Journal of Cellular Biochemistry 65:430–442 (1997)

Inhibition of Cerebellar Nitric Oxide Synthase and Cyclic GMP Production by Melatonin Via Complex Formation With Calmodulin

David Pozo,[1] Russel J. Reiter,[2] Juan R. Calvo,[1] and Juan M. Guerrero[1]*

[1]Department of Medical Biochemistry and Molecular Biology, The University of Seville School of Medicine and Virgen Macarena Hospital, Seville, Spain
[2]Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas

Abstract Constitutive rat cerebellar nitric oxide synthase (NOS) activity is shown to be inhibited by physiological concentrations of the pineal hormone melatonin. The inhibition was dose-dependent and was coupled to an inhibition of the cyclic GMP production activated by L-arginine. Results also show that calmodulin appears to be involved in this process because its presence in the incubation medium was able to prevent the effect of melatonin on both NOS activity and cyclic GMP production. Moreover, polyacrylamide gel electrophoresis studies suggest that melatonin can interact with calmodulin modifying the binding of the peptide to the synthetic NOS peptide encompassing the calmodulin-binding domain of constitutive NOS from rat cerebellum, the natural mechanism by which calmodulin activates cerebellar NOS. J. Cell. Biochem. 65:430–442. © 1997 Wiley-Liss, Inc.

Key words: melatonin; pineal gland; cerebellum; nitric oxide; nitric oxide synthase; calmodulin

Nitric oxide (NO), originally identified as the principal endothelium-derived relaxing factor, is now recognized also as a neuromodulator in the central nervous system (CNS) [Snyder and Bredt, 1991; Moncada et al., 1991]. In the CNS, NO is considered as a mediator of the excitatory neurotransmitter glutamate. After binding of glutamate by postsynaptic NMDA receptors, L-arginine is converted to NO by a calmodulin/Ca^{++}/O_{2-} and NADPH-dependent soluble neuronal NO synthase (NOS) which requires tetrahydrobiopterin (H_4biopterin) for the expression of enzyme activity [Knowles et al., 1989]. Additionally, NO is also synthesized in the endothelial cells of the blood vessel irrigating the CNS by a membrane bound endothelial NOS [Faraci and Brian, 1994]. NO may serve multiple functions within the CNS [Bruhwyler et al., 1993]. In cerebellum, NO might be involved in coordination and balance. Recent evidence indicates that NO also participates in synaptic plasticity in the cerebellum, where it may be involved in long-term depression of Purkinje cell output [Shibufi and Okada, 1991]. This system has been implicated as the cellular mechanism for cerebellar motor learning [Matthies, 1989].

The pineal gland, and its major hormone melatonin, is capable of translating environmental lighting information into signals that modulate reproductive, adrenal and other neuroendocrine interactions as well as immune function [Reiter, 1991; Guerrero and Reiter, 1992]. A considerable body of evidence has accumulated suggesting that the mammalian pineal is capable of influencing CNS function [Miles and Philbrick, 1988]. Thus, Philo and Reiter [1978] have shown that pinealectomy induces convulsions that seem to parallel alterations in midbrain dopamine, serotonin, and norepinephrine levels. Melatonin itself may be involved in the pineal effect on brain excitability, as suggested by the ability of this hormone to suppress seizure activity in humans, a concept supported by the generation of epileptiform activity in rats that have received intraven-

Contract grant sponsor: CICYT, contract grant number PB91-0616; Contract grant sponsor FISSS, contract grant number 93/0210.

*Correspondence to: Juan M. Guerrero, Department of Medical Biochemistry and Molecular Biology, The University of Seville School of Medicine, Avda Sanchez Pizjuan 4, 41009-Seville, Spain.

Received 19 October 1995; accepted 15 March 1996

© 1997 Wiley-Liss, Inc.

tricular injections of antimelatonin sera [Fariello et al., 1977]. Additionally, melatonin is capable of inducing sedation in adult males [Lerner and Case, 1960], and subsequent studies of melatonin administration to humans have shown this hormone capable of modifying electroencephalographic patterns and rhythms in sleep and motor activity [Moskowitz and Wurtman, 1977]. Also, various aspects of human performance have also been found to be influenced by the daytime administration of melatonin, i.e., reduced vigor and increased fatigue and confusion as measured by the Profile of Mood States (POMS), Standford Sleepiness Scale (SSS), and the Wilkinson auditory vigilance task [Lieberman et al., 1984; Dollins et al., 1994]. These and other functions of melatonin are consistent with the existence of high affinity melatonin receptors in different areas of the CNS [Stankov et al., 1991].

In this paper, we present new data showing that physiological concentrations of melatonin inhibit rat cerebellar NOS activity and, consequently, cyclic GMP production. The inhibitory effect of melatonin appears to be mediated by a mechanism in which binding of melatonin to calmodulin prevents activation of NOS by calmodulin. Results suggest an important role of melatonin in modulating NO production in cerebellum.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade and obtained from commercial sources. L-arginine, L-citrulline, bovine brain calmodulin (>98% SDS-PAGE), tris(hydroxymethyl)-aminomethane (Tris-HCl), N-[2-hydroxyethyl]piperazine-N′-[2-hydroxypropanesulfonic acid] (HEPES), DL-dithiothreitol (DTT), leupeptin, aprotinin, pepstatin, phenylmethylsulfonylfluoride (PMSF), hypoxanthine-9-β-D-ribofuranosid (inosine), ethylene-glycol bis-(β-aminoethyl ether)-tetraacetic acid (EGTA), bovine serum albumin (BSA), Dowex-50W (50 × 8-200), guanosine 5′-triphosphate (GTP), 3-isobutyl-1-methyl-xanthine (IBMX), phosphocreatine, creatine phosphokinase (EC 2.7.3.2) were purchased from Sigma (St. Louis, MO). FAD and NADP were purchased from Boehringer Mannheim GmbH (Germany). 6(R,S)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (H_4biopterin) was purchased from ICN (Costa Mesa, CA). L-[^{3}H]arginine (60-70 Ci/mmol) was obtained from Amersham (Amersham, Bucks, UK). The 23-residue neuronal NOS peptide, KRRAIGFKKLAEAVKFSAKLMGQ, which corresponds to the amino acid sequence of residues 725–747 in soluble rat cerebellar NOS [Bredt et al., 1991] was obtained commercially from Affiniti RPL (Nottingham, UK), and shares 57% sequence identity with the endothelial NOS peptide [Sessat et al., 1992]. The peptide was judged to be ≥95% pure by high performance liquid chromatography. Electrophoretic chemicals were from BioRad (Richmond, CA).

Animals

Male Wistar rats (120–150 g) born in our animal facilities were used. Animals received food and water *ad libitum* and were exposed to an automatically regulated light:dark cycle of 14:10; the lights were turned off daily from 2000 through 0600 h. On the day of the experiment, animals were killed by decapitation between 1200 and 1400 h and cerebella were quickly collected and immediately used to measure NOS activity or for cyclic GMP studies.

Tissue preparation. Animals were killed by cervical dislocation and the cerebella were rapidly removed and cooled in ice-cold homogenization buffer (25 mM Tris/1 mM DTT/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml pepstatin/1 mM PMSF, pH 7.6). Subsequent procedures were carried out at 0–4°C. Two cerebella were placed in 2 ml of the same buffer, finely minced, and sonicated (10 s × 6). The crude homogenate was centrifuged 5 min at 1,000*g*, and aliquots of the supernatant were either stored at −20°C for total protein determination [Lowry et al., 1951] or used immediately to measure NOS activity or for cyclic GMP studies.

