Negative regulation of cytosolic phospholipase A_2 by melatonin in the rat pineal gland

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In this paper evidence that supports a new role for melatonin as a negative endogenous regulator of cytosolic phospholipase A_2 ($cPLA_2$) is presented. When rat pineal glands were incubated in culture, time-dependent release of arachidonic acid (AA) was observed, which was significantly inhibited by a known 85-kDa $cPLA_2$ inhibitor, methyl arachidonyl fluorophosphonate. Co-incubation with melatonin inhibited the AA release in a concentration-dependent manner, and this decrease was accompanied by a reduction of $cPLA_2$ protein and mRNA expression. Melatonin-receptor agonists, 2-iodo-*N*-butanoyl-5-methoxytryptamine and 5-methoxycarbonylamino-*N*-acetyltryptamine, also decreased AA release and $cPLA_2$ protein and mRNA levels, while pre-incubation with the melatonin receptor antagonists luzindole and 2-phenylmelatonin abolished the melatonin effect. *In vivo*, as melatonin production reflected a typical diurnal variation, endogenous non-esterified AA and $cPLA_2$ mRNA levels in the rat pineal gland showed an off-phase diurnal pattern in relation to melatonin levels. Intravenous administration of isoproterenol, which has been shown to elevate melatonin production, also decreased the levels of non-esterified AA and $cPLA_2$ mRNA significantly. Direct administration of melatonin to rats by intravenous injection decreased the levels of non-esterified AA, $cPLA_2$ protein and mRNA in rat pineal glands. In conclusion, melatonin endogenously down-regulates $cPLA_2$ expression, presumably through melatonin-receptor-mediated processes.

Key words: arachidonic acid, gas chromatography/mass spectrometry, isoproterenol, melatonin receptor.

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine), a circulating indoleamine hormone, is biosynthesized primarily in the mammalian pineal gland from tryptophan and secreted into the bloodstream [1,2]. Production and secretion of melatonin is under control of a circadian clock, with the highest levels occurring during the dark period of the light/dark cycle. Melatonin has been suggested to be involved in many physiological and pathophysiological processes such as synchronizing biological rhythms, seasonal control of reproduction, mood and body temperature, as well as immune and neuroendocrine responses [1–3]. However, its definite biochemical and biological functions are not clearly understood.

Recent reports have indicated a role for melatonin in the transcriptional regulation of proteins involved in the production of eicosanoids [4–7]. It has been reported that melatonin represses 5-lipoxygenase (LOX) gene expression in human B lymphocytes [4]. This finding has been further supported by observations that levels of hippocampal 5-LOX mRNA increased in melatonin-deficient pinealectomized rats [5], and pineal 5-LOX mRNA levels decreased during the dark phase [6]. In the rat pineal gland, 12-LOX is the major LOX expressed, producing 12-hydroxyeicosatetraenoic acid (12-HETE) from arachidonic acid (AA) [8]. Recently, we have reported that melatonin also down-regulates 12-lipoxygenation in the rat pineal gland, as 12-HETE and 12-LOX mRNA/protein levels decreased significantly after melatonin treatment *in vitro* as well as *in vivo* [7]. The observed reduction of 12-HETE levels may well be the consequence of the negative control of 12-LOX expression. However, it is also possible that availability of the precursor AA is decreased by melatonin.

The release of AA is an important step for the generation of a variety of eicosanoids, many of which are bioactive mediators [9–12]. Free AA has also been implicated in numerous physiological processes such as mediating signals of neurotransmitters [13], cytokines and growth factors [14] as well as modulation of ion-channel activities [15]. Therefore, a tight control of the levels of AA is critical for proper cellular function in biological systems. AA can be released from membrane phospholipids by phospholipase A_2 (PLA_2). Mammalian cells contain various forms of PLA_2 [16,17], including a 14-kDa secretory PLA_2 ($sPLA_2$) [18,19], an 85-kDa cytosolic PLA_2 ($cPLA_2$) [9,20,21] and a calcium-independent high-molecular-mass PLA_2 ($iPLA_2$) [22]. These enzymes provide differently regulated pathways for the important process of fatty acid turnover [9]. Among these PLA_2s, the 85-kDa $cPLA_2$ has unique specificity for agonist-induced AA release from the *sn*-2 position of phospholipids with the concomitant

Abbreviations used: AA, arachidonic acid; d_8-AA, deuterium-labelled AA; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent high-molecular-mass PLA_2; GR-135,531, 5-methoxycarbonylamino-*N*-acetyltryptamine; 12-HETE, 12-hydroxyeicosatetraenoic acid; 2IbMT, 2-iodo-*N*-butanoyl-5-methoxytryptamine; LOX, lipoxygenase; 2-PMT, 2-phenylmelatonin; MAFP, methyl arachidonyl fluorophosphonate; MAP kinase, mitogen-activated protein kinase; GC-NCI/MS, GC-negative-ion chemical ionization/MS; PKC, protein kinase C; RT, reverse transcriptase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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production of lysophospholipids, and is generally thought to play an important role in mediating AA release and maintaining cellular AA levels [9,20,23,24].

