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Characterization of Binding Sites for N-Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) in Rat Brain

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ZADINA, J. E., A. J. KASTIN, E. F. KRIEG JR. AND D. H. COY. *Characterization of binding sites for N-Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) in rat brain.* PHARMAC. BIOCHEM. BEHAV. 17(6) 1193–1198, 1982.—Binding sites for N-Tyr-Pro-Leu-Gly-NH_2 (Tyr-MIF-1), a novel peptide structurally related but immunoreactively different from Pro-Leu-Gly-NH_2 (MIF-1), were investigated. The presence of Tyr-MIF-1-like material in brain tissue has previously been demonstrated by RIA and its levels were shown to vary with the diurnal cycle and after pinealectomy. We now demonstrate a high affinity binding site in rat brain that is saturable and specific for Tyr-MIF-1. Crude P2 synaptosomal fractions from rat brains were incubated with ^{125}I-Tyr-MIF-1 in the presence or absence of 10 μM unlabeled Tyr-MIF-1 (nonspecific binding). Binding reached equilibrium at 30–40 min at 23°C and at about 4 hr on ice, after which it was relatively stable for at least 18 hr. None of the other peptides (including MIF-1) or amino acid residues tested were found to effectively compete for ^{125}I-Tyr-MIF-1 binding. Binding was linear with protein from 280 μg to at least 1.1 mg protein per tube. Scatchard analysis of the striatum-thalamus revealed the presence of binding sites with an apparent K_D of 91 nM and maximum number of sites in the range of 45 fmol/mg tissue. Analysis of several brain areas revealed a differential distribution of the binding sites with relatively high concentrations in cortex, striatum, and amygdala and low concentrations in pons-medulla. Together with the previously published RIA results, the demonstration of a receptor for Tyr-Pro-Leu-Gly-NH_2 supports the concept of the presence of this novel peptide and its receptor in the brain.

Tyr-MIF-1 MIF-1 Receptors

Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2)-immunoreactive material was demonstrated by radioimmunoassay (RIA) in the rat pineal gland [8] and was subsequently shown to be differentially distributed in the brain [9]. The RIA for this peptide was developed during an attempt to establish an RIA for melanocyte-stimulating hormone inhibiting factor-1 (MIF-1), a peptide whose effects on the CNS have been extensively reviewed [7,10]. A rabbit injected with MIF-1 conjugated to thyroglobulin produced an antibody with high immunoreactivity for Tyr-MIF-1, but very low cross-reactivity with MIF-1. The possibility of a physiological role for Tyr-MIF-1 increased with the demonstration that the levels of Tyr-MIF-1 immunoreactivity in whole brain were higher after pinealectomy and showed a diurnal rhythmicity with highest levels during the dark period [9]. In this report, we demonstrate the presence of saturable, specific, high affinity binding sites for ^{125}I-Tyr-MIF-1 and describe their distribution in the rat brain.

METHOD

Tissue Preparation

Brains from male (200–300 g) Sprague-Dawley rats (Zivic Miller) were rapidly removed and dissected on ice. For studies of brain area, the dissection method of Glowinski and Iverson [4] was used with the following modifications: the cut posterior to the mamillary bodies was extended through the cortex, separating the midbrain from the thalamus and the parietal from the occipital cortices. The striatum was separated from the tissue (frontal cortex) anterior to the cut through the anterior commissure, and after the hypothalamus, amygdala and surrounding cortex ("amygdala"), parietal cortex and hippocampus were removed, a cut through the internal capsule separated the thalamus from the remainder of the striatum. After the demonstration of the presence of high levels of Tyr-MIF-1 like material by RIA in the striatum and thalamus [9], some of the binding studies were conducted with a block of tissue including both of these areas ("striatum-thalamus").

Crude synaptic plasma membranes were prepared by homogenizing tissues in 15–20 volumes of 0.32 M sucrose with a Brinkman Polytron (setting 6 for 20 sec) and centrifuging at 1000 g for 10 min at 4°C to separate the crude nuclear (P1) pellet. The supernatant was then centrifuged at 30,000 g for 10 min. The resulting crude mitochondrial-synaptosomal (P2) pellet was washed once in buffer (25 mM sodium phosphate, pH 7.4 at 23°C), centrifuged again at 30,000 g for 10 min, and reconstituted in 10 volumes of buffer.

