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SOLUBILIZATION OF RAT KIDNEY BENZODIAZEPINE BINDING SITES

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The high-affinity binding site for [^{3}H]Ro 5-4864 has been solubilized from rat kidney using 1% Triton X-100. After lowering the concentration of detergent and using a poly(ethylene glycol) γ-globulin assay, it has been possible to demonstrate solubilization of about 90% of the binding sites. A single soluble class of binding sites with a K_d of 1.8 nM is found. The order of potency of benzodiazepines is identical for the solubilized receptor and the membrane-bound form. Gel filtration revealed a major peak of binding activity with apparent molecular weight of 215000 and a Stokes' radius of 5.03 nm.

Introduction

Benzodiazepines bind with high affinity to pharmacologically relevant receptors in the mammalian brain, but they also bind with high affinity to membranes of a variety of peripheral tissues [1], including the kidney. The benzodiazepine receptor present in peripheral tissues differs from those in the brain. In fact clonazepam, a potent inhibitor of [^{3}H]flunitrazepam binding to brain membranes, is virtually inactive in dispacing [^{3}H]flunitrazepam from kidney membranes. On the other hand, the benzodiazepine Ro 5-4864, a clinically active anxiolytic devoid of anxiolytic-like activity in most animal tests [2], is a potent inhibitor of the kidney binding sites but fails to show significant activity in the brain. However, it has been reported that high-affinity, saturable binding sites of [^{3}H]Ro 5-4864 are present in rat cerebral cortical membranes [3]. The exact chemical structure of these peripheral receptors can be determined only when receptor molecules are available in a soluble and highly purified form. As a first step toward this end, we report the solubilization of a macromolecular moiety which has high binding affinity for [^{3}H]Ro 5-4864 and present data which suggest that this moiety may be the peripheral benzodiazepine receptor.

Materials and Methods

[^{3}H]Ro 5-4864 (73.8 Ci/mmol) and other non-radioactive benzodiazepines were generously provided by Hoffman-La Roche Inc. All other compounds were purchased from chemical sources. γ-Globulin was a gift of Biagini, Pisa.

Male Sprague-Dawley rats (approx. 200–250 g) were decapitated. The kidneys were removed and homogenized in 10 vol. (w/v) 0.32 M ice-cold sucrose containing protease inhibitors [4]. The homogenate was centrifuged at 2000 × *g* for 5 min at 4°C; the pellet (P_1) was discarded and the supernatant was recentrifuged at 30000 × *g* for 30 min at 4°C. The membrane were lysed by resuspension in 50 mM Tris-HCl buffer (pH 7.4) containing the inhibitors and recentrifuged at 30000 × *g* for 30 min at 4°C (P_2). The receptor was either assayed

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in the membrane-bound state or solubilized by adding Triton X-100 to a final concentration of 1% (using 7 ml medium/g original tissue), this being followed by brief homogenization and shaking at 4°C for the time noted in the text. The resultant extract was spun at $105\,000 \times g$ for 30 min. Then Triton X-100 was removed from solubilized membranes by treatment with Bio-Beads SM-2 for the time reported in the text. The resulting suspension was centrifuged again at $30\,000 \times g$ for 30 min at 4°C. The clear supernatant is referred to as the solubilized fraction and was used for various experiments.

