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Specific Binding of an Immunoreactive and Biologically Active ^{125}I-Labeled Substance P Derivative to Mouse Mesencephalic Cells in Primary Culture

J. C. BEAUJOUAN, Y. TORRENS, A. HERBET, M.-C. DAGUET, J. GLOWINSKI, AND A. PROCHIANTZ

Groupe NB, Institut National de la Santé et de la Recherche Médicale U.114, Collège de France, 75231 Paris Cedex 05, France

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SUMMARY

Binding characteristics of ^{125}I-labeled Bolton-Hunter substance P ([^{125}I]BHSP), a radioactive analogue of substance P, were studied with mesencephalic primary cultures prepared from embryonic mouse brain. Nonspecific binding represented no more than 20% of the total binding observed on the cells. In contrast, significant specific binding—saturable, reversible, and temperature-dependent—was demonstrated. Scatchard analysis of concentration-dependent binding saturation indicates a single population of noninteracting sites with a high affinity (K_d = 169 pM). Substance P and different substance P analogues were tested for their competitive potencies with regard to [^{125}I]BHSP binding. BHSP itself, substance P, (Tyr^8)-substance P, and (nor-Leu^{11})-substance P strongly inhibited the binding. Good inhibition was also obtained with physalaemin and eledoisin, two peptides structurally related to substance P. When substance P C-terminal fragments were tested for their ability to compete with [^{125}I]BHSP binding, a good relationship was found between competitive activity and peptide length. Regional distribution of [^{125}I]BHSP binding sites was found using primary cultures obtained from different regions of embryonic mouse brain. Mesencephalic, hypothalamic, and striatal cultures had the highest [^{125}I]BHSP binding capacities, whereas cortical, hippocampal, and cerebellar cells shared only little binding activity. Finally, when mesencephalic cells were grown under conditions impairing glial development, [^{125}I]BHSP binding was not affected, demonstrating that binding sites are located on neuronal cells.

INTRODUCTION

Several attempts have been made to characterize substance P receptors in brain and peripheral tissues. Nakata *et al.* (1) first described binding of ^{3}H-labeled substance P to membrane preparations from rabbit brain. However, they observed no antagonism with physalaemin, a naturally occurring substance P-related peptide, and different authors who used similar experimental conditions failed to replicate their results (2, 3). Mayer *et al.* (4) reported the existence of specific binding of ^{125}I-labeled (Tyr^8)-substance P to a synaptic vesicle fraction from rat brain, but its relevance to the known biological activity of the peptide is questionable since substance P C-terminal fragments inhibited ^{125}I-labeled (Tyr^8)-substance P binding only when used at micromolar concentrations. No antagonism was observed with eledoisin, and physalaemin enhanced rather than inhibited binding of the labeled substance P analogue. Using 4-^{3}H-labeled (Phe^8)-substance P, Hanley *et al.* (2) indicated that this ligand binds specifically and reversibly to a saturable population of sites in rat brain particulate fractions. This specific binding was antagonized by structurally related peptides and substance P C-terminal fragments exhibiting biological activity. Moreover, a parallelism was found between the distributions of ^{3}H-labeled substance P binding sites and substance P immunoreactivity in several brain structures. However, in the study by Hanley *et al.* (2) most of the experiments were performed with a concentration of ^{3}H-labeled substance P (2 nM) at which relatively high nonspecific binding occurs, and specific binding was arbitrarily defined by the displacement induced with 10 μM substance P. Studies on substance P receptors were also carried out on peripheral dispersed cells. Thus, specific high-affinity binding sites were detected on pancreatic or parotid acinar cells using [^{125}I]physalaemin (5, 6), ^{125}I-labeled (Tyr^8)-substance P (7), and [^{125}I]BHSP[1] (8). In their study on dispersed acinar cells from guinea pig pancreas, Jensen and Gardner (5) observed that [^{125}I]

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[1] The abbreviation used is: [^{125}I]BHSP, ^{125}I-labeled Bolton-Hunter substance P.

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physalaemin binding was saturable, temperature-dependent, and reversible and that it was restricted to a single class of sites interacting with physalaemin, eledoisin, and substance P.

The present study was undertaken to explore further the properties of central substance P receptors. Since the mesencephalon is densely innervated by the habenulo-interpeduncular and striato-nigral substance P pathways (for a review see ref. 3), we first examined the ability of [^{125}I]BHSP to bind to mouse embryonic mesencephalic cells grown in primary culture (9). Our results revealed the presence on the cells of high-affinity specific binding of this immunoreactive and biologically active ligand (10). This binding was saturable, reversible, and competitively inhibited by substance P, C-terminal fragments of substance P exhibiting biological activity, physalaemin, and eledoisin. Furthermore, [^{125}I]BHSP high-affinity specific binding was still detected when mesencephalic cells were grown under culture conditions in which glial cells could be virtually eliminated, indicating that binding occurs on neuronal elements. Finally, comparative studies conducted with cell cultures obtained from various brain structures revealed that embryonic mesencephalic and hypothalamic cells contain the highest levels of [^{125}I] BHSP specific binding sites.

