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PHARMACOLOGICAL INHIBITION OF FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY IN RAT BRAIN BY MELATONIN, ITS ANALOGS, AND DIAZEPAM

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Abstract—Preincubation of rat forebrain membranes for 30–60 min with micromolar concentrations of the pineal hormone, melatonin, significantly inhibited forskolin-stimulated adenylate cyclase (AC) activity. Melatonin had an EC_{25} (concentration which inhibited AC activity by 25%) of 600 μM and caused a maximal inhibitory effect of ~30% at a concentration of 1000 μM. A comparison of the effects of melatonin and its analogs, 6-chloromelatonin and 2-iodomelatonin, in the striatum revealed that these halogenated drugs were 2–3 times more potent than melatonin in inhibiting AC activity. The EC_{25} values were 611, 226 and 189 μM for melatonin, 6-chloromelatonin and 2-iodomelatonin respectively. The receptor antagonists phentolamine (α-adrenergic), propranolol (β-adrenergic), and metergoline (serotonergic) did not block the effect of melatonin in forebrain membranes. The central-type benzodiazepine (BZ) antagonist, Ro 15-1788 (flumazenil), also failed to block the inhibitory effects of melatonin, and the benzodiazepines, diazepam and Ro 5-4864, on AC activity. Evidence that inhibition of adenylate cyclase activity may be involved in the prevention of seizures suggests that the reported anticonvulsant effect of large doses of melatonin may be due to this mechanism. The greater potency of the halogenated melatonin analogs in inhibiting AC suggests that further study of their potential usefulness as anticonvulsants would be worthwhile.

Pharmacological doses of the pineal hormone, melatonin, produce anticonvulsant effects in humans and other mammals [1–3] but the mechanisms involved are unknown. Like benzodiazepines (BZs†), such as diazepam, melatonin enhances γ-aminobutyric acid (GABA) binding in the CNS [4, 5]. Enhancement of central GABAergic function via the allosteric modulation of $GABA_A$ receptors is thought to underlie the tranquilizing effects of BZ drugs [6]. Recent evidence that diazepam inhibits adenylate cyclase activity in the rat brain by acting at a site distinct from the central-type BZ site, which is allosterically linked to $GABA_A$ receptors [7], suggests that more than one site and mechanism is involved in the neuropharmacological actions of BZs.

Since melatonin exhibits micromolar affinity for both central-type [8] and peripheral-type [9] BZ binding sites in the rat brain, its pharmacologic effects may also involve modulation of central adenylate cyclase (AC) activity. Therefore, we examined the effects of micromolar concentrations of melatonin on forskolin-stimulated AC activity in rat brain membranes.

MATERIALS AND METHODS

Reagents and drugs. [α-^{32}P]ATP (800 Ci/mmol) and [2,8-^{3}H]cAMP (30 Ci/mmol) were obtained from New England Nuclear. Diazepam, Ro 15-1788 and Ro 5-4864 were donated by Hoffmann-LaRoche. 2-Iodomelatonin was purchased from RBI. Melatonin and all other chemicals were obtained from Sigma.

Membrane preparation. Male Sprague–Dawley rats were maintained under a 12L:12D lighting regimen (lights on from 7:00 a.m.) with free access to food and water. Animals were decapitated about 4 hr after lights-on and brains were rapidly removed on ice. In some experiments, male Wistar rat brain tissues were used. These animals were stunned by a blow to the head and decapitated, and brain tissues were dissected on ice. Fresh tissues were immediately used for enzyme assays or frozen for about 1–2 weeks at −20° before use. Forebrains or various brain regions were homogenized in 10 vol. of 0.32 M sucrose containing 5 mM Tris–HCl and 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 800 *g* for 10 min and the supernatant spun at 10,000 *g* for 30 min. The pellet was resuspended in 20 vol. of 5 mM Tris–HCl buffer (pH 7.4) and left on ice for 30 min. Membranes were washed twice by centrifugation (20,000 *g* for 30 min) and resuspension before use in assays.

Adenylate cyclase assay. Membrane aliquots containing about 50–100 μg protein were preincubated with melatonin or various drugs at 30° for the indicated periods. Following preincubation, a reaction mixture containing 4 mM $MgCl_2$, 100 μM GTP, 1 mM EGTA, 1 mM Dithiothreitol, 0.5 mM cAMP, 5 mM theophylline, 1 mg/mL BSA, 5 mM creatine phosphate and 20 units/mL creatine phosphokinase was added to a final volume of 150 μL.