NOS activity determination. Total constitutive NOS activity (soluble plus membrane bound isoforms) was measured by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described [Bredt and Snyder, 1989]. The final incubation volume was 100 μl and consisted of 10 μl crude homogenate added to prewarmed (37°C) buffer to give (final concentration) of 25 mM Tris/1 mM DTT/30 μM H_4biopterin/10 μM FAD/0.5 mM inosine/0.5 mg/ml BSA/0.1 mM $CaCl_2$/10 μM L-arginine/40 nM [^{3}H]arginine, pH 7.6. When required, increasing concentrations of melatonin (up to 1 mM) and EGTA (0.5 mM) were also added to the incubation medium. The reaction was started by the addition of 10 μl NADP (1 mM final) and

continued for 30 min at 37°C. Control incubations were performed by omission of NADP. The reaction was terminated by the addition of 400 μl cold 0.1 M HEPES/10 mM EGTA/0.175 mg/ml L-citrulline, pH 5.5. The reaction mixture was decanted onto a 1.5 ml column packed with Dowex-50W ion exchange resin (Na^+ form) and eluted with 1.2 ml water. L-[^{3}H]citrulline was quantified by liquid scintillation spectroscopy. The retention of L-[^{3}H]arginine in this process was greater than 98%. Specific enzymatic activity was determined by subtracting the control value, which usually amounted to less than 1% of the radioactivity added. NOS activity is referred to as picomoles of L-[^{3}H]citrulline produced per mg protein/min.

Cyclic GMP studies. To study the effect of melatonin on rat cerebellar cyclic GMP production activated by L-arginine, incubations were initiated by the addition of 10 μl of crude homogenate to prewarmed (37°C) buffer to give (final concentration) 25 μl of 50 mM Tris/2 mM DTT/30 μM H_4biopterin/10 μM FAD/0.5 mM inosine/0.5 mg/ml BSA/0.1 mM $CaCl_2$/5 mM GTP/10 mM phosphocreatine/2.5 U/100 μl creatine phosphokinase/5 mM $MgCl_2$/1 mM IBMX/3 μM L-arginine, pH 7.6, in a total volume of 100 μl. When required, increasing concentrations of melatonin (up to 1 mM) were also added to the incubation medium.

To study the effect of melatonin on cerebellar cyclic GMP production activated by NO, incubations were initiated by addition of 50 μl of crude homogenate to prewarmed (37°C) buffer to give (final concentration) 25 mM Tris/5 mM GTP/5 mM $MgCl_2$/1 mM 3-isobutyl-1-methylxanthine, pH 7.6 in a total volume of 100 μl. When required, increasing concentrations of melatonin (up to 1 mM) and sodium nitroprusside (100 μM), as a donor of NO, were also added to the incubation medium.

Incubations were performed at 37°C for 30 min and terminated by the addition of 20 μl of 20% $HClO_4$ and cooling on ice. Following centrifugation (12,000*g*, 2 min), the cyclic GMP was determined following the non-acetylation procedure using commercially available radioimmunoassay kits with [^{125}I]cyclic GMP (Amersham, Bucks, UK) in aliquots of supernatants diluted 1:100 in 25 mM Tris/4 mM EDTA, pH 7.2 at 0–4°C. Cyclic GMP in the incubation medium is referred to as picomoles per mg protein/h.

Electrophoresis studies. Urea-polyacrylamide gel electrophoresis was performed as described [Zhang and Vogel, 1994]. To study calmodulin electrophoretic migration or calmodulin-NOS peptide complexes, slab gels (1.5 mm thickness) of 15% polyacrylamide/4 M urea/0.375 M Tris, pH 8.8, and 0.1 mM $CaCl_2$ or 2 mM EGTA, in the absence or presence of 1 mM melatonin, were run at a constant current of 16 mA for 18 h (Protean II xi Cell, BioRad, Richmond, CA). Electrode buffer consisted in 25 mM Tris/192 mM glycine, pH 8.3, and 0.1 mM $CaCl_2$ or 2 mM EGTA, in the absence or presence of 1 mM melatonin. Samples containing 10 μg calmodulin and increasing amounts of NOS peptide in 100 mM Tris/4 M urea, pH 7.2, and 0.1 mM $CaCl_2$ in the absence or presence of 1 mM melatonin were incubated at 25°C for 60 min in a total volume of 60 μl. One half-volume of 50% glycerol with tracer bromphenol blue was added and samples were applied to wells. The gels were fixed in 43% (v/v) methanol/1.6 M acetic acid, stained in 0.1% (w/v) Coomasie brilliant blue R-250, and detained in the solution described above. When required, gels were scanned using the BioImage system from Millipore Corp. (Bedford, MA).

RESULTS

NOS Activity Studies

We initially studied the effect of increasing concentrations of melatonin (up to 1 mM) on rat cerebellar NOS activity. As shown in Figure 1 (bottom), melatonin inhibited in a dose-dependent manner enzyme activity. However, 1 nM melatonin (≈230 pg/ml), the physiological concentration of the hormone at night, significantly inhibited NOS activity. The inhibitory effect of melatonin on the synthesis of citrulline from L-arginine was observed in the presence of Ca^{++}. Figure 1 (top) shows that 0.5 mM EGTA, a specific Ca^{++} chelator, inhibited completely NOS activity. Consequently, the inhibitory effect of melatonin was observed exclusively in the absence of EGTA and in the presence of Ca^{++}.

Cerebellar NOS activity was also proportional to the substrate concentration, as shown in Figure 2 (top), and showed a saturable pattern. However, the enzyme activity was always significantly lower in the presence of 1 mM melatonin. Double reciprocal plots of the data are represented in Figure 2 (bottom), and show kinetic constant values of Km = 1.64 ± 0.23 μM

