Characterization of 2-[^125]I Iodomelatonin Binding Sites in Syrian Hamster Peripheral Organs

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ABSTRACT

The neurohormone melatonin is a key agent in synchronizing the circadian rhythms. At least three types of binding sites have been described for melatonin: the G-coupled, seven-transmembrane domain receptors mt1 and MT2 and a putative binding site called MT3. The latter has been described in hamster brain membranes, and its binding capacity is optimum at 4°C. We further characterized this binding site on other peripheral hamster tissues, including intestine, liver, kidney, lung, muscle, and heart. We found a high level of binding sites (>30 fmol/mg of protein) in intestine and kidney. Furthermore, we completed the existing pharmacological profile of this site, which can now be described as 2-iodomelatonin > 6-chloromelatonin > melty-isobutyl-amiloride > acidine orange > 5-methylcarbonylamino-N-acetyltryptamine > prazosin > N-acetylseryotonin > melatonin. This profile was found in all the hamster organs tested that had a large number of binding sites, namely, brain, intestine, kidney and liver. Furthermore, when comparisons were possible, the MT3 pharmacological characteristics were similar to those described in the literature for hamster brain and tests. This profile was compared to the pharmacology obtained on human cloned mt1 and MT2 receptors and proved to be completely different, as expected. We provide new evidence for an alternate melatonin binding site not only in hamster brain but also in some peripheral organs.

Melatonin (5-methoxy-N-acetyltryptamine) is a neurohormone synthesized during the night by the pineal gland. Its secretion is regulated by circadian and seasonal variations in daylight length. Melatonin acts through the blood circulation as an internal synchronizer of circadian rhythms and informs the organism about the photoperiod. All the structures, central and peripheral, that present melatonin receptors or binding sites will receive this information. In addition to resetting or resynchronizing effects, melatonin has been proposed to have many other functions, notably in the central nervous system, cell metabolism, cardiovascular system, immune system, and cell proliferation in cancer (for review, see Brzezinski, 1997).

In 1994, cDNAs encoding melatonin receptors were cloned from human and other species (Reppert et al., 1994, 1995). The mt1 and MT2 receptors (Dubocovich et al., 1998) correspond to the high-affinity binding site described previously (Dubocovich, 1988). Indeed, the characterization and distribution of melatonin sites have been studied since the discovery of the specific radioligand 2-[^125]I Iodomelatonin. Specific melatonin binding sites have been identified in many species (Morgan et al., 1994; Delagrange and Guardiola-Lemaire, 1997), mainly in the central nervous system but also at the peripheral level, such as in spleen (Poon and Pang, 1992), thymus (Lopez-Gonzalez et al., 1993), prostate (Gilad et al., 1996), liver (Acuna-Castroviejo et al., 1994), lung, and heart (Pang et al., 1993). These findings suggest an ubiquitous distribution of melatonin binding sites. Most of these binding sites are characterized by high-affinity states (picomolar affinity). In contrast to this group, a so-called low-affinity (nanomolar affinity) type of melatonin binding site has been identified (Dubocovich, 1995) and named MT3, according to the IUPHAR nomenclature (Dubocovich et al., 1998). In addition to affinity characteristics, the two groups of melatonin binding sites are clearly discriminated by kinetic parameters (temperature and ion dependence) and pharmacological profile. In first approximation, MT3 class is recognized for its fast kinetics of association and dissociation, with peak melatonin specific binding reached at 4°C. In contrast, the kinetics of association and dissociation are slow for the high-affinity melatonin receptors mt1 and MT2, with an increase in affinity with temperature (Dubocovich, 1995). To better discriminate MT3 from mt1 and MT2, a ligand specific for MT3, 5-methoxy carbonylamino-N-acetyltryptamine (5-MCA-NAT), has been developed.
oped (Moliniari et al., 1996). Pharmacological studies have shown that prazosin, an α1-adrenergic antagonist, is one of the most potent inhibitors of $2^{-[125I]}$iodomelatonin binding to $MT_3$ (Pickering and Niles, 1990).

Several sites with melatonin binding activity at 4°C have been described in hamster brain (Duncan et al., 1988; Duncan et al., 1989; Pickering and Niles, 1990) and in RPMI 1846 melanoma cells (Pickering and Niles, 1992). Additional melatonin binding sites at 0°C were also reported in rat liver nuclei (Acuna-Castroviejo et al., 1994) and at 4°C in Siberian hamster brown adipose tissue (Le Coué et al., 1997) but their pharmacology and binding kinetics differed from those of $MT_3$.