Iodination

Tyr-MIF-1 was iodinated by the chloramine-T method as

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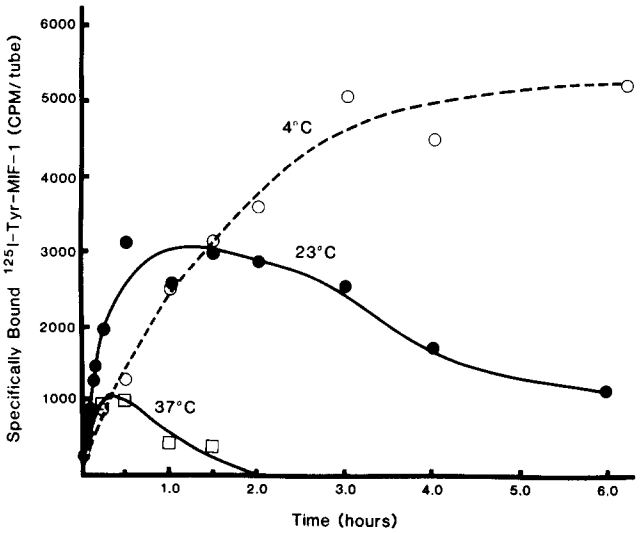

FIG. 1. Effect of incubation temperature on time to reach equilibrium. Membranes from rat cortex were incubated at one of three temperatures with ^{125}I-Tyr-MIF-1 in the presence or absence of 10^{-6} M unlabeled Tyr-MIF-1. The reaction was terminated at various times by filtration and the specifically bound ^{125}I-Tyr-MIF-1 was determined. Data points up to 2 hr are the mean of two experiments in triplicate.

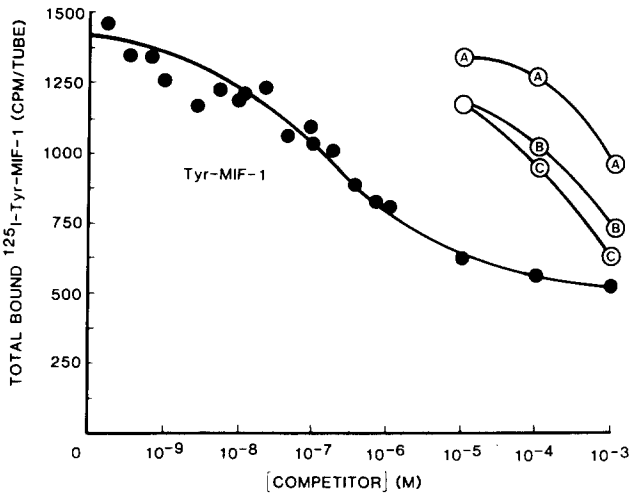

FIG. 2. Competition for ^{125}I-Tyr-MIF-1 binding sites by unlabeled Tyr-MIF-1 and various competitors listed in Table 1. Membranes from striatum-thalamus were incubated (23°C, 40 min) with 1 nM ^{125}I-Tyr-MIF-1 and various concentrations of competitors. Data points for unlabeled Tyr-MIF-1 are the mean of 2 experiments in triplicate except for the $\log_{10}$ doses, which represent 3 experiments, the third conducted in the assay with the other competitors.