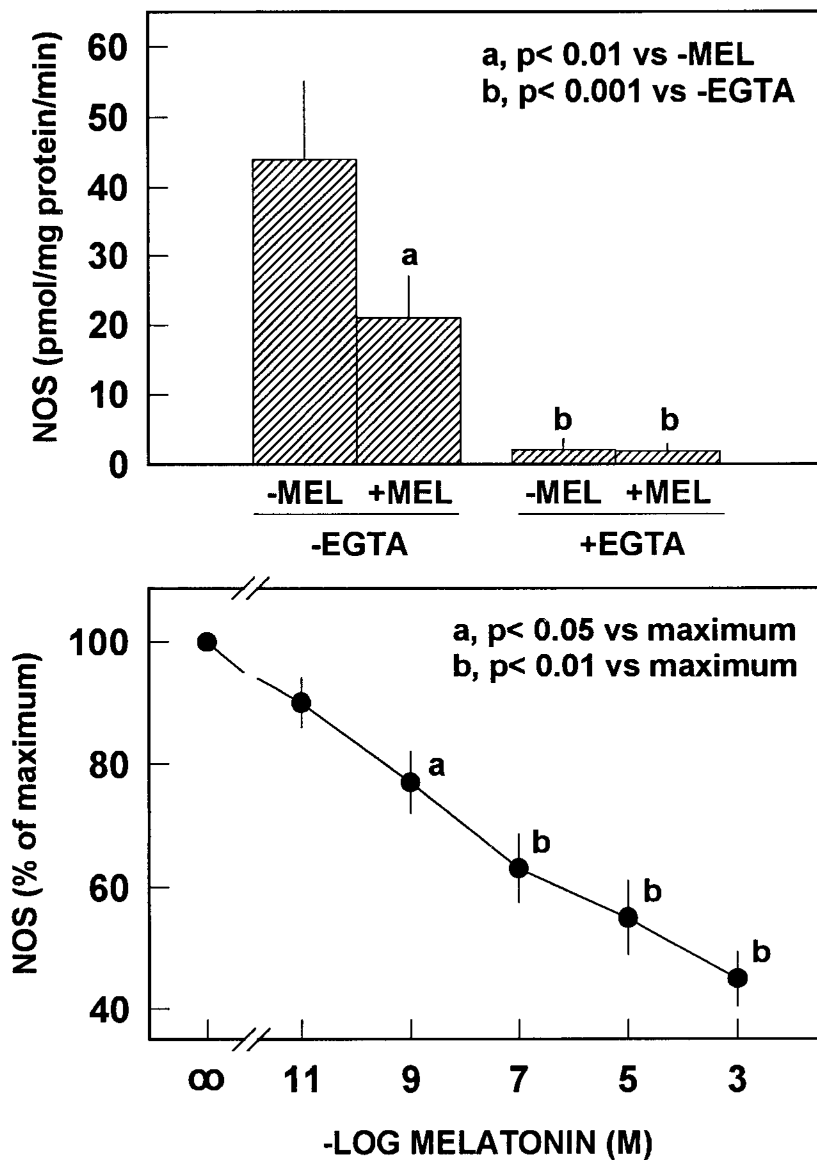

Fig. 1. NOS activity in rat cerebellar homogenates. **Top:** Effect of melatonin and EGTA on rat cerebellar NOS activity. NOS activity was determined in the absence or the presence of melatonin (1 mM) and/or EGTA (0.5 mM). Data are the means ± SE of six experiments performed in duplicate. **Bottom:** Effect of melatonin on rat cerebellar NOS activity. Rat cerebellar homogenates were incubated at 37°C for 30 min in the presence of increasing concentrations of melatonin. Data are the means ± SE of five experiments performed in duplicate. Specific activity in the absence of melatonin was 47.8 ± 10.8 pmol/mg protein/min.

and Vmax = 90.9 ± 10.5 pmol/mg protein/min in the absence of the hormone. In the presence of melatonin, although the Km value was similar (1.54 ± 0.18 μM), the Vmax was significantly lower (65.5 ± 8.3 pmol/mg protein/min) than that found in controls. The results suggest that melatonin behaves as a non-competitive inhibitor on rat cerebellar NOS activity.

The inhibitory effect of melatonin on rat cerebellar NOS activity was partially reversed by adding to the incubation medium exogenous calmodulin. As shown in Figure 3, the addition of 0.1 μM calmodulin completely prevented the inhibitory effect of 1 nM, melatonin, while partially preventing the effect of 1 μM melatonin. The effect of calmodulin appears to be dose-dependent because the addition of 0.5 μM calmodulin not only completely prevented the inhibitory effect of melatonin at 1 nM and 1 μM, but partially prevented the effect of 1 mM mela-

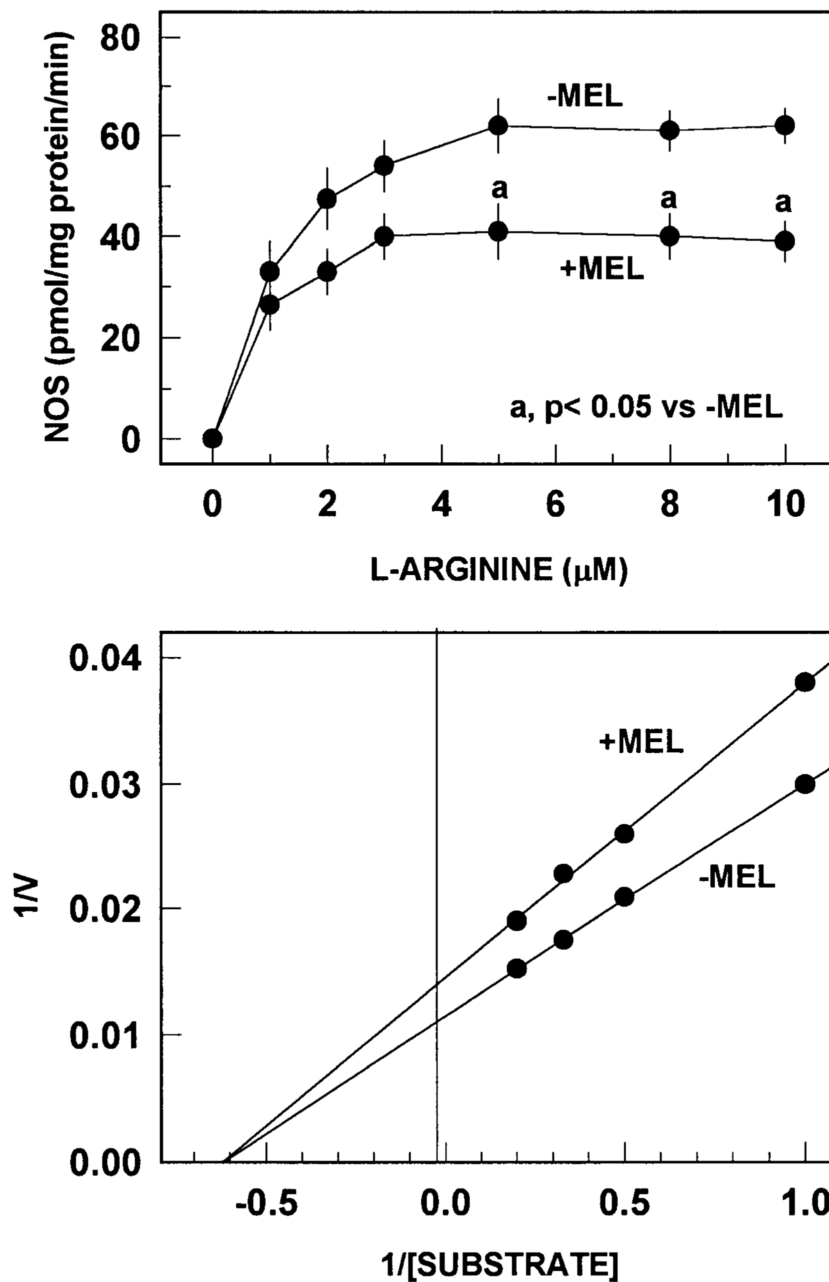

Fig. 2. **Top:** Effect of melatonin on rat cerebellar NOS activity. Rat cerebellar homogenates were incubated for 30 min at 37°C in the absence (−MEL) or presence (+MEL) of 1 mM melatonin and increasing concentrations of L-arginine. Each point is the means ± SE of five experiments performed in duplicate. **Bottom:** Double reciprocal plot of the data.

tonin. The results suggest that the inhibitory effect of melatonin on NOS activity might be mediated by a calmodulin-mediated mechanism.

