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A novel specific binding site for anxiolytic homophthalazines in the rat brain

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Radioligand binding studies were performed in order to elucidate the mechanism of action of anxiolytic-neuroleptic homophthalazines. Rat striatal membrane preparations were found to bind ^{3}H-EGIS 6775 [^{3}H-GYKI-52 322, ^{3}H-(1-(4-aminophenyl)-4-methyl-7,8-dimethoxy-5H-homophthalazine)] in a specific and displaceable manner. Several other brain regions tested were devoid of similar binding activity. Saturation analysis revealed that binding affinity was in the 10^{-8}-10^{-7} M range. Binding was enhanced by Mg^{2+} ions and, to a smaller extent by Ca^{2+} ions. The binding principle was sensitive to heat or trypsin treatment. This specific binding site appears, according to competition studies, different from the receptors whose presence in the rat striatum has been reported earlier.

Specific homophthalazine binding; Anxiolytic drug; Atypical neuroleptic drug; (Rat striatum)

1. INTRODUCTION

Several members of the family of homophthalazines (formerly called 2,3-benzodiazepines), such as tofisopam (Grandaxin), EGIS 6775 (GYKI-52 322) [1] or girisopam (EGIS 5810, GYKI-51 189) [2] proved anxiolytic in man and in various animal models. Moreover, GYKI-52 322 could also be characterized as an atypical neuroleptic agent. In spite of the structural similarity, their pharmacological profiles differ significantly from that of the 'classical' 1,4-benzodiazepines. Importantly, these drugs also do not have an addiction potential.

Their mechanism of action has still not been unraveled after several years of investigation. They do not appear to bind to the serotonin, dopamine or diazepam receptors ([1] and unpublished data), or to influence biogenic amine levels in the striatum [1]. Therefore, we have decided to search for a putative novel binding entity in the rat brain, using [^{3}H]EGIS 6775, which may be responsible for mediating the action of these drugs.

2. MATERIALS AND METHODS

[^{3}H]EGIS 6775 [^{3}H](1-(4-aminophenyl)-4-methyl-7,8-dimethoxy-5H-homophthalazine has been prepared by reductive dehalogenation of a suitable precursor to be published elsewhere (Zólyomi et al., in preparation). Its specific radioactivity was 0.845 TBq/mmol.

The non-radioactive analogs were prepared and analyzed in our laboratories as in [3]. Trypsin (206 E/mg) and trypsin inhibitor were purchased from Worthington Biochem. Co. (Freehold, NJ). All other chemicals used were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Protein was determined by the Lowry method [4] using bovine serum albumin as a standard.

For preparation of the membrane fractions, male OFA rats (250-300 g body weight, obtained from LATI, Gödöllö) were used. The brains were removed quickly after decapitation and placed on an ice-cold plate. Brain regions of interest were isolated and homogenized in 40 vols. of 0.05 M Tris-HCl buffer, pH 7.4, filtered through glass wool and centrifuged at 48,000 × *g* for 15 min at 4°C. The pellet was resuspended in buffer, incubated at 25°C for 10 min and then washed twice. Finally, it was resuspended in 50 vols. of Tris-HCl buffer containing 120 mM NaCl, 2.5 mM KCl, 0.1% ascorbic acid, 10 μM pargylin and varying concentrations of divalent cations. The suspension was aliquoted and stored at −20°C for no longer than 2 weeks before use.

Binding studies were performed in a final volume of 1.0 ml with about 1.0 mg protein/tube. [^{3}H]EGIS 6775 was used at a concentration of 1-200 nM. Non-labeled EGIS 6775 (10^{-5} M) was added to some of the samples for determination of non-specific binding. Samples were incubated at 25°C for 30 min to reach equilibrium, then they were centrifuged at 48,000 × *g* for 5 min and washed three times to remove the excess radioligand. The pellet was then solubilized with 2% sodium dodecylsulfate for 18 h and the radioactivity determined by an LKB Wallac 1410 radiospectrofluorimeter.

3. RESULTS

Membrane suspensions originating from rat striatum showed affinity for [^{3}H]EGIS 6775. Binding was displaceable by non-labeled EGIS 6775. On the other hand, no specific binding was detected if the membrane suspension was prepared from the hippocampus, frontal cortex or cerebellum (Table I). These findings were corroborated by quantitative autoradiography (manuscript in preparation).