$cPLA_2$ is widely expressed in various tissues and is under the control of phosphorylation, G-protein activation and calcium-dependent translocation to membranes as well as transcriptional regulation [14,17,25]. In this study we tested whether negative transcriptional regulation of the eicosanoid production system by melatonin can be extended to the level of AA generation and $cPLA_2$ expression. The effect of melatonin on AA release and the nature of this effect were first examined using cultured rat pineal glands with exogenous addition of melatonin, a $cPLA_2$ inhibitor (methyl arachidonyl fluorophosphonate, MAFP) and melatonin-receptor agonists and antagonists. Subsequently, the endogenous levels of non-esterified AA, $cPLA_2$ protein and mRNA were examined in the rat pineal gland during the light/dark cycle. The effect of melatonin *in vivo* was investigated after elevating melatonin levels during the daytime by intravenous administration of melatonin or a β-adrenergic receptor agonist, isoproterenol [26,27]. Both *in vitro* and *in vivo* results strongly suggested a new role for melatonin as an endogenous negative modulator of pineal $cPLA_2$.

EXPERIMENTAL

Chemicals and animals

Deuterium-labelled arachidonic acid (d_8-AA) was purchased from Biomol Research Laboratory (Rockford, IL, U.S.A.). Melatonin was obtained from Sigma, and d_4-melatonin [*N*-acetyl-5-methoxy(a,a,b,b-d_4)tryptamine] was a generous gift from Dr S. P. Markey (National Institute of Mental Health, NIH, Bethesda, MD, U.S.A.). BGJb medium and other tissue-culture reagents were purchased from Life Technologies (Rockville, MD, U.S.A.). $cPLA_2$ inhibitor MAFP and other kinase inhibitors were obtained from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). Melatonin-receptor agonists, 2-iodo-*N*-butanoyl-5-methoxytryptamine (2IbMT) and 5-methoxy-carbonylamino-*N*-acetyltryptamine (GR-135,531), and antagonists, luzinodole and 2-phenylmelatonin (2-PMT) were purchased from Tocris Cookson (Ballwin, MO, U.S.A.). Luzindole was first dissolved in DMSO and 2IbMT, GR-135,531 and 2-PMT in ethanol, and then they were diluted in PBS to the designated concentration immediately before use.

Typically, male Sprague–Dawley rats (200 ± 10 g body weight) were conditioned for 7 days upon arrival with a controlled light cycle (light from 7:00 to 19:00 h) and free access to a rat chow diet (NIH-07) and water.

Pineal-gland culture

Rat pineal glands were collected immediately after the animals were decapitated [28], then placed in a 24-well culture dish and incubated with 300 μl of BGJb medium supplemented with 0.2 mM L-glutamine, 0.1 mg/ml ascorbic acid and 100 μg/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2. The pineal glands were conditioned for 48 h with the medium changed every 24 h. At the end of the conditioning period, the medium was changed and the glands were treated with various agents, followed by the collection of medium or pineal glands at specified time points. It is well established that in the mammalian pineal gland noradrenaline (norepinephrine) is released from post-ganglionic sympathetic nerve terminals. The released noradrenaline can activate β-adrenergic receptors, which will ultimately increase melatonin synthesis. During the conditioning period, nerve endings degenerate and stored noradrenaline is emptied, allowing better control of pineal glands for subsequent melatonin treatments [29,30]. Separately, for melatonin-receptor antagonist studies, the conditioned rat pineal glands were pre-incubated with 10 nM antagonists for 1 h, and then incubated with 10 nM melatonin for an additional 3 h.

Analysis of AA

For the analysis of AA release from pineal glands, AA was extracted from culture medium according to the method of Bligh and Dyer [31] in the presence of d_8-AA as an internal standard. The free AA was then converted to pentafluorobenzyl esters by reacting with 1 % di-isopropylethylamine and 0.5 % pentafluorobenzyl bromide in acetonitrile at 45 °C for 20 min. The pentafluorobenzyl esters were then analysed by GC-negative-ion chemical ionization/MS (GC-NCI/MS) using an HP 5890 gas chromatograph equipped with an SPB-1 [Supelco bonded poly(dimethylsiloxane)]-fused silica capillary column (30 m $\times$ 0.32 mm, 0.25 μm film thickness; Supelco, Bellefonte, PA, U.S.A.) and coupled to an HP 5989A mass spectrometer. The oven temperature was raised from 70 to 220 °C at 30 °C/min, and from 220 to 280 °C at 5 °C/min. The temperatures of the mass spectrometer source and quadrupole were set at 150 and 105 °C, respectively. The spectrometer was operated in the selected-ion monitoring mode to record ions of m/z 303 (AA) and m/z 311 (d_8-AA).

In vivo modulation of non-esterified AA and $cPLA_2$ expression by melatonin

For studies performed during the regular light/dark cycle, a group of six animals was killed and the pineal glands were collected at 10:00 (day) and 0:00 h (night) for day and night studies, respectively. Non-esterified AA levels and $cPLA_2$ expression were measured directly from the collected pineal glands.