The inhibitory effect of melatonin on NOS activity was abolished by increasing the homogenate concentration in the incubation medium. Previous experiments were performed at a homogenate concentration in the range of 1–5 mg/mL of protein. However, when protein concentration was higher than 6 mg/ml, melatonin was incapable of inhibiting NOS activity, as show in Figure 4 (top and bottom), where a concentration of 24 mg/ml was used. In Figure 4 (top), the inhibitory effect of melatonin on NOS activity is observed only after a dilution of

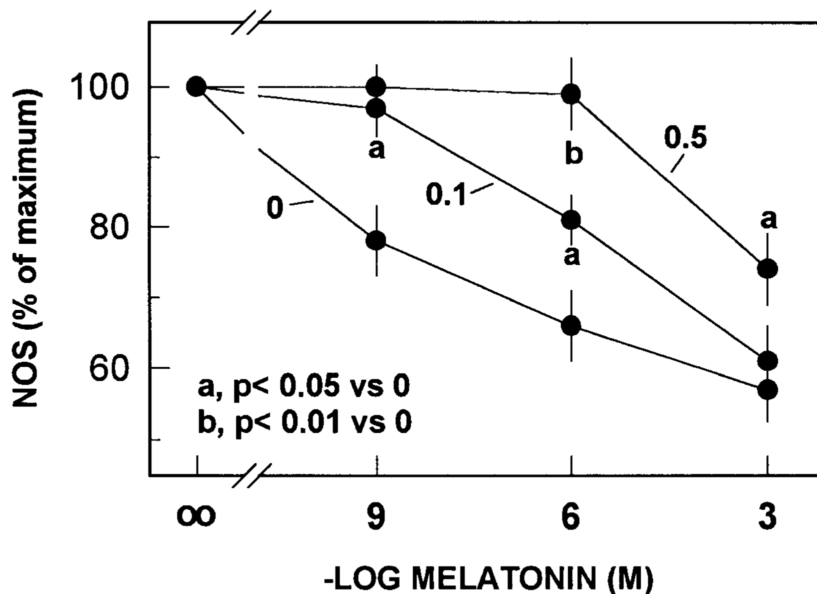

Fig. 3. Effect of calmodulin on the inhibitory effect of melatonin on rat cerebellar NOS activity. Rat cerebellar homogenates were incubated at 37°C for 30 min in the presence of increasing concentrations of melatonin without the addition of calmodulin (0) or after the addition of 0.1 and 0.5 μM exogenous calmodulin. Data are the means ± SE of five experiments performed in triplicate. Specific activity in the absence of melatonin and calmodulin was 52.8 ± 9.7 pmol/mg protein/min.

the homogenate to reach 3 mg/ml of protein concentration.

In Figure 4 (bottom), the concentration of homogenate was also 24 mg/ml in the incubation medium. In this experiment, increasing concentrations of the 23-residue NOS peptide, which corresponds to the amino acid sequence of residues 725–747 in the constitutive rat cerebellar NOS [Bredt et al., 1991], were added to the incubation medium. Because the NOS peptide is the proposed calmodulin-binding domain of constitutive NOS from rat cerebellum, increasing concentrations of the peptide should trap calmodulin and, consequently, should inhibit NOS activity. Figure 4 (bottom) shows that the presence of NOS peptide in the incubation medium inhibited NOS activity in a dose-dependent manner. Melatonin exhibited no effect on NOS activity at low doses of the peptide. However, at 10 μM NOS peptide, when calmodulin is partially trapped by the peptide, the inhibitory effect of melatonin on NOS activity was observed. These and previous results suggest that the inhibitory effect of melatonin on NOS activity can be observed only when calmodulin concentration is relatively low. Then, melatonin appears to antagonize the activating effect of calmodulin on NOS activity.

The pharmacological characterization of the inhibition of rat cerebellar NOS activity was carried out with melatonin or different metabolites involved in the melatonin synthesis pathway. Results show that the inhibition of NOS activity is highly specific for melatonin. Table I shows that only melatonin was able of inhibiting the enzyme activity. Other indoles, 5-hydroxyindoles, or 5-methoxyindoles were shown to be ineffective.

Cyclic GMP Studies

Melatonin not only inhibited NOS activity in rat cerebellum homogenates, but also cyclic GMP production. Figure 5 (top) shows that melatonin inhibited cyclic GMP production in dose-dependent manner. Maximal inhibition was achieved at 1 mM melatonin, although a statistically significant inhibition was obtained at 1 nM. As it occurred with NOS activity, the inhibitory effect of melatonin on cyclic GMP production was prevented by adding exogenous calmodulin to the incubation medium. The experiment was performed in the presence of 3 μM L-arginine as activator, via NO, of cyclic GMP production. However, when cyclic GMP production was activated by sodium nitroprusside (SNP), which releases of NO, and in the

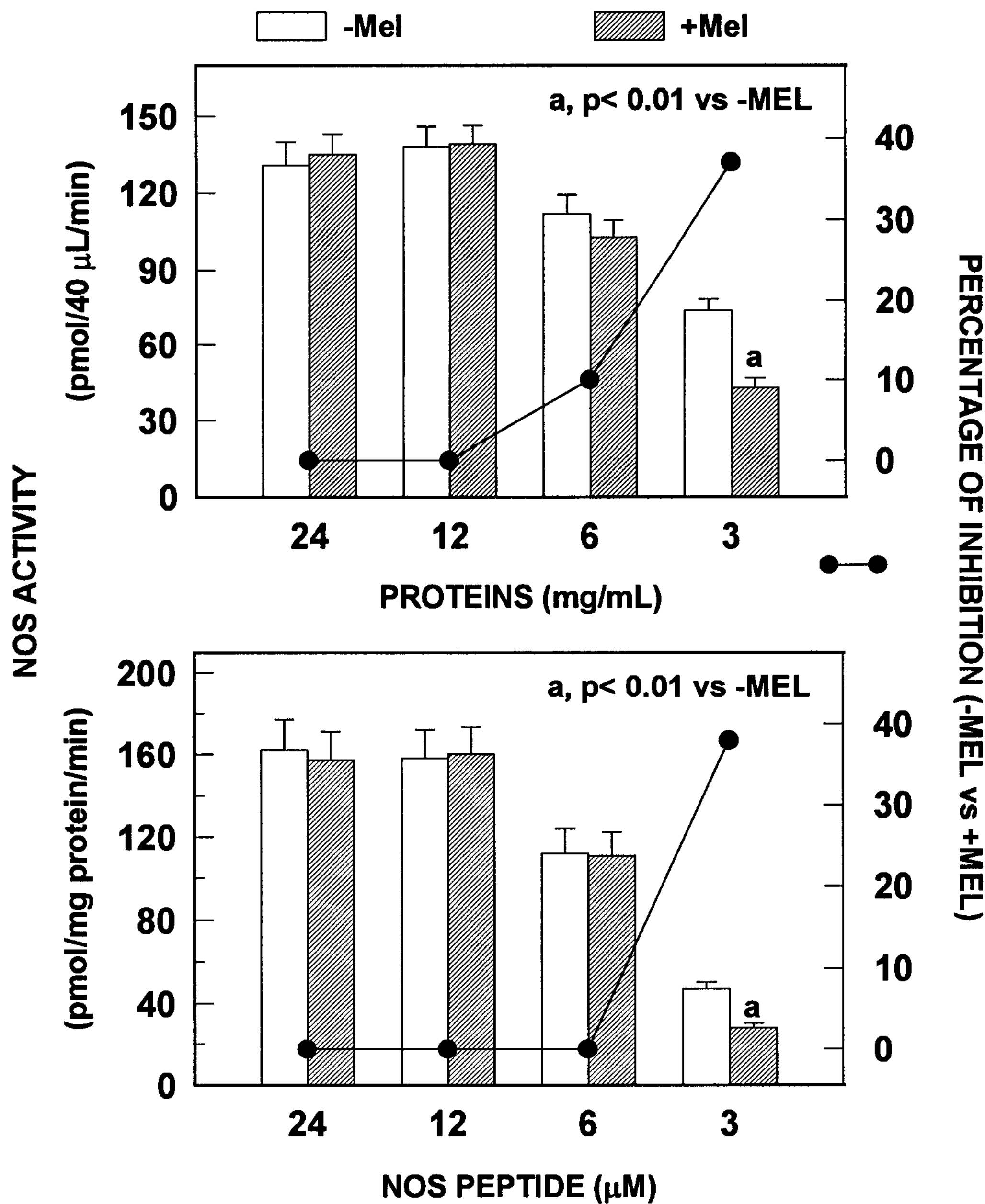

Fig. 4. Effect of melatonin on rat cerebellar NOS activity. **Top:** Effect of homogenate concentration on the inhibitory effect of melatonin on cerebellum NOS activity. Rat cerebellar homogenates (3–24 mg protein/ml) were incubated at 37°C for 30 min in the absence (−MEL) or presence of 1 mM melatonin (+MEL). Specific activity at 24 mg protein/ml in the absence of melatonin was 130.1 ± 28.5 pmol/mg protein/min. **Bottom:** Effect of the NOS peptide on the inhibitory effect of melatonin on cerebellum NOS activity. Rat cerebellar homogenates (24 mg protein/ml) were incubated at 37°C for 30 min in the absence (−MEL) or presence of 1 mM melatonin (+MEL) and in the presence of increasing concentrations of the NOS peptide (up to 10 μM). Results are expressed as NOS activity (bars) or percentage of inhibition by melatonin (points). Data are the means ± SE of four experiments performed in triplicate.

