Characterization of central melatonin receptors using ^{125}I-melatonin

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The binding of ^{125}I-melatonin, a potent analog of melatonin, to rat brain synaptosomal preparations was investigated. ^{125}I-melatonin bound with high affinity ($K_d = 38$ nM) to a single class of sites ($B_{max} = 81$ fmol/mg protein). Kinetic studies indicated that binding was time-dependent and reversible. Specific ^{125}I-melatonin binding was inhibited by melatonin, and was unaffected by other structurally related compounds including serotonin. Binding of ^{125}I-melatonin was greatly reduced if the synaptosomal preparations were pretreated by heat or trypsin but was unaffected by freeze-thawing. These results suggest that ^{125}I-melatonin may serve as a valuable probe for studying melatonin receptors.

Melatonin Receptor (Brain)

1. INTRODUCTION

A large body of evidence supports the notion that the pineal gland and its major product, melatonin, play a role in coordinating mammalian reproduction [1], but the mode of action of the hormone is unclear. We have recently shown that melatonin inhibits the stimulated release of dopamine from rat hypothalamus in vitro [2], by the reduction of Ca^{2+} entry into the presynaptic nerve endings [3]. The effect of melatonin on dopamine release varied significantly within the various brain regions [4], and was dependent on the prevailing cyclical stage of the animal [5] and on the circadian time [6]. These phenomena were compatible with the existence of melatonin receptors in the brain. The characterization of melatonin receptors has been limited due to the unavailability of adequate receptor probes. The existence of high-affinity binding sites for [^{3}H]melatonin in membrane and cytosolic fractions of brain and peripheral tissues has been reported [7,8]. However, the findings are incomplete and sometimes conflicting, and there are difficulties in replicating these studies using the presently available [^{3}H]melatonin. We have recently observed that iodomelatonin could mimic the effects of melatonin in inhibiting the evoked release of dopamine from rat hypothalamus in vitro (to be described elsewhere). Here we describe the binding of radiolabeled iodomelatonin to synaptosomal preparations from rat brain.

2. MATERIALS AND METHODS

^{125}I-melatonin was prepared by iodination of melatonin with ^{125}I (Amersham) (20 Ci/mmol) in the presence of Iodo-Gen. The principal iodination product, 2-[^{125}I]iodomelatonin was separated from unsubstituted melatonin and purified by TLC as described by Vakkuri et al. [9]. Melatonin, serotonin, 5-hydroxyindoleacetic acid, 5-methoxytryptamine and *N*-acetylserotonin were purchased from Sigma.

Male rats of the CD strain (3–4 months old, weighing 190–250 g) were maintained on a 14 h light : 10 h dark schedule (lights on at 05.00 h). Food and water were supplied ad libitum. The rats were decapitated (between 09.00 and 11.00 h),

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their brains rapidly removed and suspended in 10 vols ice-cold 0.32 M sucrose. Crude synaptosomal pellets were prepared as described [7] and suspended in 2 vols of 50 mM Tris-HCl buffer, pH 7.4, containing 4 mM $CaCl_2$. Protein content was determined according to Markwell et al. [10] with bovine serum albumin (Sigma) as a standard.

Aliquots of the synaptosomal preparations (200 μg protein/20 μl) were incubated with 40 μl Tris buffer containing 5–50 nM ^{125}I-melatonin for 0–30 min at 37°C on a shaking water bath, in the absence or presence of unlabeled melatonin (50 μM). Membranes were then collected by vacuum filtration using GF/C glass fiber filters and washed with 3×4 ml buffer at 4°C. The filters containing bound ^{125}I-melatonin were assayed for radioactivity using a Packard gamma counter. All samples were run in duplicates and each experiment repeated 4 times, using different batches of ^{125}I-melatonin. Specific binding was defined as that displaced by 50 μM non-radioactive melatonin and ranged from 50 to 60% of the total binding at 20 nM ^{125}I-melatonin. Analysis of the equilibrium binding data was performed utilizing a distribution-free computer method [11] as well as traditional graphical methods [12].

In some experiments, the synaptosomal preparations were pretreated by heat (65°C, 10 min), with trypsin (0.1 mg/ml trypsin, 22°C, 10 min) or freezing (−20°C, 24 h) and thawing prior to the binding assay.

3. RESULTS AND DISCUSSION

3.1. *Equilibrium studies*

The concentration dependence of ^{125}I-melatonin binding to rat brain synaptosomal preparations was investigated (fig.1). Specific binding at equi-