Isoproterenol (Sigma), a potent β-adrenergic receptor agonist, was used to stimulate melatonin production during the daytime [26,27]. A total of 12 rats were divided into two groups: vehicle control (0.9 % NaCl, six animals) and isoproterenol-injection group (six animals). Isoproterenol was dissolved in 0.9 % NaCl solution and injected into the tail vein (5 mg/kg of body weight). The injection was performed between 10:00 and 14:00 h since the stimulation of melatonin production by isoproterenol would be more pronounced during the daytime. After 3 h, rats were killed by decapitation and the pineal glands collected immediately.

To study the effect of melatonin injection on $cPLA_2$, melatonin was first dissolved in DMSO and then diluted 50 times using 0.9 % NaCl to a final concentration of 1.5 mg/ml in 2 % DMSO. The freshly prepared melatonin solution was injected into the rat tail vein (0.5 mg/kg of body weight) using rat restrainers (Lab Products). For this experiment 12 rats were used in two groups: vehicle control (2 % DMSO, six animals) and melatonin-injection group (six animals). After 1 h, rats were killed and the pineal glands collected immediately.

Analysis of endogenous melatonin and non-esterified AA

For the analysis of endogenous melatonin and non-esterified AA in pineal glands, rats were decapitated and pineals were collected within 40 s into 1 ml of cold methanol containing 50 μg/ml butylated hydroxytoluene on dry ice for immediate analysis or stored at -80 °C for later use. Rapid pineal-gland collection has previously been shown to yield consistent results [28]. Each

pineal gland was homogenized in butylated hydroxytoluene/methanol with d_4-melatonin and d_8-AA. AA and melatonin were extracted according to the method of Bligh and Dyer [31].

Aliquots of pineal extracts (typically one-fifth to one-third of a pineal gland) were dried down under nitrogen and melatonin was derivatized with pentafluoropropionic anhydride in ethyl acetate for 10 min at 60 °C. Non-esterified AA was isolated by solid-phase extraction as described previously [28] before derivatization. Quantitative determination of melatonin or AA was performed using GC-NCI/MS in the selected-ion monitoring mode as described previously [7,28].

Western-blot analysis of $cPLA_2$ protein

Pineal glands were collected in sterilized 1.5-ml Eppendorf centrifuge tubes on dry ice for immediate analysis or stored at −80 °C for later use. Each pineal gland was homogenized and lysed in 50 μl of RIPA buffer (1% igepal, 0.5% sodium deoxycholate and 0.1% SDS in 1 × PBS) and the supernatant was collected after centrifugation at 14000 ***g*** for 20 min. A protein assay was performed using the BCA reaction [32] (BCA protein assay kit, Pierce, Rockford, IL, U.S.A.). Pineal proteins (10 μg) were electrophoresed by SDS/PAGE (13% gels), and transferred on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked with 5% non-fat dried milk and then incubated with a 1:500 dilution of rabbit polyclonal anti-$cPLA_2$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 4 °C overnight. The membranes were incubated subsequently for 1 h at room temperature with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Sigma), washed with Tris-buffered saline containing 0.1% Tween 20 and subjected finally to enhanced chemiluminescence detection (ECL®, Amersham, Arlington Heights, IL, U.S.A.). The $cPLA_2$ protein levels were quantified by densitometry, and statistical analysis was performed using the unpaired Student's *t* test.

$cPLA_2$ mRNA determination via reverse transcriptase (RT)-PCR

Total rat pineal RNA was isolated from each gland, which was collected immediately according to the procedures described earlier [7]. Isolated RNA (1 μg) was used to reverse-transcribe the first-strand cDNA using Superscript II RT (Life Technologies) in a total volume of 20 μl. Template cDNA (1–2 μl) was mixed with PCR reaction buffer (PCR Master Mix, Boehringer Mannheim, Indianapolis, IN, U.S.A.) and 40 pmol of the following primers and amplified for 35 cycles as described earlier [7]. The upstream primer used was 5′-GCTCCACATG-GTACATGTCA-3′ (20-mer, positioned from nt 638 to 658) and the downstream primer was 5′-CTTCAAGCTACTCAAGGT-CG-3′ (20-mer, positioned from nt 896 to 879 to give a PCR product of 259 bp). The glyceraldehyde-3-phosphate dehydrogenase primer (G3PDH, 20 pmol; Clontech, Palo Alto, CA, U.S.A.) was used as a positive control.

RESULTS

Time-dependent release of AA and dose-dependent inhibition by melatonin

When rat pineal glands were incubated in the medium for up to 5 h, AA release was increased in a time-dependent manner (Figure 1, black bars). Co-incubation of pineal glands with 10 nM melatonin significantly inhibited the observed increase of AA release (Figure 1, grey bars). Treatment of pineal glands with melatonin in the concentration range between 10 pM and 250 nM resulted in a dose-dependent decrease of AA release, as shown for the 3 h incubation period in Figure 2. At a melatonin concentration as low as 10 pM, AA release was decreased by $40.7 \pm 6.3\%$ ($P < 0.01$) in comparison to the control, and at 1 nM, a $69.1 \pm 2.4\%$ reduction ($P < 0.001$) was observed. At melatonin concentrations above 1 nM, no further reduction in AA release was detected.

