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Melatonin binding proteins identified in the rat brain by affinity labeling

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N-Bromoacetyl-2-iodo-5-methoxytryptamine (BIM), a novel derivative of the biologically active melatonin analog, 2-iodomelatonin, was prepared and used to identify melatonin binding proteins in rat brain synaptosomes. Incubation of the synaptosomes with BIM resulted in a time and concentration dependent, irreversible inhibition of 2-[^{125}I]iodomelatonin binding. In parallel, the radioactive form of BIM, *N*-bromoacetyl-2-[^{125}I]iodo-5-methoxytryptamine ([^{125}I]BIM) became incorporated into the synaptosomes. The incorporation of [^{125}I]BIM was inhibited by BIM, 2-iodomelatonin and melatonin but not by 5-methoxytryptamine or *N*-acetyl serotonin. [^{125}I]BIM became covalently attached to three polypeptides with apparent molecular weight values of 92, 55 and 45 kDa; the labeling of all three proteins was markedly inhibited by melatonin. These results indicate that the 92, 55 and 45 kDa polypeptides are melatonin binding proteins.

Melatonin; Iodomelatonin; Affinity labeling; Brain

1. INTRODUCTION

The nocturnal production and secretion of melatonin by the pineal gland of vertebrates provides a signal which coordinates the neuroendocrine system with the environmental lighting cycles [1]. The use of 2-[^{125}I]iodomelatonin ([^{125}I]melatonin), a potent melatonin analog enabled the characterization and quantitation of low (K_d ca. 40–70 nM) and high affinity (K_d 20–40 pM) melatonin binding sites in synaptosomes and slices from the brains of various species [2–6], and enabled the elucidation of some of their properties such as: their modulation by guanyl nucleotides [7] and cations [8], diurnal variations in binding site density and their regulation by melatonin, photoperiod and sex steroids [3,10–15] and determination of the apparent molecular mass of melatonin receptors by target size analysis [9], but the molecular structure of these sites has not been elucidated. We have developed a novel, chemically reactive, [^{125}I]melatonin derivative, *N*-bromoacetyl-2-[^{125}I]iodo-5-methoxytryptamine ([^{125}I]BIM), and report here on the identification of melatonin binding proteins using this compound as an affinity label.

2. MATERIALS AND METHODS

Preparation of *N*-bromoacetyl-2-iodo-5-methoxytryptamine (BIM). To a solution of 5-methoxytryptamine in dimethylformamide containing 2 equivalents of triethylamine, 3 equivalents of $BrCH_2COCl$ were added and the reaction was allowed to proceed at room temperature in the dark for 60 min. The pH was then brought to >7 with $NaHCO_3$ and the mixture was left for another 20 min and then centrifuged (5 min 10000×*g*). The supernatant was evaporated to dryness and subjected to thin-layer chromatography (TLC) using silicagel plate with ethyl acetate as a solvent. The major product, bromoacetyl-5-methoxytryptamine (BM; $R_F = 0.6$) was extracted with ethanol and iodinated according to Vakkuri et al. [16,17]. Briefly, BM in ethanol and KI (2 equivalents in H_2O) were added simultaneously to an Eppendorf tube precoated with Iodo-gen. After 1 min the products were extracted into chloroform and subjected to TLC using silicagel plates with ethyl acetate as a solvent. The major iodination product, BIM ($R_F = 0.73$) was extracted with ethanol. [^{125}I]BIM was prepared similarly, from BM and carrier-free Na^{125}I (Amersham). BIM and [^{125}I]BIM co-migrated as single spots on silicagel TLC plates with ethylacetate (Fig. 1) or chloroform (not shown) as solvents. The spots were detected by iodine vapors, oxidation of the indole moiety in UV light and autoradiography (Fig. 1). The chemical composition of BIM was verified by ^{1}H-nuclear magnetic resonance spectrum taken on a Bruker WM-360 NMR instrument in (CDC13 + CD3OD) solution. $^\delta$H (ppm relative to internal TMS): 7.62s (H-1), 7.28dd (J = 9,04 Hz, H-7), 6.95dd (J = 2.9,0.4 Hz, H-4), 6.85dd (J = 9.0, 2.9 Hz, H-6), 6.70t (J = 5.5 Hz, H-3′), 3.86s (5-OCH_3), 3.74s(H_2-5′), 3.58dt (J = 6.9, 5.5 Hz, H_2-2′), 2.82t (J = 6.9 Hz, H_2-1′).