Despite studies suggesting the coupling of phosphoinositide hydrolysis to $MT_3$ binding site (Eison and Mullins, 1993; Popova and Dubocovich, 1995; Mullins et al., 1997), no physiological function has been linked to $MT_3$ yet. The aim of the present study was to investigate the presence of 2-$[125I]$iodomelatonin binding sites in Syrian hamster peripheral tissues and to compare them with $MT_3$.

### Experimental Procedures

**Materials.** 2-$[125I]$iodomelatonin (2200 Ci/mmol) was purchased from NEN (Boston, MA). 5-methoxy carbonylamino N-acetyltryptamine (5-MCA-NAT) was obtained from Tocris (Langford, UK) as GR135531. Other drugs and chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

**Cell Culture.** Human embryonic kidney cell line HEK293 stably expressing mt1 or MT2 human melatonin receptors (provided by A.D. Strosberg, Paris, France) were grown as monolayers at 37°C (95% O2 / 5% CO2) in Dulbecco’s modified Eagle’s medium glutamax-1 (Gibco 31966–036; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin, and streptomycin (1%) in the presence of the selection agent geneticin G-418 (4%) (Gibco 11811–31966–036; Gibco Laboratories, Grand Island, NY). Human embryonic kidney cell line HEK293 was purchased from Charles River Breeding Laboratories, Inc. (Saint Aubin les Elbeuf, France) from male Syrian hamsters weighing 120 to 130 g. The tissues were thawed and homogenized in 15 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride with a Polytron homogenizer. The homogenate was centrifuged at 45,000 g for 20 min. Pellets were washed by repeating the homogenization and centrifugation procedure. Membrane pellets were suspended by passing back and forth through a 26-gauge needle connected to a syringe and finally adjusted to a concentration of 3 mg/ml.

**Hamster Organ Membrane Preparations.** Hamster frozen tissues were prepared by Charles River Breeding Laboratories, Inc. (Saint Aubin les Elbeuf, France) from male Syrian hamsters weighing 120 to 130 g. The tissues were thawed and homogenized in 15 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride with a Polytron (Kinematica GmbH, Lucerne, Switzerland) set at 4 to 5 for 15 s. The homogenate was centrifuged at 45,000 g for 20 min. Pellets were washed by repeating the homogenization and centrifugation procedure. Membrane pellets were suspended by passing back and forth through a 26-gauge needle connected to a syringe and finally adjusted to a concentration of approximately 5 mg/ml in homogenization buffer. The membrane fractions were filtered through cheesecloth, flash frozen in dry ice, and stored at −80°C until use.

**Binding Assays.** In saturation experiments, membranes suspensions of mt1 (0.04 mg/ml) and MT2 (0.04 mg/ml) were incubated for 2 h at 37°C in 0.25 ml (final volume) of 50 mM Tris-HCl containing 5 mM of MgCl2, at pH 7.40, with varying concentrations of 2-$[125I]$iodomelatonin (2200 Ci/mmol) from 0.005 to 1.5 nM for mt1 and from 0.02 to 3 nM for MT2 in the absence or presence of melatonin (10 μM), which determines the nonspecific binding. Competition studies for 2-$[125I]$iodomelatonin binding (radioligand concentration, 0.025 nM for mt1 studies and 0.200 nM for MT2 studies) were performed in the presence of reference substances to determine their affinities on the two human melatonin subtype receptors.

Binding assay conditions were essentially as previously described (Pickering and Niles, 1990). Briefly, membranes (50–100 μg) were incubated at 4°C in 250 μl total volume per sample with 96-well assay blocks (Corning-Costar, Corning, NY). Unless otherwise stated, the binding of 2-$[125I]$iodomelatonin (0.2 nM) was routinely measured in 50 mM Tris-HCl buffer (pH 7.4) and was initiated by addition of 200 μl membrane preparations. After incubation at 4°C for 30 min, reactions were terminated by filtration with a cell harvester (Brandel M-48; Gaithersburg, MD) connected to a vacuum pump (Edwards 18; Gennevilliers, France) through glass-fiber filters (GF/B; Brandel) soaked in 0.5% (w/v) polyethyleneimine. Filters were washed three times with 1 ml of ice-cold 50 mM Tris-HCl buffer. Total filtration time was less than 5 s. Radioactivity was measured in a gamma counter (Auto-Gamma 5000 series, Packard). Nonspecific binding was estimated as binding in the presence of 30 μM of melatonin (Duncan et al., 1988; Molinari et al., 1996). For competition studies, 2-$[125I]$iodomelatonin was incubated in the presence of increasing concentrations of drug from $10^{-11}$ to $10^{-6}$ M. For saturation binding assays, 0.125 to 20 nM of 2-$[125I]$iodomelatonin was used. Protein contents were determined with Coomassie blue dye reagent (Bio-Rad Laboratories, Inc., Richmond, CA) as previously described (Bradford, 1976), with BSA as standard.