Studies of [^{3}H]Ro 5-4864 binding to kidney membrane preparation were performed by incubating aliquots of the P_2 fraction (0.3 mg protein) for 90 min at 0°C in 500 μl 50 mM Tris-HCl buffer (pH 7.4)/0.8 nM [^{3}H]Ro 5-4864 in the absence or presence of 5 μM of unlabelled diazepam. Incubation was terminated by filtration through Whatman GF/B glass fiber filters under suction. The filters were washed twice with 5 ml 50 mM Tris-HCl (pH 7.4) and the radioactivity was counted in 8 ml HP Beckman scintillation cocktail containing 0.4 ml 0.01 M KOH. The binding assay for solubilized fraction was performed as follows. The solubilized fraction was incubated with 0.8 nM [^{3}H]Ro 5-4864 in 50 mM Tris-HCl buffer (pH 7.4) for 90 min at 0°C. After incubation the samples were assayed according to the method of Cuatrecasas [5] mixing first with 0.5 ml 0.5% γ-globulin (w/v) and then with 0.5 ml 40% (w/v) poly(ethylene glycol) 6000, both in 50 mM Tris-HCl buffer (pH 7.4) at 4°C. The mixture was immediately filtered under low vacuum through GF/B filters. The filters were rinsed twice with 4 ml 8% poly(ethylene glycol) 6000 in 50 mM Tris-HCl buffer (pH 7.4) at 4°C. Specific [^{3}H]Ro 5-4864 binding is defined as total [^{3}H]Ro 5-4864 binding minus binding obtained in the presence of 5 μM diazepam.

Ultrogel chromatography was performed as follows. Elution volumes of the binding activity were determined by adding 1 ml (0.25–0.51 mg protein) of sample to a 1.5×97 cm. Ultrogel AcA 34 column pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.4)/0.05% Triton X-100, at a flow rate of 8 ml/h at 20°C. Soluble receptor was assayed as above on 0.4 ml aliquots of each 2.0 ml column fraction using 2 nM [^{3}H]Ro 5-4864. Marker enzymes were lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), cytochrome c (CyC). The Stokes' radius was evaluated as described by Axelsson [9].

Protein was determined by the method of Lowry et al. [6].

Results

Specific binding of [^{3}H]Ro 5-4864 to rat kidney membranes was observed and the data reported by Yamamura et al. [3] were confirmed. At pH 7.4 (0°C) the sites in the crude kidney membrane fraction exhibited a dissociation constant (K_d) of 1.4($\pm$0.2) nM for the drug, with a single, saturable class of sites.

TABLE I

BINDING OF [^{3}H]Ro 5-4864 IN RAT KIDNEY MEMBRANE (P_2) AND THEIR EXTRACTS IN 1% TRITON X-100

Binding assays for membrane and solubilized fraction were carried out in triplicate as described under Materials and Methods using 0.8 nM [^{3}H]Ro 5-4864. Values are means $\pm$S.E.

Receptor state	Time of extraction (min)	Relative binding (fmol/mg protein)		
			Before treatment with Bio-Beads	After treatment with Bio-Beads (120 min)
Membrane-bound	–	155 $\pm$ 30	–	–
Solubilized	2	–	3.3 $\pm$ 0.5	431 $\pm$ 50
	15	–	2.8 $\pm$ 0.5	605 $\pm$ 70
	30	–	2.2 $\pm$ 0.4	650 $\pm$ 30
	60	–	0.4 $\pm$ 0.1	480 $\pm$ 10

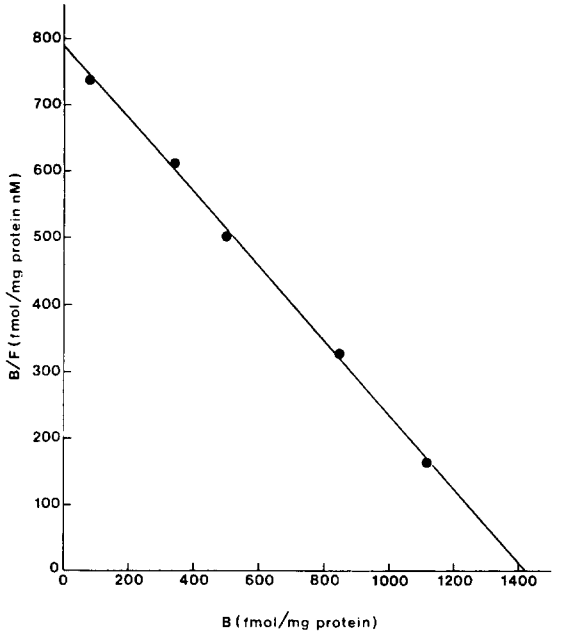

Fig. 1. Scatchard plot of [^{3}H]Ro 5-4864 binding to the soluble receptor. The assay was carried out using [^{3}H]Ro 5-4864 between 0.1 and 10 nM. *B*, specifically bound [^{3}H]Ro 5-4864 (fmol/mg protein); *F*, [^{3}H]Ro 5-4864 concentration of unbound [^{3}H]Ro 5-4864 in the incubation medium (nM). The apparent dissociation constant derived from at least four such experiments is 1.8($\pm$0.1) nM.