MATERIALS AND METHODS

Monolayer primary cultures from various brain structures. Mesencephalic cells (6×10^5) from 14-day-old Swiss mouse embryos (Iffa Credo) were dissociated and plated in the presence (10%) or absence of serum in multi-tray Falcon culture dishes (16-mm diameter wells) precoated with polyornithine, 1.5 μg/ml (40,000 mol wt; Sigma Chemical Company, St. Louis, Mo.) as previously described (11). Briefly stated, the medium consisted of a mixture (1:1) of minimal essential medium and F-12 nutrient (GIBCO, Grand Island Biological Company, Grand Island, N. Y.) supplemented with glucose (33 mM, 6 mg/ml), glutamine (2 mM), sodium bicarbonate (3 mM), and fetal calf serum (10%, Serumaid). In order to eliminate glial elements, mesencephalic cells were grown in the absence of serum using a medium which was conditioned for 48 hr on glial cells and supplemented with insulin (25 μg/ml; Sigma Chemical Company), transferrin (100 μg/ml; Sigma), progesterone (20 nM; Sigma), putrescine (60 μM; Sigma), and selenium salt (30 nM; Merck). This conditioned medium was filtered (Millex; Millipore) before use (12). Striatal, hippocampal, hypothalamic, cortical, and cerebellar cells were prepared and grown similarly but they originated from 15-day-old embryos. In all cases before binding assays were carried out the cells were cultured for 5 days without changing the medium.

Binding assays. The labeled ligand used was obtained by coupling the ^{125}I-labeled Bolton-Hunter reagent (Amersham; monoiodo-derivative, 1500 Ci/mmole) to substance P (Peninsula Laboratories, San Carlos, Calif.) as previously described (10). This ligand is immunoreactive and biologically active (10). Routinely, 5-day-old cultures were incubated for 30 min at 20° with [^{125}I] BHSP (10,000 cpm = 40 pM) in the presence or absence of substance P (1 μM). The incubating medium (0.2 ml) consisted of a Krebs-Ringer phosphate buffer (NaCl, 120 mM; KCl, 4.8 mM; $CaCl_2$, 1.2 mM; $MgSO_4$, 1.2 mM, and NaH_2PO_4/Na_2HPO_4, 15.6 mM; pH 7.4) containing bovine serum albumin (0.4 mg/ml; Calbiochem, San Diego, Calif.), bacitracin (30 μg/ml; Sigma), and glucose (6 mg/ml). At the end of the incubation period the supernatant was discarded and the cells were washed three times with cold (4°) Krebs-Ringer phosphate buffer. Cells were then scraped off using Triton X-100 (0.25 ml, 0.2%) containing bovine serum albumin (1 mg/ml). Radioactivity bound to tissues was estimated with a Packard Gamma counter. All assays were made in quadruplicate.

In some experiments, in order to verify that [^{125}I]BHSP was not inactivated by peptidases during the 30-min incubation period, the identity of the radioactive material recovered in the supernatant at the end of the incubation period was checked by high-pressure liquid chromatography using C_{18} μ-Bondapak columns (Waters Instruments, Rochester, Minn.) and methanol/ammonium acetate (50 mM, pH 4.0) (540:460) as solvent. Most of the radioactivity (>90%) migrated as authentic [^{125}I]BHSP. Furthermore, it was verified that the radioactive ligand present in the supernatant at the end of the incubation period was still able to bind specifically to mesencephalic cells. Similar results were obtained when cells were grown in the presence or absence of serum.

RESULTS

Binding of substance P radioactive analogue to mesencephalic cells. Substantial binding was detected when mesencephalic cells grown for 5 days *in vitro* were incubated with [^{125}I]BHSP for 30 min at 20°. Competition studies with increasing concentrations of unlabeled sub-

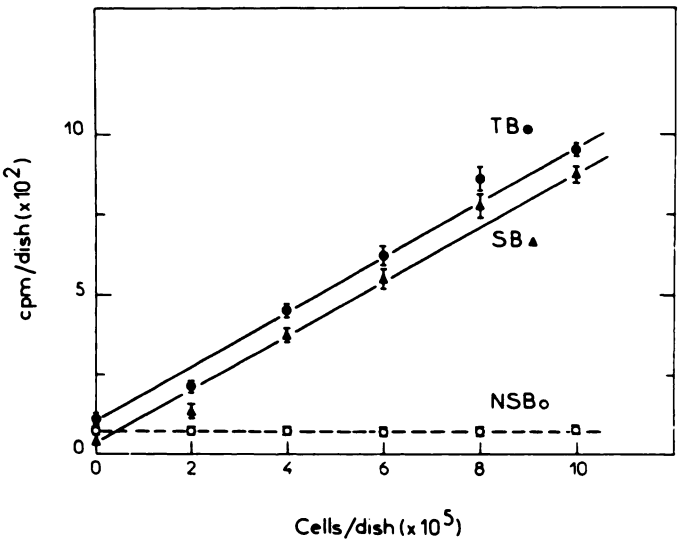

FIG. 1. *[^{125}I]BHSP binding at various mesencephalic cell concentrations*

Mesencephalic cells grown for 5 days at different concentrations were exposed to the ligand (31 pM) for 30 min at 20° as described under Materials and Methods. Nonspecific binding (○– – –○) was determined by co-incubating the radioactive ligand with 1 μM substance P. Specific values (▲——▲) were calculated by substracting nonspecific binding from total binding (●——●). All values are means ± standard error of the mean of four determinations.