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† Abbreviations: AC, adenylate cyclase; ATP, adenosine-5′-triphosphate; BSA, bovine serum albumin; BZ, benzodiazepine; cAMP, adenosine 3′,5′-cyclic monophosphate; EGTA, ethylene glycolbis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GABA, γ-aminobutyric acid; and GTP, guanosine-5′-triphosphate.

Forskolin (10 μM) was added to all tubes except basal and background samples and 0.5 mM ATP containing ~1 μCi [α-^{32}P]ATP was then added to all tubes and incubation carried out at 30° for 10 min. The reaction was stopped by adding 4 mM cAMP and boiling for 5 min. Samples were centrifuged at 12,000 *g* for 10 min in a refrigerated Mikro rapid centrifuge. [^{3}H]cAMP (~20,000 cpm) was added to monitor recovery and the [^{32}P]cAMP formed was separated from supernatants by sequential chromatography on Dowex and alumina columns [10].

Protein concentrations were measured as described by Lowry *et al.* [11] using bovine serum albumin as the standard.

Statistical methods. Data were analyzed by one-way analysis of variance and Scheffe's test or by Student's *t*-test when appropriate.

RESULTS

Effects of preincubation. Preliminary experiments indicated that preincubation of rat forebrain membranes with melatonin (500 μM) or diazepam (100 μM), for periods of 30–120 min, caused a significant decrease in adenylate cyclase activity. However, extended preincubation for more than 60 min decreased basal AC activity and the response of the enzyme to forskolin (data not shown).

Concentration–response studies. Following preincubation with melatonin for 30 min, a biphasic concentration–response curve for AC activity was observed. A plateau of about 12–15% inhibition occurred between 250–500 μM melatonin while higher concentrations of the hormone caused further decreases in enzyme activity with a maximal effect (~22%) at 1000 μM melatonin. After preincubation for 60 min, the effect of melatonin on AC activity was monophasic with an EC_{25} (concentration which inhibited enzyme activity by 25%) of 600 μM and a maximal inhibition of about 30% observed at 1000 μM (Fig. 1). As a result of these observations, a 60-min preincubation was used in subsequent experiments.

A comparison of the effects of melatonin, 6-chloromelatonin and 2-iodomelatonin revealed that these analogs were about 2–3 times more potent than melatonin in inhibiting AC activity in the striatum. The EC_{25} values were 611, 226 and 189 μM for melatonin, 6-chloromelatonin and 2-iodomelatonin respectively (Fig. 2). The analogs also exhibited greater efficacy than melatonin with a maximal effect of about 80% inhibition in the striatum as compared with about 40% by melatonin at a concentration of 1000 μM (Fig. 2).

Regional effects of melatonin. The effects of melatonin on forskolin-stimulated AC activity in various brain regions are presented in Table 1. Melatonin caused a significant ($P < 0.05$) decrease in AC activity in the striatum, hippocampus, cortex, hypothalamus and pons-medulla. Non-significant decreases in AC activity were observed in the midbrain and cerebellum. The effect of forskolin was most pronounced in the striatum where it increased AC activity by 10- to 12-fold, as compared with 2- to 5-fold in other regions.

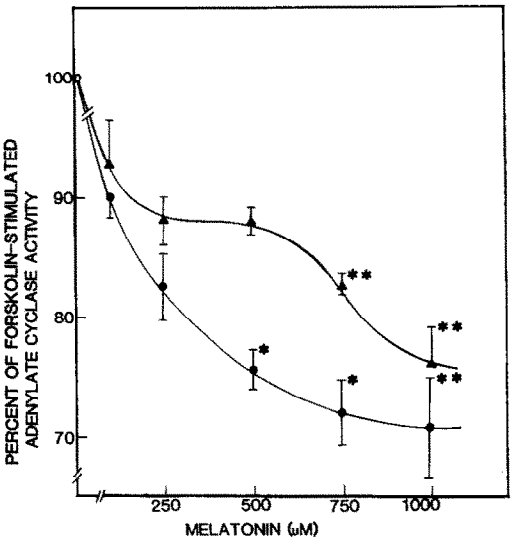

Fig. 1. Concentration-dependent effects of melatonin on forskolin (10 μM)-stimulated adenylate cyclase activity in rat forebrain. Membranes were preincubated with melatonin at 30° for 30 (▲) or 60 (●) min before measuring enzyme activity. Basal and forskolin-stimulated enzyme activities were 34 ± 7 and 108 ± 18 pmol cAMP/mg protein/min (30-min preincubation) and 26 ± 2 and 73 ± 6 pmol cAMP/mg protein/min (60-min preincubation) respectively. Means ± SE of three separate experiments conducted in triplicate are presented. Key: * $P < 0.05$, and ** $P < 0.01$ vs control.