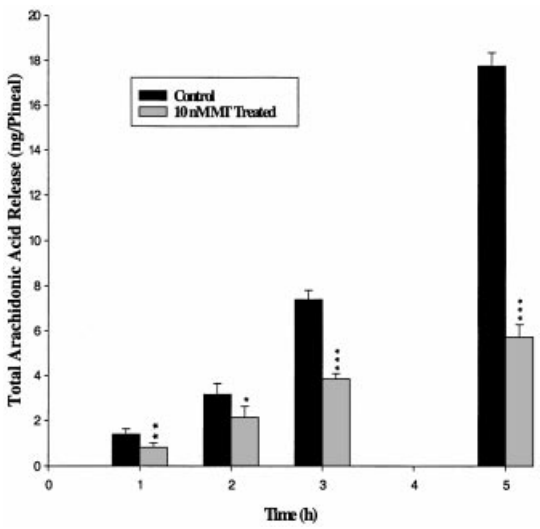

Figure 1 Time courses for AA release in cultured rat pineal glands treated with 0.08% DMSO as vehicle control (black bars) and 10 nM melatonin (MT, grey bars)

Rat pineal glands were collected immediately after decapitation and then conditioned for 48 h at 37 °C with 500 μl of working medium (BGJb medium supplemented with 0.2 mM L-glutamine, 0.1 mg/ml ascorbic acid and 100 μg/ml penicillin/streptomycin). Subsequently, the glands were treated with vehicle or 10 nM melatonin, and the medium was collected at 1-h time intervals up to 5 h. The AA release was determined by GC-NCI/MS. Data are representative of two experiments and are expressed as means ± S.D. from three–five individual pineal glands for each time point. Unpaired Student's *t* test was performed for each time point in comparison with the respective control; $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Effect of $cPLA_2$ inhibitor on AA release

When the cultured pineal glands were incubated with 25 μM MAFP, a $cPLA_2$ and $iPLA_2$ inhibitor, the AA release was decreased by $50.6 \pm 7.5\%$ ($P < 0.001$). This result suggested that $cPLA_2$ may be involved in the release of AA from the pineal gland, although other pathways may also have contributed to the AA release.

Effect of melatonin on $cPLA_2$ protein and mRNA levels in cultured pineal glands

As a mechanism by which melatonin inhibits the release of AA in cultured pineal glands, the expression of $cPLA_2$ was examined. The $cPLA_2$ protein levels in pineal glands that were treated with 10 nM melatonin for 3 h decreased by $50 \pm 4\%$ ($P < 0.01$) in comparison with the control (Figure 3A). Steady-state levels of $cPLA_2$ mRNA, measured by RT-PCR of total RNA extracted from cultured pineal glands, were also decreased significantly after the melatonin treatment (Figure 3B).

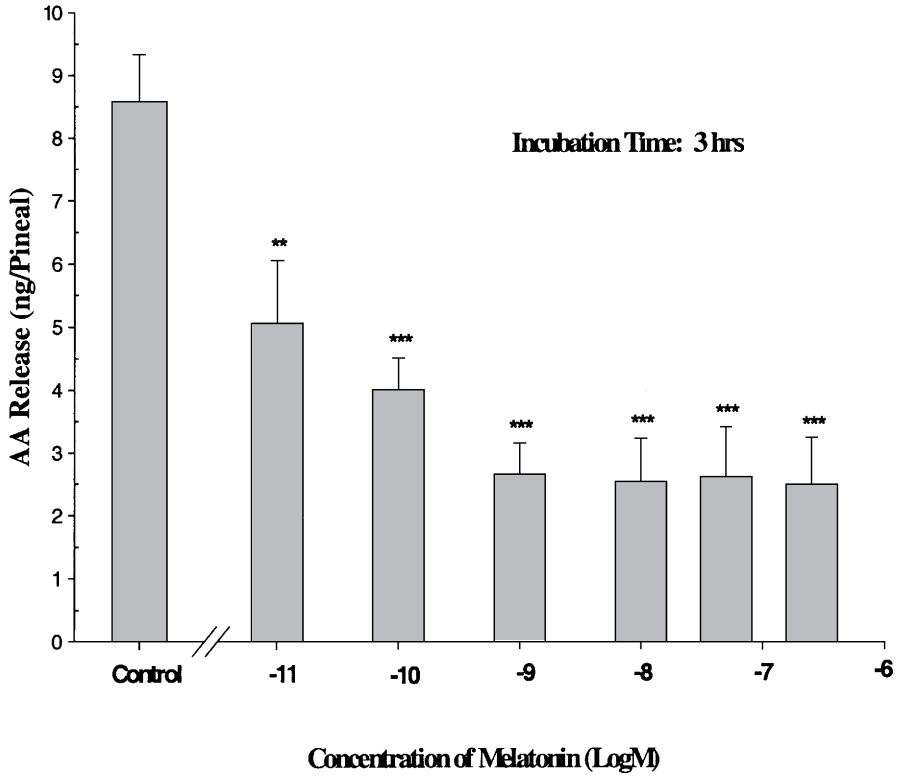

Figure 2 Dependence of the AA release on the concentration of melatonin in cultured rat pineal glands

