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A New Type of Tachykinin Binding Site in the Rat Brain Characterized by Specific Binding of a Labeled Eledoisin Derivative

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SUMMARY

A new ligand for investigating tachykinin-binding site subtypes was synthesized by coupling the ^{125}I-Bolton and Hunter reagent to eledoisin (^{125}I-BHE). Using a synaptosomal preparation (P_2 fraction) of rat cerebral cortex, ^{125}I-BHE was shown to bind with apparent high affinity (apparent K_d = 15.3 nM). When concentrations of up to 30 nM ^{125}I-BHE were used, ^{125}I-BHE binding was specific, saturable, reversible, and temperature-dependent. In contrast to [^{3}H]dopamine, ^{125}I-BHE was not taken up within synaptosomes by an ouabain-sensitive process. Eledoisin, kassinin, and substance P were examined for their ability to inhibit specific ^{125}I-BHE binding to cortical synaptosomes. Eledoisin and kassinin were considerably more potent than substance P, in contrast to the order of potency observed for specific ^{125}I-Bolton-Hunter substance P (^{125}I-BHSP) binding. Specific ^{125}I-BHE binding was highest in the cerebral cortex and hypothalamus; intermediate in the hippocampus, striatum, and thalamus; low in the mesencephalon, septum, and substantia nigra; and absent in the cerebellum. Comparison of these data with those previously obtained for ^{125}I-BHSP binding to synaptosomes indicated that ^{125}I-BHE-labeled binding sites differ markedly from those of ^{125}I-BHSP-labeled binding sites. Therefore, tachykinin receptors other than substance P receptors seem to be present in the central nervous system.

INTRODUCTION

Several structurally related nonmammalian peptides, termed tachykinins, have been isolated which exhibit biological activity similar to that of substance P (1). This similarity is due to a common COOH-terminal region, whereas the specific action of each tachykinin could depend essentially on its unique NH_2-terminal sequence. For several years, among members of the tachykinin family, substance P has been considered the only representative tachykinin in mammals. Recently, however, a few studies have suggested that other tachykinins also could be present in mammalian tissues. Thus, Lazarus and colleagues (2) reported the existence of a physalaemin-like immunoreactivity in lung, gastrointestinal tract, and spinal cord of guinea pig, mouse, or rat. Furthermore, this group has indicated that small-cell carcinoma of human lung contains a physalaemin-like peptide with structural and biological properties similar to those of physalaemin (3). Two new decapeptides, with sequences and ileum-contracting activities comparable to those of substance P and the tachykinins, have been isolated from porcine spinal cord. These two tachykinin-like peptides were termed neurokinin α and β (4). Neurokinin β, however, is identical with neuromedine K, discovered separately by another group (5). Finally, by cloning DNA sequences complementary to the bovine striatal mRNAs coding for substance P precursors, Nawa and co-workers (6) have shown that the bovine brain substance P precursors are encoded by at least two distinct mRNAs. One of them encodes not only the substance P sequence but also a kassinin-like sequence, similar to neurokinin α, and termed substance K by Nawa *et al.*[6] In a way similar to that observed with the endorphins, the occurrence of several tachykinins in mammalian nerve tissues suggests the existence of multiple central tachykinin receptors. In fact, experiments made on peripheral preparations have revealed that, depending on the bioassay used, eledoisin or kassinin can be more potent than substance P (intestinal and genitourinary smooth muscle) or vice versa (blood pressure, vascular smooth muscle) (1). Recently, Iversen *et al.* (7) have introduced the terminology of substance P-P and substance P-E receptors to describe two main subtypes of substance P receptors that may be present in smooth muscle. The rank order of potency for the substance P-P receptor subtype is physalaemin ≥ substance P ≥ eledoisin ≈ kassinin, whereas that for the

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substance P-E subtype is eledoisin ≈ kassinin ≫ substance P ≈ physalaemin. Since electrophysiological studies have demonstrated that eledoisin can be more potent in depolarizing some central neurons than can substance P (8, 9) and that similar differences were found in some behavioral tests (10–12), tachykinin receptors distinct from substance P receptors could be present in the brain. In the present report, we show the existence of a tachykinin receptor exhibiting characteristics different from those of substance P receptors, which were defined by binding studies performed on embryonic mesencephalic neurons of the mouse (13), and synaptosomes (14, 15) or membrane preparations (16–18) of the rat brain using ^{125}I-BHSP[2] or ^{3}H-labeled substance P. For this purpose, a new ligand obtained by coupling the ^{125}I-Bolton Hunter reagent with eledoisin was used in conjunction with synaptosomal preparations from several rat brain structures.

MATERIALS AND METHODS

Synthesis of unlabeled ^{127}I-BHE. Unlabeled BHE was synthesized according to the procedure previously used for the synthesis of unlabeled BHSP (19). ^{127}I-Bolton and Hunter reagent (3 mg) was mixed with eledoisin (1.1 mg; Bachem) in the presence of dimethylformamide (300 μl) and borate buffer (0.5 M, pH 8.5, 1.5 ml) at room temperature for 1 hr with constant agitation. The reaction was terminated by the addition of dimethylformamide (1.5 ml) and acetic acid (10%, v/v; 1.5 ml). ^{127}I-BHE was purified on an HPLC column: HIBAR (Merck) CAT 50994 Lichrosorb RP 18 (7 μm), using methanol/water/trifluoroacetic acid as the mobile phase (600/400/3, v/v) and then isolated following methanol evaporation and lyophilization.

