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European Journal of Pharmacology 301 (1996) 159–168

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2-[^{125}I]Iodo-5-methoxycarbonylamino-*N*-acetyltryptamine: a selective radioligand for the characterization of melatonin ML_2 binding sites

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Received 15 December 1995; accepted 19 December 1995

Abstract

We now describe the preparation and characterization of a novel radioligand, 2-[^{125}I]iodo-5-methoxy-carbonylamino-*N*-acetyltryptamine (2-[^{125}I]MCA-NAT), with high affinity and pharmacological selectivity for melatonin ML_2 receptor sites. 2-[^{125}I]MCA-NAT was prepared by introducing an [^{125}I]iodine molecule on carbon 2 of 5-MCA-NAT (5-methoxycarbonylamino *N*-acetyltryptamine), a selective melatonin ML_2 receptor ligand. The specific binding (88%) of 2-[^{125}I]MCA-NAT (50 pM) to whole washed hamster brain membranes showed rapid kinetics of association/dissociation, and was of high affinity and saturable (K_d value = 116 ± 14 pM; B_{max} value = 15.5 ± 1.8 fmol/mg protein, $n = 3$). 2-[^{125}I]MCA-NAT showed no affinity for melatonin ML_1 receptors of chicken retina. Competition curves of various melatonin analogues for 2-[^{125}I]MCA-NAT binding to hamster brain, testes and kidney were monophasic and showed a pharmacological order of affinities (K_i values for brain, nM) identical to that of the ML_2 sites [2-iodomelatonin (0.77) > 6-chloro-2-methyl-melatonin (2.56) > 6-chloromelatonin (6.8) > prazosin (21.7) ≥ *N*-acetylserotonin (23.3 nM) ≥ 5-MCA-NAT (29.5) ≥ melatonin (83.9) > luzindole (1687) > serotonin (2120)]. Affinity constants for competition of melatonin analogues on [^{125}I]MCA-NAT binding to hamster brain, testes, and kidney correlated significantly [$r = 0.962$, $P < 0.001$, $n = 9$; $r = 0.982$, $P < 0.0001$, $n = 13$; $r = 0.975$, $P < 0.0001$, $n = 9$, respectively) with the affinities determined on 2-[^{125}I]iodomelatonin binding to ML_2 sites (hamster brain) but not to ML_1 sites (chicken retina, $r = 0.33$, $P > 0.05$, $n = 16$). In conclusion, 2-[^{125}I]MCA-NAT is a specific radioligand for the identification and characterization of ML_2 binding sites in brain and peripheral tissues.

Keywords: 2-[^{125}I]MCA-NAT (2-[^{125}I]iodo-5-methoxy-carbonylamino-*N*-acetyltryptamine); Melatonin ML_2 receptor; Melatonin; Brain, hamster; Kidney; Testes; 2-[^{125}I]Iodomelatonin

1. Introduction

The hormone, melatonin, mediates a variety of cellular, neuroendocrine, and physiological processes through activation of receptor sites in target tissues. The nocturnal production of melatonin by the pineal gland is controlled by the suprachiasmatic nucleus of the hypothalamus and synchronized by the light:dark cycle to a 24 h period (Reiter, 1991; Krause and Dubocovich, 1990; Morgan et al., 1994). Activation of melatonin receptors in the retina, suprachiasmatic nucleus and pars tuberalis of the pituitary gland is involved in the modulation of visual, circadian and reproductive function, respectively (Morgan et al., 1994; Dubocovich, 1995). Recent evidence suggests that melatonin can also access both the cell cytoplasm to interact with key target enzymes and structural proteins (Benítez-King and Antón-Tay, 1993), and the cell nucleus to act as a free radical scavenger and to regulate gene expression (Menendez-Pelaez and Reiter, 1993; Acuña-Castroviejo et al., 1994; Becker-Andre et al., 1994; Weisenberg et al., 1995).

Membrane and cytosolic melatonin binding sites were first identified using [^{3}H]melatonin (Cohen et al., 1978; Niles et al., 1979; Cardinali et al., 1979). The synthesis of the radiolabelled melatonin analog with high specific activity, 2-[^{125}I]iodomelatonin (Vakkuri et al., 1984), allowed the characterization and localization of high-affinity melatonin binding sites in brain, retina and peripheral tissues

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SSDI 0014-2999(95)00870-5

from vertebrate species (Laudon and Zisapel, 1986; Duncan et al., 1986; Dubocovich and Takahashi, 1987; Pickering and Niles, 1990; Stankov et al., 1991; Viswanathan et al., 1990; Yu et al., 1991; Song et al., 1993; Acuña-Castroviejo et al., 1993; Kennaway and Hugel, 1992). 2-[^{125}I]Iodomelatonin binds to at least two distinct groups of sites defined as the melatonin ML_1 and ML_2 subtypes. These melatonin binding sites show different binding kinetics and affinity, regulation by ions and temperature (Dubocovich, 1988a, 1995). Furthermore, the two sites show different pharmacological profiles. Melatonin ML_1 receptors bind 2-[^{125}I]iodomelatonin with picomolar affinity, and are characterized by the following rank of pharmacological affinities: 2-iodomelatonin ≥ melatonin > 6-hydroxymelatonin ≫ *N*-acetylserotonin ≫ prazosin > serotonin (Dubocovich, 1988a, 1995). In contrast, the melatonin ML_2 sites first described in Syrian hamster brain membranes using 2-[^{125}I]iodomelatonin show low nanomolar affinity and a pharmacological profile (2-iodomelatonin > prazosin ≥ *N*-acetylserotonin ≥ 6-hydroxymelatonin ≥ melatonin ≫ serotonin) different from that of the ML_1 site (Dubocovich, 1988a, 1995; Duncan et al., 1988, 1989; Pickering and Niles, 1990).