**Miscellaneous.** Cytochrome P-450 content in membrane preparations and cytochrome P-450 spectrum shift assay in the presence of 1 μM of melatonin were done as described by Omura and Sato (1964). Incubation in the presence of UDP-glucuronic acid was done according to the method of measurement for UDP-glucuronosyl transferase activity as described by Boutin et al. (1993). Serotonin N-acetyltransferase activities were measured with the methodologies described by De Angelis et al. (1998).

### Results

2-$[125I]$iodomelatonin Binding Capacity of Different Tissues. Melatonin binding sites were identified in crude preparations of various tissue membranes from Syrian hamsters. The binding assay for each tissue was carried out at 4°C with increasing protein quantities ranging from 0.025 to 1.6 mg/ml. For the tissues showing significant binding capacities, the specific signal was linear over the range of concentrations up to 0.5 mg/ml. Binding capacities of the different organ membranes are displayed in Table 1. The high binding capacities were found in kidney and liver. Although lower, binding was measured in intestine at a level similar to that of brain. The lowest radioactivity signals associated with binding of 2-$[125I]$iodomelatonin were obtained with lung and heart. Binding activity with rear thigh skeletal muscle membrane.

**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Binding Activity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>9.0</td>
</tr>
<tr>
<td>Liver</td>
<td>6.1</td>
</tr>
<tr>
<td>Brain</td>
<td>3.4</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.0</td>
</tr>
<tr>
<td>Lung</td>
<td>2.2</td>
</tr>
<tr>
<td>Heart</td>
<td>0.5</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Binding assays were carried out as described in Experimental Procedures. 2-$[125I]$iodomelatonin concentration in the experiments was 0.2 nM. Protein concentrations used in the assays were 0.2 and 0.8 mg/ml. Calculated results were similar in both experimental conditions. Values are means of two independent determinations performed in duplicate.
branes was detectable under our conditions only when the concentration of tested protein was more than 1.5 mg/ml.

Characterization of 2-[125I]Iodomelatonin Binding on Kidney and Intestine Membranes. 2-[125I]Iodomelatonin binding characteristics were studied on kidney and intestine membrane preparations, which presented high binding capacities among the tested organs. Kinetic studies with 2-[125I]iodomelatonin showed that binding at 4°C to kidney and intestine membranes was saturable and reversible (Fig. 1). Association of 2-[125I]iodomelatonin proceeded at a high rate during the first seconds of incubation. For both tissues, binding equilibrium was reached within 20 s and remained stable for at least 2 h (Fig. 1A). Therefore, we used 30 min as maximum incubation time for routine binding assays. After 30 min of incubation with 2-[125I]iodomelatonin, dissociation was initiated by addition of 30 μM of unlabeled melatonin. Dissociation of the radioactive ligand was fully and immediately completed (Fig. 1B).

The effect of temperature on 2-[125I]iodomelatonin specific binding on kidney and intestine membranes was examined. For both tissues, maximal binding was reached when incubation mixture was ice cooled. Under these conditions, binding in kidney was 4- and 5-fold higher than when carried out at 25 or 37°C, respectively (Fig. 2). In intestine, binding at 4°C was increased 2.5- and 6.5-fold compared with the binding at 25 and 37°C, respectively (Fig. 2). In the course of the same experiment, the effect of prazosin, a specific inhibitor of MT2 (Pickering and Niles, 1990), was measured. Whereas 2-[125I]iodomelatonin residual binding activity was 77 and 70% that of controls for intestine and kidney, respectively, at 37°C, the binding was reduced to 45 and 34% by prazosin at 4°C. In additional experiments, kidney and intestine membranes were preincubated separately in the presence of 2-[125I]iodomelatonin, dissociation was initiated by addition of 30 μM of unlabeled melatonin. Each point represents the average of two determinations obtained in a representative experiment. The experiment was repeated twice with similar results.