Synthesis of ^{125}I-BHE. ^{125}I-BHE was obtained by coupling the ^{125}I-labeled Bolton and Hunter reagent (Amersham: monoiodo derivative, 2000 Ci/mmol) with eledoisin (50 μg) in dimethylformamide (25 μl) and borate buffer (50 mM, pH 8.5, 90 μl) at 4° for 30 min with constant agitation. The reaction was stopped by the addition of dimethylformamide (50 μl) and acetic acid (2 N, 9 μl).

^{125}I-BHE was purified by HPLC on a C-18 μ-Bondapak column (Waters Instruments, Rochester, Minn.) with methanol/ammonium acetate (50 mM, pH 4) as the solvent (540/460, v/v, rate 2 ml/min). When ^{127}I-BHE was dissolved in dimethyl sulfoxide, then mixed with ^{125}I-BHE (80,000 cpm) and analyzed by HPLC as described above, ^{127}I-BHE and ^{125}I-BHE co-migrated in Fractions 25–26 (Fig. 1). Similar results were obtained following modification of the mobile phase: methanol/ammonium acetate (50 mM, pH 4) (510/490, v/v), the compounds being detected then in Fractions 53–54.

Preparation of the crude synaptosomal fraction (P_2). The crude synaptosomal (P_2) fraction was prepared by using slight modifications (14) of the procedure previously reported by Gray and Whittaker (20). Briefly stated, male Sprague-Dawley rats (200–250 g; Charles River France, Cleon, France) were killed by decapitation, and synaptosomes were prepared from the cerebral cortex. The final pellet was resuspended in Krebs-Ringer phosphate buffer (NaCl, 120 mM; KCl, 4.8 mM; $CaCl_2$, 1.2 mM; $MgSO_4$, 1.2 mM; NaH_2PO_4, 15.6 mM; pH 7) containing bovine serum albumin (0.4 mg/ml; Calbiochem, San Diego, Calif.), bacitracin (30 μg/ml, Sigma Chemical Company, St. Louis, Mo.), and glucose (1 mg/ml); 2.5 ml of this solution were used for 1 g of original tissue (wet weight).

In vitro ^{125}I-BHE binding assays. Specific ^{125}I-BHE binding was determined in Eppendorf tubes (1.5 ml) precoated with a Krebs-Ringer phosphate buffer containing bovine serum albumin (0.5%). Routinely, 20 μg of the P_2 fraction (200 μg of protein) were incubated in 200 μl (final volume) of Krebs-Ringer phosphate buffer enriched with bovine serum albumin, bacitracin, and glucose (as described above) for 15 min at 20° with ^{125}I-BHE (25,000 cpm ≈ 40 pM). At the end of the incubation, radioactivity bound to synaptosomes was separated from that free in solution by centrifugation (Eppendorf minicentrifuge) for 30 sec at 10,000 × *g*. The pellet was washed once with the same medium used for the incubation (1 ml). Radioactivity bound to the pellet was estimated by using a Packard gamma counter (efficiency 70%). Assays were performed in quadruplicate, and specific ^{125}I-BHE binding was defined by incorporation of unlabeled eledoisin (1 μM). Proteins were determined according to the method of Lowry *et al.* (21).

The identity of the radioactive material recovered in the supernatant fluid at the end of the 15-min incubation was checked to verify the authenticity of ^{125}I-BHE. For this purpose, HPLC analysis was performed using C-18 μ-Bondapak columns and methanol/ammonium acetate (50 mM, pH 4) (540/460, v/v) as solvent.

[^{3}H]Dopamine uptake. Crude synaptosomes (200 μg of protein) were preincubated for 20 min at 20° in Krebs-Ringer phosphate buffer (180 μl) and then incubated for 15 min at 20° with [2,5,6-^{3}H]dopamine (10 nM; Amersham International plc; 6 Ci/mmol) in the presence (blank) or absence of benztropine (50 μM; Merck Sharp & Dohme, West Point, Pa.), a specific inhibitor of dopamine uptake in dopaminergic neurons (22). When ouabain (Sigma Chemical Company) was used (0.1 or 1 mM), it was added at the beginning of the preincubation. At the end of the incubation, tissues were separated from the incubating medium by rapid centrifugation (30 sec, 10,000 × *g*) and washed once with ice-cold incubation medium (1 ml). Radioactivity bound to tissues was estimated by liquid scintillation spectrometry following addition of Aquasol (10 ml; New England Nuclear Corporation, Boston, Mass.) at a counting efficiency of 32–35%.

Drugs and materials. ^{127}I-BHE and ^{125}I-BHE were synthesized as previously described. Some batches of eledoisin were obtained from Bachem Feinchemikalien AG. All other peptides used in the study and

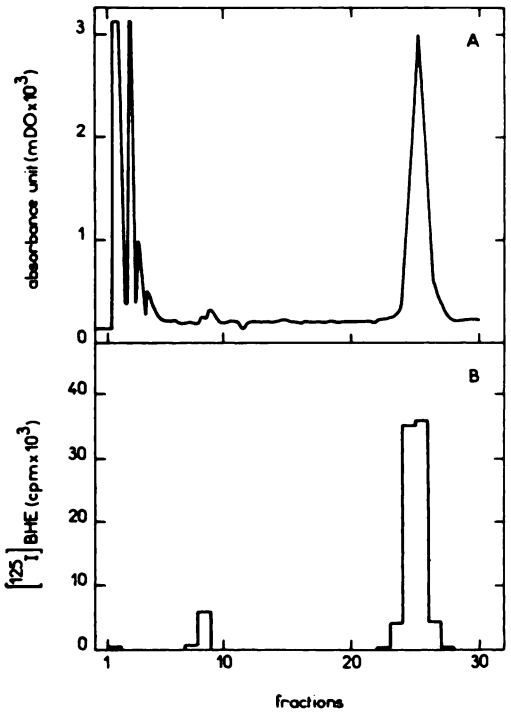

FIG. 1. *Co-migration of ^{127}I-BHE and ^{125}I-BHE using HPLC*
Chromatographic conditions are described under Materials and Methods. A sample containing both ^{127}I-BHE and ^{125}I-BHE (87,000 cpm) (20 μl) was injected through a C-18 μ-Bondapak column. The volume of each collected fraction was 2 ml. Profiles of elution were analyzed simultaneously using UV detection (A) at 254 nm (^{127}I-BHE) and radiometric estimation (B) with a gamma counter (^{125}I-BHE).