In order to determine the affinity and capacity of the binding sites, a saturation analysis was performed. As is seen in Fig. 1, a single class of sites was observed with an affinity of 97 nM (B_{max}=3.85 pmol/mg protein). The composition of the buffer dit not affect binding exces-

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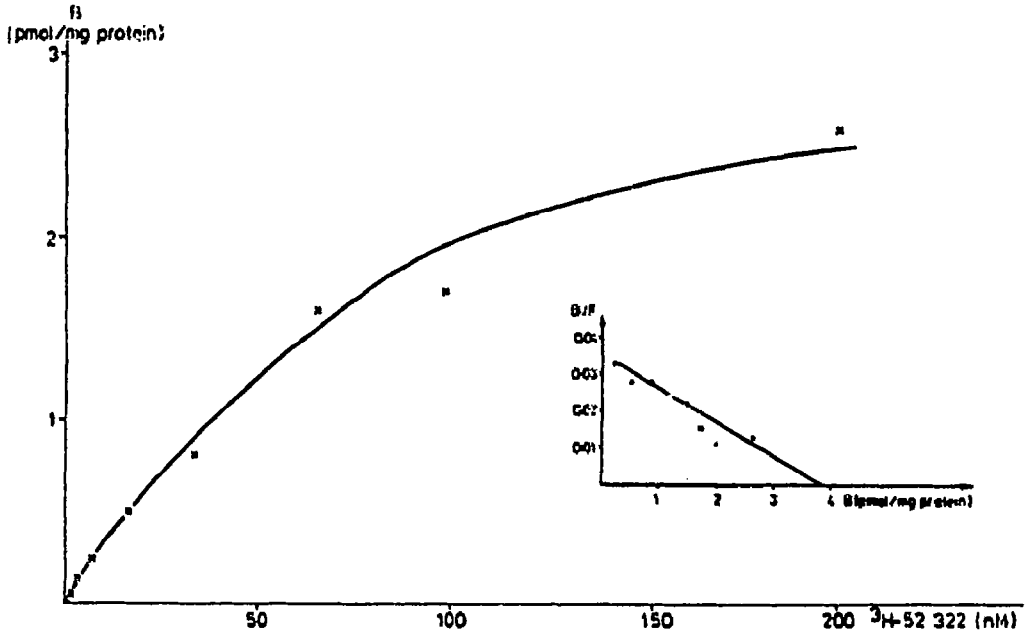

Fig. 1. Saturation of the striatal radioligand binding sites. Mg^{2+} ion concentration was 1.5 mM. The inset shows Scatchard transformation of the data.

sively, except for the bivalent ions. Both Ca^{2+} and Mg^{2+} increased binding: Mg^{2+} ion concentration dependence is shown in Fig. 2. The half-maximum effect is observed below 2 mM Mg^{2+}, whereas much larger concentrations of Ca^{2+} are required for comparable (or smaller) effects (not shown).

Binding activity of the membrane preparations could be destroyed by either heat or trypsin treatment (Fig. 3).

Specificity of [^{3}H]EGIS 6775 binding was also studied in displacement experiments. In these cases, equilibration of membranes with the radioligand was carried out in the presence of increasing concentrations of the competitor (Fig. 4). Tofisopam, girisopam and GYKI-6775 in the 10^{-7} M range all displaced [^{3}H]EGIS 6775, whereas diazepam was of much lower activity. However, serotonin, GABA, dopamine or glutamic acid were ineffective up to 10^{-5} M (Fig. 4). These results demonstrate the novelty of the specific binding site.

4. DISCUSSION

Disclosure of the presence in the rat striatal membrane fractions of a binding site which displays high affinity and remarkable specificity for the homophthalazines offers an explanation of their hitherto ill-understood pharmacological activity. This binding principle is probably of a proteinaceous nature, as testified by the results in Fig. 3.

The interesting Mg^{2+} ion dependence of binding deserves further study. The use of radioligand displacement may permit a new approach towards the design of novel anxiolytic and/or neuroleptic drugs in the future.

Table I

Regional distribution of [^{3}H]EGIS-6775 binding sites

Brain region[a]	Total binding (dpm/sample)	Non-specific binding (dpm/sample)	Specific binding[b] (pmol/mg protein)
Cerebellum	13,594 ± 1660	13,013 ± 1453	0.028 ± 0.006 (3)
Frontal cortex	14,785 ± 1477	13,833 ± 0629	0.030 ± 0.011 (3)
Hippocampus	15,026 ± 1836	14,078 ± 1086	0.031 ± 0.008 (3)
Striatum	23,252 ± 768	8,326 ± 333	0.42 ± 0.023 (5)

[a]Membrane fractions were prepared from the regions indicated.
[b]Specific binding was determined using 6 nM of tritiated ligand. Means ± S.E.M. are shown with the number of independent experiments in parentheses.