Ion effects on 2-[125I]iodomelatonin binding were also investigated. Addition of CaCl2, NaCl, KCl, MgCl2, CuSO4, MnCl2, or 2 mM EDTA did not significantly affect the binding amount (data not shown).

Saturation studies were performed at 4°C on both tissues at various concentrations of radioligand, as shown for representative experiments (Fig. 3). Specific binding increased
linearly with increasing concentrations of 2-[^125]Iiodomelatonin from 0.125 to 1.5 nM and reached saturation at higher concentrations for kidney and intestine membranes. Scatchard analyses of the specific binding data obtained in the range of concentrations were linear and showed dissociation constants in the nanomolar range. \( K_d \) and \( B_{\text{max}} \) values obtained for crude kidney membranes were 1.90 ± 0.45 nM and 89.5 ± 18.0 fmol/mg of protein, respectively. In intestines, \( K_d = 1.75 ± 0.13 \) nM, and the maximal number of binding sites was \( B_{\text{max}} = 31.0 ± 3.7 \) fmol/mg of protein. These constants were the average of values obtained from four independent experiments.

Furthermore, because these organs are rich in drug-metabolizing enzymes, we tentatively speculate that the \( MT_3 \) binding site might be an enzyme. UDP-glucuronosyl transferases and cytochrome P-450 were obvious candidates as potential binding sites. Direct (cytochrome P-450 spectrum shift) and indirect (incubation in UDP-glucuronosyl transferase activity conditions) experiments showed that neither of these enzyme families could bind or catalyze the biotransformation of iodomelatonin and melatonin.

**Pharmacological Characterization.** A pharmacological characterization of 2-[^125]Iiodomelatonin binding to hamster kidney and intestine membranes was carried out with 0.2 nM radioligand and various competing agents. Table 2 shows the affinity constants \( K_i \) of 5 melatonin analogs and 14 other drugs for \( MT_3 \), as well as for the cloned human \( m_3 \) and \( MT_3 \) receptors. The relative order of potency of the tested ligands inhibiting specific 2-[^125]Iiodomelatonin binding corresponds to that reported for \( MT_3 \) binding sites in hamster brain membranes (Duncan et al., 1989; Pickering and Niles, 1990; Molinari et al., 1996). The rank order was 2-iodomelatonin > 6-chloromelatonin > 5-MCA-NAT = prazosin = N-acetylseryerotonin = melatonin for kidney and intestine membranes (Table 2). Acridine orange and the amiloride derivative 5-(N-methyl-N-isobutyl)amiloride (MIA) were new potent inhibitors of 2-[^125]Iiodomelatonin binding in both tissues (Table 2). A similar affinity was observed in brain membranes \( K_i = 5.9 \) and 6.9 nM for MIA and acridine orange, respectively. These affinity constants were also similar to that of prazosin \( K_i = 7.2 \) nM in hamster brain. 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), another amiloride analog, displaced 2-[^125]Iiodomelatonin binding in kidney, intestine (Table 2), and brain as well \( K_i = 32.0 \) nM. Other compounds with various properties, such as channel ligands (amiloride, cimetidine), adrenergic-receptor ligands (isoproterenol, phentolamine, idazoxan), or enzyme inhibitors (rolipram for phosphodiesterase, octadecynoic acid for cytochrome P-450, tryanlycypromine for monoamine oxidase) were tested as potential inhibitors of 2-[^125]Iiodomelatonin binding. Except for amiloride, which efficiently lowered the binding in both tissues, none of them proved to be good competitors.

For the most recently discovered compounds, a brief pharmacology profile was done in membranes derived from hamster brain (Table 3). Melatonin, 5-MCA-NAT (GR135531), and N-acetylseryerotonin were included as references. The results obtained corresponded to those described by Molinari et al. (1996). Interestingly, the other compounds showed a potency in brain membrane similar to the potencies measured in kidney and intestine membrane preparations.

Furthermore, the pharmacological profile of these compounds was measured in human \( m_3 \) and \( MT_3 \) cloned receptors (Table 2). None of them showed any displacement capacity toward any of these human receptors. The values for the reference compound toward either \( m_3 \) or \( MT_3 \) compared well with the reference work of Dubocovich et al. (1997), despite minor discrepancies due to subtle methodological differences.