2-[^{125}I]iodomelatonin ([^{125}I]melatonin) and unlabeled 2-iodomelatonin were prepared by iodination of melatonin as described [16,17]. 2-[^{125}I]iodomelatonin was diluted radioisotopically with unlabeled 2-iodomelatonin to yield a specific radioactivity of 20 Ci/mmol. 5-Methoxytryptamine, $BrCH_2COCl$, melatonin and Iodogen were purchased from Sigma, St. Louis, MO.

Male rats of the CD strain (2-months-old), maintained on a daily 14 h light:10 h darkness schedule (lights-on 05.00 h; cool white fluorescent illumination) at 24 ± 2°C, were decapitated between 09.00–11.00 h and their brains were rapidly removed and suspended in 10 ml/g ice-cold 0.32 M sucrose. Crude synaptosomal pellets were prepared as described [2] and suspended in 2 vols of 50 mM Tris-HCl buffer, pH 7.4 containing 5 mM $CaCl_2$. The following experiments were performed:

(a) Aliquots of the synaptosomal preparations (0.4 mg protein/ml; [18]) were incubated at 37°C with 10^{-14}–10^{-3} M BIM, for 1–30 min. Membranes were then collected by centrifugation (10000×*g*, 10 min), and washed extensively by repeated cycles of resuspension in the Tris buffer and centrifugation. The washed membranes were resuspended in the Tris buffer and the reversible equilibrium binding of [^{125}I]melatonin was assessed: aliquots of the membranes were in-

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Fig. 1. Thin-layer chromatography (silicagel plate, in ethylacetate) of BIM. The plate was exposed to iodine vapors (a) and oxidation in UV light (b). (c) Autoradiogram of a chromatogram of [^{125}I]BIM.

cubated in triplicates with 50 nM [^{125}I]melatonin at 37°C for 30 min in the absence or presence of unlabeled melatonin (50 μM). Membranes were then collected by vacuum filtration on GF/C filters as described [2] and the radioactivity determined. Specific binding was defined as that displaced by 50 μM melatonin.

(b) Aliquots of the synaptosomal preparations were incubated with 5 nM [^{125}I]BIM (2100 Ci/mmol) in the absence or presence of various indoles ($0-10^{-4}$ M) for 1–30 min at 37°C. Membranes were then collected by vacuum filtration using GF/C filters and washed extensively with 3×4 ml for 10 min at 4°C. The filters were assayed for radioactivity using a gamma counter.

(c) Synaptosomes were incubated with [^{125}I]BIM as in (b). The reaction was terminated by the addition of sodium-dodecyl sulfate (SDS) sample buffer (36 mM Tris-HCl, pH 6.8, 3% wt/vol SDS, 5% v/v 2-mercaptoethanol and 10% v/v glycerol) and subjected to electrophoresis on 7% polyacrylamide gels [19] followed by autoradiography.

3. RESULTS AND DISCUSSION

Fig. 2A shows a time course of inhibition of [^{125}I]melatonin binding, following preincubation of the rat brain synaptosomes with BIM (1 μM) and extensive washing. Complete loss of the specific [^{125}I]melatonin binding was observed within 30 min of incubation with BIM. The decrease followed apparent first order kinetics ($k = 0.094 \pm 0.02$ min^{-1}; Fig. 2A, inset). The time course of incorporation of [^{125}I]BIM (5 nM) into the synaptosomes is shown in Fig. 2B. The incorporation followed apparent first order kinetics ($k = 0.136 \pm 0.04$ min^{-1}; Fig. 2B, inset).