[2] The abbreviations used are: BHSP, Bolton-Hunter substance P; BHE, Bolton-Hunter eledoisin; HPLC, high-pressure liquid chromatography.

other batches of eledoisin were purchased from commercial sources (Peninsula Laboratories, San Carlos, Calif.).

RESULTS

Binding of ^{125}I-BHE to synaptosomes. Total, specific, and nonspecific binding of ^{125}I-BHE were found to increase linearly with increasing (0.1–0.7 mg/200 μl assay volume) protein concentration. When 0.2 mg of protein was used, specific ^{125}I-BHE binding represented 68% of the total binding to synaptosomes and 2% of the total radioactivity added to the incubating medium. HPLC analysis demonstrated that 82% of the radioactivity recovered at the end of the 15 min incubation migrated as authentic ^{125}I-BHE. Furthermore, when this supernatant was reincubated with a fresh P_2 preparation, specific binding was 89% of that obtained in the first assay; thus, only minor peptide inactivation occurred during the binding assays.

Specific ^{125}I-BHE binding was temperature-dependent, as it was decreased by 72% when the 15-min incubation was carried out at 4°.

Influence of ouabain on ^{125}I-BHE binding. Preincubation of synaptosomes for 20 min at 20° in the presence of ouabain (0.1 or 1 mM) immediately prior to the addition of the labeled ligand did not alter total or specific ^{125}I-BHE binding, (data not shown). In contrast, using identical conditions, ouabain (1 mM) reduced [^{3}H]dopamine uptake into synaptosomes by 95%. This indicated that ^{125}I-BHE was not taken up into synaptosomes by a Na^+, K^+-dependent ATPase process.

Kinetic characteristics of ^{125}I-BHE binding. The association of ^{125}I-BHE with synaptosomal preparations of rat cerebral cortex was rapid and reached equilibrium within the 15-min incubation at 20° (Fig. 2A). Transformation of these data (Fig. 2B) according to the method of Frost and Pearson (23) provided an estimate of the association velocity constant, $k_{+1} = 5.2 \times 10^9$ M^{-1} min^{-1}.

The specific binding of ^{125}I-BHE was reversible; rapid dissociation of the labeled ligand was observed when unlabeled eledoisin (1 μM) was added to the incubation tubes following an initial 15-min incubation at 20° in the presence of ^{125}I-BHE (Fig. 3A). First-order reaction analysis of the receptor ligand dissociation was curvilinear (Fig. 3B). The first phase of dissociation was very rapid, and the rate of dissociation (k_{-1}) was estimated to be 0.81 min^{-1} with an apparent half-life ($t_{½}$) of 0.86 min after allowing for the contribution of the slow phase, using the feathering technique. The second phase of dissociation was much slower: $k'_{-1} = 0.068$ min^{-1} and $t'_{½} = 10.3$ min. Thus, the apparent equilibrium dissociation constant ($K_d = k_{-1}/k_{+1}$) estimated from these kinetic studies was found to be 0.156 nM (for $k_{-1} = 0.81$ min^{-1}).

Equilibrium binding studies. Saturation studies of the binding of ^{125}I-BHE to rat cerebral cortex synaptosomal preparations were made with concentrations of the ligand between 2 and 30 nM (because of technical difficulties, higher concentrations of the ligand could not be used). These studies revealed that nonspecific binding defined using unlabeled eledoisin (1 μM) was a linear function of the labeled ligand concentration, in contrast to specific ^{125}I-BHE binding, which was saturable (Fig. 4A). In six independent experiments, each performed in

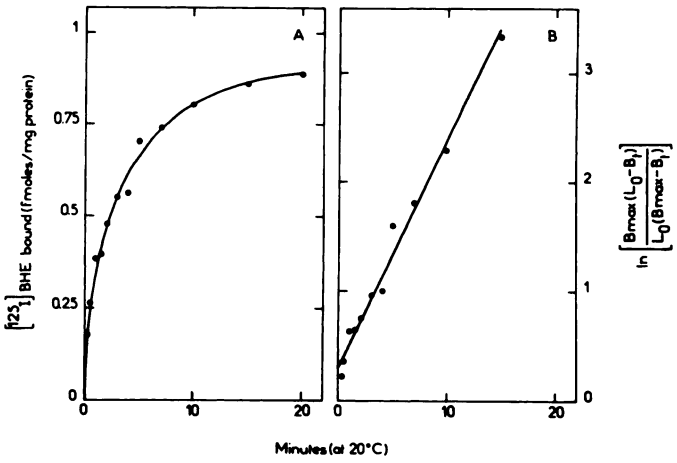

FIG. 2. *Association of ^{125}I-BHE with rat cerebral cortex synaptosomes*

A. Synaptosomes (185 μg of protein) were incubated at 20° for different time periods with ^{125}I-BHE (39.8 pM) in Krebs-Ringer phosphate buffer (0.2 ml, pH 7) containing bovine serum albumin, bacitracin, and glucose as described under Materials and Methods in the presence (nonspecific binding) or absence (total binding) of 1 μM eledoisin. Specific binding was calculated by subtracting nonspecific binding from total binding. Each point corresponding to specific binding represents the mean of data obtained in three separate experiments.