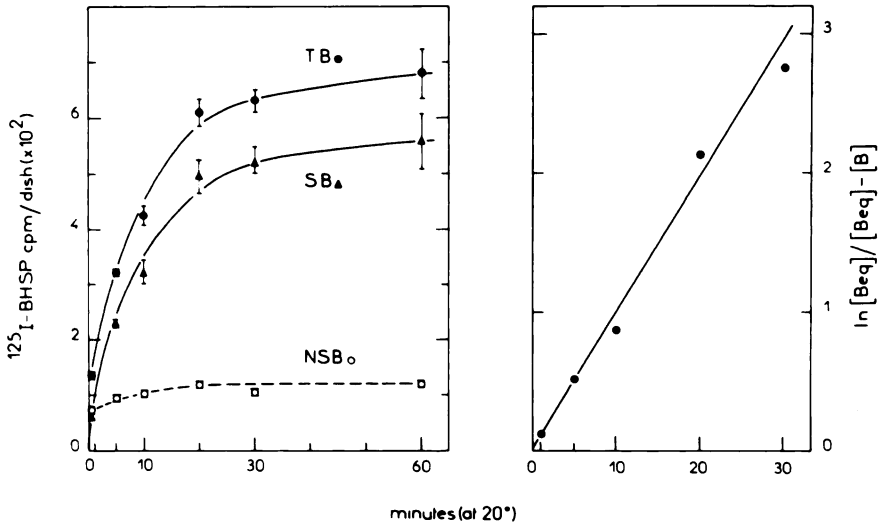

FIG. 2. *Association time course of [^{125}I]BHSP binding on mesencephalic cells*

Mesencephalic cells grown for 5 days were incubated with the ligand (37 pM) for different time periods. Left, nonspecific (*NSB*) (○- - -○), total (*TB*) (●——●), and specific (*SB*) (▲——▲) binding values. All values are means ± standard error of the mean of four determinations. Right, the data obtained for specific binding are plotted as ln $[B_{eq}]/[B_{eq}]-[B]$ versus time, where $[B_{eq}]$ represents the concentration of ligand bound at equilibrium and $[B]$ represents the amount of ligand bound at the time considered.

stance P showed that 1 μM substance P was sufficient to inhibit specific binding completely so that nonspecific binding could be measured (Fig. 6). Since total and specific binding increased linearly with mesencephalic cell concentrations ranging from 2×10^5 to 10^6 cells/dish (Fig. 1), 6×10^5 cells were routinely used for binding assays. Specific binding of [^{125}I]BHSP was about 80% of the total binding and represented 6% of the total number of counts per minute added to the incubating medium. In fact, most of the [^{125}I]BHSP binding to the cells was specific in nature since nonspecific binding was not dependent on the number of cells, suggesting that it also occurred on the cell culture dishes. [^{125}I]BHSP specific binding appeared to be temperature-dependent since it was decreased by 75% when incubation was carried out at 4°.

Kinetic binding data. The time course of [^{125}I]BHSP association to mouse embryonic mesencephalic cells in dissociated primary cultures is illustrated in Fig. 2. Nonspecific binding was maximal within 5 min. In contrast, total and specific binding increased slowly with time up to 20 min, when a plateau was reached. Specific binding was reversible, since dissociation of the labeled ligand was observed when fresh medium containing 1 μM unlabeled substance P was substituted for the culture medium after 30 min of incubation at 20° (Fig. 3). By using the procedure of Kitabgi *et al.* (13), the association ($k_1 = 4 \times 10^6$ M^{-1} sec^{-1}) and dissociation (initial slope $k_{-1} = 3.2 \times 10^{-4}$ sec^{-1}) rate constants were calculated and allowed the determination of the equilibrium dissociation constant, which was found to be 80 pM ($K_d = k_{-1}/k_{+1}$).

Equilibrium binding studies. When the concentration

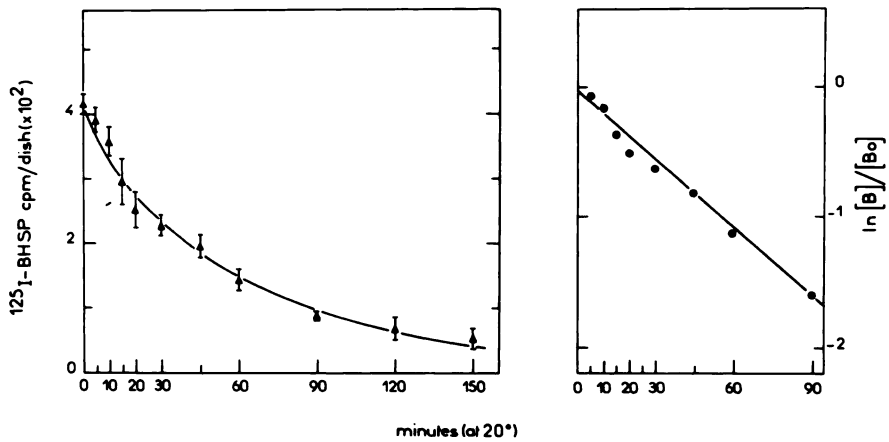

FIG. 3. *Dissociation time course of [^{125}I]BHSP specifically bound to mesencephalic cells*

Radioactive ligand (27 pM) was incubated for 30 min at 20° with mesencephalic cells after 5 days in culture. The incubation medium was then removed and replaced by fresh medium containing 1 μM substance P. Left, the concentration of bound ligand was followed with time. Each point represents the mean of four determinations ± standard error of the mean. Right, the results are represented as ln $[B]/[B_0]$ versus time, where $[B_0]$ represents the concentration of ligand bound before dissociation and $[B]$ represents the concentration of bound ligand at the time considered.