**Discussion**

2-[^125]Iiodomelatonin specific binding at 4°C has previously been described in hamster brain membranes (Duncan et al., 1988, 1989; Pickering and Niles, 1990), in RPMI 1846 melanoma cells (Pickering and Niles, 1992), in Siberian hamster brown adipose tissue (Le Guic et al., 1997) and at 0°C in rat liver (Acuna-Castroviejo et al., 1994). 2-[^125]I]MCA-NAT was used to characterized binding sites at low temperatures in Siberian hamster brain and kidney (Molinari et al., 1996). Our study confirmed the presence of specific melatonin binding sites in peripheral tissues from Syrian hamsters at 4°C, and we have extended this investigation by characterizing 2-[^125]Iiodomelatonin binding sites in intestine and kidney as well as identifying potent inhibitors with structures not related to melatonin.

Binding of iodomelatonin was tissue dependent. To iden-
tify new 2-[125I]iodomelatonin binding sites, the binding capacity was evaluated and compared to that from brain, as already described (Duncan et al., 1988; Pickering and Niles, 1990), with increasing amounts of tissue membrane protein. Seven different tissues were tested (Table 1), only four of which had a significant and linear increase in radioactive signal with protein concentration and were therefore suitable to use in our study.

Because 2-[125I]iodomelatonin specific binding at 4°C has already been described in several rodent tissues such as hamster brain (Duncan et al., 1988, 1989; Pickering and Niles, 1990) and rat liver (Acuna-Castroviejo et al., 1994), we focused our study on hamster kidney and intestine. At 0.2 mg/ml (50 μg protein/well), binding activity in kidney was more than 2-fold higher than in intestine. At 200 μg kidney membrane protein per well, binding was 3-fold higher than in intestine. In subsequent assays, final protein concentrations of 0.2 and 0.4 mg/ml of intestine protein were used for kidney and intestine, respectively.

2-[125I]iodomelatonin specific binding was saturable and reversible. The time-course study shows that association of the radioligand reached a maximum level within 10 to 20 s and was stable for at least 2 h (Fig. 1A). The dissociation was initiated by adding unlabeled melatonin and obtained within the first seconds. Such fast kinetics did not allow us to accurately measure association and dissociation rate constants (harvesting procedure lasts about 5 s), preventing the calculation of kinetic K_i's for both kidney and intestine. The time range of association and dissociation in our study was comparable to that reported for MT_2 sites but slightly faster (10–20 s) than we observed in brain (1–2 min), as also described by Dubocovich (1995). A more significant discrepancy is observed with 2-[125I]iodomelatonin binding in hamster brown adipose tissue, where maximum association is reached within 60 min (Le Gouic et al., 1997), and more than 20 min is needed to fully dissociate the binding complex with rat liver cell nuclei (Acuna-Castroviejo et al., 1994). Such kinetic features are unusually fast at this temperature for receptors. Although slower, rapid kinetics of association and dissociation have been reported for adenosine receptors (Zocchi et al., 1996), glycine receptor (Popik et al., 1995), neurotensin receptor (Mazella et al., 1998), and phencyclidine receptor (Vincent et al., 1979). However, these receptors bound ligands at higher temperatures. Because 2-[125I]iodomelatonin binding to both kidney and intestine was not affected by ions and quickly reached equilibrium at low temperatures (Fig. 2A), these binding sites are not mt or MT_2 receptors. Indeed, maximum binding of 2-[125I]iodomelatonin at 37°C in chicken retina (Dubocovich and Takahashi, 1987) and ovine pars tuberalis (Morgan et al., 1989) was obtained after 2 h incubation. In stably transfected mt or MT_2 receptors in HEK293 cells (Conway et al., 1997), we observed the same association kinetics at 37°C and no detectable binding at 4°C for 30 min incubation (unpublished data). The low amount of binding observed at 37°C might be due to mt/MT_2 receptors. We evaluated this possibility by adding 10 nM of prazosin (K_i ~ 10 nM; Table 2), a specific inhibitor of MT_2 (Pickering and Niles, 1990). Whereas 55 and 65% of 2-[125I]iodomelatonin binding was inhibited at 4°C for intestine and kidney, respectively, a little more than 20% inhibition was obtained at 37°C (Fig. 2B), suggesting that part of the signal is probably of the same nature as that observed at 4°C. Furthermore, under our conditions (4°C for 30 min), binding related to mt/MT_2 re-
ceptors would not be detectable and therefore would not overlap with $MT_3$ binding. Preincubation of the preparation at 37°C before 4°C did not affect the binding amount, suggesting that the radioligand and the binding site were not degraded or metabolized at 37°C, at least under these experimental conditions.