B. According to the equation (23) $\ln [B_{max}(L_0-B_t)/L_0(B_{max}-B_t)] = k_1 \cdot (L_0-B_{max})t$, results are represented as $\ln [B_{max}(L_0-B_t)/L_0(B_{max}-B_t)]$ versus time, where B_{max} represents the maximal concentration of ligand specifically bound at equilibrium; B_t, the concentration of ligand specifically bound at time t; and L_0, the initial ^{125}I-BHE concentration in the incubating medium.

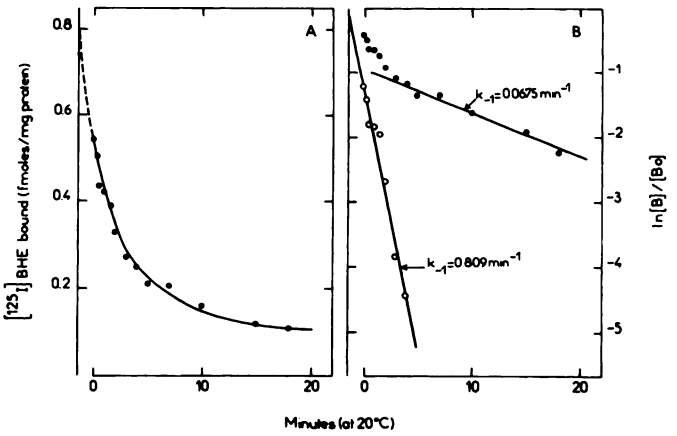

FIG. 3. *Dissociation of ^{125}I-BHE from rat cerebral cortex synaptosomes*

A. Synaptosomes were incubated to equilibrium for 15 min at 20° with ^{125}I-BHE (40 pM). Eledoisin (1 μM) was then added and the concentration of ^{125}I-BHE specifically bound with time was followed. Each point represents the mean of data obtained in three separate experiments. The initial *broken line* corresponds to the duration of centrifugation immediately following the addition of eledoisin (1 μM).

B. Results are represented as $\ln[(B)/(B_0)]$ versus time, where B_0 represents the concentration of ligand before dissociation and B the concentration of bound ligand at the time considered. The dissociation curve can be resolved into two different phases, the first one (○——○) being obtained by subtracting the contribution of the slow process (●——●).

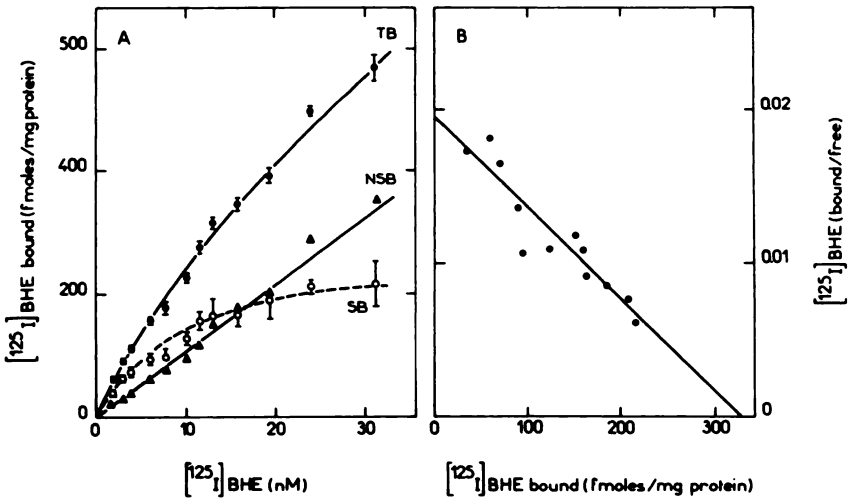

FIG. 4. *^{125}I-BHE binding to a crude synaptosomal preparation (P_2) from rat cerebral cortex as a function of the radiolabeled peptide concentration*

A. Radioactive ligand at different concentrations was incubated with synaptosomes (P_2) for 15 min at 20°c. Total (*TB*, ●), nonspecific (*NSB*, ▲), and specific (*SB*, ○) binding were estimated as indicated in the legend to Fig. 2. Each point represents the mean of quadruplicate determinations obtained in a single representative experiment.

B. Scatchard plot of specific ^{125}I-BHE binding data.

quadruplicate on different synaptosomal preparations, the apparent equilibrium dissociation constant (K_d) was estimated by Scatchard analysis (Fig. 4B) to be 15.3 ± 1.4 nM and the maximal number of ^{125}I-BHE binding sites (B_{max}) to be 297 ± 36 fmol/mg of protein. In addition, since Scatchard analysis may have some limitation when a relatively narrow range of ligand concentrations is used, data represented in Fig. 4A were analyzed further by the method of Homburger *et al.* (24). This consisted of a nonlinear least-squares curve-fitting procedure involving the Minuit routine for function analysis described by James and Roos (25). It can be concluded that ^{125}I-BHE is bound to a single site in the range of concentrations used (2–30 nM) (Fischer coefficient 53.25), and binding parameters were found to be 14.9 nM (K_d) and 328 fmol/mg of protein (B_{max}), values closely related to those estimated by the Scatchard analysis. When attempts were made to fit the data to a two-site model, this resulted in a reduction of the Fischer coefficient. Hill analysis of data from eledoisin competition with ^{125}I-BHE suggested that the ligand bound to noninteracting sites (n_H = 0.91 ± 0.02) with an estimate to the K_d = 14 nM.