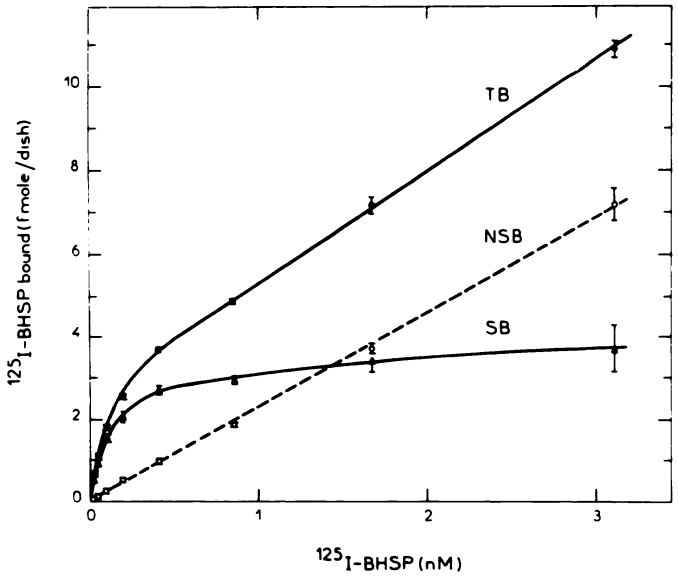

FIG. 4. *[^{125}I]BHSP binding on mesencephalic cells as a function of radiolabeled peptide concentration*

Radioactive ligand at different concentrations was incubated with mesencephalic cells grown for 5 days *in vitro*. Total (*TB*) (●——●), nonspecific (*NSB*) (○- - -○), and specific (*SB*) (▲——▲) binding was measured as indicated in the legend to Fig. 1. Each point represents the mean ± standard error of the mean of four determinations.

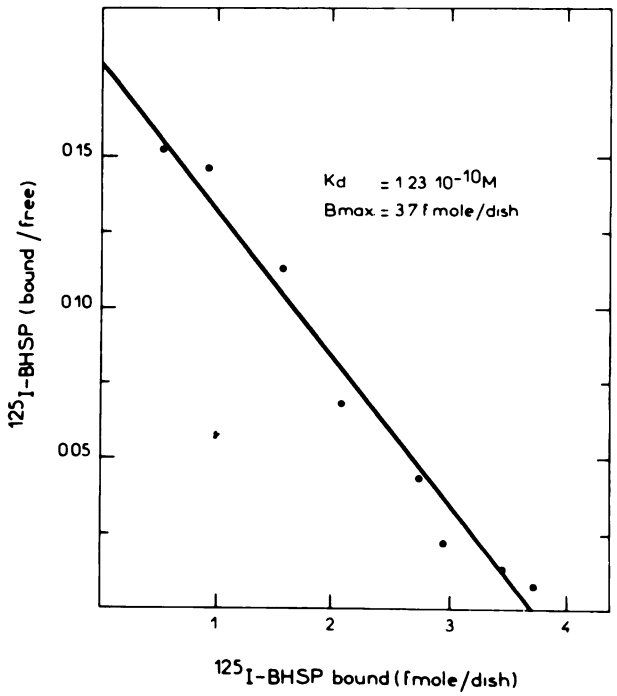

FIG. 5. *Scatchard plot of specific [^{125}I]BHSP binding on mesencephalic cells*

Mesencephalic cells after 5 days *in vitro* were incubated in the presence of the ligand as described under Materials and Methods and in the legend to Fig. 1. Points shown are those obtained in a single experiment, each point representing the mean value of four determinations.

dependence of [^{125}I]BHSP binding to mesencephalic cells was investigated, nonspecific binding was found to be a linear function of the concentration of labeled ligand (Fig. 4). In contrast, [^{125}I]BHSP specific binding was saturable. Scatchard plots of [^{125}I]BHSP binding data were linear, a finding compatible with the presence of a single class of binding sites (Fig. 5). Average values for K_d and B_{max} calculated from results obtained in four experiments were 169 ± 22 pM and 2.72 ± 0.35 fmoles/dish, respectively. The Hill plot of the data (n_H = 1.00 ± 0.03; n = 4) indicated that [^{125}I]BHSP was bound to noninteracting sites.

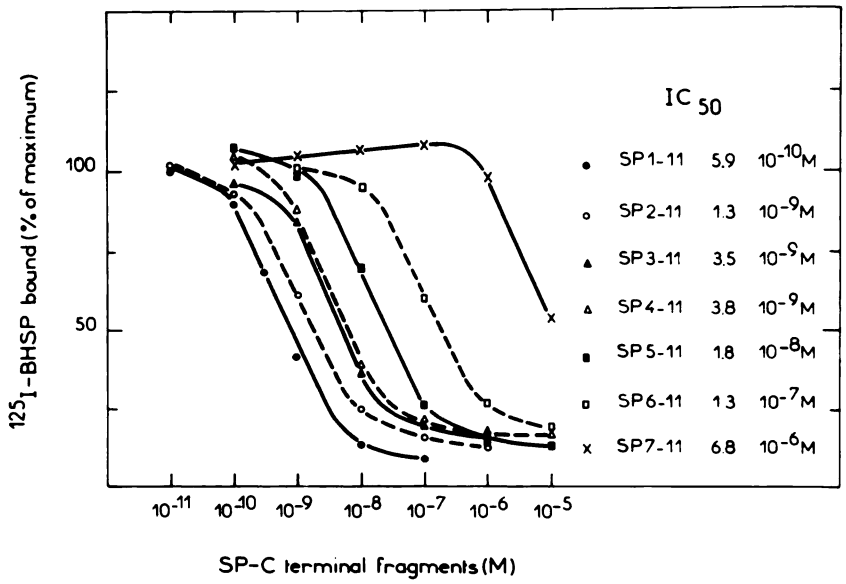

FIG. 6. *Competitive inhibition of [^{125}I]BHSP binding by unlabeled substance P (SP) and its shorter C-terminal fragments*

Radioactive ligand and increasing concentrations of cold competitors were mixed and incubated with 5 day-old cultures of mesencephalic cells. Incubation conditions are described under Materials and Methods. Results are expressed as the percentage binding of [^{125}I]BHSP alone. IC_{50} values for which 50% inhibition was obtained are indicated for each tested compound. All points represent the mean of four determinations made within a single experiment. For all compounds tested, the experiment was repeated two or three times and the results obtained were similar to those represented in the figure. In each experiment the IC_{50} value of substance P and the compound tested were compared.