We tested lubrol and Triton X-100 for their ability to solubilize this moiety on kidney membranes which has high binding affinity for [^{3}H]Ro 5-4864. This lubrol treatment seems to be less effective than Triton X-100 in solubilizing the binding sites (30% against 90%). The solubilized fraction was tested for [^{3}H]Ro 5-4864 binding. Low recovery of the binding was observed. The activity was recovered by lowering of Triton X-100 using Bio-Beads SM-2, a neutral porous styrene-divinyl benzene copolymer.

Table I shows the binding of [^{3}H]Ro 5-4864 in rat kidney membranes (P_2) in in their 1% Triton X-100 extracts obtained with various periods of extraction followed by the treatment with Bio-Beads.

Specific binding was saturable and Scatchard analysis of the data indicated a single binding component with an apparent affinity constant of 1.8($\pm$0.1) nM (Fig. 1).

Inhibition studies with several benzodiazepines

TABLE II

INHIBITION OF SPECIFIC [^{3}H]Ro 5-4864 BINDING TO MEMBRANES AND THE SOLUBILIZED FRACTION BY VARIOUS BENZODIAZEPINES

Values are means $\pm$S.E. IC_{50} is that concentration causing 50% inhibition of specific [^{3}H]Ro 5-4864 binding.

Benzodiazepines	IC_{50} (M)	
	Membrane fraction	Solubilized fraction
Diazepam	$(2.1\pm0.3)\cdot10^{-8}$	$(2.6\pm0.5)\cdot10^{-8}$
Flunitrazepam	$(2.9\pm0.1)\cdot10^{-8}$	$(3.3\pm0.6)\cdot10^{-8}$
Clonazepam	$(8.4\pm3.0)\cdot10^{-6}$	$(9.8\pm2.0)\cdot10^{-6}$
Chlordiazepoxide [a]		–

[a] Less than 30% inhibition at $5.0\cdot10^{-6}$ M was observed.

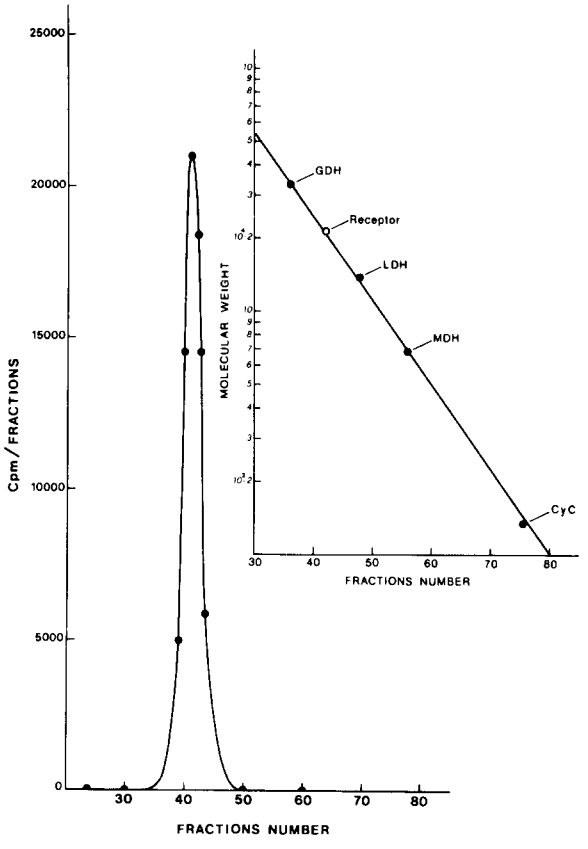

Fig. 2. Gel filtration of soluble [^{3}H]Ro 5-4864 binding. The apparent molecular weight was determined by the means of at least three similar experiments.

were performed using both P_2 and solubilized fractions (Table II). The IC_{50} values of benzodiazepines obtained using the solubilized fraction agreed well with those obtained using the membrane fraction.