Competitive inhibition of specific ^{125}I-BHE binding. Various tachykinins and related peptides inhibited competitively the high-affinity specific binding of ^{125}I-BHE to rat cerebral cortex synaptosomes in a concentration-dependent manner (Fig. 5; Table 1). The Hill coefficients for some of these compounds were close to unity, but for others the n_H values were less than unity (Table 1).

The most potent peptides were BHE, kassinin, and eledoisin; the concentrations required to inhibit 50% of the specific binding (IC_{50}) were 4.7, 5.9, and 14.0 nM, respectively. The inhibitory effects of physalaemin and substance P were only 25% and 10%, respectively, that of eledoisin. Apparent inhibition constants (K_i) were obtained according to the Cheng-Prusoff formula, $K_i = IC_{50}/[1 + (L_0/K_d)]$. Under these circumstances, IC_{50} and

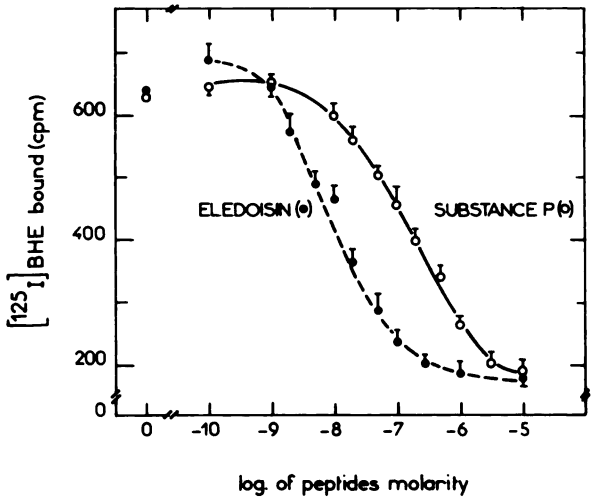

FIG. 5. *Competitive inhibition of ^{125}I-BHE binding with eledoisin and substance P*

Synaptosomes were incubated with ^{125}I-BHE (40 pM) and increasing concentrations of unlabeled competitive agents. Incubation conditions were those described under Materials and Methods. Each point represents the mean of quadruplicate determinations obtained in a single experiment. At least four independent experiments were performed to provide the data in Table 1.

apparent K_i values were found to be virtually the same, since the ligand concentration used in these studies was so low.

Finally, unrelated peptides, such as angiotensin II, neurotensin, bombesin, Phe-Met-Arg-Phe-NH_2 (FMRF amide), met-enkephalin, somatostatin, melanocyte-stimulating hormone release-inhibiting factor, endorphin, litorin, thyrotropin-releasing hormone, and cholecystokinin-8 sulfate (up to 1 or 10 μM) were devoid of inhibitory activity on ^{125}I-BHE binding.

Regional distribution. Specific ^{125}I-BHE binding, defined using unlabeled eledoisin (1 μM) was most extensive in cerebral cortex synaptosomes, but was found also in

TABLE 1

Inhibition characteristics of tachykinins on specific ^{125}I-BHE and ^{125}I-BHSP binding

The inhibitory effects of the unlabeled BHE and of various tachykinins on specific ^{125}I-BHE binding to rat cerebral cortex synaptosomes were determined as described in the legend to Fig. 7. Inhibition constants were obtained using the formula $K_i = IC_{50}/[1 + (L_0/k_d)]$, where L_0 represents the ^{125}I-BHE concentration in the incubating medium and K_d represents the dissociation constant calculated from equilibrium studies. IC_{50} and Hill (n_H) values were derived from original data plotted according to log $[(B_0 - B)/B]$ versus log peptide concentration, where B_0 represents the concentration of ligand specifically bound in the absence of competitor and B is the concentration of ligand specifically bound in the presence of competitive agents.

Data represent the mean of results obtained from 4–13 independent experiments performed in triplicate or quadruplicate. The standard deviation did not exceed 64% of the mean. Results were compared with those obtained in a previous study (14) in which the inhibitory effects of tachykinins on ^{125}I-BHSP specific binding were estimated on whole brain (minus cerebellum and cerebral cortex) synaptosomes.

Compound	^{125}I-BHE		^{125}I-BHSP		Ratio K_i BHE/ K_i BHSP
	K_i	n_H	K_i	n_H	
	nM		*nM*		
BHE	4.7	0.91	1500	1.03	0.003
Kassinin	5.9	0.92	980	0.83	0.006
Eledoisin	14	0.91	98	0.95	0.14
Physalaemin	56	0.78	2.1	0.81	27
Substance P	130	0.81	0.57	1.01	230

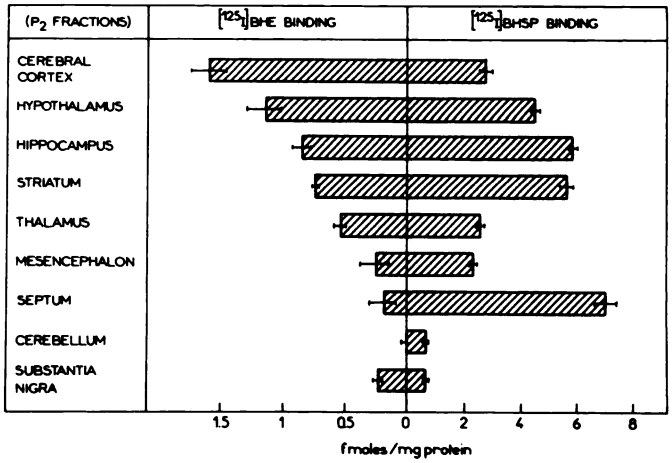

FIG. 6. *Regional distribution of specific ^{125}I-BHE binding activity in rat brain*

