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Melatonin binding sites in interstitial cells from immature rat testes

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Melatonin binding sites in rat testes interstitial cells were identified using 2-[^{125}I]-iodomelatonin. Saturation studies of cells revealed a single class of high affinity binding sites, with an apparent equilibrium dissociation constant (K_d) of 100 ± 20 pM, and a total binding capacity (B max) of 3.0 ± 0.2 x 10^3 melatonin molecules per cell. Binding was reversible and inhibited by non radioactive melatonin. These results suggest that interstitial cells from immature rat testes have specific receptors for melatonin.

INTRODUCTION

It has been demonstrated that the hormone melatonin secreted from the pineal gland mediates the reproductive response of various species including humans (Reiter, 1980; Lang *et al.*, 1990). Several possible sites of pineal melatonin action in the reproductive system include the hypothalamus (Reiter *et al.*, 1981) and the pituitary (Martin *et al.*, 1977). There are numerous reports showing melatonin binding in a number of peripheral organs, such as retina (Dubocovich and Takahashi, 1987), spleen (Lang *et al.*, 1981), gastrointestinal tract (Lee and Pang, 1992), although most studies have been performed on neural tissues. With the development of a biologically active radioiodinated agonist, ^{125}I-labelled melatonin, high-affinity melatonin binding sites have been identified in nervous tissue from chicks (Rivkees *et al.*, 1989), rats (Laitinen and Saavedra, 1990) and humans (Reppert *et al.*, 1988).

The possibility of a direct gonadal effect of melatonin is suggested by previous work in our laboratory (Olivares *et al.*, 1989). The daily administration of melatonin to male rats during sexual maturation, modified testicular function only when the treatment was extended up to the end of the juvenile period (Olivares *et al.*, 1989). Furthermore, recently we have found an inhibitory effect of melatonin on hCG-induced testosterone production in isolated interstitial cells from immature rats testes (Valladares *et al.*, 1992). In the present studies, we describe the presence of [^{125}I]iodomelatonin-binding sites in interstitial cells from immature rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (35 - 40 days old) from our colony were used. They were maintained under controlled conditions of 12 h light: 12 h darkness, with rat chow and water available *ad libitum*.

Interstitial cells preparation

Decapsulated testes were dispersed with collagenase according to previously described (Dufau *et al.*, 1974). After treatment, interstitial cells were suspended in Medium 199-0.1% Bovine Serum Albumin (M-199-0.1% BSA). Total nucleated cell concentration was determined by counting aliquots in a haemocytometer.

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Leydig cell number was established by a histochemical reaction for 3ß- hydroxysteroid dehydrogenase (3ß-HSD). Cell viabilities, assessed by trypan blue-exclusion, and the percentage of cells 3ß-HSD positive were 95% and 25%, respectively.

Binding assays

2-[^{125}I]-iodomelatonin (Sp. Act. 2000 Ci/mmol) from Amersham, was dissolved in 100% ethanol and diluted in M-199-0.1% BSA to a desired concentration. Binding reaction was initiated by the addition of 100 µl aliquots of interstitial cells (2 - 6 x 10^6 cells) to tubes containing 10 µl 2-[^{125}I]-iodomelatonin (225 pM) in a 0.2 ml final incubation volume. The binding assay was measured after incubation in a water bath at 4° C for 40 min. Bound ^{125}I-melatonin was separated from the unbound radioactive hormone by vacuum filtration through Whatman GB-F filter (pore size 1.0 µm) (Ayre *et al.*, 1992). Filters were washed with 5 ml of ice-cold PBS and counted in a gamma counter (Beckman Biogamma, Fullerton, Ca). Non specific binding was detected in presence of 0.25 mM of melatonin (Sigma Chemical Co). For binding studies, two experiments run in triplicate were performed. Results were expressed as fmol/10^6 cells.

Data analysis

The specific binding data from saturation studies were evaluated for Scatchard analyses. Line slopes and intercepts were determined by simple linear regression. The ENZFIT program for data analyses was used.