absence of exogenous L-arginine, melatonin was unable to inhibit cyclic GMP production. Figure 5 (bottom) shows that 100 μM SNP clearly activated cyclic GMP production, while at all doses used melatonin failed to alter this response. Results suggest that the inhibitory effect of melatonin on cyclic GMP production is due to inhibition of NOS activity rather than to inhibition of the guanylate cyclase system.

Electrophoresis Studies

To further study the mechanism of the inhibitory effect of melatonin on cerebellar NOS activity, urea-polyacrylamide gel electrophoresis of

TABLE I. Pharmacological Profile of the NOS Activity Inhibition in Rat Cerebellum*

Drugs	NOS activity (% of control)
5-methoxyindoles	
Melatonin	58 ± 8
6-hydroxymelatonin	101 ± 9
5-methoxyindole-3-acetic acid	105 ± 7
5-methoxytryptamine	110 ± 7
5-hydroxyindoles	
N-acetylserotonin	98 ± 8
5-hydroxyindole-3-acetic acid	102 ± 4
5-hydroxytryptophol	100 ± 9
5-hydroxytryptamine	95 ± 8
5-hydroxy-L-tryptophan	101 ± 6
Indoles	
Tryptamine	104 ± 7
DL-tryptophan	102 ± 5

*Rat cerebellar homogenates were incubated in the presence of each drug (1 mM) at 37°C for 30 min. Results are the mean ± SE of three experiments performed in duplicate. Results are expressed as percentages of the control value in the absence of drugs (52.8 ± 14.7 pmol/mg protein/min).

calmodulin in the absence and presence of melatonin was performed. As shown in Figure 6, the electrophoretic migration pattern of calmodulin was studied in the absence or presence of 1 mM melatonin and/or 2 mM EGTA, a specific Ca^{++} chelator. The presence of melatonin gives rise to a slower migration of calmodulin on the gel. The slowest migration of calmodulin was observed in the presence of EGTA. In the presence of EGTA, melatonin did not modify the migration pattern of calmodulin. Results suggest that a possible interaction between calmodulin and melatonin might be responsible for the slower migration of the peptide. Moreover, the interaction is Ca^{++}-dependent because it is not observed in the presence of EGTA.

The following experiments were performed to determine whether the calmodulin-melatonin interactions affect the activation of rat cerebellum NOS by calmodulin. The binding of the synthetic NOS peptide encompassing amino acid residues 725–747 of rat cerebellar NOS to calmodulin was studied using polyacrylamide gel electrophoresis in 4 M urea. In this method, the calmodulin-NOS peptide complexes, in the absence and presence of melatonin, are loaded at different ratios into the gel slots and subjected to electrophoresis (Fig. 7). A band shift is observed for calmodulin in some lanes; this results from the binding of the peptide to the protein. Binding of the NOS peptide to calmodulin gives rise to a slower migration on the gel (Fig. 7, top). At 0:1 ratio of peptide to calmodulin, all the protein remains in the free form (lane A). Increasing the ratio of peptide to calmodulin results in the formation of calmodulin-peptide complex (lanes B and C). At 1:1 ratio, only the calmodulin-peptide complex is observed (lane D), indicating that the NOS peptide binds calmodulin with a well described 1:1 stoichiometry [Zhang and Vogel, 1994]. In the presence of melatonin (Fig. 7, bottom), the peptide-calmodulin complex is not observed at 0.4:1 ratio of peptide to calmodulin (lane B), while the complex formed at 0.8:1 ratio is clearly less than that formed in the absence of melatonin (lane C). Figure 8 shows the densitometric study of the peptide-calmodulin complexes formed at the indicated ratios of peptide to calmodulin. Melatonin significantly prevented the formation of the complex when the ratio was <1. The results strongly suggest that binding of melatonin to calmodulin partially prevents binding of calmodulin to the NOS peptide.

DISCUSSION

Preliminary results showing the inhibitory effect of melatonin on cerebellar NOS activity have been previously reported by our group [Pozo et al., 1994]. Herein, further studies have been performed to investigate the mechanisms of this inhibition. In this paper, we show the inhibition of rat cerebellar NOS activity by physiological concentrations of the pineal hormone melatonin. The inhibition induced by melatonin was dose-dependent and was coupled to an inhibition of the cyclic GMP production activated by L-arginine. Results also show that calmodulin appears to be involved in this process because increasing its presence in the incubation medium prevented the effect of melatonin on both NOS activity and cyclic GMP production. Moreover, polyacrylamide gel electrophoresis studies suggest that melatonin interacts with calmodulin and modifies the binding of the peptide to the synthetic NOS peptide encompassing the calmodulin-binding domain of constitutive NOS from rat cerebellum, the natural mechanism by which calmodulin activates cerebellar NOS [Bredt et al., 1991].

Kinetic studies showed that the effect of melatonin can not be prevented by increasing the concentration of L-arginine, as the natural substrate of the enzyme, suggesting that melatonin behaves as a non-competitive inhibitor of