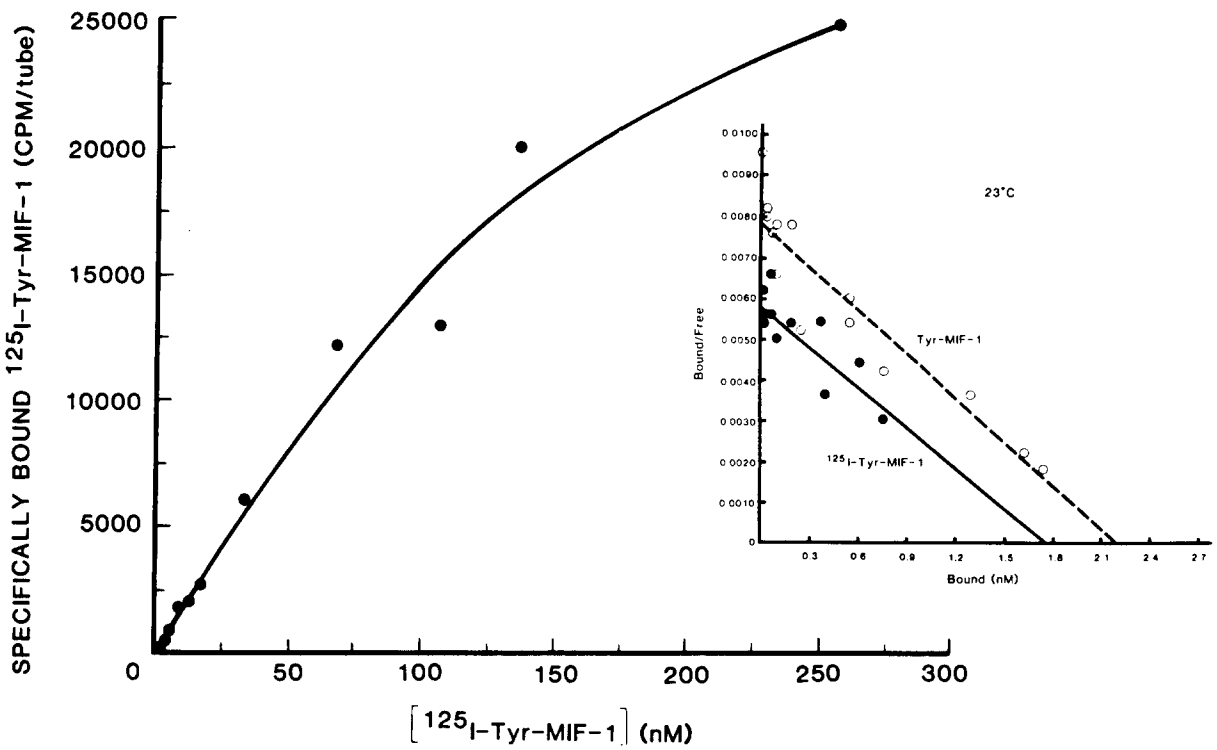

FIG. 3. Saturation curve for binding of ^{125}I-Tyr-MIF-1 to striatum-thalamus membranes. Various concentrations (1–256 nM) of ^{125}I-Tyr-MIF were incubated in the presence or absence of 10^{-5} Tyr-MIF-1. Inset shows Scatchard plot of these data (filled circles) together with a plot generated in the same experiment by varying the concentration of unlabeled peptide in the presence of 1 nM ^{125}I-Tyr-MIF-1 (open circles).

TABLE 1

B/Bo AFTER VARIOUS CONCENTRATIONS OF COMPETITOR

	Competitior	Molar Concen. 10^{-5}	10^{-4}	10^{-3}
	Lys-vasopressin	1.0		
	Isotocin	0.94		
A	cycLeu-Gly	1.02	1.05	0.96
A	TRH	1.20	1.05	0.96
A	pGlu-Leu-Gly-NH_2	0.92	0.97	0.70
A	MIF-1 (Pro-Leu-Gly-NH_2)	0.84	0.74	0.70
A	Pro-Leu	0.95	0.91	0.54
A	Oxytocin	0.95	0.91	0.54
B	Pro-Tyr	0.80	0.72	0.27
B	LHRH	0.92	0.64	0.23
B	L-Tyr	0.73	0.50	0.35
B	Arg-vasotocin	0.68	0.58	0.29
C	Met-Enk	0.87	0.53	0.15
C	Leu-Enk	0.85	0.53	0.14
C	Somatostatin	0.67	0.52	0.19
	Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2)	0.19	0.06	0.00

Competition for ^{125}I-Tyr-MIF-1 binding sites by various peptides and amino acids.

One to three concentrations (10^{-5}–10^{-3} M) of each of the substances listed was incubated with 1 nM ^{125}I-Tyr-MIF. The ratio of CPM bound in the presence of the substance to that bound in the absence of competitor (B/Bo) was computed. Substances were grouped as (A) those failing to reach a B/Bo ratio of 0.50 at any concentration, (B) those reaching a B/Bo ratio of 0.50 and (C) those competing beyond a B/Bo of 0.20 at 10^{-3} M. Lysine-vasopressin and isotocin were tested only at the 10^{-5} M concentration, showed no evidence of competition, and were not included in any group.

described elsewhere [8]. The specific activity of the iodinated peptide was estimated using the antibody to Tyr-MIF-1 and by self-displacement in the radioreceptor assay. Both methods produced similar estimates of the specific activity (about 150 Ci/mmole).