In a first experiment, rat brains were dissected into eight structures, and corresponding structures from several animals (3–10) were pooled to prepare P_2 fractions. In a second experiment, the substantia nigra was carefully dissected alone from serial slices (500 μm thick) of the mesencephalon. Tissues from 12 rats were used to prepare the corresponding P_2 fraction. Specific binding was determined following a 15-min incubation at 20° in the presence of ^{125}I-BHE (60 pM). Results are the means ± standard error of the mean from data obtained in three independent experiments performed in quadruplicate. Data corresponding to the regional distribution of specific ^{125}I-BHSP binding activity in the rat brain (given for comparison) have been published previously (15). The quantity of ^{125}I-BHSP used was 100 pM, and synaptosomes were incubated for 5 min at 20°.

synaptosomal preparations from eight other rat brain regions (Fig. 6). High-density specific binding occurred also in the hypothalamus, with intermediate binding (density) in the hippocampus, striatum, and thalamus and low-density in the mesencephalon, substantia nigra, and septum. The cerebellum was totally devoid of ^{125}I-BHE binding sites. The relative order of ^{125}I-BHE binding in these various brain regions differed from that demonstrated previously for ^{125}I-BHSP (Fig. 6) (15).

DISCUSSION

In previous studies, we have characterized central substance P receptors using ^{125}I-BHSP as a ligand and a synaptosomal preparation, which allows the minimization of unspecific binding and peptidasic inactivation of the ligand (14, 15). ^{125}I-BHSP characteristics were identical with those previously obtained using murine intact embryonic mesencephalic neurons grown in primary cultures (13). These data have been confirmed by other authors using membrane preparations or slices with ^{3}H-labeled substance P as the ligand (16, 18). The present results, also obtained using synaptosomes and ^{125}I-BHE, indicate that tachykinin receptors other than substance P receptors are present in rat brain. Indeed, specific ^{125}I-BHE binding sites exhibited pharmacological characteristics and a regional distribution which differed from those of ^{125}I-BHSP binding sites.

The characteristics of ^{125}I-BHE binding to synaptosomes prepared from the rat cerebral cortex were determined following incubation at 20° for 15 min in the presence of bacitracin, conditions which resulted in a minimal peptidasic cleavage of the ligand. Under such conditions, high-affinity ^{125}I-BHE binding to synaptosomes was linearly dependent on the tissue concentration, and it was specific, saturable, and reversible. Furthermore, the ligand binding was temperature-dependent. As previously shown for ^{125}I-BHSP binding to synaptosomes, ^{125}I-BHE binding was unaltered by ouabain in concentrations (0.1 or 1 mM) that inhibited [^{3}H] dopamine or substance P[5–11] uptake (26). This suggested that the ligand was not transported by a high-affinity uptake process within the "pinched off" nerve endings.

Analysis of the apparent rate of association for ^{125}I-BHE binding to cerebral cortex synaptosomes indicated that the ligand-receptor interaction was extremely rapid (apparent $k_{+1} = 5.2 \times 10^9$ M^{-1} min^{-1}). This estimate was obtained assuming that the ligand-receptor interaction obeys pseudo-first order reaction kinetics. Obviously, this simplification for the estimation of apparent K_{+1} is subject to error (27). Theoretically, determination of the dissociation rate constant is much less complicated, since k_{-1} is a first-order rate constant (27). However, the dissociation of ^{125}I-BHE appeared to be complex, since the displacement of the ligand bound at equilibrium by unlabeled eledoisin was biphasic. Thus the use of the rapid phase of dissociation only for the estimation of K_d from kinetic studies would result in further error. Several factors might account for such a biphasic phenomenon, which has been observed previously with other ligands (14, 28) and discussed extensively by Cuatrecasas and Hollenberg (29).

Scatchard analysis of the concentration-dependent,

saturable, specific ^{125}I-BHE binding was linear over the narrow range of concentrations used. Estimation of the binding parameters revealed a high ^{125}I-BHE binding affinity (apparent K_d = 15.3 nM) and a relatively large receptor density of 297 fmol/mg of protein on cerebral cortex synaptosomes. However, there are fundamental problems associated with the use of Scatchard analysis to estimate binding parameters, as emphasized by Klotz (30). Therefore, we analyzed our saturation data (Fig. 4A) by nonlinear curve fitting to provide a further estimate of binding parameters. All of the estimates of K_d using Scatchard analysis, nonlinear curve fitting, and Hill analysis of competition studies were found to be in the same range (14.0–15.3 nM). It becomes apparent, therefore, that the discrepancy of the K_d value obtained from reaction kinetic studies (0.16 nM) results from problems connected with measurements of the apparent rates of association and dissociation. Interestingly, the apparent dissociation constant from equilibrium studies for ^{125}I-BHE binding to cerebral cortex synaptosomes was different from that obtained for ^{125}I-BHSP binding to rat brain or cerebral cortex synaptosomes (K_d values were 0.47 nM and 1.1 nM, respectively) (14, 15).