After the 48 h conditioning period with 300 μl of working medium, the glands were treated with 0.08% DMSO as vehicle control or different concentrations of melatonin (10 pM–250 nM). AA levels were determined by GC-NCI/MS. Data are expressed as means $\pm$ S.D. from three–five individual pineal glands for each point. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control values (unpaired Student's t test). The concentrations used for the two right-most bars were 50 nM (-7.3 logM) and 250 nM (-6.6 logM).

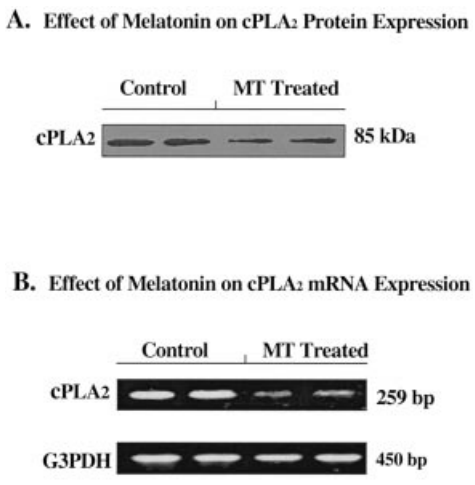

Figure 3 Effect of melatonin on cPLA$_2$ protein (A) and mRNA (B) levels in cultured pineal glands

The pineal glands were collected after 3 h of incubation with 0.08% DMSO as vehicle control or 10 nM melatonin (MT). cPLA$_2$ protein levels were determined by Western blotting. Steady-state levels of cPLA$_2$ mRNA were measured by RT-PCR of total RNA extracted from cultured pineal glands. G3PDH was used as an internal control for mRNA determination. Each lane of the Western-blot analysis represents a separate pineal gland treated individually. The data presented are representative of three experiments, each of which were performed using four–six pineal glands per group.

Effect of melatonin-receptor agonists and antagonists on AA release and cPLA$_2$ protein and mRNA levels in cultured pineal glands

Like melatonin, melatonin-receptor agonists 2IbMT or GR-135,531 also attenuated the AA release in cultured pineal glands. When the pineal glands were incubated with 2IbMT or GR-135,531 at 10 nM for 3 h, AA release was decreased by 64.2 ± 10.1 and $70.2 \pm 9.3\%$, respectively ($P < 0.001$; Figure 4A). When pineal gland was pre-incubated with melatonin-receptor antagonists luzindole or 2-PMT at 10 nM for 1 h, the attenuating effect of melatonin on AA release observed during the 3-h incubation period was abolished. These data indicated that inhibition of AA release by melatonin was exerted through a melatonin-receptor-mediated mechanism.

In agreement with the AA release presented in Figure 4(A), melatonin and 2IbMT or GR-135,531, melatonin-receptor agonists, decreased the cPLA$_2$ protein levels by 56 ± 11 ($P < 0.01$), 71 ± 6 ($P < 0.001$) and $59 \pm 10\%$ ($P < 0.01$), respectively, in comparison with the control level. However, in the presence of melatonin-receptor antagonists, luzindole or 2-PMT, melatonin-induced reduction of cPLA$_2$ protein was not observed (Figure 4B). The cPLA$_2$ protein levels were maintained at 101 ± 13 and $101 \pm 9\%$ of the control level, respectively. cPLA$_2$ mRNA levels also showed results consistent with the cPLA$_2$ protein levels, providing further evidence that inhibition of AA release by melatonin is regulated by cPLA$_2$ expression.