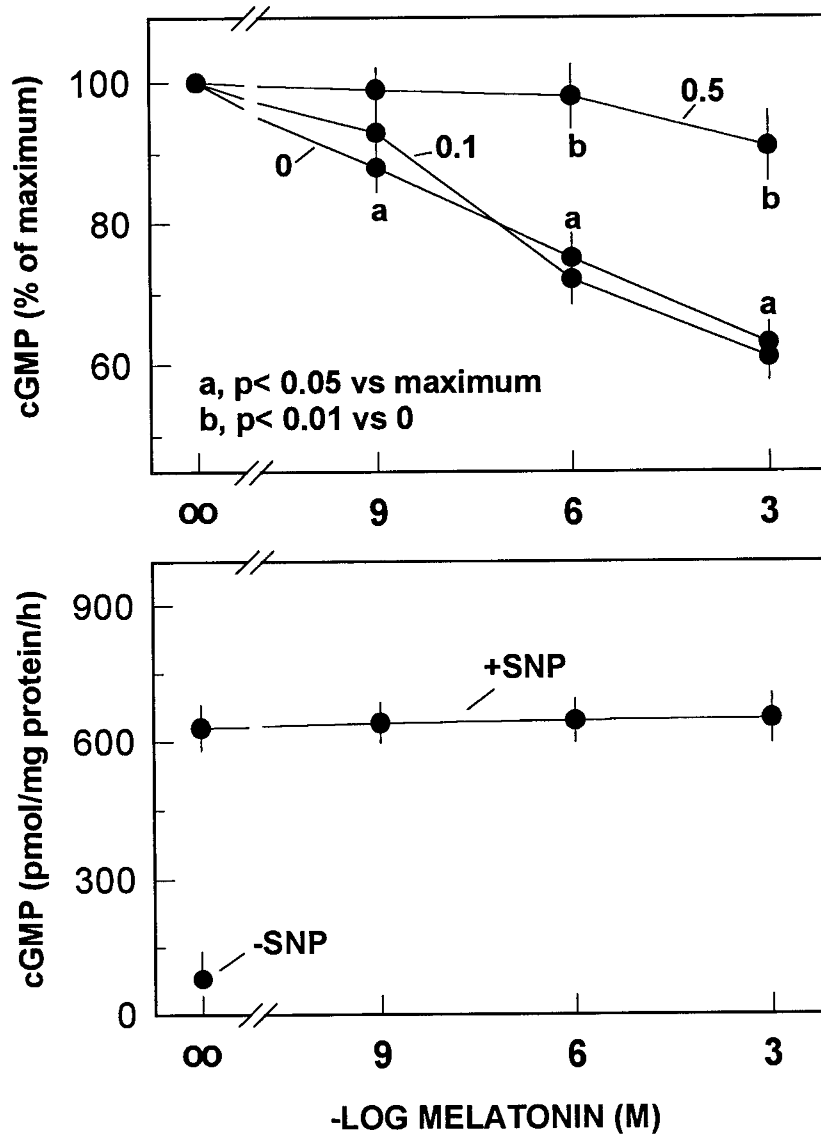

Fig. 5. **Top:** Effect of calmodulin on the inhibitory effect of melatonin on rat cerebellar cyclic GMP production. Rat cerebellar homogenates were incubated at 37°C for 30 min in the presence of increasing concentrations of melatonin without (0) or with the addition of 0.1 and 0.5 μM exogenous calmodulin. Data are the means ± SE of six experiments performed in duplicate. Data are expressed as the percentage of maximum in the absence of melatonin. Cyclic GMP production in the absence of melatonin and calmodulin was 352.8 ± 89.7 pmol/mg protein/h. **Bottom:** Effect of melatonin on the cyclic GMP production activated by sodium nitroprusside. Rat cerebellar homogenates were incubated at 37°C for 30 min in the absence (−SNP) and presence of 100 μM sodium nitroprusside (+SNP) and increasing concentrations of melatonin. Data are the means ± SE of three experiments performed in duplicate.

cerebellar NOS activity (Fig. 2). Stoichiometric studies also indicated that the maximal effect was observed at 1 mM melatonin (≈55% inhibition), a pharmacological concentration of the hormone (Fig. 1, bottom). However, the possible physiological role of melatonin in modulating NOS activity derives from the fact that 1 nM melatonin significantly inhibited the enzyme activity (>20%). Melatonin concentrations in the nanomolar range are present in the rodent and human sera at night [Vakkuri et al., 1984; Claustrat et al., 1986]. Moreover, the pharmacological characterization of the inhibitory effect of melatonin on NOS activity indicated that this effect is highly specific for the hormone (Table I). Other indoles including 5-hydroxy- and 5-methoxyindoles were ineffective. This pharmacological profile resembles those obtained when studied the binding of melatonin to membranes receptors [Calvo et al., 1995].

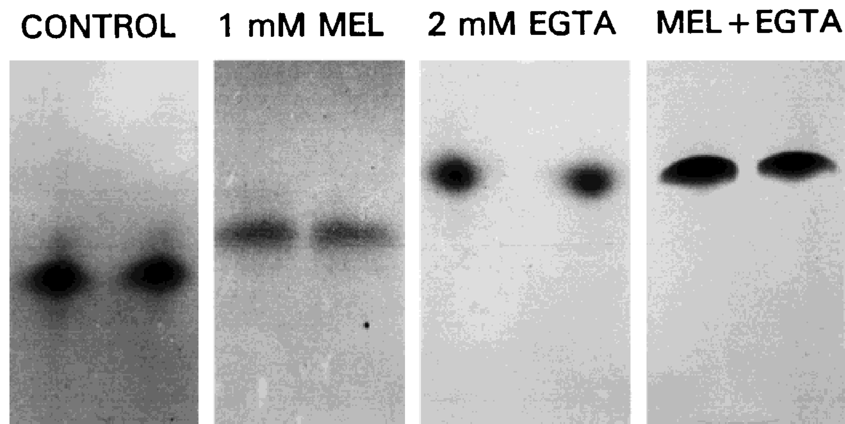

Fig. 6. Effect of melatonin on polyacrylamide gel electrophoresis of calmodulin. Gels were obtained in the presence of 0.1 mM Ca^{2+} (CONTROL), 1 mM melatonin (MEL), 2 mM EGTA, or melatonin plus EGTA (MEL + EGTA). Two additional experiments gave similar results.

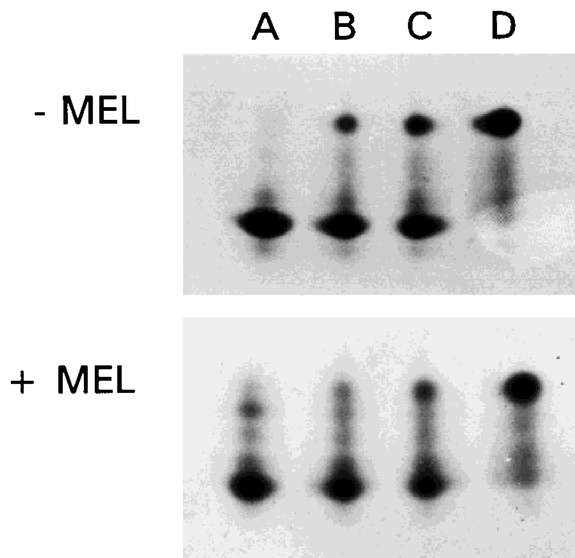

Fig. 7. Effect of melatonin on polyacrylamide gel electrophoresis of calmodulin/NOS peptide mixtures at different ratios. The peptide to calmodulin ratio from left to right is 0:1 (**lane A**); 0.4:1 (**lane B**); 0.8:1 (**lane C**); and 1:1 (**lane D**). The gel with lanes A–D were obtained in the presence of 0.1 mM Ca^{2+} and in the absence (−MEL) or the presence (+MEL) of 1 mM melatonin. Three other experiments gave similar results.

While trying to find a functional measurement of the inhibitory effect of melatonin on rat cerebellar NOS activity, production of cyclic GMP by L-arginine and NO was studied. In cerebellum, following the conversion of L-arginine to NO and citrulline, NO acts by binding to iron in the heme portion of soluble guanylate cyclase, stimulating the formation of cyclic GMP [Knowles et al., 1989; Bredt and Snyder, 1989]. As described, we also have shown that either L-arginine (Fig. 5, top) or sodium nitroprusside, as donor of NO (Fig. 5, bottom), activated cyclic GMP production. The results showed that cyclic GMP production stimulated by NO was unaffected by melatonin, while cyclic GMP production stimulated by L-arginine was inhibited by melatonin in a dose-dependent manner, suggesting that the inhibitory effect of melatonin on cyclic GMP production is due to inhibition of the NO-forming enzyme rather than to inhibition of guanylate cyclase. Thus, inhibition of NOS activity by melatonin appears to be coupled to inhibition of cyclic GMP production.

