Properties of a $^{125}$I-Substance P Derivative Binding to Synaptosomes from Various Brain Structures and the Spinal Cord of the Rat

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Summary. Using crude synaptosomal fractions (P$_2$ fractions) and $^{125}$I-Bolton and Hunter substance P ($^{125}$I-BHSP) as a ligand, the characteristics of specific binding sites were examined in various brain structures and in the spinal cord (dorsal and ventral parts) of the rat. Scatchard plots revealed the occurrence of a single class of binding sites in the various structures studied with comparable $K_d$ values (from 0.46 to 1.10 nmol/l in the brain and 0.51, 0.56 nmol/l in the spinal cord dorsal and ventral parts respectively) and of marked differences in the number of binding sites ($B_{max}$) (septum > striatum > hippocampus, hypothalamus > mesencephalon > cerebral cortex and dorsal part of the spinal cord > ventral part). In the brain no correlation was found between the number of $^{125}$I-BHSP binding sites and the amount of substance P levels (substance P-like immunoreactivity) in synaptosomes, particularly in the hippocampus and the substantia nigra since the former structure was characterized by its low substance P content and its high number of binding sites and the reverse was observed in the substantia nigra. The ability of several C- and N-terminal fragments of substance P and of tachykinins to compete with $^{125}$I-BHSP binding to synaptosomes from the hippocampus, the hypothalamus and the dorsal part of the spinal cord was then determined. Results obtained were closely similar from one structure to another and comparable to those previously reported using whole brain synaptosomes. Although the presence of various types of central substance P receptors cannot be excluded, the present results indicate that only one class of sites can be demonstrated using $^{125}$I-BHSP as a ligand.

Key words: Substance P receptors - $^{125}$I-BHSP binding - Synaptosomes - Brain structures - Spinal cord - Rat

Introduction

Recently, we have demonstrated the occurrence of a specific binding of $^{125}$I-labeled Bolton-Hunter substance P ($^{125}$I-BHSP) on a crude synaptosomal fraction (P$_2$) obtained from the rat brain (Viger et al. 1983). This binding was saturable, reversible, temperature-dependent and not affected by ouabain thus excluding the intervention of an uptake process in pinched-off nerve endings. Scatchard analysis has revealed the existence of a single population of sites exhibiting a high affinity ($K_d$: 0.47 nmol/l). Substance P C-terminal fragments competed with $^{125}$I-BHSP binding and a good relationship was found between competitive activity and peptide length, the longer fragments being more potent. In contrast, N-terminal fragments were ineffective. Among various tachykinins, physalaemin was the most potent but its efficacy was only one fourth that of substance P. The synaptosomal preparation was chosen for two main reasons. First, in our hands technical difficulties were found with membranes, i.e. the nonspecific binding was important and peptidase inactivation of the labeled ligand was pronounced. Secondly, successful results had been previously obtained in our laboratory with mesencephalic embryonic cells from the mouse in primary cultures suggesting that closed systems were preferable (Beaujouan et al. 1982). In fact, the characteristics of $^{125}$I-BHSP binding on synaptosomes and embryonic cells were comparable in several ways. The present study was first undertaken to determine the distribution of $^{125}$I-BHSP binding sites on synaptosomes prepared from several structures of the rat brain and to investigate whether or not the properties of these binding sites could differ from one structure to another. Such an analysis seemed to be required since discrepancies were found concerning the distribution of $^3$H-substance P binding sites on crude membranes from various rat brain structures as described by Hanley et al. (1980) and Bittiger (1982) and no attempts were made in these studies to compare the pharmacological properties of these binding sites in the various brain areas examined. Furthermore, we have also examined the properties of $^{125}$I-BHSP binding on synaptosomes prepared from the spinal cord, since this structure is densely innervated by substance P fibres originating mainly (but no exclusively) from primary sensory afferents (Jessell et al. 1979; Hökfelt et al. 1980; Senba et al. 1982) and may contain physalaemin as suggested in a biochemical study (Lazarus et al. 1980).

Material and Methods

Materials. All peptides used in this study were purchased from Peninsula Laboratories (San Carlos, CA, USA) except for (α-Pro$^2$, β-Trp$^9$) substance P and substance P$^{1-9}$ amide which were synthesized by Dr. S. Lavielle (University Paris VI). The unlabeled Bolton and Hunter derivative of substance P was obtained by coupling 1.5 mg of $^{127}$I-Bolton and Hunter reagent (prepared as described by Michelot et al. 1980) with 5 mg of substance P in 600 μl of dimethylformamide, 200 μl water and 1 ml of triethylamine at 4°C during 90 min. The product was evaporated under vacuum and solubilized in 700 μl acetic acid (10%). $^{127}$I-BHSP was purified by HPLC.
on a Merck C18 column (semi-preparative) with methanol/water/trifluoroacetic acid as solvent (400/275/2 v/v/v). 125I-BHSP, a biologically active ligand, was obtained by coupling the 125I-labeled Bolton-Hunter reagent (Amersham: monoiodo derivative 2000 Ci/mmol) to the lysine amino-acid of substance P (Michelot et al. 1980). Bovine serum albumin was obtained from Calbiochem (San Diego, CA, USA); bacitracin and histones (type II, AS from calf thymus) were provided by Sigma (St. Louis, MO, USA); (--)boeflon was purchased from Ciba-Geigy (Rueil-Malmaison, France).