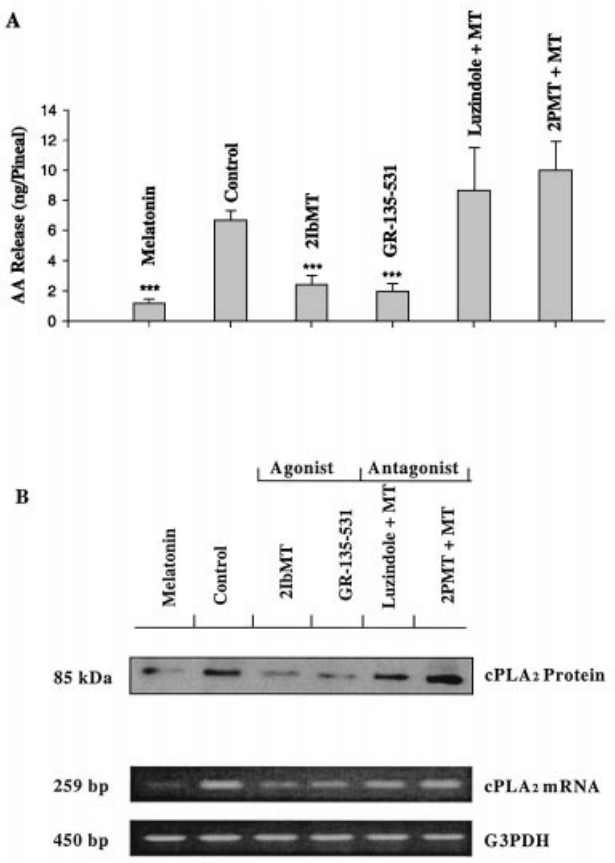

Figure 4 Effect of melatonin-receptor agonists or antagonists on AA release, $cPLA_2$ protein and mRNA levels in cultured pineal glands

After the 48-h conditioning period, the glands were treated with 10 nM each of melatonin (MT) and melatonin-receptor agonists, 2IbMT or GR-135,531, for 3 h using 0.08% DMSO and 0.08% ethanol as vehicle control. To examine the effect of melatonin-receptor antagonists, the pineal glands were pre-incubated with 10 nM luzindole or 2-PMT for 1 h, and then incubated with 10 nM melatonin for an additional 3 h. After these treatments, the medium was collected for AA analysis (**A**), and pineal glands were collected for the determination of $cPLA_2$ protein or mRNA levels (**B**). Data in (**A**) is expressed as means $\pm$ S.D. from four individual pineal glands for each data point. $^{***}P < 0.001$ versus control values (unpaired Student's *t* test). Each lane of the Western blot and RT-PCR analysis in (**B**) represents a separate pineal gland treated individually. The data presented are representative of two experiments, each of which was performed using four pineal glands per group.

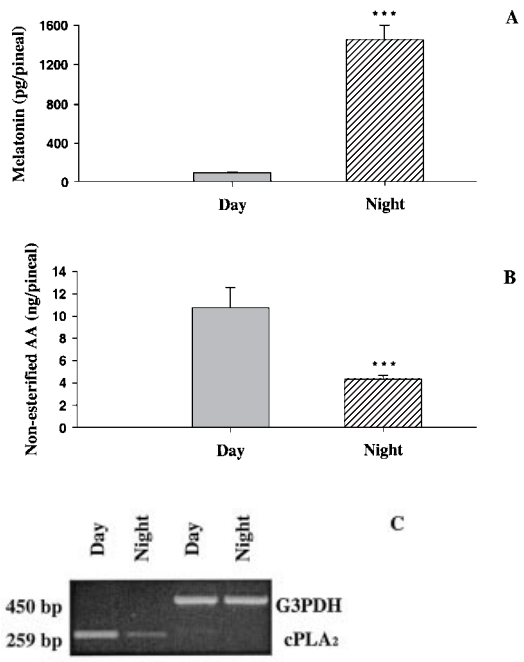

Figure 5 Modulation of endogenous levels of melatonin (A), non-esterified AA (B) and $cPLA_2$ mRNA (C) during the light/dark cycle

Pineal glands were collected immediately before analysis during 10:00–12:00 h for the light-phase study, and collected during 0:00–2:00 h for the dark-phase study. Data are expressed as means $\pm$ S.D. from six individual animals for each data point. $^{***}P < 0.001$ versus control values (unpaired Student's *t* test). The data presented are representative of three experiments, each of which was performed using six animals per group.

Diurnal variations in the endogenous levels of melatonin, non-esterified AA and $cPLA_2$ expression in the pineal glands

As expected, pineal melatonin levels were much higher at night (0:00 h, 1.44 ± 0.15 ng/pineal gland) in comparison to those during the daytime (10:00 h, 90.4 ± 9.3 pg/pineal gland; Figure 5A). In contrast, the endogenous non-esterified AA level observed at night (4.2 ± 0.3 ng/pineal gland) was significantly lower than that of the daytime (10.7 ± 1.8 ng/pineal gland; Figure 5B), indicating a negative correlation ($r = -0.96$; $P < 0.001$) between levels of melatonin and non-esterified AA. $cPLA_2$ expression assessed by $cPLA_2$ mRNA levels also showed a significant nocturnal reduction, correlating the AA levels observed during the day/night cycle (Figure 5C).

Effect of intravenous administration of isoproterenol on endogenous levels of melatonin, non-esterified AA and $cPLA_2$ expression in pineal glands

It has been well established that stimulation of the β-adrenergic receptor increases melatonin synthesis in the rat pineal gland [26,27]. As shown in Figure 6(A), melatonin levels were significantly increased (> 11-fold increase over the basal level) in the rat pineal gland at 3 h after the intravenous administration of isoproterenol. Conversely, endogenous levels of non-esterified AA were reduced by $50.3 \pm 2.6\%$ ($P < 0.001$) in comparison with the control level (Figure 6B). $cPLA_2$ mRNA levels were also significantly reduced after the isoproterenol injection, as shown in Figure 6(C). These results were in agreement with the results observed during the day/night cycle (Figure 5).