Melatonin ML_1 sites function as receptors in that their activation has been linked to functional responses (Heward and Hadley, 1975; Dubocovich, 1983, 1985, 1988b; Dubocovich and Takahashi, 1987; Sugden, 1991; Vanecek and Klein, 1992; Morgan et al., 1994). More recently, c-DNAs encoding melatonin ML_1 type receptors were cloned from *Xenopus laevis*, sheep, and human tissue (Ebisawa et al., 1994; Reppert et al., 1994). Binding of 2-[^{125}I]iodomelatonin to ML_2 sites was identified in hamster brain and the RPMI 1846 melanoma cell line (Duncan et al., 1988, 1989; Pickering and Niles, 1990, 1992). Although activation of ML_2 sites appears to be associated with increases in phosphoinositide hydrolysis, the physiological responses linked to this signalling pathway are not known (Eison and Mullins, 1993; Mullins and Eison, 1994; Popova and Dubocovich, 1995).

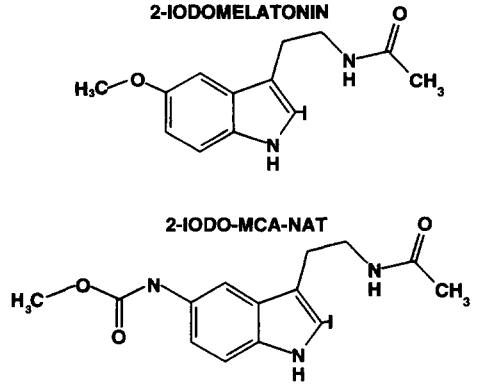

Fig. 1. Chemical structures of the radioligands 2-iodomelatonin and 2-iodo-MCA-NAT.

Here, we describe a novel radioligand, 2-[^{125}I]iodo-5-methoxycarbonylamino-*N*-acetyltryptamine (2-[^{125}I]MCA-NAT) (Fig. 1), for the identification and characterization of melatonin ML_2 receptor sites. 2-[^{125}I]MCA-NAT has a number of advantages over 2-[^{125}I]iodomelatonin in that it shows higher affinity and selectivity for the melatonin ML_2 site.

2. Materials and methods

2.1. Materials

6-Chloromelatonin and *N*-acetyltryptamine were donated by Eli Lilly Laboratories (Indianapolis, IN, USA); spiperone and ketanserine by Janssen Pharmaceutica (Piscataway, NJ, USA); fluphenazine by E.R. Squibb and Sons (Princeton, NJ, USA); methysergide by Sandoz Pharmaceuticals (East Hanover, NJ, USA); D-600 by Knoll (Chemische Fabriker); 5-methoxyluzindole by Nelson Research (Irvine, CA, USA); 5-MCA-NAT, 2-Br-MCA-NAT (2-Br-5-methoxycarbonylamino *N*-acetyltryptamine), and luzindole by Glaxo Group Research (Ware, UK). 6-Chloro-2-methylmelatonin and 6-methoxymelatonin were obtained from Research Biochemicals Int. (Wayland, MA, USA) as part of the Chemical Synthesis Program of the National Institute of Mental Health [Contract 278-90-0007 (BS)]. 2-Iodomelatonin and prazosin were purchased from Research Biochemicals Int. (Wayland, MA, USA). Other drugs and reagents were from Sigma Chemical Company (St. Louis, MO, USA). 2-[^{125}I]Iodomelatonin (specific activity 2200 Ci/mmol) was purchased from Dupont NEN Research Products (Boston, MA, USA).

2.2. Preparation of 2-[^{125}I]MCA-NAT

5-MCA-NAT was prepared by the direct acetylation of the primary tryptamine derivative using excess acetic anhydride and pyridine in tetrahydrofuran at room temperature for 18 h (Coates et al., 1982). Iodination of 5-MCA-NAT was performed using a modification of the procedure described previously for the preparation of 2-[^{125}I]iodomelatonin (Vakkuri et al., 1984). Briefly, 10 μl of 5-MCA-NAT (1 mg/ml in phosphate buffer, pH 6) and 7 μl of Na^{125}I (New England Nuclear, Boston, MA, USA) were added to Eppendorf tubes coated with 2 μg of Iodo-Gen (Pierce, Rockford, IL, USA) for 1 min. The reaction was stopped with 5 μl of sodium metabisulfite (0.4 mg/ml), and the product was extracted with chloroform and purified on silica gel TLC plates (Kodak 13179). The TLC solvent used was ethyl acetate/methanol/triethylamine (90:10:0.005). The purified 2-[^{125}I]MCA-NAT (specific activity = 2175 Ci/mmol) was extracted with methanol and stored at −20°C.

2.3. Animals

Siberian hamsters (2 months old) were raised in our colony at Northwestern University under a 16/8 h light/dark cycle. Chicken brain and retinas were obtained from a slaughterhouse. Adult Sprague-Dawley rats, C3H/HeN mice, HSD-DH guinea pigs, New Zealand white rabbits, and Syrian hamsters were obtained from commercial sources (Harlan, Indianapolis, IN, USA). All animals were killed in the middle of the light cycle. Brains, retinas, testes, and kidneys were dissected and stored at −80°C until use.

2.4. Membrane preparation

Frozen tissue was thawed and homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25°C) containing $MgCl_2$ (10 mM) with a Brinkmann Polytron PT-5 at setting 5 for 10 s. Homogenates were centrifuged at 44 000 $\times g$ for 10 min at 4°C. Pellets were washed once by resuspension and centrifugation in ice-cold Tris-HCl buffer. Membrane pellets were resuspended by homogenization at a concentration of approximately 1.7 mg protein/ml.

2.5. Binding assays

Radioligands (2-[^{125}I]MCA-NAT or 2-[^{125}I]iodomelatonin) were diluted in Tris-HCl buffer with 0.01% bovine serum albumin following evaporation of the methanol. Drugs were dissolved in 1 mM HCl with 0.1% bovine serum albumin. Binding of radioligands was initiated by addition of 220 μl aliquots of membranes (≃ 370 μg protein) in Tris-HCl buffer to tubes containing 20 μl of appropriate concentrations (10 pM–600 pM) of radioligands (i.e., [2-[^{125}I]MCA-NAT or 2-[^{125}I]iodomelatonin) and 20 μl of vehicle or drugs. Unless otherwise indicated, the binding of either radioligand was routinely measured in duplicate, and samples were incubated at 4°C for 1 h. Reactions were terminated by addition of 5 ml of ice-cold Tris-HCl buffer, and the contents were immediately filtered through glass-fiber filters (Schleicher&Schuell No. 30) soaked in 0.5% (v/v) polyethylenimine solution. Each filter was washed twice with 5 ml of ice-cold buffer. Total filtration time was less than 5 s. Radioactivity was determined in a gamma counter. Non-specific binding, unless otherwise indicated, was defined as binding in the presence of 10 μM melatonin. Specific binding was calculated by subtracting non-specific binding from total binding and was expressed as fmol/mg of protein. In a typical experiment, the total 2-[^{125}I]MCA-NAT (50 pM) binding was 5563 ± 193 dpm ($n = 4$), and the non-specific binding defined with 10 μM melatonin was 1070 ± 105 dpm ($n = 4$). The total radioactivity bound to the filters in the absence of protein was 821 ± 124 dpm ($n = 8$). Protein contents were determined by the method of Bradford (1976).