Binding Assay

For the standard assay, 250 μl/ml of the membrane preparation were added to triplicate tubes containing the labeled peptide, 3.3 μg/ml Bestatin HCl (a potent inhibitor of enzymes which degrade MIF-1 [5]) and either unlabeled Tyr-MIF-1 (10^{-6} (initial studies) or 10^{-5} M) or buffer alone. All ingredients were in buffer containing 0.1% BSA. Bound and free ^{125}I-Tyr-MIF-1 were separated by rapid filtration over Whatman GF/B filters with 4 washers of 4 ml of ice-cold buffer. Specific binding was defined as the amount of ^{125}I-Tyr-MIF-1 bound in tubes containing no competitor minus that in tubes containing excess unlabeled Tyr-MIF-1. Protein was measured by the method of Lowry *et al.* [6] using bovine serum albumin as the standard.

Statistical Analysis

Differences between brain areas in concentration of Tyr-MIF-1 binding sites were analyzed by analysis of variance followed by Duncan's Multiple Range Test.

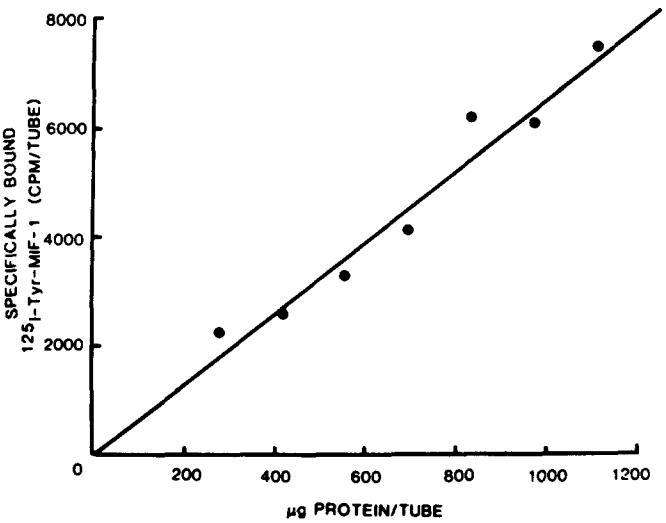

FIG. 4. Dependence of specifically bound ^{125}I-Tyr-MIF-1 on amount of protein added to the incubation. Membranes (280–1100 μg protein) were incubated at 23°C for 40 min with 1 nM ^{125}I-Tyr-MIF-1 in the presence or absence of 10^{-5} M Tyr-MIF-1 at a final volume of 0.5 ml.

RESULTS

Optimization of Incubation Conditions

Buffer: Total and specific binding in initial studies (using 10^{-6} M unlabeled Tyr-MIF-1) were higher when incubations were conducted in sodium phosphate (25 mM) than in an equivalent concentration of HEPES or in isotonic (50 mM) TRIS. In preliminary experiments, 25 mM sodium phosphate was superior to 10, 20, or 50 mM concentrations and was used in all subsequent experiments.

Time to equilibrium at various temperatures: As shown in Fig. 1, specific binding at 37°C reached a low peak and then rapidly declined, indicating considerable degradation of tracer and/or receptors at this temperature. At 23°C, equilibrium was reached at about 30–60 min and binding remained relatively stable for about 2 hr before declining. At 4°C, equilibrium was approached between 3 and 6 hr, and in a separate experiment, no decrease in binding was observed for up to 18 hr. Subsequent incubations were conducted either at room temperature for 40 min or on ice for 18 hr.

Saturation, Specificity and Choice of Appropriate Blank

Varying concentrations (10^{-9} to 10^{-3} M) of unlabeled Tyr-MIF were incubated (23°C, 40 min) with approximately 1 nM ^{125}I-Tyr-MIF-1. As shown in Fig. 2, saturation of binding sites for Tyr-MIF-1 was approached by concentrations of the unlabeled peptide in the range of 10^{-5} M. Competition for these sites by several amino acids and peptides including analogs of MIF-1 was also tested. The substances are listed in Table 1. For simplicity of illustration, Fig. 2 shows them divided into 3 groups based on their potency in competing with ^{125}I-Tyr-MIF-1 for the sites. All of these substances are about 3 or more orders of magnitude less potent than unlabeled Tyr-MIF-1 in competing for ^{125}I-Tyr-MIF-1, indicating that the assay is specific for Tyr-MIF-1. At concentrations greater than 10^{-5}M, however, some of the substances began to compete for sites binding ^{125}I-Tyr-MIF-1, indicating that low affinity, nonspecific sites may be involved in