Competitive inhibition of [^{125}I]BHSP binding. The ability of substance P and of its C-terminal fragments exhibiting biological activity to compete with [^{125}I]BHSP binding was first analyzed. Results obtained indicated that substance P inhibits [^{125}I]BHSP binding in a competitive manner and with very high affinity. Fifty per cent of specific [^{125}I]BHSP binding (IC_{50}) was inhibited with a substance P concentration equal to 374 ± 57 pM (n = 6). When substance P C-terminal fragments were used, a relationship was found between their length and their ability to compete with [^{125}I]BHSP binding (Fig. 6). For example, substance P^{3-11} and substance P^{6-11} inhibited [^{125}I]BHSP binding with one-tenth and one-two hundredth the potency of substance P, respectively. Substance P^{7-11}, the pentapeptide which exhibits very low biological activity in various tests (14, 15) was much less potent than other substance P C-terminal fragments in inhibiting competitively [^{125}I]BHSP binding.

Further experiments indicated that synthetic substance P analogues used as ligands after appropriate labeling were more potent [(Tyr^8)-substance P] or as potent (BHSP) in inhibiting [^{125}I]BHSP binding (Fig. 7). However, this was not the case with (nor-Leu^{11})-substance P and with two natural substance P-related peptides, physalaemin and eledoisin, which were less potent. Nevertheless, the latter three compounds were at least as active as substance P^{5-11} (Fig. 7).

When compounds devoid of biological activity, such as free acid substance P or the NH_2-terminal tetrapeptide of substance P, were tested they did not inhibit [^{125}I]BHSP binding even when used at a concentration of 1 μM. Similar negative results were obtained with peptides not related to substance P, such as carnosine, thyrotropin-releasing hormone, melanocyte-stimulating hormone release-inhibiting factor, Met-enkephalin, Leu-enkephalin, luteinizing hormone-releasing hormone, and vasoactive intestinal polypeptide (all of which were tested at 1 μM).

[^{125}I]BHSP binding to cells from various brain structures. The binding of [^{125}I]BHSP to mesencephalic cells was compared with that observed with cells obtained from different parts of embryonic brain and grown under the same conditions (Fig. 8). The highest binding per dish was found with mesencephalic and hypothalamic cultures. Striatal cultures were also rich in [^{125}I]BHSP binding sites. Lower but consistent binding was detected with cortical, hippocampal, and cerebellar cells. Nonspecific binding was identical with all cell types tested.

Neuronal localization of [^{125}I]BHSP binding sites. Mesencephalic cells can also be grown using a conditioned synthetic medium which avoids the proliferation of glial cells. Previous studies have shown that under these conditions the number of glial elements is very much reduced and that the neurons constitute more than 95% of the total cell population (16). When [^{125}I]BHSP was incubated for 30 min at 20° with dissociated embryonic mesencephalic neurons grown for up to 5 days in the absence of serum, specific [^{125}I]BHSP binding was comparable to that found with mesencephalic cells grown in the presence of serum. Nonspecific binding was almost identical. Kinetic analysis revealed that the characteristics of [^{125}I]BHSP binding on mesencephalic neurons were similar to those obtained with a mixed population of neurons and glial cells (Table 1).

DISCUSSION

The present results indicate that embryonic mesencephalic cells in primary culture have the capacity to bind specifically a labeled substance P analogue which exhibits a biological activity identical with that of substance P on guinea pig ileum (10). The characteristics of [^{125}I]BHSP specific binding on mesencephalic cells

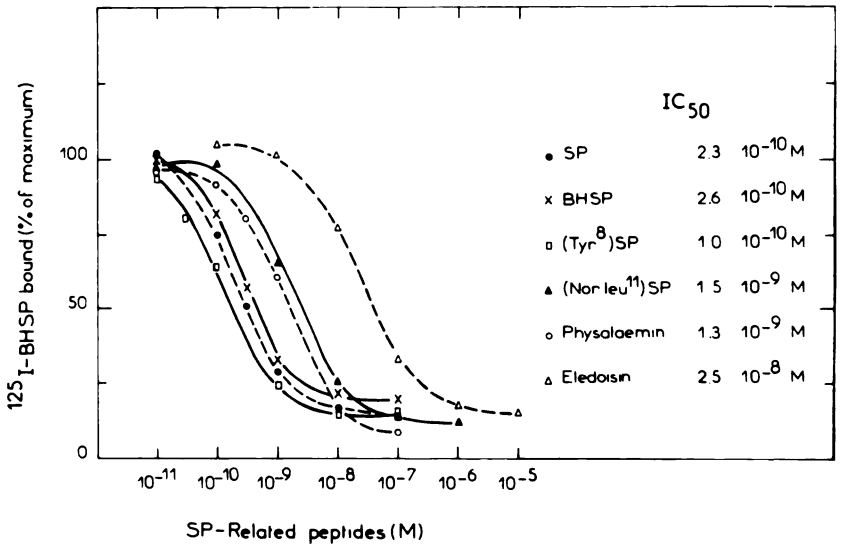

FIG. 7. *Competitive inhibition of [^{125}I]BHSP binding by substance P (SP) and structure-related peptides or derivative*