Effect of intravenous administration of melatonin on endogenous non-esterified AA levels and $cPLA_2$ expression in pineal glands

To obtain direct evidence for melatonin negatively modulating pineal $cPLA_2$ *in vivo*, rats were injected with melatonin (0.5 mg/kg of body weight) during the daytime (between 10:00 and 12:00 h). As shown in Figure 7(A), melatonin injection decreased the endogenous levels of non-esterified AA by $46 \pm 11\%$ ($P < 0.01$) in as little as 1 h. The melatonin injection also significantly decreased the endogenous levels of $cPLA_2$ protein (by $33 \pm 8\%$, $P < 0.01$) and mRNA in pineal gland (Figure 7B), indicating that melatonin indeed negatively modulates $cPLA_2$ expression *in vivo*.

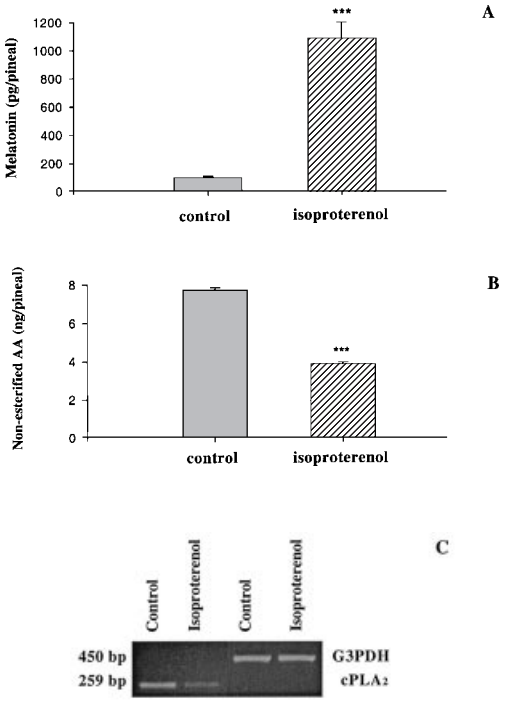

Figure 6 Effect of isoproterenol on endogenous levels of melatonin (A) and non-esterified AA (B) and cPLA$_2$ mRNA expression (C) in the rat pineal glands after intravenous administration of isoproterenol

Isoproterenol was dissolved in saline solution (0.9% NaCl, w/v), and injected into the tail vein (5 mg/kg of body weight) between 10:00 and 12:00 h. Rat pineal glands were collected 3 h after the injection, and the analyses were performed immediately after the pineal collection. Data are expressed as means ± S.D. from six individual animals for each data point. $^{***}P < 0.001$ versus control values (unpaired Student's *t* test). The data presented are representative of three experiments, each of which was performed using six animals per group.

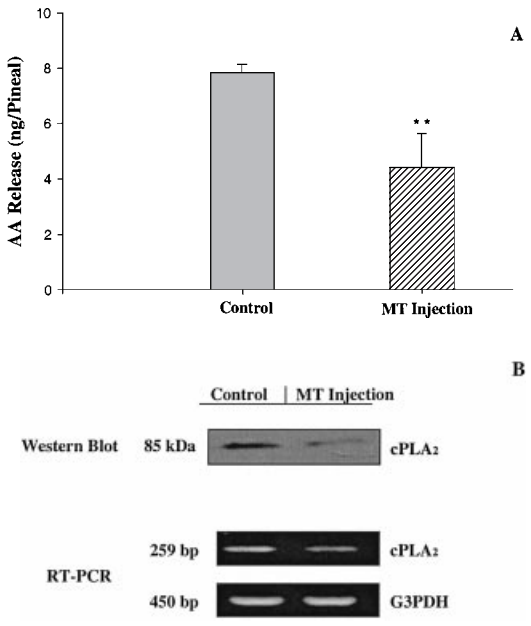

Figure 7 Effect of melatonin injection on endogenous levels of non-esterified AA (A), cPLA$_2$ protein and mRNA (B)

Rats were injected intravenously with melatonin (MT, 0.5 mg/kg of body weight), which was dissolved in saline solution (0.9% NaCl, w/v) containing 2% DMSO (v/v) between 10:00 and 12:00 h. Rat pineal glands were collected 1 h after the injection, and immediately before the analysis. Data are expressed as means ± S.D. from six individual animals for each data point. $^{**}P < 0.01$ versus control values (unpaired Student's *t* test). Each lane of the Western-blot and mRNA analysis represents a separate pineal gland treated individually. The data presented are representative of two experiments, each of which was performed using six animals per group.

DISCUSSION

In this study, we evaluated the role of melatonin in the regulation of AA release by 85-kDa cPLA$_2$ in rat pineal glands. Here, we demonstrate for the first time that melatonin suppresses the release of AA and cPLA$_2$ gene expression in the pineal gland through a receptor-mediated process. Consistent results obtained from *in vivo* models strongly suggest that melatonin may be an endogenous negative regulator of cPLA$_2$.