The concentration dependency of the BIM-mediated decrease in [^{125}I]melatonin binding is shown in Fig. 3A. The percentage of inhibition of [^{125}I]melatonin binding following 20 min incubations increased with increasing BIM concentration, approaching a plateau of ca. 50% inhibition between 5 and 100 nM. Further elevation of BIM concentration between 0.1–10 μM resulted in a further concentration-dependent loss of [^{125}I]melatonin binding capacity. The biphasic concentration dependency indicates that BIM forms two types of reversible complexes with melatonin binding sites, prior to the covalent modification: a high and a low affinity type, each accounting for 50% of the [^{125}I]melatonin binding. The fact that the kinetics of inactivation by 1 μM BIM was not biphasic, suggests that the bromoacetyl group of BIM reacted with similar nucleophilic groups in the two types of complexes. In addition, the kinetic data indicated that the apparent first order rate constant of the incorporation of 5 nM [^{125}I]BIM was similar to the rate constant of the decrease in [^{125}I]melatonin binding produced by 1 μM BIM. This implies that the covalent modification is slow relative to the reversible association of BIM with the binding sites.

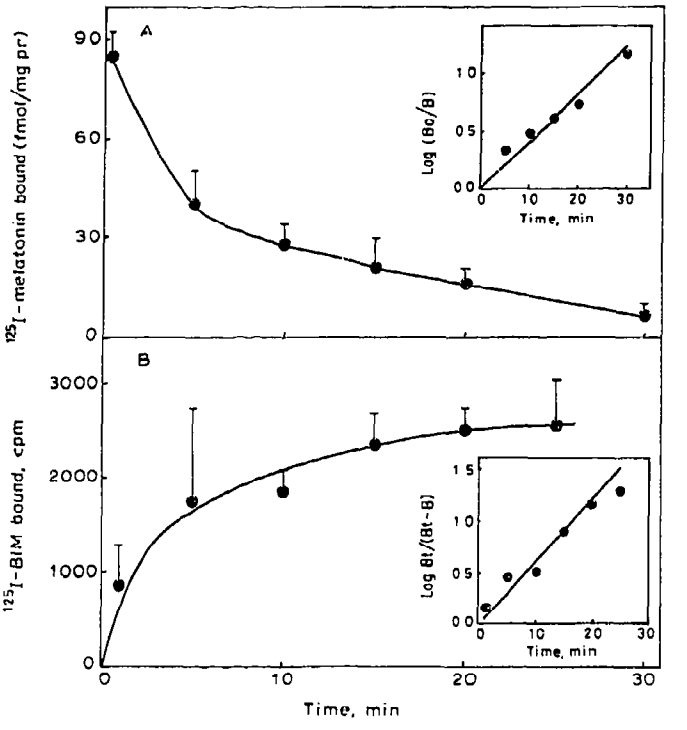

Fig. 2. (A) Effect of BIM treatment (1 μM; 0–30 min) on the binding of 50 nM [^{125}I]melatonin to rat brain synaptosomes. Inset: The loss of [^{125}I]melatonin binding is plotted according to first order kinetics. B and B_o are the binding at a specific time and zero, respectively. (B) Kinetics of the incorporation of [^{125}I]BIM (5 nM) into rat brain synaptosomes. Inset: Incorporation (B) is plotted according to first order kinetics. B and B_t are the amounts of radioactivity retained in the synaptosomes at a specific time and infinity, respectively.

The ability of various indole compounds to protect melatonin binding sites against the incorporation of [^{125}I]BIM (5 nM) during 10 min incubation periods was investigated (Fig. 3B). Unlabeled BIM prevented the incorporation of [^{125}I]BIM in a concentration-dependent manner, with 50% protection around 5 nM. This suggests that a 1:1 complex of BIM with the 'high affinity' type of sites. Among the reversible competitors studied, 2-iodomelatonin and melatonin but not 5 methoxytryptamine or *N*-acetyl serotonin, significantly slowed down the incorporation of [^{125}I]BIM into the synaptosomes.