Melatonin is not the only natural substance capable of inhibiting NOS [Knowles and Moncada, 1994]. Thus, N^{ω} -monomethyl-L-arginine (L-NMMA) and asymmetaric $N^{\omega}N^{\omega}$-dimethyl-L-arginine (L-ADMA) are naturally occurring compounds that are derived from methylated protein arginine residues during protein turnover [Vallance et al., 1992]. Characteristically, L-NMMA is a non-selective competitive (with L-arginine) inhibitor of all the NOS so far examined; this inhibition is enantiomer-specific. The normal plasma concentrations of both inhibitors are low (<1 μM) but, under some circumstances, e.g., during renal failure, these compounds accumulate in sufficient quantities to inhibit NO synthesis [Knowles and Moncada, 1994]. Melatonin, like these two compounds, also inhibits NOS in cerebellum. However, some differences should be mentioned because, unlike the other two natural inhibitors

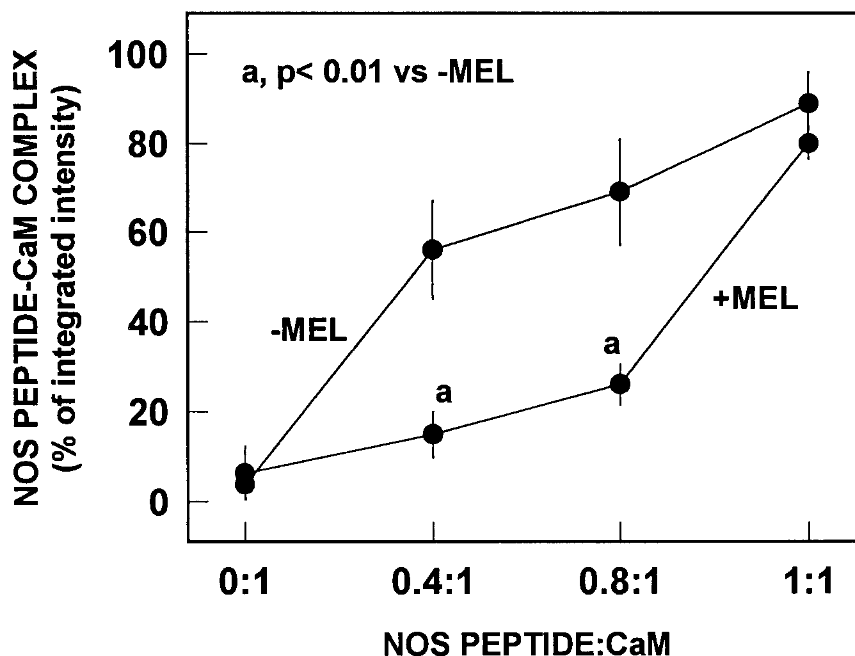

Fig. 8. Effect of melatonin on NOS peptide-calmodulin complexes. NOS peptide and calmodulin were incubated, at the indicated peptide to calmodulin ratios, in the absence (−MEL) or presence (+MEL) of 1 mM melatonin. Then, polyacrylamide gel electrophoresis was performed as in Figure 7, and the presence of the NOS peptide-calmodulin complexes was quantified by densitometric scanning and expressed as percentage of the total integrated intensity in each condition. Data are the means ± SE of four experiments.

described, melatonin is a noncompetitive inhibitor of cerebellar NOS activity and can act at nocturnal physiological concentrations.

Cerebellar NOS activity has been shown to be dependent on Ca^{++}/calmodulin. Thus, it is also known that calmodulin antagonists such as trifluoperazine, chlorpromazine, calmidazolium, W7, and W13 inhibit some NOS isoenzymes, including the constitutive Ca^{++}-dependent neuronal synthase [Förstermann et al., 1991]. In this context, one possibility is that melatonin acts as a calmodulin antagonist. In the present paper, several experiments are summarized which explored this possibility, and the results appear to support it. First, the inhibitory effect of melatonin on rat cerebellar NOS activity and cyclic GMP production was prevented, in a dose-dependent manner, by adding to the incubation medium exogenous calmodulin (Figs. 3 and 5). In subsequent experiments, we showed that increasing the protein concentration of the homogenate resulted in loss of the inhibitory effect of melatonin on NOS activity (Fig. 4, top). The reason for this loss was not clear but, under the same circumstances, adding to the incubation medium the synthetic NOS peptide, which binds calmodulin [Bredt et al., 1991], the inhibitory effect of melatonin was recovered (Fig. 4, bottom). Calmodulin is a 16.7 kDa protein present at high concentration in the interior of cells. Approximately 50% of the intracellular calmodulin is associated with membranes (including the plasma membrane), and the remainder is present in the cytosol [Paul and Ebadi, 1993]. Our results suggest that in the presence of a high homogenate concentration, which presumably contains high quantities of calmodulin, melatonin is ineffective in inhibiting NOS activity. However, when calmodulin concentration decreases because it is trapped by the NOS peptide added to the incubation medium, the inhibitory effect of melatonin is restored.

Further studies were performed to investigate whether the inhibitory effect of melatonin on cerebellar NOS activity may be mediated through binding to calmodulin. Urea-polyacrylamide gel electrophoresis of calmodulin in the absence and presence of melatonin was performed (Fig. 6). Results showed that the presence of melatonin gives rise to a slower migration of calmodulin on the gel. However, the slowest migration of calmodulin was observed in the presence of EGTA, a Ca^{++} chelator. When

EGTA was present, melatonin did not modify the migration pattern of calmodulin. Results suggest that a possible interaction between melatonin and the calmodulin/Ca^{++} complex might be responsible for the slower migration of the peptide. In fact, it has been reported that melatonin binds and antagonizes calmodulin [Benitez-King et al., 1993]. The interaction between melatonin and calmodulin was initially deduced from observation that both MDCK and N1E-115 cell cultures incubated with melatonin showed microfilament and microtubule rearrangements compatible with changes in intracellular calmodulin activity [Benitez-King et al., 1990; Huerto-Delgadillo et al., 1994]. Moreover, melatonin inhibited calmodulin-dependent phosphodiesterase activity in these cells [Benitez-King et al., 1991]. Therefore, if this is true, we can speculate that many calmodulin-dependent proteins and enzymes in the CNS might be modulated by melatonin as it has been previously suggested [Anton-Tay et al., 1993]. Thus, calcineurin, synapsin-1-kinase, brain phosphodiesterase, calmodulin/Ca^{++}-dependent protein kinase, and Ca^{++}/Mg^{++} ATPase are, among other enzymes, possible indirect targets for melatonin.

Finally, additional experiments were performed to determine whether melatonin-calmodulin interactions would affect the binding of the synthetic NOS peptide to calmodulin. The presence of calmodulin-NOS peptide complexes were studied by loading calmodulin and the NOS peptide at different ratios into lanes; these were then subjected to electrophoresis (Figs. 7 and 8). Increasing the ratio of peptide to calmodulin results in the formation of calmodulin-peptide complex. At a 1:1 ratio, only calmodulin-peptide complex is observed, indicating that NOS peptide binds calmodulin with a well described 1:1 stoichiometry [Zhang and Vogel, 1994]. In the presence of melatonin, the peptide-calmodulin complex is not observed at 0.4:1 ratio of peptide to calmodulin, while the complex formed at 0.8:1 ratio is clearly less than that formed in the absence of melatonin, suggesting that binding of melatonin to calmodulin partially prevents binding of calmodulin to the NOS peptide.

The results could represent a new mechanism of action for melatonin, in addition to others previously described. Thus, specific binding sites for melatonin located on the plasma membrane have been amply described in the CNS [Stankov et al., 1991] and in peripheral tissues [Guerrero et al., 1994]. Recently, a protein with seven transmembrane-spanning hydrophobic domains was identified as the receptor for melatonin [Ebisawa et al., 1994]. Additionally, other studies have shown nuclear localization of melatonin in different mammalian tissues, suggesting that melatonin may have a function at the nuclear level in these tissues [Menendez-Pelaez and Reiter, 1993]. Furthermore, specific melatonin binding sites in rat liver cell nuclei have been described [Acuña-Castroviejo, et al., 1994]. At the nuclear level, recent studies show that melatonin binding site is an orphan receptor called RZRβ, which binds melatonin with a Kd in the nanomolar range; this receptor may participate in the nuclear signaling of this hormone [Becker-Andre et al., 1994]. In this sense, the effect of melatonin on NOS activity described in this paper, is not mediated by a membrane or nuclear receptor. Moreover, other effects of melatonin also appear to be independent of its ability to bind to a receptor. Thus, it was recently shown that melatonin is a potent hydroxyl radical scavenger; indeed, compared to glutathione and mannitol, two well known scavengers [Tan et al., 1993], it was considerably more effective. Thus, melatonin, besides its well-known actions via membrane receptors, has now been shown to have intracellular actions which are both receptor-independent and receptor-dependent.

