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CHARACTERIZATION OF SOLUBILIZED "PERIPHERAL TYPE" BENZODIAZEPINE BINDING SITES FROM RAT ADRENALS BY USING [^{3}H]PK 11195, AN ISOQUINOLINE CARBOXAMIDE DERIVATIVE

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Abstract—"Peripheral type" benzodiazepine binding sites have been solubilized with digitonin. Binding site density for the solubilized material is increased 1.7 times compared to membranes. A decrease in the affinity for [^{3}H]-PK 11195 (a new ligand for the peripheral type benzodiazepine binding sites) was also observed. Pharmacological specificity of displacing agents was conserved during solubilization. The apparent molecular weight determined by gel filtration was 215,000 ± 20,000. The high B_{max} value of the solubilized preparation (>50 pmole/mg protein) makes it advantageous as the starting point for a purification procedure.

"Peripheral type" benzodiazepine binding sites were first characterized by Braestrup *et al.* [1]. These sites were very abundant in peripheral tissues such as kidney [2] and heart [3]. Further studies demonstrated their presence in brain [4] and adrenals [5]. In contrast to brain benzodiazepine receptors, which are coupled to GABA receptors and are related to the classical pharmacological properties of benzodiazepines (anticonvulsant, anxiolytic, sedative and myorelaxant) [6], the physiological relevance of the "peripheral type" binding sites is almost unknown. However, the antiangina [7] and antiarrhythmic [8] actions of benzodiazepines, as well as the inhibition of cell proliferation [9], may be related to such peripheral binding sites.

Peripheral binding sites for benzodiazepines can be specifically labelled with [^{3}H]RO5-4864 (a benzodiazepine without affinity for the central type receptor) and [^{3}H]PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide[10].

Thermodynamic studies on the effect of temperature in the affinity of these ligands suggested that RO5-4864 and PK 11195 were respectively agonist and antagonist of a hypothetical receptor coupled to the peripheral binding sites for benzodiazepines [3].

Although this interpretation was only made by analogy to better characterized receptors [11], it is in agreement with behavioural and electrophysiological data. RO5-4864 induces an increase in sensitivity of DBA/2 mice to audiogenic seizures, and this effect is readily and specifically antagonized by PK 11195 [12]. Moreover, the decrease in the intracellular action potential duration induced by RO5-4864 was specifically antagonized by PK 11195 in a guinea-pig heart preparation [13]. These results show the pharmacological relevance of these binding sites.

Peripheral binding sites for benzodiazepine have been characterized in adrenals [5]. The high density of [^{3}H]PK 11195 binding sites in adrenals makes this the most suitable tissue from which the sites purify. Indeed, the density of binding sites in adrenals is at least 25 times higher than in the kidney membrane fraction used by Martini *et al.* [14] to solubilize "peripheral type" benzodiazepine binding sites labelled with [^{3}H]RO5-4864. In this paper, we have used digitonin to solubilize these binding sites from rat adrenals, using [^{3}H]PK 11195 as a ligand.

MATERIALS AND METHODS

The solubilization procedure was carried out on ice. Adrenals from male Sprague–Dawley rats (200 g body weight) were homogenized in 10 vol. 0.25 M sucrose with a glass–vinyl homogenizer and stored until use at −20°. Binding to frozen membranes remains stable for at least 2 months. Before solubilization, the membranes (10 mg protein) were thawed and centrifuged at 100,000 *g* for 1 hr. The pellet was resuspended in 3 ml 50 mM Tris HCl, pH 7.4 containing the detergent with a glass-vinyl homogenizer, and shaken slowly for 1 hr. Following solubilization, the suspension was centrifuged at 150,000 *g* for 60 min. Binding to the membrane fraction was assayed at 25° in 1 ml Tris HCl, pH 7.4 containing 1 nM [^{3}H]PK 11195. Binding was terminated after 30 min by quickly filtering through a GF/C filter under vacuum. The filters were washed twice with 5 ml of buffer and radioactivity measured by liquid scintillation spectroscopy. Binding to the solubilized preparation was performed as indicated for membranes, but the solubilized material (0.5–

Table 1. Solubilization of [^{3}H]PK 11195 binding sites from rat adrenal membranes

Detergent used	Proteins solubilized (%)	Binding in solubilized material (pmole/mg prot)
Chaps 1%	41	1.0
Triton X-100 1%	46	0
Trisoctan 1%	3	0
Digitonin 1%	48.5	5.1
Digitonin 0.75%	46	2.6
Digitonin 0.5%	39	1.1
Digitonin 0.25%	29	0.4
Digitonin 0.1%	20.5	0.1

Binding was measured in the presence of 1 nM [^{3}H]PK 11195. Results do not take into account possible changes in affinity due to solubilization. Each value is the mean of 3 experiments. S.E. was less than 10% in all the experiments. Binding to the membrane fraction was 5.2 pmole/mg prot.