2.6. Identity of bound radioligand

Immediately following filtration, filters were removed and placed in 4 ml of water. The filters were disintegrated by vortexing, and radioactivity was extracted from the water with 2 ml of chloroform. The chloroform extract was evaporated under nitrogen to a volume of 100 μl. Extracts were applied to a silica gel TLC plate (Kodak 13179). Chromatograms were run with chloroform/methanol/acetic acid (93 : 7 : 2) and developed on a phosphoimaging plate (Fuji Photo Film Co., Japan) (Fig. 2).

2.7. Calculations

Kinetic data were analyzed by the method of Bennett and Yamamura (1985), using pseudo-first-order conditions to estimate the association rate constant (k_{+1}) and following the addition of melatonin (10 μM) to estimate the dissociation rate constant (k_{-1}). Data from saturation (K_d and B_{max} values) and competition experiments (IC_{50} and K_i values) were analyzed by means of the INPLOT program (ISI, Philadelphia, PA, USA). K_i values were calculated from IC_{50} values by the method of Cheng and Prusoff (1973).

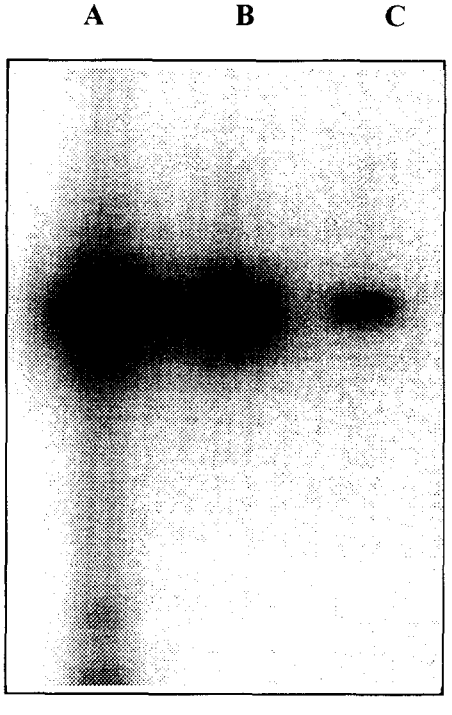

Fig. 2. Identity of bound 2-[^{125}I]MCA-NATto hamster brain membranes. Washed Siberian hamster brain membranes were incubated with 2-[^{125}I]MCA-NAT (200 pM) for 1 h at 4°C. Free radioligand was separated from membrane bound by filtration through glass-fiber filters (Schleicher &Schuell No. 30). Filters were disintegrated by vortexing in 4 ml water and the radioactivity was extracted with chloroform. Chloroform extracts were loaded and ran on a silica gel TLC plate. Free 2-[^{125}I]MCA-NAT (≃ 5000 cpm) (A); radioactivity bound to the membranes in the absence (B) and presence (C) of 10 μM melatonin.

3. Results

3.1. Selectivity of 5-MCA-NAT to compete for 2-[^{125}I]iodomelatonin binding to the ML_2 site of Siberian hamster brain

The melatonin analogue, 5-MCA-NAT, competed with high affinity ($K_i = 35.4 \pm 10.2$ nM) for 2-[^{125}I]iodomelatonin binding to the ML_2 binding site of Siberian hamster brain membranes (Table 1). In contrast, 5-MCA-NAT showed 550 times lower affinity for the ML_1 binding site of chicken retinal membranes ($K_i = 19.4 \pm 4.4$ μM) (Dubocovich et al., submitted; Table 1). Similarly, prazosin, the well known α_1 receptor antagonist, which also is a putative ML_2 melatonin receptor analogue, showed 938-fold higher affinity for the ML_2 than the ML_1 site. Substitution on carbon 2 of 5-MCA-NAT with a bromine yielded 2-Br-5-MCA-NAT, which showed a greater affinity for the ML_2 site 15 times than did the parent compound and very low affinity for the ML_1 site (Table 1). The high affinity and selectivity of 5-MCA-NAT for the ML_2 site, as well as the increase in affinity observed following halogenation of this compound on carbon 2, provided the rationale for the preparation and characterization of 2-[^{125}I]MCA-NAT as a selective radioligand for the ML_2 site (Fig. 1).

3.2. Characterization of 2-[^{125}I]MCA-NAT binding to brain membranes of different species

The radioligand 2-[^{125}I]MCA-NAT obtained by iodination of 5-MCA-NAT was tested for its ability to bind specifically to brain membranes obtained from different species. Binding of 2-[^{125}I]MCA-NAT (50 pM) to washed brain membranes was determined after 1 h incubation in Tris-HCl buffer (pH 7.4) at 4°C. The binding of 2-[^{125}I]MCA-NAT to Siberian and Syrian hamster brain membranes defined with 100 μM melatonin was 88% and 63% specific, respectively (Table 2). A high level of 2-[^{125}I]MCA-NAT-specific binding was also found in membranes of C3H/HeN whole mouse brain (Table 2). In contrast, both total and specific 2-[^{125}I]MCA-NAT binding to membranes of guinea pig, rat, rabbit, chicken brain, and chicken retina was significantly lower than in hamster brain (Table 2).