RESULTS AND DISCUSSION

The time course of specific ^{125}I-melatonin binding to interstitial cells is shown in Figure 1. The binding increased rapidly for the first 15 min and equilibrated after about 30 min of incubation time (Fig 1A). Following equilibration for 60 min with 225 pM ^{125}I-melatonin, a large excess of unlabelled melatonin (0.25 mM) was able to displace 85% of specific binding within 15 min (Fig 1B). Total and non specific binding of ^{125}I-melatonin to interstitial cells was determined using a range of ligand concentration from 0.03 nM to 3.3 nM as shown in Figure 2. Specific binding of ^{125}I-melatonin was concentration dependent and reached saturation at 0.45 nM of ^{125}I-melatonin (Fig 2A). Scatchard analysis indicated a single class of binding sites (Fig 2B). The Hill coefficient was close to 1.0 (0.94), further supporting non co-operativity between binding sites. Computer assisted analysis of the data yielded an apparent K_d of 100 ± 20 pM. The maximal number of binding sites (B_{max}) was 3.0 ± 0.2x10^3 melatonin molecules per interstitial cell.

These studies describe, for the first time, melatonin binding sites in interstitial cells of immature rat testes, and suggest that the observed inhibitory effect of melatonin in steroidogenic pathway (Valladares *et al.*, 1992) may be receptor mediated. It is important to have in mind that in addition to Leydig cells, our interstitial cell preparation may contain other types of cells (germinal cells, fibroblasts, macrophages, erythrocytes and endothelial cells).

Although saturable melatonin binding sites have been described previously (Duncan *et al.*, 1988), mainly in the central nervous system, the search for physiological receptor sites has been elusive. With the use of ^{125}I- labelled iodomelatonin to identify high affinity binding sites, it appears that melatonin receptors of pmolar affinity are very discretely localized within the brain (Williams, 1989), retina and pituitary (Krause and Dubocovich, 1991). The recent reports regarding the pharmacological characteristics of specific receptor sites that show high affinity for melatonin, can be now correlated to a functional response. In this sense, our data of high affinity melatonin binding sites in interstitial cells of immature rats testes, demonstrated by the K_d values in the pmolar range, suggest that they could be physiologically relevant.

Additional experiments demonstrate that labelled melatonin is not displaced by serotonin, N-acetylserotonin, 6-OH melatonin and 5-methoxy-tryptamine (unpublished observations).