As in Fig. 6, radioactive ligand and increasing concentrations of the tested compounds were mixed before addition to the cell culture dishes containing mesencephalic cells after 5 days *in vitro*. Experimental conditions were as described under Materials and Methods. Results are expressed as in Fig. 6. IC_{50} values for which 50% inhibition was obtained are indicated for each tested compound. All points represent the mean of four determinations in a single experiment.

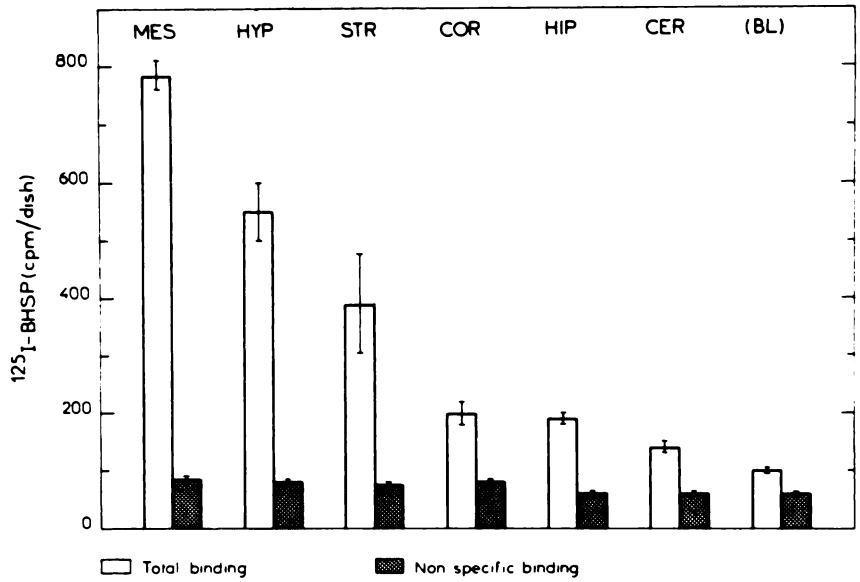

FIG. 8. *[^{125}I]BHSP binding on 5-day-old cell cultures prepared from different brain regions*

Cells were prepared as described under Materials and Methods and grown for 5 days. Radioactive ligand (36 pM) was then added to the cells, and total (*open bars*) and nonspecific (*hatched bars*) binding values were determined as described under Materials and Methods and in the legend to Fig. 1. Mesencephalic cell cultures (*MES*) were compared with hypothalamic (*HYP*), striatal (*STR*), cortical (*COR*), hippocampal (*HIP*), and cerebellar (*CER*) cells. Control dishes devoid of cells but treated as for cell culturing were incubated with the radioactive ligand for blank value determination (*BL*). Each bar represents the mean ± standard error of the mean of four determinations. Each point represents the mean of four determinations made within a single experiment. For all compounds tested, the experiment was repeated two or three times and the results obtained were similar to those represented in the figure. In each experiment the IC_{50} values of substance P and the compound tested were compared.

strongly suggest that the ligand is bound on substance P receptors. Since [^{125}I]BHSP binding was still observed and exhibited similar properties when mesencephalic cells were grown under conditions in which glial development was impaired, it can be concluded that specific binding sites are present on neurons. Nevertheless, it cannot yet be excluded that such binding sites are also located on glial membranes when mesencephalic cells are grown in the presence of serum.

Under our experimental conditions, in which no significant peptidic cleavage of [^{125}I]BHSP occurs, a low concentration of the ligand can be used (<40 pM). More than 80% of the total binding on mesencephalic cells was inhibited by 1 μM substance P, indicating that it was mainly represented by specific binding. These results differ from preliminary data obtained with bovine and rat brain membranes in which a large proportion of the total binding was found to be nonspecific,[2] a finding which in some ways resembles results obtained by Hanley *et al.* (2) and Mayer *et al.* (17) in their studies with rat brain membranes.

[^{125}I]BHSP binding on mesencephalic cells was linearly dependent on the cell concentration in the culture dish, and was saturable and reversible. Moreover, [^{125}I]BHSP was found to bind with high affinity to a single class of noninteracting sites. The dissociation constants calculated from equilibrium binding (K_d = 169 pM), competition (253 pM), and kinetic binding (80 pM) studies show good agreement. These data can be compared with those reported by Hanley *et al.* (2) in their study on rat brain membranes. Indeed, these authors reported K_d values of 380 and 460 pM when dissociation constants were calculated from equilibrium and kinetic binding studies, respectively. Values obtained with mesencephalic cells were lower than those indicated for the high-affinity specific binding of various substance P-related ligands on dispersed pancreatic and salivary acinar cells (5–8). As demonstrated for the binding of ^{125}I-labeled (Tyr^8)-substance P (7) and [^{125}I]physalaemin (5) on pancreatic acinar cells, specific binding of [^{125}I]BHSP on embryonic mesencephalic cells was temperature-dependent, since it was reduced at 4°.