Specific 2-[^{125}I]MCA-NAT binding was detected in Siberian hamster brain membranes obtained from various brain regions (i.e., hippocampus, cerebellum, frontal cortex, thalamus, hypothalamus, striatum, pons medulla, olfactory tubercle) (Table 3). The highest and lowest levels of 2-[^{125}I]MCA-NAT specific binding were detected in hippocampus and olfactory tubercle, respectively. Based on these data, all studies on characterization of 2-[^{125}I]MCA-NAT were carried out using whole Siberian hamster brain membranes.

Table 2
2-[^{125}I]MCA-NAT binding to brain and retina membranes from different species

Species	2-[^{125}I]MCA-NAT binding		
	Total binding [a]	Specific binding [b]	
	fmol/mg protein	fmol/mg protein	%
Siberian hamster brain	3.69 ± 0.13	3.25 ± 0.13	88 ± 2
Syrian hamster brain	3.40 ± 0.12	2.14 ± 0.11	63 ± 1
C3H/HeN mouse brain	2.41 ± 0.22	1.30 ± 0.19	54 ± 2
Guinea pig brain	1.54 ± 0.04	0.43 ± 0.05	28 ± 3
Rat brain	1.06 ± 0.11	0.19 ± 0.09	18 ± 7
Rabbit brain	2.06 ± 0.16	0.27 ± 0.12	13 ± 4
Chicken brain	1.04 ± 0.07	0.31 ± 0.07	30 ± 4
Chicken optic tectum	1.47 ± 0.12	0.35 ± 0.12	24 ± 1
Chicken retina	1.17 ± 0.08	0.21 ± 0.08	18 ± 3

[a] Total binding of 2-[^{125}I]MCA-NAT (50 pM) to membranes from various tissues was measured after incubation for 1 h at 4°C. [b] Specific binding was defined with 100 μM melatonin and expressed as fmol/mg protein and percentage. Values shown represent the means ± S.E.M of 3 independent determinations performed in duplicate.

Table 1
Affinities of various compounds to compete for 2-[^{125}I]iodomelatonin binding to chicken retina and Siberian hamster brain membranes

Compounds	2-[^{125}I]Iodomelatonin binding		
	Chicken retina (ML_1) K_i value (nM)	Hamster brain (ML_2) K_i value (nM)	Affinity ratios ML_1/ML_2
2-Br-5-MCA-NAT	2116 ± 527	2.4 ± 0.9	882
2-Iodomelatonin	0.104 ± 0.006	3.1 [a]	0.03
6-Chloromelatonin	0.520 ± 0.18	5.7 [a]	0.09
Prazosin	6950 ± 1107	7.4 ± 1	938
5-Hydroxytryptamine	1450 ± 230	29.6 [a]	50
5-MCA-NAT	19400 ± 4400	35.4 ± 10	548
Melatonin	0.56 ± 0.16	73 ± 7	0.01
Serotonin	> 100000	3620 [a]	> 28

Competition of the various compounds for 2-[^{125}I]iodomelatonin binding to chicken retina and Siberian hamster brain membranes was carried out at 25°C and 4°C, respectively, for 1 h. K_i values were calculated from IC_{50} values obtained from competition curves, by the method of Cheng and Prusoff (1973). [a] K_i values for Siberian hamster brain membranes were taken from Duncan et al. (1989). All K_i values shown represent the means of 3–7 independent determinations performed in duplicate.

Table 3
Distribution of 2-[^{125}I]MCA-NAT binding sites in various Siberian hamster brain areas

Tissue	Specific 2-[^{125}I]MCA-NAT binding (fmol/mg protein)
Hippocampus	2.13 ± 0.06
Cerebellum	1.98 ± 0.16
Frontal cortex	1.95 ± 0.16
Thalamus	1.87 ± 0.05
Hypothalamus	1.76 ± 0.20
Striatum	1.71 ± 0.02
Pons medulla	1.36 ± 0.11
Olfactory tubercle	1.20 ± 0.15

2-[^{125}I]MCA-NAT (50 pM) binding to membranes from various areas of Siberian hamster brain was determined in the absence and presence of melatonin (10 μM). Values shown represent the means ± S.E.M of 3 independent determinations performed in duplicate.

3.3. Kinetic studies

Association of 2-[^{125}I]MCA-NAT to hamster brain membranes at 4°C was rapid, reaching equilibrium following 20 min of incubation and was stable for 90 min (Fig. 3). Specific 2-[^{125}I]MCA-NAT binding was reversed by the addition of excess competing ligand (melatonin, 10 μM) at equilibrium (60 min). Dissociation of 2-[^{125}I]MCA-NAT from hamster brain membranes was rapid and essentially complete within 5 min (Fig. 3). Kinetic analysis of data from three experiments demonstrated that the association rate constant was $k_{+1} = 0.00119 \pm 0.00023$ pM^{-1} min^{-1}, and the dissociation rate constant was $k_{-1} = 0.259 \pm 0.093$ min^{-1}. The K_d was calculated as $k_{-1}/k_{+1} = 218 \pm 86$ pM ($n = 3$).

3.4. Saturation studies

Concentration-dependent binding of 2-[^{125}I]MCA-NAT (10–600 pM) to hamster brain membranes was saturable

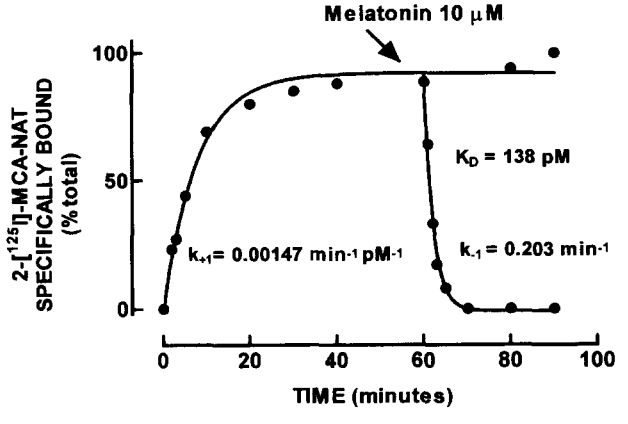

Fig. 3. Time course of association and dissociation of 2-[^{125}I]MCA-NAT binding to Siberian hamster brain membranes at 4°C. Association of 2-[^{125}I]MCA-NAT (200 pM) was rapid and was completely reversible upon addition of 10 μM melatonin (arrow). Each point represents the average of duplicate determinations obtained in a representative experiment, which was repeated 3 times with comparable results.