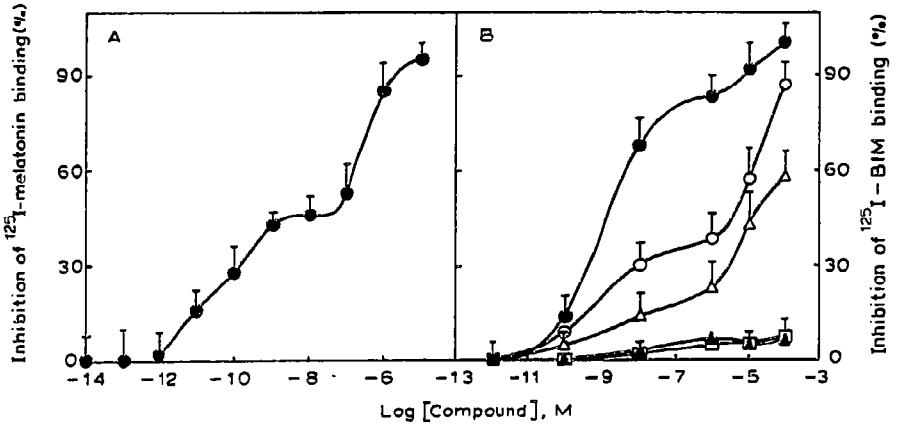

Fig. 3. (A) Concentration dependency of the decrease in binding of 50 nM [^{125}I]melatonin following treatment of synaptosomes with 0-10 μM BIM for 20 min. (B) Inhibition of incorporation of [^{125}I]BIM (5 nM, 10 min) into rat synaptosomes by BIM (●), 2-iodomelatonin (○), melatonin (△), *N*-acetyl serotonin (▲) and 5 methoxytryptamine (□). The results are expressed as 100 ($1 - B_i/B_o$) where B_i and B_o are the amounts incorporated in the presence and absence of the indole, respectively.

2-iodomelatonin was more potent than melatonin, which suits well the order of potency of these ligands at inhibiting reversible [^{125}I]melatonin binding in rat brain synaptosomes [2].

Following incubation of the rat brain synaptosomes with 5 nM [^{125}I]BIM for 30 min, the synaptosomal polypeptides were separated by SDS-polyacrylamide gel electrophoresis and the radiolabeled proteins were detected by autoradiography (Fig. 4). Only three polypeptides, with apparent molecular masses of 92, 55 and 45 kDa repeatedly incorporated the label. An additional labeled polypeptide with apparent molecular weight of 37 kDa was not always present. The 90 and 45 kDa proteins were normally less abundant than the 55 kDa protein. However, preliminary peptide-mapping studies indicated that the three proteins were distinct proteins rather than degradation products of the 92 kDa protein. The labeling of all these proteins was markedly reduced in the presence of melatonin (10 μM; Fig. 4) indicating that non-specific labeling under these conditions was very low.

The results demonstrate the potency of BIM as a selective affinity label for melatonin binding proteins according to the following criteria: (1) formation of reversible complexes with the sites; (2) specific protection against labeling by melatonin and 2-iodomelatonin, in an order of potency corresponding to their relative affinity towards melatonin receptors; (3) exclusive labeling of proteins which also recognize melatonin.

Interestingly, by using the technique of irradiation inactivation, the molecular mass of melatonin receptors in the chick retina was estimated to be ca. 44 kDa [9] which agrees well with one of the BIM target proteins found in our study; however, in the same study, the molecular mass of the receptor in the hamster hypothalamus was estimated at 30 kDa [9] suggesting an additional type of site in the rodent brain. Such a site might be the type which becomes inactivated by micromolecular concentrations of BIM and was not characterized here. It remains to be determined whether the 92, 55 and 45 kDa polypeptides represent melatonin receptor subunits or distinct subtypes.

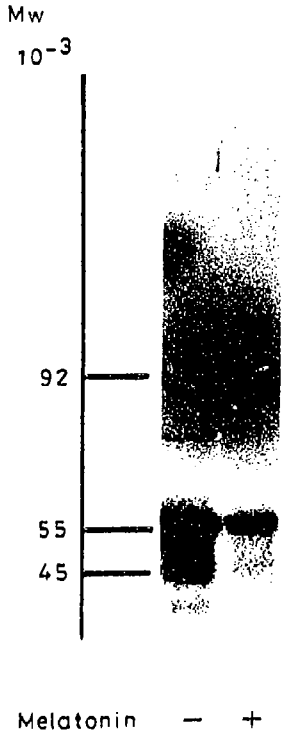

Fig. 4. Autoradiographic identification of the synaptosomal proteins labeled irreversibly with [^{125}I]BIM (5 nM, 30 min) in the absence and presence of melatonin (10 μM) as indicated. The proteins were resolved by polyacrylamide gel electrophoresis. The apparent molecular weight values of the labeled proteins are depicted.

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