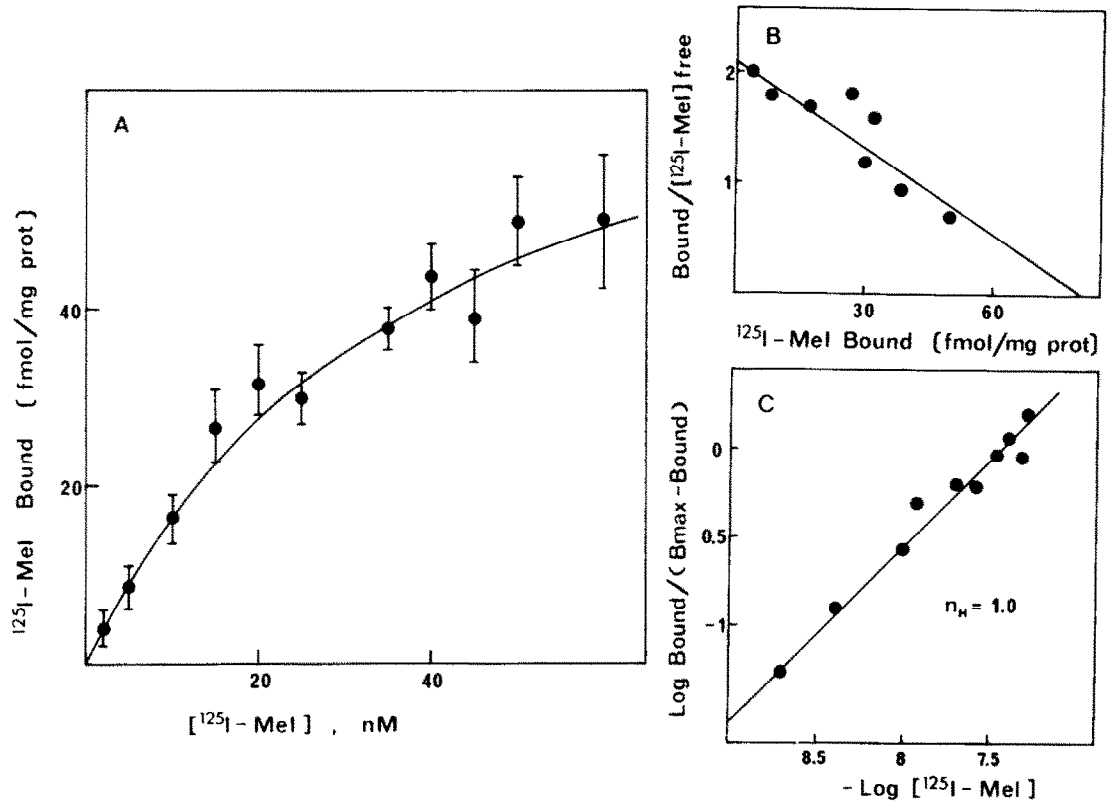

Fig.1. ^{125}I-melatonin binding to rat brain synaptosomal preparations as a function of ^{125}I-melatonin concentration. (A) Specific binding was determined at equilibrium (30 min incubations). The solid line is a theoretical line reconstructed from the K_d and V_{max} values obtained by distribution-free analysis of the data. (B) Scatchard plot of the specific ^{125}I-melatonin-binding data. The line drawn represents the best fit assuming a single class of sites; the K_d and B_{max} values obtained are the same as in A. (C) Hill plot of the same data. The value of B_{max} obtained from the Scatchard analysis was used.

librium increased greatly at low and slightly at higher concentrations of ^{125}I-melatonin approaching saturation (fig.1A). Scatchard analysis of the equilibrium experiments suggested that there is only a single class of binding sites for ^{125}I-melatonin (fig.1B). Distribution-free analysis of the data indicated that ^{125}I-melatonin was bound with a dissociation constant (K_d) of 38.7 ± 2.0 nM and a number of binding sites at saturation (B_{max}) of 81 ± 14 fmol/mg protein (fig.1A,B). Analysis of the binding data according to the Hill equation (fig.1C) yielded a Hill coefficient of 1.00 ± 0.05, thus indicating a non-cooperative binding mode (fig.2C). Thus, the binding capacity for ^{125}I-melatonin in rat brain synaptosomes (81 nmol/mg protein) is very similar to that reported for [^{3}H]melatonin-binding sites in these preparations (i.e. 62–94 fmol/mg protein [7]).

Freeze-thawing of the synaptosomal preparations had no effect on the binding of ^{125}I-melatonin. Heat and trypsin treatment abolished almost all specific binding (not shown). These data suggested that ^{125}I-melatonin was bound to a synaptosomal membrane-associated protein.

3.2. *Kinetic studies*

Fig.2A shows the association of ^{125}I-melatonin with the synaptosomal preparations. Specific binding increased during the first 15 min and equilibrated after approx. 20 min. The association rate constant ($k_1 = 1.82 \times 10^6$ min$^{-1} \cdot$M^{-1}) was determined from the pseudo-first-order plot of the time course (fig.2A, inset). Following equilibration with 40 nM ^{125}I-melatonin for 30 min at 37°C, a 1000- fold excess of melatonin was added. Displacement of all specific binding of ^{125}I-melatonin occurred within 30 min (fig.2B) indicating that the specific binding of ^{125}I-melatonin to the synaptosomal preparations was reversible. The