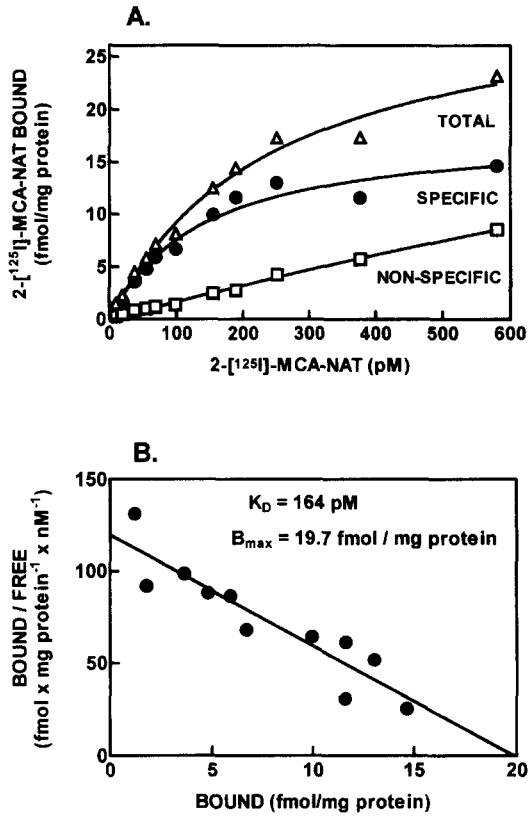

Fig. 4. Binding isotherms (A) and Scatchard plot (B) of 2-[^{125}I]MCA-NAT binding to Siberian hamster brain membranes. Membranes were incubated with various concentrations of 2-[^{125}I]MCA-NAT (10–600 pM) for 1 h at 4°C. Nonspecific binding (□) was determined in the presence of 10 μM melatonin. Specific binding (●) was defined as total binding (△) minus non-specific binding (□). Each point represents the average of duplicate determinations obtained in a representative experiment, which was repeated 3 times with comparable results.

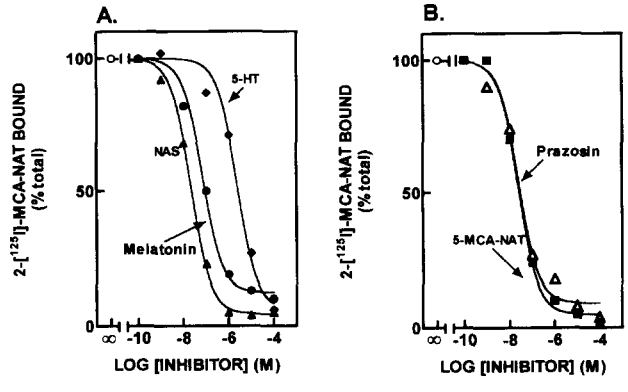

Fig. 5. Competition of various melatonin analogues and prazosin for 2-[^{125}I]MCA-NAT binding to Siberian hamster brain membranes. Washed membranes were incubated with 2-[^{125}I]MCA-NAT (50 pM) and 7 concentrations of drugs at 4°C. Control (○); *N*-acetylserotonin (▲: NAS; $K_i = 18$ nM); melatonin (●; $K_i = 63$ nM); serotonin (◆: 5-HT; $K_i = 1964$ nM); 5-MCA-NAT (△; $K_i = 22$ nM) and prazosin (■; $K_i = 25$ nM). Each point represents the average of duplicate determinations obtained in a representative experiment, which was repeated at least 3 times with comparable results.

and resulted in linear Scatchard plots, suggesting binding to a single class of sites (Fig. 4). The apparent K_d for 2-[^{125}I]MCA-NAT derived from Scatchard analysis was 116 ± 14 pM ($n = 3$) and the total number of binding sites was 15.5 ± 1.8 fmol/mg of protein ($n = 3$). The K_d values determined in kinetic (218 ± 86 pM, $n = 3$) and saturation experiments were in close agreement.

3.5. Pharmacological characteristics

The pharmacological characterization of 2-[^{125}I]MCA-NAT binding to hamster brain membranes was carried out with tracer concentrations of radioligand (50 pM) and various competing agents. Fig. 5A shows that competition of *N*-acetylserotonin, melatonin, and serotonin for 2-[^{125}I]MCA-NAT binding displays the pharmacological profile of a typical ML_2 site (Dubocovich, 1988a, 1995). Compounds previously reported as showing high affinity and selectivity for the ML_2 site, such as prazosin (Niles et al., 1987; Pickering and Niles, 1989, 1990) and 5-MCA-NAT (Table 1; Dubocovich, 1995), showed high affinity for competition with 2-[^{125}I]MCA-NAT binding to hamster brain membranes (Fig. 5B).

Table 4 shows the affinity constants (K_i values) of 16 putative melatonin analogues and other drugs from various chemical classes determined from the binding of 2-[^{125}I]iodomelatonin and 2-[^{125}I]MCA-NAT to hamster brain membranes. The affinity of compounds competing for inhibition of 2-[^{125}I]MCA-NAT binding to hamster brain membranes did not correlate with their affinity to inhibit 2-[^{125}I]iodomelatonin binding to melatonin ML_1 receptors in chicken retinal membranes ($r = 0.33$; $P < 0.05$; $n = 16$) (Fig. 6A). In contrast, a highly significant correlation (slope = 0.862; $r = 0.962$; $P < 0.001$; $n = 19$) was found between the affinity of compounds to compete for binding of 2-[^{125}I]MCA-NAT and 2-[^{125}I]iodomelatonin binding to Siberian hamster brain membranes (Duncan et al., 1988, 1989) (Table 4; Fig. 6B). Selected antagonists for serotonin receptors (spiperone, ketanserine, methysergide), dopamine receptors (fluphenazine), α-adrenoceptors (phentolamine), and Ca^{2+} channels (D-600, verapamil)

Table 4
Pharmacological profile of the 2-[^{125}I]iodomelatonin and 2-[^{125}I]MCA-NAT binding to hamster brain, testes, and kidney membranes