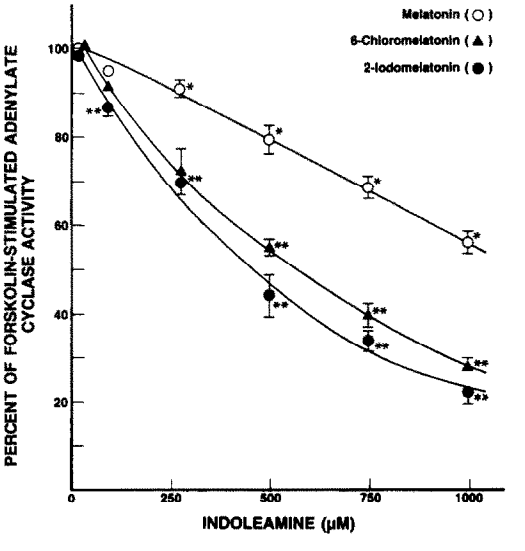

Fig. 2. Inhibition of forskolin-stimulated adenylate cyclase activity in rat striatal membranes by melatonin (○), 6-chloromelatonin (▲), and 2-iodomelatonin (●). Membranes were preincubated with the above indoleamines at 30° for 60 min. Basal and forskolin-stimulated enzyme activities were 50 ± 8 and 422 ± 23 pmol cAMP/mg protein/min respectively. Means ± SE of three separate experiments are presented. Key: * $P < 0.05$, and ** $P < 0.01$ vs control.

Table 1. Regional effects of melatonin on forskolin-stimulated adenylate cyclase activity in rat brain

Region	Adenylate cyclase activity (pmol cAMP/mg protein/min)		% Inhibition
	Forskolin	Forskolin + Melatonin	
Cortex	58 ± 16 (4)	51 ± 15* (4)	12
Striatum	170 ± 27 (6)	136 ± 16* (6)	20
Hippocampus	27 ± 2 (3)	21 ± 2* (3)	22
Hypothalamus	83 ± 22 (5)	73 ± 20* (5)	12
Midbrain	31 ± 7 (3)	28 ± 8 (3)	10
Pons-medulla	24 ± 6 (3)	21 ± 5* (3)	12
Cerebellum	80 ± 17 (3)	75 ± 18 (3)	6

Means ± SE for (N) experiments conducted in triplicate are presented. Basal enzyme activities, which ranged from 20 to 40 pmol/mg protein/min in various regions, were subtracted from all values presented. Samples were preincubated with melatonin (750 μM) for 60 min. Forskolin (10 μM) was then added and enzyme activity measured as described in Materials and Methods.

* $P < 0.05$ vs respective controls.

Table 2. Effects of melatonin and benzodiazepines on forskolin-stimulated adenylate cyclase activity in the absence or presence of flumazenil

	Adenylate cyclase activity (pmol cAMP/mg protein/min)		Adenylate cyclase activity (pmol cAMP/mg protein/min)	
	Solvent	% Inhibition	+ Flumazenil (10 μM)	% Inhibition
Forskolin (10 μM)	84.4 ± 3.9		78.9 ± 4.3	
Melatonin (100 μM)	76.2 ± 3.8	9.7	61.7 ± 1.0*	21.8
Melatonin (500 μM)	69.1 ± 3.9*	18.1	62.2 ± 3.2*	21.2
Diazepam (10 μM)	33.9 ± 1.0†	59.8	33.8 ± 2.5†	57.1
Ro 5-4864 (10 μM)	68.9 ± 1.1*	18.4	65.4 ± 2.2*	17.1

Fresh forebrain membranes were preincubated with the indicated drugs (except forskolin) at 30° for 60 min. Forskolin was then added and following incubation at 30° for 10 min, enzyme activity was measured. Means ± SE are presented for N = 4.

* $P < 0.05$ vs appropriate control.

† $P < 0.01$ vs appropriate control.

Effects of receptor antagonists. Various receptor antagonists including phentolamine (α-adrenergic), propranolol (β-adrenergic) and metergoline (serotonergic) failed to block the effect of melatonin on AC activity (data not shown).

Ro 15-1788 (flumazenil), a central-type BZ receptor antagonist, also failed to block the inhibitory effects of both melatonin and diazepam on forskolin-stimulated AC activity (Table 2). These findings indicate that if melatonin's effect involves BZ receptors, then these are of a non-central type. In keeping with this possibility, Ro 5-4864, an agonist at peripheral-type and other BZ receptors, was found to inhibit stimulated AC activity in rat brain (Table 2).