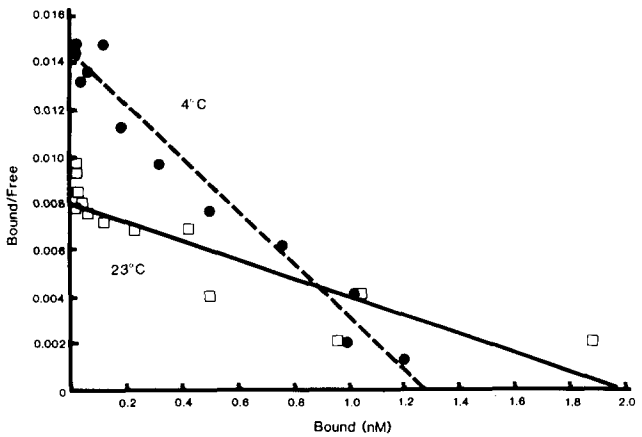

FIG. 5. Effect of incubation temperature on apparent Kd of ^{125}I-Tyr-MIF-1 binding. Striatum-thalamus membranes were incubated with 1 nM ^{125}I-Tyr-MIF-1 and various concentrations (1–1000 nM) of unlabeled Tyr-MIF-1 at 4°C for 18 hr (filled circles) or at 23°C for 40 min (open squares).

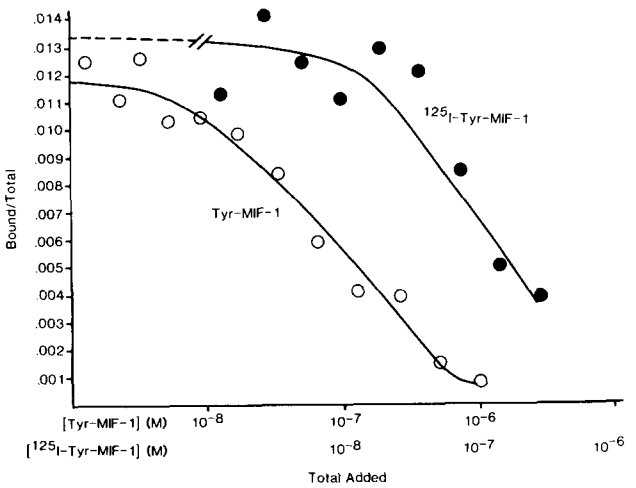

FIG. 6. Competition by unlabeled and self-competition by labeled Tyr-MIF-1 at 4°C. Varying concentrations (1–1000 nM) of unlabeled Tyr-MIF-1 were incubated (4°C, 18 hr) with 1 nM ^{125}I-Tyr-MIF (open circles). In the same experiment, varying concentrations (1–256 nM) of ^{125}I-Tyr-MIF-1 were incubated with 10^{-5} M Tyr-MIF (filled circles).

competition at these high concentrations. Since the 10^{-5} M concentration was sufficient for Tyr-MIF-1 to occupy most of the saturable sites but insufficient for other substances to compete for binding, this concentration was chosen as the appropriate blank for further studies. To ensure that ligand concentrations were well beyond half maximal binding, a 1–1000 nM range of Tyr-MIF-1 was used in all experiments involving Scatchard analysis after varying the concentration of unlabeled peptide in the presence of a constant amount of labeled peptide.

Saturation experiments were also conducted (23°C, 40 min) with varying concentrations of labeled peptide as shown in Fig. 3. The quantity of labeled peptide available from a single iodination precluded extending the concentration range of ^{125}I-Tyr-MIF-1 as high as that used to saturate the binding site with unlabeled peptide. Nevertheless, the approach to saturation and, as shown in the inset to Fig. 3, the Scatchard plot generated in such an experiment, were similar to curves generated using the same tissue preparation but varying the concentration of unlabeled peptide in the presence of a constant, low concentration of labeled peptide. The two procedures produced similar estimates of the Kd (296 and 277 nM for varying labeled and unlabeled peptide, respectively) and Bmax (69.8 and 87.1 fmols/mg tissue), indicating that iodination did not appreciably alter the affinity of the ligand for the binding site.

Linearity of Specific Binding with Tissue Concentration

Varying amounts of tissue (5–20 mg wet weight, corresponding to 280–1100 μg protein) were incubated with 10 nM ^{125}I-Tyr-MIF-1. As shown in Fig. 4, the amount of specifically bound ^{125}Tyr-MIF-1 was linearly related (r=0.98) to the amount of protein present in the incubation tubes. All experiments described were conducted using a tissue concentration at the midpoint (about 700 μg) of this range.