Gel filtration of [^{3}H]Ro 5-4864 binding component on a calibrated Ultrogel AcA 34 column revealed a peak with an apparent molecular weight of 215 000 ± 20 000 (Fig. 2).

The Stokes' radius calculated according to Axelsson was 5.03 nm.

Discussion

The presence of specific benzodiazepine binding sites has been demonstrated in the brain and in some peripheral tissues [1,7,8]. The benzodiazepine receptors in the brain appear to be correlated with the pharmacologic activity of the compounds. The significance of the peripheral receptors is at present not known. Of particular interest are the benzodiazepine binding sites present in the kidney which are labelled with Ro 5-4864 and found also in the brain. Benzodiazepines do not exert known pharmacological effects on the kidney, nor there is a correlation between anxiolytic activity and their binding potency in the kidney. Nevertheless, the binding is specific and saturable.

We report the solubilization of the peripheral benzodiazepine binding site by Triton X-100 and subsequent treatment with Bio-Beads SM-2. Specific [^{3}H]Ro 5-4864 binding to the solubilised fraction was saturable, with an apparent dissociation constant, K_d, of 1.8(±0.1) nM. The ligand specificity of the soluble and the membrane-bound binding sites is very similar. Ro 5-4864, diazepam and flunitrazepam potently inhibited [^{3}H]Ro 5-4864 binding with IC_{50} values in the nanomolar range. In constrast to its potent inhibition of [^{3}H]flunitrazepam binding to brain membranes, clonazepam was only weakly active in inhibiting [^{3}H]Ro 5-4864 binding. [^{3}H]Clonazepam at 0.9 nM shows no binding to kidney membrane or soluble receptors (data not shown). The binding of [^{3}H]Ro 5-4864 to benzodiazepine kidney membrane or soluble receptors is not affected by γ-aminobutyric acid (data not shown). The molecular weight of this peripheral receptor was estimated 215 000 ± 20 000 with Ultrogel column. However it is not known how many molecules of Triton X-100 or other proteins remain associated with the receptor; thus this may represent a somewhat overestimated molecular weight. So we evaluated the Stokes' radius, which is 5.03 nm.

While further analysis of the soluble non-neuronal binding site is required, these results suggest that the pharmacological differences in the two types of benzodiazepine binding site are due to the inherent properties of the binding sites, since an intact membrane is not required.

The solubilization of the kidney membrane-bound benzodiazepine binding component in an active state opens the way to its purification.

Studies are in progress to synthesize a specific adsorbent to purify the peripheral receptor by affinity chromatography according to the procedure described by us for the purification of the central type receptor [4].

References

1 Braestrup, C. and Squires, R.F. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3805–3809
2 Randall, L.O., Schallek, W., Sternbach, L.H. and Ning, R.Y. (1974) in Psychopharmacological Agents (Gordon, M. ed.), pp. 175–198, Academic Press, New York
3 Schoemaker, H., Bliss, M. and Yamamura, H.I. (1981) Eur. J. Pharmacol. 71, 173–175
4 Martini, C., Lucacchini, A., Ronca, G., Hrelia, S. and Rossi, C.A. (1982) J. Neurochem. 38, 15–19
5 Cuatracasas, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 318–322
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
7 Davies, L.P. and Huston, V. (1981) Eur. J. Pharmacol. 73, 209–211
8 Regan, J.W., Yamamura, H.Y., Yamada, S. and Roeske, W.R. (1981) Life Sci. 28, 991–998
9 Axelsson, I. (1978) J. Chromatogr. 152, 21–32