1 mg prot/ml) was diluted 50-fold into binding medium containing 0.1% Trisoctan.* At the end of the experiment, protein was precipitated by addition of 500 μl of 0.3% w/v solution of bovine γ-globulin and 500 μl 40% w/v solution of polyethylene-glycol (M.W. $\simeq$ 8000 D) [15]. After shaking for 15 min, the precipitated binding sites were separated by filtration through GF/B filters under vacuum. Filters were washed twice with 5 ml incubation medium containing 8% polyethylene-glycol. Non specific binding was determined in the presence of 1 μM PK 11211 (a PK 11195 analogue with F substituted for Cl). Although the specific binding of [^{3}H]PK 11195 could be displaced equally by RO5-4864 as by PK 11211, the latter was used routinely by token of its superior affinity for the solubilized binding sites (see Table 2). Polyethylene-glycol 8000 and digitonin were obtained from sigma and bovine γ-globulin from Fluka. [^{3}H]PK 11195 was prepared by catalytic tritiation of 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-2-propenyl)-3-isoquinolinecarboxamide. Gel exclusion chromatography was performed in an Ultrogel AcA 22 column (1.6 × 75 cm) pre-equilibrated with 50 mM Tris HCl buffer containing 0.05% digitonin, 0.1 M NaCl and 0.01% NaN_3. One ml of digitonin solubilized membranes (3 mg protein) was applied and the column run at 10 ml/hr at 25°. Molecular weight marker proteins were thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and aldolase 158,000). Molecular weight was calculated as described by Axelsson [16]. Trisoctan-*N*-Tris-(hydroxymethyl)methyloctanamide was synthetized by reaction of octanoyl chloride with Tris-(hydroxymethyl) aminomethane in isopropanol. The reaction product (Trisoctan) was recrystallized in acetonitrile and had a melting point of 106°. Protein concentration was measured as described by Lowry *et al.* as modified by Dulley and Grieve [17] to avoid interference from detergents.

* Abbreviations used: Trisoctan, N-Tris (hydroxymethyl)methyloctanamide; BZ, benzodiazepine; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio-]-1-propanesulfonate.

RESULTS

The binding of 1 nM [^{3}H]PK 11195 to the material solubilized by several detergents has been measured (Table 1). Although all the detergents solubilized an identical amount of protein, the material solubilized by digitonin contained the highest amount of [^{3}HPK 11195 binding. Solubilization of binding sites as detected by 1 nM [^{3}H]PK 11195 binding was dependent on the digitonin concentration, but at low digitonin concentrations, negligible binding could be detected in the soluble material even though 20% of proteins were solubilized. Membrane protein was not solubilized by Trisoctan but 0.1% of this detergent avoided further aggregation without inhibiting the binding and was routinely present in experiments on solubilized membranes. Solubility of binding sites in these experimental conditions was demonstrated by the passage of the sites through 0.22 μ filters and by the absence of precipitation after 1 hr centrifugation at 160,000 *g* [18]. [^{3}H]PK 11195 binding

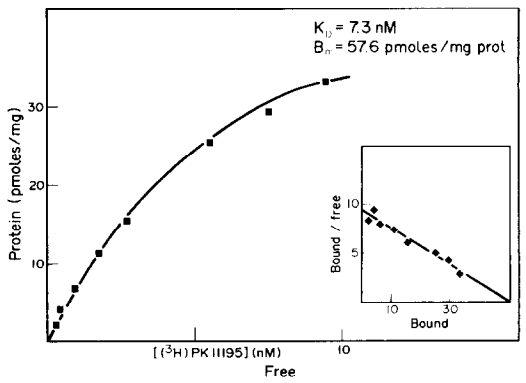

Fig. 1. Specific binding of [^{3}H]PK 11195 to solubilized rat adrenals: saturation studies. Membranes solubilized with 1% digitonin (10 μg protein/ml) were incubated in the presence of increasing concentrations of [^{3}H]PK 11195 for 30 min at 25°. Non specific binding (determined with 1 μM PK 11211) was always less than 10% of total binding. Results are the mean of 3 experiments.