Preparation of Crude Synaptosomal Fractions (P2). As previously reported (Viger et al. 1983), the procedure of Gray and Whittaker (1962) was used with slight modifications to prepare crude synaptosomes from various brain structures and the spinal cord. Charles River (Sprague Dawley) male rats (200–250 g) were killed by decapitation and their brain were immediately dissected into cerebellum, cerebral cortex, hypothalamus, striatum, hippocampus, mesencephalon and septum as described by Glowinski and Iversen (1966). The spinal cord was also dissected in its ventral and dorsal parts. All subsequent operations were carried out at 4°C. Tissues from several animals (7–10) were pooled and homogenized in 10% sucrose (wt/vol) (1 g of tissue per 10 ml sucrose) using a teflon Potter homogenizer (10 strokes of the pestle). Homogenates were centrifuged at 800 × g for 20 min, the pellet was then discarded and the supernatant was centrifuged at 9,000 × g for 20 min. The pellet (P2) was resuspended (same volume as that used for sucrose) in a Krebs-Ringer phosphate buffer (in mmol/l): NaCl, 120; KCl, 4.8; CaCl₂, 1.2; MgSO₄, 1.2; NaH₂PO₄, 15.6; pH 7.4) containing bovine serum albumin (0.4 mg/ml); bacitracin (30 µg/ml) and glucose (1 mg/ml), and centrifuged again at 9,000 × g for 20 min. The final pellet was then resuspended in the same Krebs-Ringer phosphate buffer (containing bovine serum albumin, bacitracin and glucose; 5 ml for 1 g of initial tissues).

Binding Assays. Binding assays were performed in Eppendorf tubes (1.5 ml) precoated with a Krebs-Ringer phosphate buffer solution containing histones (0.05%). Routinely, 20 µl of the P2 fraction (~100 µg of proteins) were incubated in 200 µl (final volume) of the Krebs-Ringer phosphate buffer enriched with bovine serum albumin, bacitracin, and glucose (as described above) for 5 min at 20°C with 125I-BHSP (25,000 cpm; 0.1 nmol/l) in the presence or absence of substance P (1 µmol/l). At the end of the incubation, the tubes were centrifuged (Eppendorf minicentrifuge) for 30 s at 10,000 × g, and the pellet was washed once with a medium identical to that used for the incubation (1 ml). Finally, radioactivity bound to tissues was estimated using a Packard gamma counter (efficiency: 30%). All assays were made in quadruplicate. Proteins were determined according to the method of Lowry et al. (1951). In some experiments, the identity of the radioactive material recovered in the supernatant at the end of the 5 min incubation was checked to verify that 125I-BHSP was not inactivated by peptidases. For this purpose a high-pressure liquid chromatography analysis was made using C18 µBondapak columns (Waters Instruments, Rochester, MN, USA) and methanol:ammonium acetate (50 mmol/l; pH 4; 540:460) as solvent.

Substance P Radioimmunoassay. Substance P antiserum was raised in rabbits using synthetic substance P coupled with bovine serum albumin by carbodiimide (Michelot et al. 1979) and the labeled substance P analogue was identical to that used in binding assays (125I-BHSP). First, the substance P antiserum (final dilution: 1/300,000) was preincubated with synthetic substance P (from 1 to 100 pg) during 48 h at 4°C in a Na+/K+ phosphate buffer (25 mmol/l) containing histones (0.1%) (total volume: 300 µl). 125I-BHSP (10 pmol/l equivalent to 2000 cpm) was then added in each tube during 24 h at 4°C. The separation of free bound 125I-BHSP was done with charcoal (Norit SXX Extra, 2.5 mg/tube) and bound 125I-BHSP was counted. The lowest quantity of substance P detectable was 3 pg. In order to estimate substance P content in different brain structures and in the spinal cord H₂PO₄ 0.1 mol/l was added to P₂ fractions (500 µl for 500 µl of synaptosomal suspension). Samples were then boiled for 10 min, neutralized, centrifuged and diluted. Aliquots of supernatants were then introduced in the radioimmunoassay. Substance P levels (substance P-like immunoreactivity) estimated were proportional to the amount of extracted tissue used. Curves obtained with various dilutions of the extracts from various brain structures were parallel with that of the standard curve made with substance P.

Results

1. Substance P Levels and 125I-BHSP Binding Sites in Various Brain Structures

In a preliminary experiment, substance P levels and the number of 125I-BHSP binding sites were simultaneously estimated on crude synaptosomes (P₂ fraction) prepared from several structures of the rat brain. Under these conditions no correlation was found between the amounts of substance P and 125I-BHSP binding (Fig. 1). Although 125I-BHSP bind-