In competition experiments, eledoisin and kassinin were more potent than substance P and physalaemin in competing with ^{125}I-BHE for binding to cerebral cortex synaptosomes. This is in contrast with the rank order of potency of these tachykinins for inhibiting the binding of ^{125}I-BHSP to synaptosomes from whole brain or from specific structures such as the hypothalamus, hippocampus, and dorsal spinal cord (14, 15) and to membrane preparations of the cerebral cortex (17). The marked differences in the values of the ratio of the apparent K_i values for inhibition of ^{125}I-BHE binding and ^{125}I-BHSP binding for each tachykinin further indicate the existence of two distinct classes of tachykinin-binding sites (Table 1). This conclusion is further justified by our regional study of the distribution of ^{125}I-BHE binding sites and its comparison to that obtained for ^{125}I-BHSP binding (15). Among the various structures tested, the cerebral cortex, which contains a moderate amount of ^{125}I-BHSP binding sites (B_{max} = 14.4 fmol/mg of protein), was found to be the structure exhibiting the highest number of ^{125}I-BHE binding sites (B_{max} = 297 fmol/mg of protein). In contrast, the septum, which is particularly rich in ^{125}I-BHSP binding sites, was almost devoid of ^{125}I-BHE binding sites. Interestingly, no ^{125}I-BHE binding was found in the cerebellum, a structure in which ^{125}I-BHSP binding is also minimal. Since, surprisingly, very small quantities of ^{125}I-BHSP binding sites were found in the substantia nigra, in agreement with results obtained by other workers (18, 31), attempts were also made to estimate ^{125}I-BHE binding in this structure. ^{125}I-BHE binding in the substantia nigra represented only 15% of that observed in the cerebral cortex.

Taking into account the differences in the apparent K_d values, the potency of various tachykinins in competitive studies, and the regional distribution, ^{125}I-BHE binding sites appear to be distinct from ^{125}I-BHSP binding sites. This indicates that, besides substance P receptors, receptors of the "eledoisin" type are present in rat brain. These observations are in agreement with results obtained in behavioral and electrophysiological studies in the rat and in other species. For instance, eledoisin was shown to induce a more pronounced stimulatory effect than substance P on drinking behavior in the pigeon (10). Furthermore, following intracerebral injection, eledoisin also is more potent than substance P in inhibiting the angiotensin II-evoked dipsogenic response in the rat (11) or in eliciting reciprocal hind limb scratching in the mouse (12). It also should be recalled that eledoisin is 10 times more active than substance P in depolarizing spinal motor neurons in the frog (8). Finally, recent investigations made by extracellular recordings from locus coeruleus neurons contained in a pontine slice preparation from the rat have indicated that the excitatory effect of eledoisin is 5 times more potent than that of substance P (9). The present results are also in agreement with several observations made in the periphery that have led to the concept of SP-P and SP-E subtypes of receptors (7). In this context, recent studies have provided further arguments for the existence of different tachykinin receptors. For instance, there is no cross-desensitization between eledoisin and substance P in the dog carotid artery (32) and in the guinea pig ileum (33). In addition, both the kassinin response in the guinea pig ileum and the eledoisin response observed in the guinea pig urinary bladder were inhibited in the presence of the alkylating agent phenoxybenzamine, whereas this latter compound was less effective or ineffective on substance P or physalaemin-evoked responses (34, 35). Finally, although the potent substance P antagonist (D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11})-substance P inhibited eledoisin and substance P responses on several smooth muscle preparations, it was without effect on the responses evoked by these peptides in the hamster urinary bladder (36, 37), thus suggesting the existence of another subtype of tachykinin receptor in this tissue.

In conclusion, the present study indicates that rat brain synaptosomes—particularly those from the cerebral cortex—have a high binding capacity for a labeled analogue of eledoisin. Several properties of specific ^{125}I-BHE binding strongly suggest that this ligand allows the characterization of a tachykinin receptor which is distinct from the substance P receptor labeled with ^{125}I-BHSP or ^{3}H-labeled substance P. Since substance K, a peptide structurally related to kassinin, is present in one of the substance P precursors recently isolated in the caudate nucleus (6), it could represent the endogenous tachykinin interacting with the receptor labeled by ^{125}I-BHE. Indeed, kassinin was more potent than eledoisin in inhibiting specific ^{125}I-BHE binding. Undoubtedly, ^{125}I-BHE appears to be a suitable ligand for further characterization of subtypes of tachykinin receptors in the central nervous system.