Compounds	K_i values (nM) for binding of			
	2-[^{125}I]Iodomelatonin	2-[^{125}I]MCA-NAT binding		
	Brain	Brain	Testes	Kidney
A. Melatonin analogues				
2-Br-MCA-NAT	2.4	0.39 ± 0.02		
6-Chloro-2-methylmelatonin	2.8	2.56 ± 0.55	2.14 ± 0.85	2.50 ± 1.29
2-Iodomelatonin	3.1 [a]	0.77 ± 0.11	2.79 ± 0.12	3.36 ± 1.58
6-Chloromelatonin	5.7 [a]	6.80 ± 0.6	14.5 ± 4.3	10.9 ± 2.6
6-Methoxymelatonin	13.5	16.8 ± 5.3		
5-MCA-NAT	28.5	29.5 ± 10.4	31.7 ± 8.8	25.7 ± 7.0
N-Acetylserotonin	29.6	23.3 ± 4.6	33.7 ± 6.0	32.9 ± 14.4
Melatonin	73	83.9 ± 11.5	78.2 ± 19.2	117 ± 19
6,7-Di-chloro-2-methylmelatonin	78.9 [a]	209 ± 5		
6-Hydroxymelatonin	83.9 [a]	106 ± 30	92.3 ± 64.8	
5-Methoxyluzindole	163	252 ± 22		
5-Methoxytryptophol	285 [a]	425 ± 41	405 ± 128	
5-Methoxy-*N*,*N*-dimethyltryptamine	340 [a]	608 ± 108		
5-Methoxytryptamine	396 [a]	641 ± 169	1926 ± 772	
N-Acetyltryptamine	1010 [a]	1261 ± 32	2367 ± 188	
Luzindole	1886	1687 ± 428	3451 ± 387	2178 ± 309
B. Other drugs				
Prazosin	7.4	21.7 ± 2.3	20.6 ± 4.0	18.9 ± 3.9
6-Methoxyharmalan	450 [b]	232 ± 6		
Spiperone	280 [a]	246 ± 68	585 ± 57	
Fluphenazine	1700 [b]	1956 ± 253		
Ketanserine	2350 [a]	1417 ± 357		
Serotonin	3620 [a]	2120 ± 124	7062 ± 1077	1401 ± 263
Methysergide	3620 [a]	4135 ± 251		
Verapamil	19555	20405 ± 1075		
Phentolamine	24482	27530 ± 2800		
D-600	25730	27180 ± 650		

K_i values were calculated from IC_{50} values obtained from competition curves using the method of Cheng and Prusoff (1973). Inhibition of specific binding of either 2-[^{125}I]iodomelatonin (80–100 pM) or 2-[^{125}I]MCA-NAT (50 pM) was determined for 7 concentrations of drugs. K_i values for competition with 2-[^{125}I]iodomelatonin to [a] Siberian and [b] Syrian hamster brain membranes were taken from Duncan et al. (1988) and Duncan et al. (1989), respectively. K_i values shown represent the means ± S.E.M of 3–5 independent determinations performed in duplicate.

showed very low affinity for 2-[^{125}I]MCA-NAT binding to hamster brain membranes.

3.6. Localization and pharmacological characterization of ML_2 binding sites in hamster peripheral tissues

2-[^{125}I]MCA-NAT was used to localize ML_2 binding sites in peripheral tissues of the Siberian hamster. 2-[^{125}I]MCA-NAT binding to hamster testes and kidney membranes was specific, and showed kinetics of association and dissociation almost identical to those reported for brain membranes. The binding of 2-[^{125}I]MCA-NAT to hamster testes and kidney membranes was saturable and of high affinity. The apparent K_d values of 2-[^{125}I]MCA-NAT binding to testes and kidney membranes, as derived from Scatchard analysis, were 247 ± 22 pM (n = 3) and 130 ± 39 pM (n = 3), respectively. The total number of binding sites was 29.1 ± 3.6 fmol/mg protein (n = 3) and 17.8 ± 3.8 fmol/mg protein (n = 3), respectively (Table 5).

The pharmacological characteristics of 2-[^{125}I]MCA-NAT binding to hamster testes and kidney membranes were identical to those of binding to hamster brain membranes. The K_i values of various compounds to compete for 2-[^{125}I]MCA-NAT binding to hamster brain membranes were significantly correlated with their affinity to

Table 5
Affinity and density of 2-[^{125}I]MCA-NAT binding to Siberian hamster brain, testes, and kidney membranes

Tissue	K_d value (pM)	B_{max} value (fmol/mg protein)	n
A. 2-[^{125}I]Iodomelatonin			
Brain	390 ± 68	19.1 ± 4.8	4
B. 2-[^{125}I]MCA-NAT			
Brain	116 ± 14	15.5 ± 1.8	3
Testes	247 ± 22	29.1 ± 3.6	3
Kidney	130 ± 39	17.8 ± 3.8	3

Affinity (K_d value) and total number of binding sites (B_{max} value) for both 2-[^{125}I]iodomelatonin (0.005–5 nM) (A) and 2-[^{125}I]MCA-NAT (10–600 pM) (B) were calculated from Scatchard plots. For saturation experiments with 2-[^{125}I]iodomelatonin (0.9–5 nM), the radioligand was diluted with 2-iodomelatonin to a lower specific activity (600 fmol/mg protein). Values shown represent the means ± S.E.M of 3 independent experiments performed in duplicate.