Melatonin has been shown to regulate intracellular concentrations of second messengers such as cAMP [33], calcium [34] and AA [35]. It has been also documented that melatonin can regulate the expression of the late-response genes such as c-*fos* and *jun*-B in pars tuberalis of the pituitary [36], suggesting a role of melatonin in transcriptional activity. Our current data strongly support the transcriptional down-regulation of cPLA$_2$ as an evident mechanism underlying the inhibitory effect of melatonin on AA release. The observed suppression of cPLA$_2$ expression is similar to the previously reported inhibitory effect of melatonin on 5- and 12-LOX expression [4,7].

Melatonin effects have been shown to be mediated by G-protein-coupled receptors localized on plasma membranes [37]. In recent reports, its binding to nuclear orphan RZR$_\beta$ receptors has also been documented [4,38]. To date, two subtypes of melatonin receptors have been detected in the mammalian system, MT1 and MT2, expressed mainly in brain and retina [39]. Although there is no direct evidence that melatonin receptors exist in the pineal gland, it has been reported that melatonin can bind to rat and bovine pineal glands [40]. Our results showed that melatonin-receptor antagonists, luzindole or 2-PMT, inhibited the melatonin effect on AA release and cPLA$_2$ expression. In contrast, melatonin-receptor agonists 2IbMT and GR-135,531 decreased the AA release and cPLA$_2$ expression in cultured pineal glands. These results are suggestive of the existence of melatonin receptors in rat pineal glands, and the observed melatonin effect may be mediated through a receptor-mediated mechanism. The specific receptor subtypes that are involved in the regulation of cPLA$_2$ expression in pineal glands, as well as their subcellular localization, remains unclear, and further investigation will be necessary.

The daytime melatonin concentration in rat serum is in the range of 8–30 pM, and the melatonin level reaches up to approx. 0.5 nM during the dark phase [41,42]. The dose-dependent suppression of AA release by melatonin was observed at the circulating melatonin concentration range during the light and dark phase. Furthermore, *in vivo* fluctuation of cPLA$_2$ expression as a function of the melatonin concentration strongly suggests that melatonin may influence AA release and subsequent production of bioactive eicosanoids under physiological conditions. No further inhibition of AA release at melatonin concentrations above 1 nM (Figure 2) may be due to the existence of melatonin-insensitive AA-release processes in pineal glands. Although the IC$_{50}$ value of MAFP has not been evaluated in our system, incomplete inhibition of AA release at 25 μM MAFP, which is significantly higher than the IC$_{50}$ of this chemical determined

for human neutrophils (10 nM) [43], was also suggestive of the existence of other pathways. Nevertheless, we could not exclude the possibility that 25 μM MAFP was not sufficient for the complete inhibition of AA release.

The AA release observed in our study was significantly inhibited by MAFP, supporting the involvement of $cPLA_2$ in this process. The activity of $cPLA_2$ may depend on many factors, such as intracellular [Ca^{2+}] concentration or phosphorylation states of $cPLA_2$ [9,16,17,20,21]. Transcriptional regulation of $cPLA_2$ expression can also influence the activity of this enzyme, as has been the case with some cytokines and growth factors [20,21]. Numerous studies have implied the involvement of protein kinase C (PKC) in the regulation of receptor-mediated AA release in various cell types [44]. For example, it has been shown that α_1-adrenergic receptors can activate $cPLA_2$ by the sequential activation of one or more forms of phospholipase C, PKC and mitogen-activated protein (MAP) kinase, which in turn phosphorylates $cPLA_2$ [45]. Some other studies showed MAP kinase can phosphorylate and activate $cPLA_2$ regardless of PKC activation [46]. We have also observed that both MAP kinase (MEK) and PKC inhibitors significantly reduced the AA release in cultured pineal glands (results not shown), implying that the activation of both MAP kinase and PKC may be important for the $cPLA_2$-mediated AA release in rat pineal glands. Although the present study does not address the phosphorylation states of $cPLA_2$, down-regulated expression of $cPLA_2$ by melatonin corroborates the observed decrease of non-esterified AA.

Our present data are the first demonstration of the role of melatonin as an endogenous negative modulator of $cPLA_2$ expression. At present, biological significance of this regulation in the pineal gland cannot be easily addressed. However, as melatonin is readily secreted into the bloodstream after its biosynthesis in the pineal gland, melatonin can reach various target cells and may exert its effect away from the pineal gland. If melatonin down-regulates $cPLA_2$ in other cells, as has been shown for the gene expression of 5-LOX in lymphocytes [4], melatonin synthesis in pineal glands may be directly coupled to the expression of $cPLA_2$, and subsequently the AA status as well as the capacity for generating eicosanoids in target cells. In such a case, the diurnal variation of melatonin levels has an important role in modulating many signalling events mediated by AA in response to stimuli, although the physiological implications of such control have yet to be seen. Under pathophysiological conditions such as inflammation, fever and immune reactions, however, melatonin, an endogenous negative regulator of $cPLA_2$, and its synthetic analogues may be explored as pharmaceutical agents to reduce abnormal activation of $cPLA_2$.

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