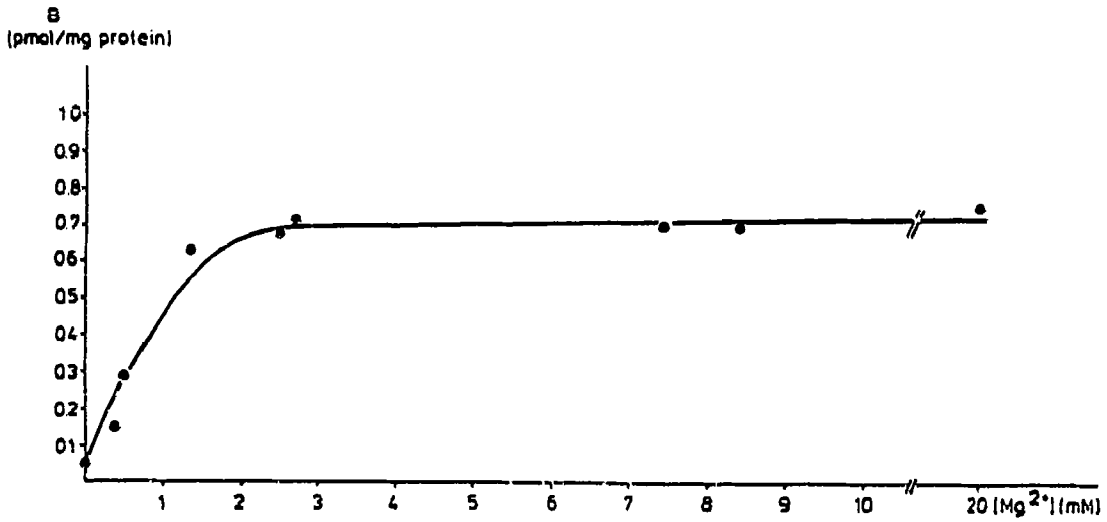

Fig. 2. Mg^{2+} ion dependence of ^{3}H-EGIS 6775 binding. Radioligand concentrations was 5 nM.

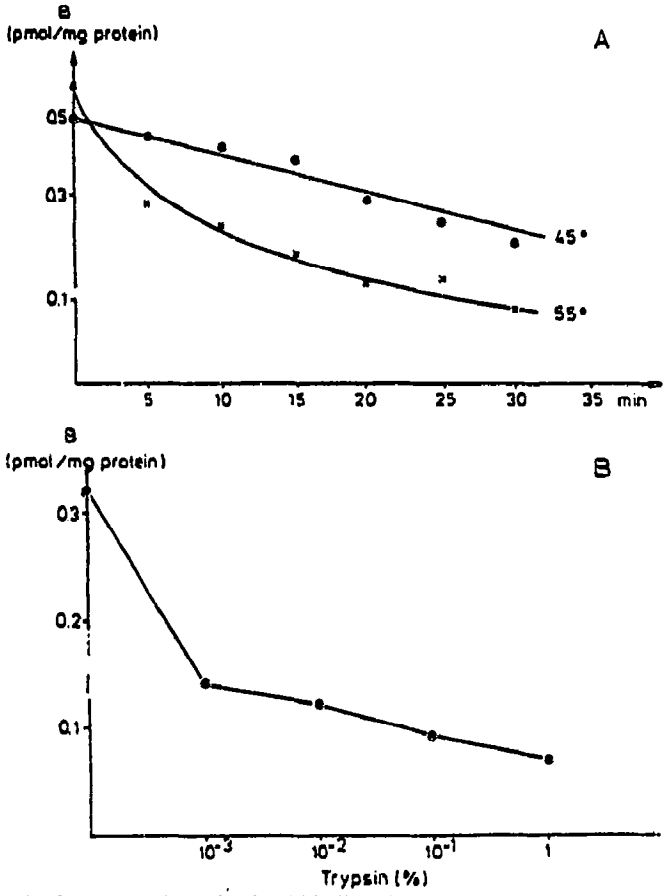

Fig. 3. Inactivation of striatal binding sites. The membrane preparations were pre-incubated at 45°C (●) or 55°C (×) for increasing lengths of time (A) or in the presence of trypsin at 37°C for 10 min (B). Trypsin digestion was done in Kreb's-Ringers' bicarbonate buffer, and was stopped by the addition of 10 mg/ml soybean trypsin inhibitor followed by centrifugation and resuspension in the binding medium (with 40 mM Mg^{2+}). Heat inactivation was performed in the binding buffer (40 mM Mg^{2+}). At the end of the treatments the temperature was adjusted to 25°C and specific binding was determined with 5 nM [^{3}H]EGIS 6775.

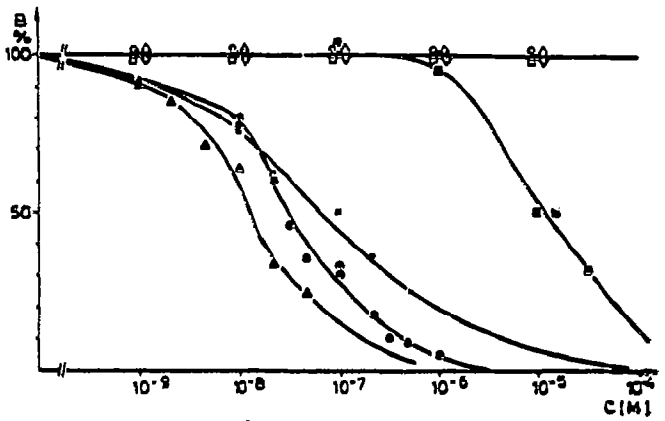

Fig. 4. Displacement of [^{3}H]EGIS 6775. Radioligand concentration 5 nM, [Mg^{2+}] = 40 mM. Symbols: Tofisopam (×), girisopam (▲), GYKI-52 322 (●), diazepam (■), dopamine (○), GABA (▽), glutamic acid (△), serotonin (□).

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