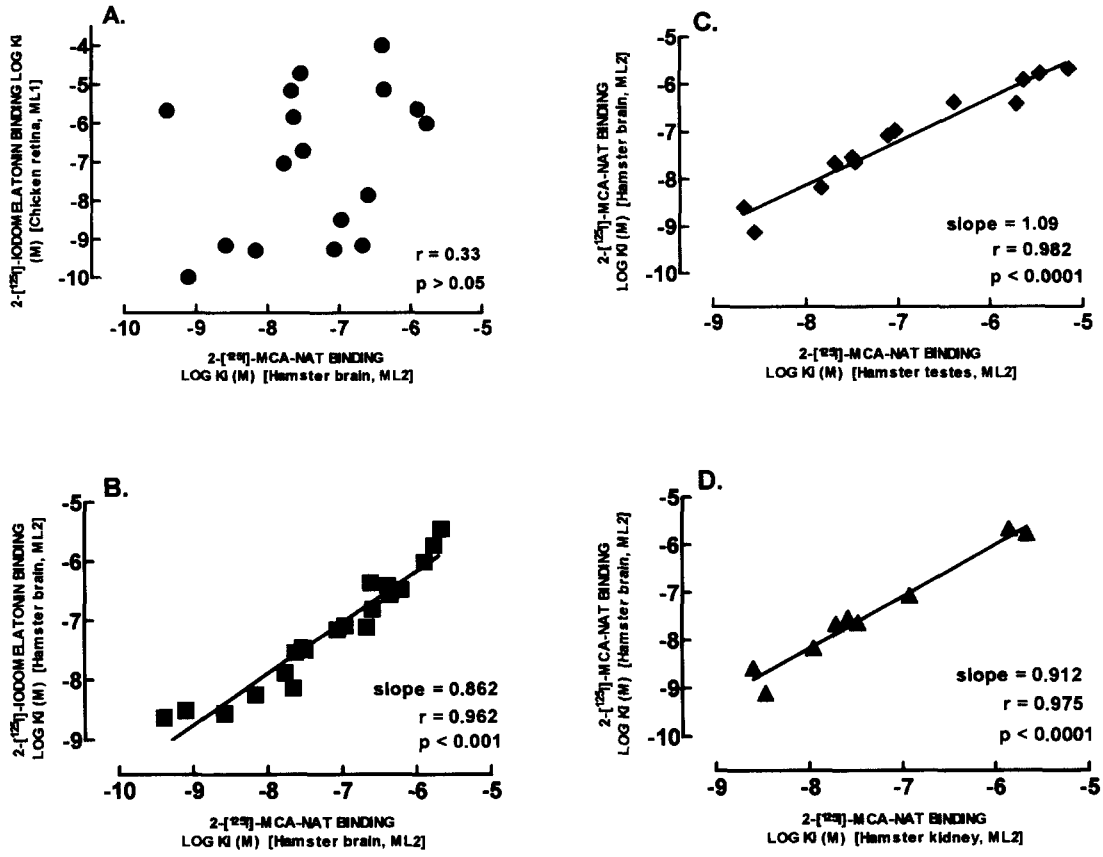

Fig. 6. Correlations between affinities of compounds to compete for 2-[^{125}I]iodomelatonin binding [chicken retina (A) or hamster brain (B)] and 2-[^{125}I]MCA-NAT binding [hamster brain (C, D)] with 2-[^{125}I]MCA-NAT [hamster brain (A, B), testes (C), and kidney (D)]. K_i values for inhibition of 2-[^{125}I]iodomelatonin binding to chicken retinal membranes incubated for 1 h at 25°C were obtained from Dubocovich (1995) and unpublished observations. K_i values for inhibition of 2-[^{125}I]iodomelatonin binding to Syrian and Siberian hamsters were obtained from Duncan et al. (1988, 1989) and Table 4. K_i values for inhibition of 2-[^{125}I]MCA-NAT to Siberian hamster brain, testes, and kidney membranes were obtained from Table 4.

compete for binding of this radioligand to both hamster testes (slope = 1.09; $r = 0.982$; $P < 0.0001$; $n = 13$) (Fig. 6C) and kidney (slope = 0.912; $r = 0.975$; $P < 0.0001$; $n = 9$) membranes (Fig. 6D).

4. Discussion

We report here that the iodination of 5-MCA-NAT resulted in a novel and selective radioligand, 2-[^{125}I]MCA-NAT, that can discriminate between the ML_1 and ML_2 binding sites for melatonin. Melatonin binding sites with the ML_2 pharmacology were first reported for hamster brain membranes from experiments with the radioligand 2-[^{125}]iodomelatonin, which binds to both ML_1 and ML_2 sites (Duncan et al., 1988, 1989; Pickering and Niles, 1990; Dubocovich, 1988a, 1995). The specific binding of 2-[^{125}I]MCA-NAT to hamster brain membranes showed rapid kinetics of association/dissociation and was of high affinity and saturable. Moreover, its pharmacological profile corresponded to that of the melatonin ML_2 binding site.

Specific 2-[^{125}I]MCA-NAT binding was demonstrated in brain and peripheral tissues where high densities of ML_2 2-[^{125}I]iodomelatonin binding sites were previously reported (Niles et al., 1987; Duncan et al., 1988, 1989; Dubocovich, 1988a; Pickering and Niles, 1990). Recently, 2-[^{125}I]iodomelatonin binding sites with characteristics of ML_2 sites were also found in the mouse brain (Bubenik et al., 1993) and a peripheral tissue, the rat liver (Acuña-Castroviejo et al., 1993, 1994). The regional distribution of 2-[^{125}I]MCA-NAT binding to Siberian hamster brain membranes was very similar but not identical to that previously reported for 2-[^{125}I]iodomelatonin to Syrian hamster brain (Duncan et al., 1988). It is conceivable that the higher level of 2-[^{125}I]iodomelatonin specific binding found in the hypothalamus of the Syrian hamster brain by Duncan et al. (1988) may represent high affinity binding to melatonin ML_1 receptors in the suprachiasmatic nucleus. Brain tissue from mammalian (i.e., rabbit, guinea pig, rat) and avian (i.e., chicken) species has lower levels of 2-[^{125}]MCA-NAT specific binding, suggesting a heterogenous distribution of ML_2 sites among species.