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REFERENCES

1. Erspamer, V. The tachykinin peptide family. *Trends Neurosci.* **4**:267–269 (1981).
2. Lazarus, L. H., R. Linnoila, O. Hernandez, and R. Di Augustine. A neuropeptide in mammalian tissues with physalaemin like immunoreactivity. *Nature (Lond.)* **287**:555–558 (1980).
3. Lazarus, L. H., R. P. Di Augustine, G. D. Jahnke, and O. Hernandez. Physalaemin: an amphibian tachykinin in human lung small-cell carcinoma. *Science (Wash. D. C.)* **219**:79–81 (1983).
4. Kimura, S., M. Okada, Y. Sugita, I. Kanazawa, and E. Munekata. Novel neuropeptides, neurokinin α and β, isolated from porcine spinal cord. *Proc. Jpn. Acad.* **56**:101–104 (1983).
5. Kangawa, K., N. Minamino, A. Fukuda, and H. Matsuo. Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. *Biochem. Biophys. Res. Commun.* **114**:533–540 (1983).
6. Nawa, H., T. Hirose, H. Takashima, S. Inayama, and S. Nakanishi. Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. *Nature (Lond.)* **306**:32–36 (1983).
7. Iversen, L. L., M. R. Hanley, B. E. B. Sandberg, C. M. Lee, R. D. Pinnock, and S. P. Watson. Substance P receptors in the nervous system and possible receptor subtypes. *Ciba Found. Symp.* **91**:186–195 (1982).
8. Konishi, S., and M. Otsuka. The effects of substance P and other peptides on spinal neurons of the frog. *Brain Res.* **65**:397–410 (1974).
9. Cheeseman, H. J., R. D. Pinnock, and G. Henderson. Substance P excitation of rat locus coeruleus neurones. *Eur. J. Pharmacol.* **94**:93–99 (1983).
10. Evered, M. D., J. T. Fitzsimons, and G. De Caro. Drinking behaviour induced by intracranial injections of eledoisin and substance P in the pigeon. *Nature (Lond.)* **268**:332–333 (1977).
11. De Caro, G., M. Massi, and L. G. Micossi. Modifications of drinking behaviour and of arterial blood pressure induced by tachykinins in rats and pigeons. *Psychopharmacology* **68**:243–247 (1980).
12. Share, N. N., and A. Rackham. Intracerebral substance P in mice: behavioral effects and narcotic agents. *Brain Res.* **211**:379–386 (1981).
13. Beaujouan, J. C., Y. Torrens, A. Herbet, M.-C. Daguet, J. Glowinski, and A. Prochiantz. Specific binding of an immunoreactive and biologically active ^{125}I-labeled substance P derivative to mouse mesencephalic cells in primary culture. *Mol. Pharmacol.* **22**:48–55 (1982).
14. Viger, A., J. C. Beaujouan, Y. Torrens, and J. Glowinski. Specific binding of a ^{125}I-substance P derivative to rat brain synaptosomes. *J. Neurochem.* **40**:1030–1039 (1983).
15. Torrens, Y., J. C. Beaujouan, A. Viger, and J. Glowinski. Properties of a ^{125}I-substance P derivative binding to synaptosomes from various brain structures and the spinal cord of the rat. *Naunyn Schmiedeberg's Arch. Pharmacol.* **324**:134–139 (1983).
16. Bittiger H. Discussion of substance P receptors. *Ciba Found. Symp.* **91**:195–205 (1982).
17. Cascieri, M. A., and T. Liang. Characterization of the substance P receptor in rat brain cortex membranes and the inhibition of radioligand binding by guanine nucleotides. *J. Biol. Chem.* **258**:5158–5164 (1983).
18. Quirion, R., C. W. Shults, T. W. Moody, C. B. Pert, T. N. Chase, and T. L. O'Donohue. Autoradiographic distribution of substance P receptors in rat central nervous system. *Nature (Lond.)* **303**:714–716 (1983).
19. Michelot, R., H. Gozlan, J. C. Beaujouan, M. J. Besson, Y. Torrens, and J. Glowinski. Synthesis and biological activities of substance P iodinated derivatives. *Biochem. Biophys. Res. Commun.* **95**:491–498 (1980).
20. Gray, E. G., and V. P. Whittaker. The isolation of nerve endings from brain: an electron microscopic study of cell fragments derivated by homogenization and centrifugation. *J. Anat. (Lond.)* **96**:79–88 (1962).
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
22. Coyle, J. T., and S. H. Snyder. Antiparkinsonian drugs: inhibition of dopamine uptake in the corpus striatum as a possible mechanism of action. *Science (Wash. D. C.)* **166**:899–901 (1969).
23. Frost, P. A., and R. G. Pearson. *Kinetics and Mechanism: A Study of Homogeneous Chemical Reactions.* John Wiley and Sons, New York (1961).
24. Homburger, V., M. Lucas, E. Rosenbaum, G. Vassent, and J. Bockaert. Presence of both $beta_1$ and $beta_2$-adrenergic receptors in a single cell type. *Mol. Pharmacol.* **20**:463–469 (1981).
25. James, F., and M. Roos. Minuit: a system for function minimization and analysis of the parameter errors and correlations. *Comput. Physics Commun.* **10**:343–367 (1975).
26. Nakata, Y., Y. Kusaka, H. Yajima, and T. Segawa. Active uptake of substance P carboxy-terminal heptapeptide (5–11) into rat brain and rabbit spinal cord slices. *J. Neurochem.* **37**:1529–1534 (1981).
27. Weiland, G. A., and P. B. Molinoff. Quantitative analysis of drug-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29**:313–330 (1981).
28. Leysen, J. E., W. Gommeren, and C. J. E. Niemegeers. (^{3}H)-Sufentanil, a superior ligand for μ-opiate receptors: binding properties and regional distribution in rat brain and spinal cord. *Eur. J. Pharmacol.* **87**:209–227 (1983).
29. Cuatrecasas, P., and M. D. Hollenberg. Membrane receptors and hormone action. *Adv. Protein Chem.* **30**:251–451 (1976).
30. Klotz, I. M. Numbers of receptor sites for scatchard graphs: facts and fantasies. *Science (Wash. D. C.)* **217**:1247–1249 (1982).
31. Shults, C. W., R. Quirion, R. T. Jensen, T. W. Moody, T. L. O'Donohue, and T. N. Chase. Autoradiographic localization of substance P receptors using ^{125}I-substance P. Peptides **3**:1073–1075 (1982).
32. Couture, R., and D. Regoli. Mini review: smooth muscle pharmacology of substance P. *Pharmacology* **24**:1–25 (1982).
33. Lee, C. M., L. L. Iversen, M. R. Hanley, and B. E. B. Sandberg. The possible existence of multiple receptors for substance P. *Naunyn Schmiedeberg's Arch. Pharmacol.* **318**:281–287 (1982).
34. Lin, C. W., and J. M. Musacchio. The determination of dissociation constants for substance P and substance P analogues in the guinea pig ileum by pharmacological procedures. *Mol. Pharmacol.* **23**:558–562 (1983).
35. Growcott, J. W., A. Jamieson, A. V. Tarpey, and L. D. Topham. Further evidence for multiple tachykinin receptors. *Eur. J. Pharmacol.* **86**:59–64 (1983).
36. Rosell, S., U. Björkroth, J. C. Xu, and K. Folkers. The pharmacological profile of a substance P (SP) antagonist: evidence for the existence of subpopulations of SP receptors. *Acta Physiol. Scand.* **117**:445–449 (1983).
37. Watson, S. P. Pharmacological characterization of a substance P antagonist, (D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11})-substance P. *Br. J. Pharmacol.* **80**:205–209 (1983).

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