Competition studies strongly suggested that specific [^{125}I]BHSP binding sites correspond to substance P receptors, since structurally related substance P analogues or substance P C-terminal fragments exhibiting biological

TABLE 1

Binding characteristics of [^{125}I]BHSP on mesencephalic cells grown with 10% fetal calf serum (FCS) or in a conditioned, chemically defined medium (CCDM)

Mesencephalic cells were incubated for 5 days under conditions allowing (FCS) or impairing (CCDM) glial cell proliferation. The radioactive ligand dissociation constant (K_d), total number of binding sites (B_{max}), and substance P IC_{50} values were determined under both conditions. Values for FCS are the means of results obtained in at least four experiments; those for CCDM are the means of results obtained in two experiments in which four determinations were made in each case.

Culture medium	K_d	B_{max}	IC_{50}
	pM	*fmoles/dish*	*pM*
FCS (neurons + glial cells)	169	2.72	374
CCDM (neurons only)	216	2.04	430

[2] J. C. Beaujouan, Y. Torrens, and J. Glowinski, unpublished observations.

activity inhibited [^{125}I]BHSP binding on mesencephalic cells. This was not the case with physiologically inactive compounds such as the substance P^{7-11} COOH-terminal fragment, the substance P free-acid derivative in which the amide residue on the C-terminal methionine has been removed, or the NH_2-terminal tetrapeptide. Similarly, other peptides not related to substance P, such as carnosine, thyrotropin-releasing hormone, melanocyte-stimulating hormone release-inhibiting factor, luteinizing hormone-releasing hormone, Met-enkephalin, Leu-enkephalin, and VIP did not compete with [^{125}I]BHSP binding.

Interestingly, BHSP was as potent as substance P in inhibiting [^{125}I]BHSP binding, but the potency of (Tyr^8)-substance P was higher. (Nor-Leu^{11}) substance P, which has also been used as ligand (18), was slightly less potent. A good relationship was found between the length of the substance P C-terminal fragments and their ability to compete with [^{125}I]BHSP, the longer fragments being the most active. These results differ slightly from those reported by Hanley *et al.* (2), since these authors observed that the potencies of substance P^{5-11} and substance P^{6-11} were higher than those of longer C-terminal fragments in inhibiting ^{3}H-labeled substance P binding on rat brain membranes. Marked differences were also seen with physalaemin and eledoisin, since these substance P-related peptides were more active under the conditions used by Hanley *et al.* than under ours. In contrast, except for the results with physalaemin, our results can be compared with those described by Liang and Cascieri (8) in their recent study on dispersed parotid cells made with [^{125}I]BHSP as ligand, since a similarity was found in the respective potencies of BHSP, C-terminal fragments, and eledoisin. However, those authors as well as Putney *et al.* (6) found that physalaemin was more potent than substance P on peripheral dispersed cells. Various factors could contribute to the discrepancies between our results and those of Hanley *et al.* (2). It cannot be excluded that these discrepancies are related to the ligand used. More probably, differences in the preparations are involved. Under our conditions, in which intact mesencephalic cells were selected, binding may occur only on substance P receptors located on the outside part of the membrane whereas experiments conducted with membrane preparations could also deal with unmasked internal binding sites. Finally, some of the properties of the substance P receptors in the embryo may differ from those of the adult animal, as has been shown for other receptors (19, 20). On the other hand, the difference in the potency of physalaemin in competing with [^{125}I]BHSP on central and peripheral dispersed cells could eventually be explained by the existence of subtypes of substance P receptors.

Marked variations were seen when [^{125}I]BHSP binding was estimated on cells from various brain structures and grown for 5 days under similar conditions. [^{125}I]BHSP specific binding on mesencephalic and hypothalamic cells was about twice that found in cells from striatum. Binding on hippocampal, cortical, and cerebellar cells was much lower. This regional distribution of [^{125}I]BHSP specific binding sites is comparable to that of endogenous substance P in adult rat brain (21, 22). It can thus be concluded that substance P receptors appear very early during ontogenesis in various brain structures.

Several properties of striato-nigral substance P neurons have already been investigated. For instance, a potassium-induced release of substance P which is calcium-dependent has been shown *in vitro* using rat substantia nigra slices (23, 24). *In vivo* studies in cat have indicated that the nigral release of substance P is dependent on nerve activity (25). As revealed by microiontophoretic electrophysiological investigations, the activity of numerous nigral cells is stimulated by substance P (26). Finally, *in vivo* release studies have allowed the demonstration that striato-nigral substance P neurons exert a tonic excitatory influence on the activity of nigro-striatal dopaminergic neurons (27). The present report, which indicates that some of the properties of substance P receptors can be studied on intact mesencephalic neurons, should help to define the functional characteristics of striato-nigral substance P neurons. Indeed, it should be possible in the future to establish whether or not [^{125}I] BHSP binding sites are restricted to specific populations of mesencephalic neurons and particularly to demonstrate their eventual presence on dopaminergic cells, which have already been studied extensively in these primary cultures (9, 11, 12, 16).