Effect of Incubation Temperature on Apparent Affinity of the Tyr-MIF-1 Binding Site

After establishing the optimal experimental conditions

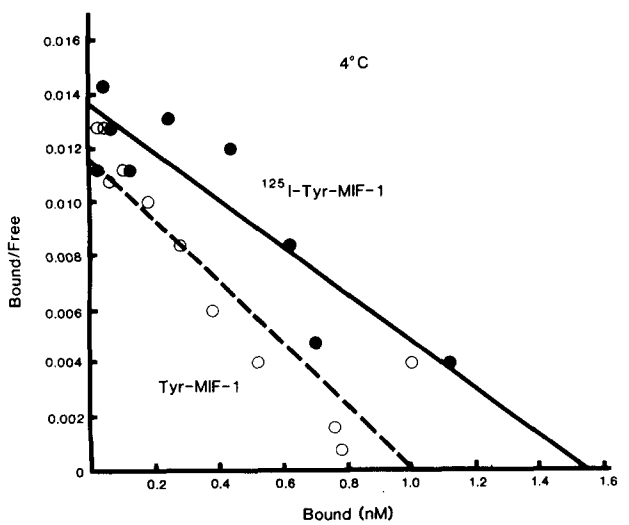

FIG.7. Scatchard plots of data from Fig. 6.

described above, we tested whether the differences in binding at 4°C and 23°C could be due, in part, to a difference in the affinity of the site for Tyr-MIF-1. We found that a higher apparent affinity (lower Kd) was measured when incubations were conducted on ice. An experiment comparing the two procedures is illustrated in Fig. 5. An apparent Kd (240 nM) similar to that observed in earlier experiments was observed after a 40 min incubation at 23°C, while that observed after 18 hr at 4°C was 89 nM. The mean apparent Kd in three such experiments at 4°C was 91.4±3.9 nM and the Bmax was 45.2±3.0 fmoles/mg tissue, corresponding to about 950 fmoles/mg protein. As shown above with the 23°C incubation, saturation of the binding site at 4°C with either labeled

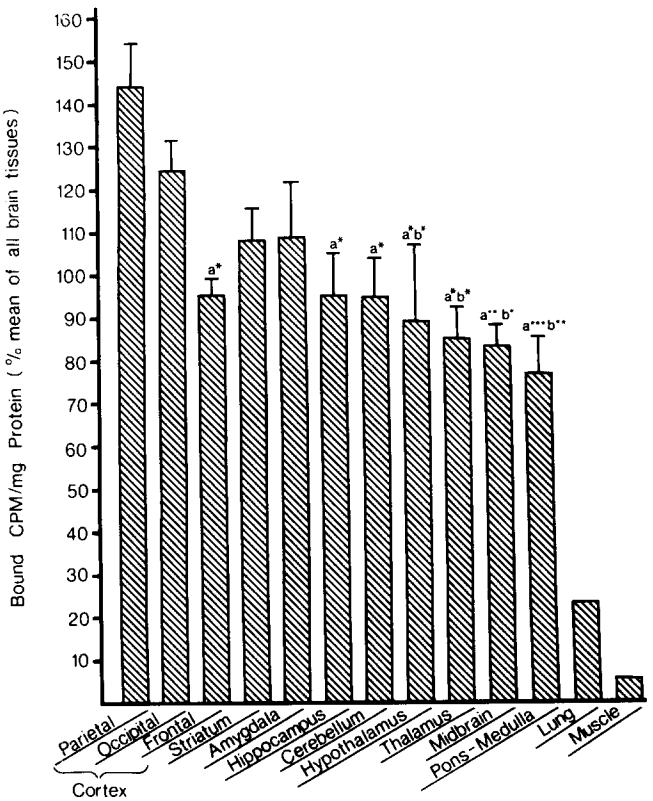

FIG. 8. Regional distribution of ^{125}I-Tyr-MIF-1 binding in rat brain. Membranes were incubated at 4°C for 18 hr with 10 nM ^{125}I-Tyr-MIF-1 with or without 10^{-5} M Tyr-MIF-1. The mean specifically bound CPM/mg protein for all tissues in a given experiment was computed and the value for each area was expressed as a percentage of this mean. Bars represent mean±SEM for 4 sets of triplicate tubes in 2 separate experiments. Letters represent significant difference from parietal (a) or occipital (b) cortex; $p<0.005$ (***), 0.01 (**) or 0.05 (*).