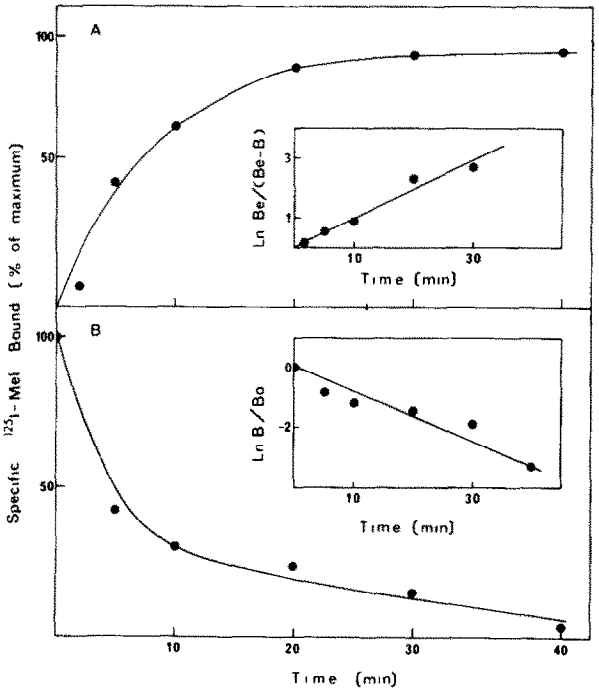

Fig.2. Time courses of ^{125}I-melatonin association with (A) and dissociation from (B) synaptosomal preparations. (A) Synaptosomes were incubated with 20 nM ^{125}I-melatonin for the indicated times. Inset: association is plotted according to a pseudo-first-order equation [12]. (B) Following equilibration with ^{125}I-melatonin (40 nM, 30 min) a 1000-fold excess of melatonin was added and the amount of specifically bound ^{125}I-melatonin remaining at the indicated times was determined. Inset: first-order plot of the dissociation data.

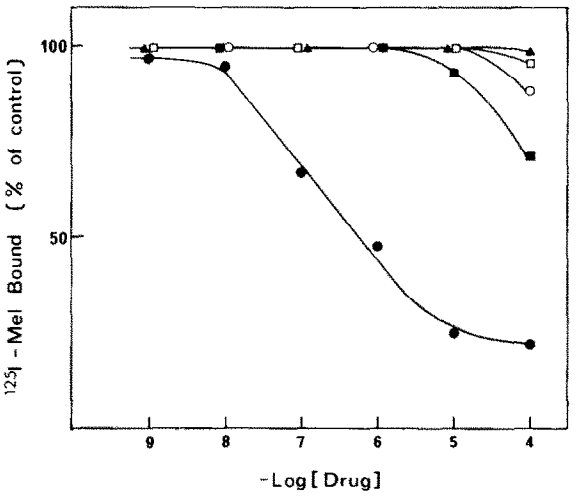

Fig.3. Inhibition of ^{125}I-melatonin (40 nM) binding to synaptosomal preparations by different concentrations of indole derivatives. Synaptosomes were incubated for 30 min with ^{125}I-melatonin in the presence of different concentrations of melatonin (●), 5-hydroxyindoleacetic acid (■), 5-hydroxytryptamine (serotonin) (○), *N*-acetylserotonin (□), 5-methoxytryptamine (▲). The specific binding of ^{125}I-melatonin was obtained by subtracting the binding observed in the presence of 50 μM iodomelatonin.

dissociation rate constant (k_{-1} = 0.082 min^{-1}) was calculated from the slope of the first-order plot of these data (fig. 2B, inset). The kinetically derived value of K_d (45.0 nM) obtained from the ratio of rate constants, k_{-1}/k_1, was in a very good agreement with that determined from equilibrium experiments (38.7 nM, fig.1). This suggested that ^{125}I-melatonin binding followed mass action principles for a simple bimolecular reaction.

3.3. *Pharmacology*

The ability of various indole compounds to inhibit ^{125}I-melatonin binding was investigated (fig. 3). Melatonin inhibited iodomelatonin binding in a dose-dependent manner. The concentrations of melatonin which inhibited the binding of ^{125}I-melatonin (40 nM) by 33 and 50% at equilibrium were 150 and 700 nM, respectively. Hence, the equilibrium dissociation constant of melatonin binding to these sites could be estimated at 150–350 nM. This value is 2–5-fold larger than that reportedly obtained using [^{3}H]melatonin as a probe (~ 75 nM [7]). Melatonin was the most potent of the indole compound tested in displacing ^{125}I-melatonin from the synaptosomal preparations; 5-hydroxyindoleacetic acid and serotonin were far less potent (fig.3). These results indicated that the specificity for melatonin of ^{125}I-melatonin-binding sites was much higher than that of the previously reported [^{3}H]melatonin-binding sites [7,8].

The use of ^{125}I-melatonin as a melatonin receptor probe has several advantages: (i) time-dependent, reversible, binding to a single class of non-cooperative sites; (ii) high specificity for melatonin; (iii) absence of radioligand instability problems; (iv) high specific activity; (v) expensive and cumbersome scintillation counting can be avoided. These advantages make the use of ^{125}I-melatonin a valuable tool in the study of melatonin receptors.

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