phospohorylated under basal conditions, and can be rapidly rephosphorylated when adrenergic stimulation is blocked in SIK1 knockdown samples, implies that another kinase is involved in this process. There is the potential that another member of the

SIK/AMPK family can cause the phosphorylation of TORC1 and controls its cellular distribution. Preliminary unpublished data suggests that SIK2 is expressed in the pineal gland and may play a role in the regulation of TORC1.

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