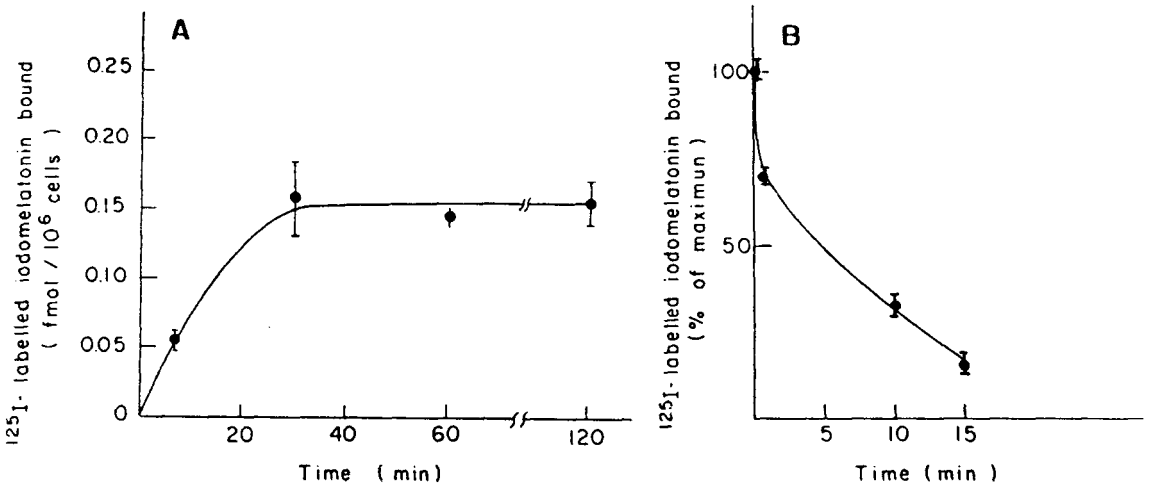

Fig 1: Time course of association and dissociation of 2-[^{125}I]iodomelatonin binding to rat interstitial cells at 4° C. A. Interstitial cells (2 x 10^6 cells) incubated with 225 pM 2-[^{125}I]iodomelatonin for indicated times. B. After equilibration for 60 min, rapid reversibility upon addition of a large excess (0.25 mM) of unlabelled melatonin. Results, means ± SD of two triplicate determinations.

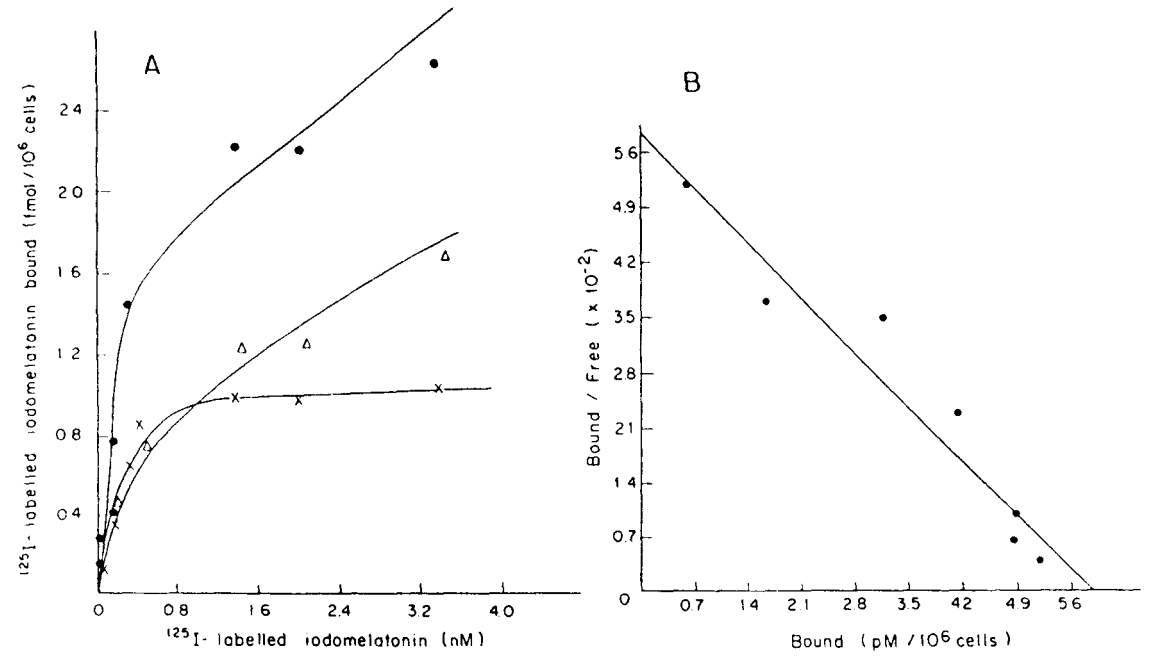

Fig 2: 2-[^{125}I]iodomelatonin binding to interstitial cells from rat testes as a function of ligand concentration. A. Interstitial cells ($6x10^6$) incubated with various concentrations of 2-[^{125}I]iodomelatonin (0.03 nM-3.3 nM) for 40 min at 4° C. Non specific binding (Δ) determined in presence of 0.25 mM non radioactive melatonin. Specific binding (x) defined as total binding (•) minus non specific binding. B. Scatchard plot of the data. Points, means of triplicate values from two representative experiments.

In conclusion, the specific binding of ^{125}I-labelled melatonin to interstitial cells of immature rats satisfied all the criteria for a binding site. It was rapid, stable, saturable, reversible, specific and of high affinity. This result may indicate the presence of putative melatonin receptors in steroidogenic cells that should be mediating the biological inhibitory response of testosterone production.

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