This new radioligand bound with picomolar affinity to sites in Siberian hamster brain, testes, and kidney and mouse brain. The pharmacological characteristics of specific 2-[^{125}I]MCA-NAT melatonin binding sites in hamster brain membranes were similar to those demonstrated for 2-[^{125}I]iodomelatonin binding to ML_2 sites (Duncan et al., 1988, 1989; Dubocovich, 1988a, 1988b, 1995). The 2-[^{125}I]MCA-NAT binding in brain and peripheral tissues of the Siberian hamster showed the same pharmacological characteristics. The specific binding of 2-[^{125}I]MCA-NAT at 4°C showed rapid binding kinetics. However, the association and dissociation rates for this radioligand were faster and slower, respectively, than those previously reported for the binding of 2-[^{125}I]iodomelatonin to the ML_2 sites of Siberian hamster brain membranes (Duncan et al., 1988). This is compatible with the higher (218 ± 86 pM, $n = 3$) affinity of 2-[^{125}I]MCA-NAT to ML_2 sites when compared with that of 2-[^{125}I]iodomelatonin (1.63 ± 0.17 nM) (Duncan et al., 1989). The rapid kinetics of association and dissociation of both 2-[^{125}I]MCA-NAT and 2-[^{125}I]iodomelatonin binding from the ML_2 site are in sharp contrast with the slow kinetics of the latter radioligand to ML_1 receptors (Dubocovich, 1995). Although the dissociation rate constant of 2-[^{125}I]MCA-NAT binding to ML_2 sites is slower than that reported for 2-[^{125}I]iodomelatonin, we were unable to demonstrate specific binding of the new radioligand to hamster brain sections using autoradiographic techniques.

The selectivity of this new radioligand, 2-[^{125}I]MCA-NAT, for ML_2 binding sites appears to be given by the methoxycarbonylamino group on carbon 5 (Dubocovich, 1985). 2-[^{125}I]Iodomelatonin, which has a methoxy group in this position, binds with high affinity to both the ML_1 and ML_2 sites. The carbon 5 substitution in 5-MCA-NAT, 2-[^{125}I]MCA-NAT or 2-Br-MCA-NAT significantly reduced the affinity for the ML_1 site, while the affinity for the ML_2 site remained intact. Similarly, *N*-acetyl-5-hydroxytryptamine, having a hydroxy group substitution on carbon 5, also shows selectivity for the ML_2 site. 2-[^{125}I]Iodomelatonin, which has a 5-methoxy group on carbon 5 and an *N*-acetoamidoethyl moiety on carbon 3, is a high affinity radioligand that labels both ML_1 and ML_2 receptors (Dubocovich, 1988a, 1995). The methoxycarbonylamino substitution on carbon 5 of 2-[^{125}I]MCA-NAT appears to confer the selectivity of this radioligand for binding to the putative melatonin ML_2 receptor sites.

2-[^{125}I]Iodomelatonin and 2-[^{125}I]MCA-NAT bind to the same ML_2 site, as demonstrated by the highly significant pharmacological correlations between the affinities of various compounds and melatonin analogues to compete for the sites labeled by each radioligand in hamster brain. The pharmacological characteristics of this site are very different from those of the site labeled by 2-[^{125}I]iodomelatonin in chicken retina (ML_1). Due to its selectivity for ML_2 melatonin sites, 2-[^{125}I]MCA-NAT offers distinct advantages over 2-[^{125}I]iodomelatonin for the characterization of these sites.

2-[^{125}I]MCA-NAT binds to ML_2 sites with higher affinity, but it defines the same number of sites as 2-[^{125}I]iodomelatonin. Both the kinetic and saturation affinity constants for 2-[^{125}I]MCA-NAT were about 3 times higher than those for 2-[^{125}I]iodomelatonin on the ML_2 binding sites of hamster brain (Figs. 3 and 4). This increase in affinity appears to result from the replacement of the 5-methoxy by a methoxycarbonylamino group of 2-iodomelatonin. This change of chemical group on carbon 5

of melatonin also increased the affinity of 5-MCA-NAT over melatonin to compete for 2-[^{125}I]iodomelatonin binding to the ML_2 site (Dubocovich, 1995; Table 1). Similarly, halogenation of MCA-NAT on carbon 2 increased the affinity for both melatonin ML_1 and ML_2 receptor sites. However, the increase in affinity conferred by this substitution was not sufficient to allow detection of 2-[^{125}I]MCA-NAT specific binding to ML_1 receptors. In previous studies, we reported a K_d value of about 1.5 nM (Duncan et al., 1989) for 2-[^{125}I]iodomelatonin on the ML_2 site of Siberian hamster brain membranes. In the present experiments, however, the affinity of 2-[^{125}I]iodomelatonin binding to Siberian hamster brain membranes was slightly higher (0.39 ± 0.07 nM, $n = 3$) (see Table 5). In addition, we found that 2-[^{125}I]iodomelatonin defined a lower number of specific ML_2 sites when compared with the results previously reported by Duncan et al. (1989). It is possible that the discrepancy in the B_{max} value resulted from underestimation of the specific activity of the radioligand in the study by Duncan et al. (1989).

2-[^{125}I]MCA-NAT binds selectively and with high affinity to ML_2 sites, which represents an advantage over 2-[^{125}I]iodomelatonin, which binds to both ML_1 and ML_2 subtypes. Up to now, the distinction between 2-[^{125}I]iodomelatonin binding to ML_1 and ML_2 sites was based on binding characteristics (kinetics of association/dissociation, affinity constants, regulation by ions) and pharmacological profile (Dubocovich, 1988a, 1995). Reported affinity constants for 2-[^{125}I]iodomelatonin have ranged from low picomolar to high nanomolar; however, only a few studies have determined the pharmacological profile of these binding sites (Dubocovich, 1988a, 1995; Laudon and Zisapel, 1986; Duncan et al., 1988, 1989; Pickering and Niles, 1990; Kennaway and Hugel, 1992). It follows that the use of 2-[^{125}I]MCA-NAT will allow the presence of ML_2 sites to be established or excluded without performing extensive binding characterization or determining the pharmacological profile in a given tissue. In addition, this radioligand will allow detection of ML_2 sites in areas expressing more than one subtype (hamster hypothalamus: Duncan et al., 1989).

In conclusion, 2-[^{125}I]MCA-NAT is a novel and selective radioligand for the characterization of ML_2 binding sites in both central and peripheral tissues. This new radioligand shows higher affinity and selectivity for ML_2 sites than does 2-[^{125}I]iodomelatonin, which makes it the ligand of choice for the detection of this melatonin receptor subtype.

Acknowledgements

We are grateful to Dr. Diana N. Krause for comments on the manuscript. This work was supported by research grants from Glaxo and USPHS R01-MH-42922 to M.L.D.

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