or unlabeled peptide produced similar results. Figure 6, which shows the change in the bound/total ratio as a function of added ligand, illustrates the similar pattern of competition for ^{125}I-Tyr-MIF-1 binding sites by unlabeled peptide and self-competition by labeled Tyr-MIF-1. The corresponding Scatchard plots are shown in Fig. 7. Since the measurements after a 4°C incubation revealed a site of higher apparent affinity and were slightly less variable than those at 23°C, the 18 hr incubation at 4°C was used to investigate the distribution of binding sites in various brain regions.

Regional Distribution of Tyr-MIF-1 Receptors in the Brain

Membranes from various brain regions, dissected as described above, were incubated at 4°C for 18 hr with or without 10^{-5} M Tyr-MIF-1 in the presence of 10 nM ^{125}I-Tyr-MIF-1. This concentration of labeled peptide does not saturate the receptor and, therefore, does not reflect the maximum number of sites; however, it is on the linear portion of the Scatchard curve and therefore provides a reliable index of relative binding in the various brain regions. A similar method has been reported for the opiate receptor [3]. The overall analysis of variance showed a significant, $F(10,33)=2.6$, $p<0.05$, differential distribution of binding sites in the brain. As shown in Fig. 8, specific binding in the parietal and occipital cortices was high; in the parietal cortex, it was significantly higher than in pons-medulla ($p<0.005$), midbrain ($p<0.01$), and all other areas ($p<0.05$) except the occipital cortex, striatum, and amygdala (which contains surrounding cortex). Binding in the occipital cortex was significantly higher than in the pons-medulla ($p<0.01$), midbrain, thalamus, and hypothalamus ($p<0.05$). Lung and muscle showed very low levels of binding and were not included in the statistical analysis. A comparable experiment conducted at 23°C produced a very similar profile except that binding in the frontal cortex was relatively higher and the differences between the cortex and the areas in the di- and mesencephalon were more pronounced.

DISCUSSION

This series of experiments provides evidence for the presence of saturable, specific, high affinity binding sites for N-Tyr-MIF-1 that appear to be differentially distributed throughout the brain. The highest concentrations of these sites were found in the cortex, followed by striatum and amygdala. The lowest concentrations in brain were found in the pons-medulla, and binding in peripheral tissues (lung and muscle) was well below that measured in brain.

Although this peptide was named for its structural similarity to MIF-1, the results from competition experiments (Fig. 2 and Table 1) indicate that MIF-1 is ineffective in competing for sites labeled by ^{125}I-Tyr-MIF-1. Similarly, cyclo-Leu-Gly and pGlu-Leu-Gly-NH_2, two structural analogs of MIF-1 reported to inhibit morphine-induced tolerance [1] and oxotremorine-induced tremors [2], respectively, were also ineffective in competing for ^{125}I-Tyr-MIF-1 binding. The C-terminus of oxytocin also contains the Pro-Leu-Gly-NH_2 sequence, but neither oxytocin nor the Pro-Leu dipeptide were effective competitors. Pro-Tyr and free tyrosine were slightly more effective in competing for these sites, but were far less potent than the tetrapeptide. These results indicate that tyrosine in conjunction with the remaining amino acids is essential for binding to the sites described here. They also suggest that while Tyr-MIF-1 shares a majority of its sequence with MIF-1 and the C-terminus of oxytocin, it may serve functions quite different from those influenced by MIF-1 or oxytocin.

The differential capacity for various brain regions to bind ^{125}I-Tyr-MIF-1 could be due to variations in the number of sites, their affinity for the ligand, or both. It is also possible that endogenous peptide, not removed by the purification steps used here, may occupy sites in areas such as the hypothalamus, where high levels of Tyr-MIF-1-like immunoreactivity were detected by RIA [9] but only moderate levels of binding were observed in the present experiment.

In this series of experiments, the following criteria for *in vitro* demonstration of a receptor for Tyr-Pro-Leu-Gly-NH_2 have been met: saturability, specificity, high affinity, a limited number of sites, and differential distribution in the brain. The correlation of biological response with binding potency awaits identification of a neuronal pool responsive to Tyr-MIF-1. Investigations into this matter can be guided by the localization and characterization of binding sites described here as well as by measurement of the levels of this peptide and their changes under various physiological states [9].

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