In conclusion, melatonin inhibits rat cerebellar NOS activity at physiological concentrations by a calmodulin-mediated mechanism. Although the physiological relevance of this inhibition remains to be clarified, the results open a new area of investigation which could clarify additional physiological actions of melatonin in CNS.

ACKNOWLEDGMENTS

Supported by grants from CICYT (PB91-0616) and FISSS (93/0210).

REFERENCES

Acuña-Castroviejo D, Reiter RJ, Menendez-Pelaez A, Pablos MI, Burgos A (1994): Characterization of high-affinity melatonin binding sites in purified cell nuclei of liver. J Pineal Res 16:100–112.

Anton-Tay F, Huerto-Delgadillo L, Ortega-Corona B, Benitez-King G (1993): Melatonin antagonism to calmodulin may modulate multiple cellular functions. In Touitou Y, Arendt J, Pevet P (eds): "Melatonin and the Pineal Gland. From Basic Science to Clinical Application." Elsevier Science Publishers BV.

Becker-Andre M, Wiesenberg I, Scaeren-Wiemer N, Andre E, Missbach M, Saurat JH, Carlberg C (1994): Pineal hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem 269:28531–28534.

Benitez-King G, Huerto-Delgadillo L, Anton-Tay F (1990): Melatonin effects on the cytoskeletal organization of MDCK and neuroblastoma N1E-115 cells. J Pineal Res 9:209–220.

Benitez-King G, Huerto-Delgadillo L, Anton-Tay F (1991): Melatonin modifies calmodulin levels in MDCK and N1E-115 cell lines and inhibits phophodiesterase activity in vitro. Brain Res 557:289–292.

Benitez-King G, Huerto-Delgadillo L, Anton-Tay F (1993): Binding of ^{3}H-melatonin to calmodulin. Life Sci 53:201–207.

Bredt DS, Snyder SH (1989): Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 86:9030–9033.

Bredt DS, Hwang PW, Glatt CE, Lowenstein C, Reed RR, Snyder SH (1991): Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351:714–718.

Bruhwyler J, Chleide E, Liegeois JF, Carreer F (1993): Nitric oxide: A new messenger in the brain. Neurosci Biobehav Rev 17:373–384.

Calvo JR, Rafii-El-Idrissi M, Pozo D, Guerrero JM (1995): Immunomodulatory role of melatonin: Specific binding sites in human and rodent lymphoid cells. J Pineal Res 18:119–126.

Claustrat B, Brun J, Garrey P, Roussel B, Sassolas G (1986): A once-repeated study of nocturnal plasma melatonin patterns and sleep recordings in six normal young men. J Pineal Res 3:301–310.

Dollins AB, Zhdanova IV, Wurtman RJ, Lynch HJ, Deng MH (1994): Effect of inducing nocturnal serum melatonin concentrations in daytime on sleep, mood, body temperature, and performance. Proc Natl Acad Sci USA 91:1824–1828.

Ebisawa T, Karne S, Lerner MR, Reppert SM (1994): Proc Natl Acad Sci USA 91:6133–6137.

Faraci FM, Brian JE (1994): Nitric oxide and the cerebral circulation. Stroke 25:692–703.

Fariello RG, Bubenik GA, Brown GM, Grota IJ (1977): Epileptogenic action of intraventricularly injected antimelatonin antibody. Neurology 27:567–570.

Förstermann U, Pollock JS, Schmidt HHHW, Heller M, Murad F (1991): Calmodulin-dependent endothelium-derived relaxing factor nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. Proc Natl Acad Sci USA 88:1788–1792.

Guerrero JM, Reiter RJ (1992): A brief survey of pineal gland-immune system interrelationships. Endocr Res 18: 91–113.

Guerrero JM, Calvo JR, Osuna C, Lopez-Gonzalez MA (1994): Binding of melatonin by lymphoid cells in humans and rodents. Adv Pineal Res 7:109–117.

Huerto-Delgadillo L, Anton-Tay F, Benitez-King G (1994): Effects of melatonin on microtubule assembly depend on hormone concentration: Role of melatonin as a calmodulin antagonist. J Pineal Res 17:55–62.

Knowles RG, Moncada S (1994): Nitric oxide synthase in mammals. Biochem J 298:249–258.

Knowles RG, Palacios M, Palmer RMJ, Moncada S (1989): Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci USA 86:5159–5162.

Lerner AB, Case JD (1960): Melatonin. Fed Proc 19:590–592.

Lieberman HR, Waldhauser F, Gardfield G, Lynch HJ, Wurtman RJ (1984): Effects of melatonin on human mood and performance. Brain Res 323:201–207.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

Matthies H (1989): In search of cellular mechanisms of memory. Prog Neurobiol 32:277–349.

Menendez-Pelaez A, Reiter RJ (1993): Distribution of melatonin in mammalian tissues: the relative importance of nuclear versus cytosolic localization. J Pineal Res 15: 59–69.

Miles A, Philbrick DRS (1988): Melatonin and psychiatry. Biol Psychiatry 23:405–425.

Moncada S, Palmer RMJ, Higgs EA (1991): Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–134.

Moskowitz MA, Wurtman RJ (1977): Pathological states involving the pineal. In Martini I, Besser GM (eds) “Clinical Neuroendocrinology.” Orlando, FL: Academic Press.

Paul S, Ebadi M (1993): Vasoactive intestinal peptide: Its interaction with calmodulin and catalytic antibodies. Neurochem Int 23:197–214.

Philo R, Reiter RJ (1978): Characterization of pinealectomy induced convulsions in the Mongolian gerbil (*Meriones unguiculatus*). Epilepsia 19:485–492.

Pozo D, Reiter RJ, Calvo JR, Guerrero JM (1994): Physiological concentrations of melatonin inhibit nitric oxide synthase in rat cerebellum. Life Sci 55:PL455–PL460.

Reiter RJ (1991): Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. Endocr Rev 12:151–180.

Sessat WC, Harrison JK, Barber CM, Zeng D, Durieux ME, D’Angelo DD, Lynch KR, Peach MJ (1992): Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. J Biol Chem 267:15274–15276.

Shibuki K, Okada D (1991): Endogenous nitric oxide release required for long term synaptic depression in the cerebellum. Nature 349:326–328.

Snyder SH, Bredt DS (1991): Nitric oxide as a neuronal messenger. Trends Pharmacol Sci 12:125–128.

Stankov B, Fraschini F, Reiter RJ (1991): Melatonin binding sites in the central nervous system. Brain Res Rev 16:245–256.

Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ (1993): Melatonin: A potent, endogenous hydroxyl scavenger. Endocrine J 1:57–60.

Vakkuri O, Leppäluoto J, Vuolteenaho O (1984): Development and validation of a melatonin radioimmunoassay using radioiodinated melatonin as a tracer. Acta Endocrinol 106:152–157.

Vallance P, Leone A, Calvert A, Collier J, Moncada S (1992): Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 339:572–575.

Zhang M, Vogel HJ (1994): Characterization of the calmodulin-binding domain of rat cerebellar nitric oxide synthase. J Biol Chem 269:981–985.