$K_d$ values calculated from saturation studies performed with kidney and intestine were in the low nanomolar range and almost identical. In addition, they were similar to the $K_d$ measured for $MT_3$ in hamster brain (Duncan et al., 1988; Pickering and Niles, 1990). The distribution of 2-[125I]iodomelatonin binding sites, evaluated by $B_{\text{max}}$, indicated a density about 3-fold greater in kidney than in intestine (Table 1). However, when higher (>10 nM) 2-[125I]iodomelatonin concentrations were tested, we observed biphasic Scatchard plots for both tissues. Mathematical treatments of such plots in two sites did not lead to reproducible fit in the different experiments. Nevertheless, they indicated the highest-affinity site with $K_d$ and $B_{\text{max}}$ to be identical to those mentioned above. The apparent multisite shape of the Scatchard plots for high 2-[125I]iodomelatonin concentrations probably resulted from nonspecific binding, especially in intestine, where the nonspecific radioactive signal is slightly higher than half of the total signal at high 2-[125I]iodomelatonin concentrations.

Prazosin has been described as one of the best inhibitors of the 2-[125I]iodomelatonin binding on $MT_3$ binding sites (Pickering and Niles, 1990). However, prazosin, initially known as a phosphodiesterase inhibitor, is used as an antagonist of $\alpha_1$-adrenergic receptor. In addition, prazosin and photoactivatable analogs were shown to bind Ca$^{2+}$ channels and organic cation exchangers (Holohan et al., 1992). Prazosin analogs were also used to affinity-label P-glycoprotein in multidrug-resistant cells (Greenberger et al., 1990). Various compounds have been found to interfere with the binding and inhibit it efficiently. MIA, an inhibitor of Na$^+$/H$^+$ antiport (Maidorn et al., 1993), and acridine orange, a dye also reported to interact with organic cation transporter (Sokol et al., 1990), were found to be slightly better inhibitors of 2-[125I]iodomelatonin binding than prazosin in both kidney and intestine. They were also comparable to 5-MCA-NAT (Table 2). EIPA, another Na$^+$/H$^+$ antiport inhibitor (Abrahamse et al., 1994), was more potent than or similar to melatonin as an inhibitor of the binding in kidney or intestine, respectively. Amiloride interfered less efficiently with the binding than its analogs MIA and EIPA. Phentolamine, an $\alpha_2$-receptor antagonist, was poorly active, as previously reported (Molinari et al., 1996). Among the compounds we tested, 2-iodomelatonin and 6-chloromelatonin remained the best competitors for 2-[125I]iodomelatonin binding as described for $MT_3$. Furthermore, MIA, acridine orange, and EIPA had no affinity for either mt$_3$ or $MT_3$ receptors. These results, together with the fast kinetics and the low temperatures required to achieve binding, fulfill the criteria characterizing $MT_3$, but raise the question whether this binding site is a true (i.e., specific) receptor for melatonin. This question has already been discussed more generally for melatonin binding sites (Kennaway and Hugel, 1992). Binding sites at low temperatures have been described in rat liver nuclei (Acuña-Castroviejo et al., 1994) and Siberian hamster brown adipose tissue (Le Gouic et al., 1997), but based on their respective pharmacology and kinetics, they are likely to differ from $MT_3$. Nevertheless, the various profiles obtained with different competitors were similar when obtained from various tissues (e.g., intestine, brain, and kidneys). We constructed the plots between the data obtained from brain and those from intestine and kidney (Fig. 4). The data are highly correlated $(r = 0.94$ and $0.97$, respectively), strongly suggesting a binding site of identical or very similar proteic nature in all the tissues.

A working but speculative hypothesis in our laboratory is that this binding site might be an enzyme, taking into account maximal binding temperature and fast equilibrium kinetics. This enzyme would recognize melatonin or its analogs but, in the absence of adequate cosubstrate, would not be able to catalyze its putative reaction. Among the possible candidates, as mentioned above, were cytochrome P-450, UDP-glucuronosyl transferases, and serotonin N-acetyltransferase. None of those tested in this study bind the melatonin derivatives. Therefore, other enzymatic candidates should be checked and purification of the binding site should be attempted (currently under study in our laboratory).

The lack of homogeneity in the observations reported in the literature concerning the $MT_3$-type binding sites highlights the difficulty of characterizing such a site. Molecular identification of $MT_3$ will bring definitive information as to its true nature.

References