Table 2. Comparison between membrane-bound and solubilized [^{3}H]PK 11195 binding sites in rat adrenals

	Solubilized	Membrane
(a) Scatchard analysis		
K_D (nM)	7.3 ± 1.3	2.9 ± 0.4
B_{max} (pmole/mg prot)	57.6 ± 11.4	34.0 ± 8.5
(b) Displacement studies	IC_{50} (nM)	
Drug	Solubilized	Membrane
PK 11195	4 ± 0.3	2.5 ± 0.2
RO5-4864	200 ± 17.5	60 ± 3.3
Diazepam	718 ± 62	320 ± 35
Clonazepam	>5 × 10^5	>5 × 10^5

K_D and B_{max} were calculated from Scatchard plots as described in Fig. 1 legend. IC_{50}s were obtained by using 6 concentrations of displacing agents. Protein concentration was 10 μg/ml. Each experiment was repeated at least 3 times on different membrane preparations.

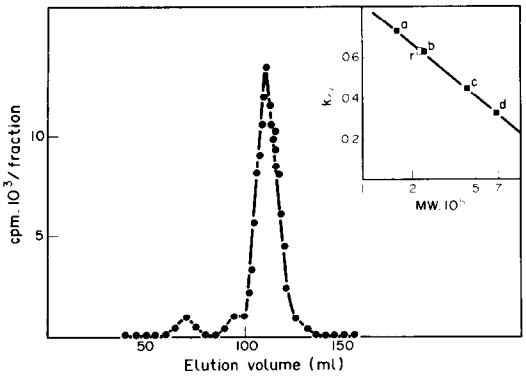

Fig. 2. Gel exclusion chromatography of solubilized [^{3}H] PK 11195 binding sites from rat adrenals. Apparent molecular weight is the mean of 3 experiments. Molecular weight marker proteins were: a, aldolase (158,000); b, catalase (232,000); c, ferritin (440,000); d, thyroglobulin (669,000); r, [^{3}H]PK 11195 binding sites.

was linear up to 40 μg protein solubilized material/ml. Although experiments with 1 nM [^{3}H]PK 11195 detected a total binding capacity similar to that of the membrane preparation, there was actually, an increase of binding site density relative to protein in the solubilized material (1.7 times) due to preferential extraction of the binding sites by the detergent. Saturation analysis of the binding to the solubilized preparation (Fig. 1) showed a decrease in the affinity of [^{3}H]PK 11195 compared to membrane bound binding sites. Table 2 shows the characteristics of the binding of [^{3}H]PK 11195 to the solubilized preparation. Displacing agent had the same relative of potencies as in membranes (PK 11195 > RO5-4864 > diazepam > clonazepam) but their IC_{50}s were increased around 3 times. Thus solubilization induces a slight, but general, decrease in the affinity for all the ligands. Solubilized or membrane bound binding sites were not affected by GABA as would be expected for the "peripheral type" of benzodiazepine binding sites (table 2).

Molecular weight estimation

Gel filtration demonstrated that the apparent molecular weight of the binding sites was 215,000 ± 20,000 (Fig. 2). A very similar view was obtained for material solubilized from bovine adrenals 210,000 ± 15,000, mean of 3 experiments, data not shown. The same molecular weight (215,000 ± 15,000) was also found for digitonin solubilized benzodiazepine binding sites in rat kidney. The molecular weight, however, can be overestimated because of an unknown number of digitonin molecules associated with the solubilized binding sites. It is likely that a membrane protein will bind more detergent molecules than the soluble proteins used as molecular weight markers.

DISCUSSION

The present results demonstrate the feasibility of solubilizing benzodiazepine binding sites from rat adrenal with digitonin. This detergent, as opposed to Triton X-100 [11] does not interfere with the binding of [3H]PK 11195 at the concentrations (0.02% w/v) present in the solubilized material. To avoid reaggregation of the solubilized material during the binding experiment, 0.1% Trisoctan was also present. This detergent does not inhibit [^{3}H]-PK 11195 binding and is unable to solubilize the membranes, but maintains the binding sites in a soluble state. Binding of [^{3}H]PK 11195 to the solubilized material was saturable and of high affinity. There was an enrichment (1.7 times) of the binding density perhaps due to a preferential extraction by the detergent. This was accompanied by a slight decrease in the affinity of [^{3}H]PK 11195.

Specific binding of [^{3}H]PK 11195 to the solubilized rat adrenals conserved its pharmacological specificity: PK 11195 > RO5-4864 > diazepam > clonazepam; GABA had no effect. The molecular weight of these binding sites was found to be 215,000 ± 20,000 Da in two species, rat and beef, similar to that found by Martini *et al.* [14] for the Triton X-100 solubilized [^{3}H]RO5-4864 binding sites from kidney. Solubilized preparations from beef and rat adrenals present a similar molecular weight and the same affinity for [^{3}H]PK 11195. This solubilization procedure yielding a solubilized preparation with a very high binding density constitutes a useful starting point for the purification of these binding sites.

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