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REFERENCES

1. Nakata, Y., Y. Kusaka, T. Segawa, H. Yajima, and K. Kitagawa. Substance P: regional distribution and specific binding to synaptic membranes in rabbit central nervous system. *Life Sci.* **22**:259–268 (1978).
2. Hanley, M. R., B. E. B. Sandberg, C. M. Lee, L. L. Iversen, D. E. Brundish, and R. Wade. Specific binding of ^{3}H-substance P to rat brain membranes. *Nature* (*Lond.*) **286**:810–812 (1980).
3. Nicoll, R. A., C. Schenker, and S. E. Leeman. Substance P as a transmitter candidate. *Annu. Rev. Neurosci.* **3**:227–268 (1980).
4. Mayer, N., F. Lembeck, A. Saria, and R. Gamse. Substance P: characteristics of binding to synaptic vesicles of rat brain. *Naunyn-Schmiedebergs Arch. Pharmacol.* **306**:45–51 (1979).
5. Jensen, R. T., and J. D. Gardner. Interaction of physalaemin, substance P and eledoisin with specific membrane receptors on pancreatic acinar cells. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5679–5683 (1979).
6. Putney, J. W., J. C. M. Van de Walle, and C. S. Wheeler. Binding of ^{125}I-physalaemin to rat parotid acinar cells. *J. Physiol.* (*Lond.*) **301**:205–212 (1980).
7. Sjodin, L., E. Brodin, G. Nilsson, and T. P. Coulon. Interaction of substance P with dispersed pancreatic acinar cells from the guinea pig: binding of radioiodinated peptide. *Acta Physiol. Scand.* **109**:97–105 (1980).
8. Liang, T., and M. A. Cascieri. Substance P receptors on parotid cell membranes. *J. Neurosci.* **1**:1133–1141 (1981).
9. Prochiantz, A., U. Di Porzio, A. Kato, B. Berger, and J. Glowinski. In vitro maturation of mesencephalic dopaminergic neurons from mouse embryos is enhanced in presence of their striatal target cells. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5387–5391 (1979).
10. 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).
11. Prochiantz, A., M. C. Daguet, A. Herbet, and J. Glowinski. Specific stimulation of in vitro maturation of mesencephalic dopaminergic neurons by striatal membranes. *Nature* (*Lond.*) **293**:570–572 (1981).
12. Di Porzio, U., M. C. Daguet, J. Glowinski, and A. Prochiantz. Effect of striatal cells on *in vitro* maturation of mesencephalic dopaminergic neurons grown in serum-free conditions. *Nature* (*Lond.*) **288**:370–373 (1980).
13. Kitabgi, P., R. Carraway, J. Van Rietschoten, C. Granier, J. L. Morgat, A. Menez, S. Leeman, and P. Freychet. Neurotensin: specific binding to synaptic membranes from rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **74**:1846–1850 (1977).
14. Bury, R. W., and M. L. Mashford. Biological activity of C-terminal partial sequences of substance P. *J. Med. Chem.* **19**:854–856 (1976).

15. Hanley, M. R., C. M. Lee, L. M. Jones, and R. H. Michell. Similar effects of substance P and related peptides on salivation and on phosphatidylinositol turnover in rat salivary glands. Mol. Pharmacol. **18**:78–83 (1980).
16. Prochiantz, A., A. Delacourte, M. C. Daguet, and D. Paulin. Intermediate filament proteins in mouse brain cells cultured in the presence or in the absence of fetal calf serum. *Exp. Cell. Res.*, in press (1982).
17. Mayer, N., A. Saria, and F. Lembeck. Binding sites of substance P in brain tissue, in *Neuropeptides and Neural Transmission* (C. Ajmone Marsan and W. Z. Traczyk, eds.). Raven Press, New York, 19–29 (1980).
18. Berger, H., K. Fechner, E. Albrecht, and H. Niedrich. Substance P: *in vitro* inactivation by rat brain fractions and human plasma. *Biochem. Pharmacol.* **28**:3173–3180 (1979).
19. Michler, A., and B. Sakmann. Receptor stability and channel conversion in the subsynaptic membrane of the developing mammalian neuromuscular junction. *Dev. Biol.* **80**:1–17 (1980).
20. Steinbach, J. H., J. Merlie, S. Heinemann, and R. Bloch. Degradation of junctional and extrajunctional acetylcholine receptors by developing rat skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* **76**:3547–3551 (1979).
21. Brownstein, M. J., E. A. Mroz, J. S. Kizer, M. Palkovits, and S. E. Leeman. Regional distribution of substance P in the brain of the rat. *Brain Res.* **116**:299–305 (1976).
22. Kanazawa, I., and T. Jessell. Postmortem changes and regional distribution of substance P in the rat and mouse nervous system. *Brain Res.* **117**:362–367 (1976).
23. Jessel, T. M. Substance P release from the rat substantia nigra. *Brain Res.* **151**:469–478 (1978).
24. Torrens, Y., J. C. Beaujouan, M. J. Besson, R. Michelot, and J. Glowinski. Inhibitory effects of GABA, L-glutamic acid and nicotine on the potassium evoked release of substance P in the substantia nigra slices of the rat. *Eur. J. Pharmacol.* **71**:383–392 (1981).
25. Michelot, R., V. Leviel, Y. Torrens, J. Glowinski, and A. Chéramy. *In vivo* release of substance P in the cat substantia nigra. *Neurosci. Lett.* **15**:141–146 (1979).
26. Davies, J., and A. Dray. Substance P in the substantia nigra. *Brain Res.* **107**:623–627 (1976).
27. Glowinski, J., R. Michelot, and A. Chéramy. Role of striato-nigral substance P in the regulation of the activity of the nigro-striatal dopaminergic neurons, in *Neural Peptides and Neuronal Communication* (E. Costa and M. Trabucchi, eds.) Raven Press, New York, 51–62 (1980).

Send reprint requests to: Dr. J. C. Beaujouan, Groupe NB, INSERM U.114, Collège de France, 11 place Marcelin Berthelot, 75231 Paris Cedex 05, France.