DISCUSSION

The present findings indicate that pharmacologic concentrations of the pineal hormone, melatonin, suppress forskolin-stimulated AC activity in diverse areas of the rat CNS. Melatonin binds to both central-type and peripheral-type BZ receptors in the rat brain with affinities of about 500 and 100 μM

respectively [8, 9]. The central-type BZ receptor is coupled to $GABA_A$ receptors in the CNS and does not affect AC activity. Moreover, as noted earlier, a central-type BZ receptor antagonist, flumazenil (Ro 15-1788), failed to block the effects of both melatonin and diazepam on AC activity, indicating the non-involvement of this receptor.

Since the central-type BZ receptor does not mediate the effects of either melatonin or BZs on AC, other sites such as the peripheral-type site [12] or a micromolar-affinity site [13] may be involved. The peripheral-type site has high affinity (K_d = ~1 nM) for Ro 5-4864, but only micromolar concentrations of this compound were effective in decreasing AC activity. Therefore, it is very unlikely that peripheral-type BZ sites are coupled to adenylate cyclase.

A micromolar BZ receptor which is kinetically and pharmacologically distinct from the nanomolar-affinity receptor has been reported [13]. The ability of BZs to inhibit maximum electric shock-induced convulsions correlates with their binding affinity for the micromolar-affinity receptor [13]. This receptor has also been implicated in the regulation of voltage-sensitive calcium channels in rat brain [14]. The fact that only micromolar concentrations of diazepam were effective in decreasing adenylate cyclase activity suggests that the micromolar-affinity receptor may be involved. It should be noted that the presence of a micromolar-affinity receptor for BZs is controversial as some investigators have not detected this receptor in rat brain membranes [15]. Nonetheless, clinical doses of diazepam produce circulating levels in the micromolar range while brain concentrations of diazepam and other BZs have been found to be significantly higher than blood levels in experimental animals [13, 14, 16, 17]. Therefore, diazepam and other BZs can readily achieve the brain concentrations which would be required for interaction with micromolar BZ receptors. Previous studies have shown that intraperitoneal injections of melatonin ranging from 150 to 200 mg/kg produce anticonvulsant effects in rats and mice [1, 2]. Brain levels of melatonin following administration of these large pharmacological doses of the hormone have not been determined. However, melatonin, like diazepam, is a lipophilic compound which can readily cross the blood–brain barrier to enter the brain [18, 19]. In addition, melatonin in doses of up to several grams is reportedly non-toxic in humans [20]; therefore, it can be administered in sufficiently high quantities to produce brain concentrations which can interact with micromolar BZ receptors. It is possible that the micromolar BZ receptor mediates the effects of large doses of melatonin on AC in the brain. Clarification of this issue will have to await the development of an antagonist for the micromolar-affinity receptor.

Since the peak concentrations of melatonin in the rat circulation are in the picomolar range [21], one would expect the physiological binding sites for this hormone to exhibit very high affinity. This is indeed the case as recent studies indicate the presence of picomolar-affinity sites for melatonin in discrete areas of the rat brain [22, 23]. Interestingly, picomolar to nanomolar concentrations of melatonin can also inhibit AC activity in the rat brain, but in contrast to the widespread inhibition caused by micromolar concentrations, the effects of low doses are restricted to discrete regions such as the basal hypothalamus and pituitary [24, 25] where high-affinity physiological receptors for melatonin are localized [22, 23]. Therefore, it is important to note that the pharmacological effects of melatonin, described in this report, involve binding sites which are quite distinct from the physiological binding sites for this hormone.

The inhibitory effect of melatonin required preincubation with brain membranes for several minutes before measuring enzyme activity. If BZ sites are involved, this is probably due to a decrease in the binding affinity of melatonin at the temperature of 30° used since elevated incubation temperatures cause a decrease in binding affinity at these sites [26]; thus, preincubation is necessary to permit sufficient binding and a pharmacological effect to occur.

There is evidence that anticonvulsant drugs block the elevation of cyclic AMP levels which is associated with chemically and electrically induced convulsions [27, 28]. The α_2-adrenoceptor agonist, clonidine, is a potent anticonvulsant [29] which inhibits adenylate cyclase activity in rat brain membranes [30]. Therefore, the reported anticonvulsant effects of large doses of melatonin [1, 2] may involve inhibition of adenylate cyclase activity in the CNS. Although the specificity of the anticonvulsant action of melatonin has been questioned [3], its inhibitory effect on AC in the present study was concentration-dependent and regionally selective indicating a specific action. Moreover, the significantly greater potency and efficacy of the melatonin analogs, 6-chloromelatonin and 2-iodomelatonin, in inhibiting adenylate cyclase activity, suggests that their potential value as anticonvulsants should be examined.

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