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Specific Labeling of Rat Brain Substance P Receptor with [^{3}H]Physalaemin[1]

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Abstract

The binding of [^{3}H]physalaemin ([^{3}H]PHY) to rat brain membranes is specific, saturable and reversible in the presence of monovalent cations and peptidase inhibitors. Monovalent cations increase the binding of [^{3}H]PHY in an ionic strength (μ)-dependent manner with an optimal effect at μ higher than 0.3. Addition of 2.5 mM $MnCl_2$ results in a 2-fold increase in the affinity (K_D) and a 40% increase in the maximal receptor density (B_{max}). Scatchard analysis under these conditions indicates the existence of a single population of noninteracting sites with K_D of 3.6 nM and a B_{max} of 76 fmol/mg of protein. Substance P (SP) and physalaemin are equipotent in inhibiting the binding of [^{3}H]PHY, whereas the potency of SP(2–11), SP(3–11), and SP(4–11) decreased in inverse proportion to their length. The relative affinity of the different tachykinins, SP, and SP fragments in competing with [^{3}H]PHY correlates with their potency to stimulate several bioassay systems, indicating that [^{3}H]PHY labels a physiologically relevant binding site that correspond to the SP-P tachykinin receptor. Guanine nucleotides completely abolish the increase in the binding of [^{3}H]PHY produced by 2.5 mM $MnCl_2$, but in its absence, the nucleotides reduce binding only by 15%. Guanine nucleotides reduce binding to the same level regardless of the presence or absence of the divalent cation. Regional distribution studies confirm that the density of SP receptors is maximal in the olfactory bulb, followed by the hypothalamus, striatum, hippocampus, cortex, and cerebellum.

The substance P (SP)-related peptide physalaemin (PHY) (pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH2) is a naturally occurring tachykinin, originally isolated from frog skin (Erspamer et al., 1964). Both peptides have a similar spectrum of physiological activities that includes depolarization of spinal cord neurons (Konishi and Otsuka, 1974), hypotension (Tregear et al., 1971), contraction of the guinea pig ileum (Bury and Mashford, 1977), and stimulation of salivary secretions (Liang and Cascieri, 1979). The results of the analysis of SP and its related peptides on various pharmacological preparations have lead to the subdivision of the tachykinin receptors into two types. One is the SP-P receptor, which interacts preferentially with PHY and SP and has lower affinity for eledoisin and kassinin (Lee et al.,1982). Tissues that have a preponderance of the SP-P receptor include rat brain and salivary glands and the guinea pig ileum. SP is the putative neurotransmitter in mammalians for the SP-P receptor. The other type is the SP-E receptor, which interacts preferentially with eledoisin and kassinin and has lower affinity for SP and PHY. Tissues that have a preponderance of the SP-E receptor include the rat vas deferens, dog duodenum, and the dog and hamster urinary bladder (Lee et al., 1982). A third tachykinin receptor, SP-K, has recently been reported with preferential affinity for neurokinin A[4] (Buck et al., 1984). Neurokinin B (Kangawa et al., 1983; Kimura et al.,1983), another recently discovered peptide, is very effective in inhibiting the binding of [^{125}I-Bolton Hunter] eledoisin (Buck et al., 1984). The recent discovery of several peptides in mammalians suggests that the tachykinin receptor classification will have to be revised to accommodate the new members of the group (Buck et al., 1984).

[^{125}I-Bolton Hunter]SP has been used successfully to label the SP receptor in rat brain synaptosomal membranes (Cascieri and Liang, 1983; Viger et al., 1983). However, the labeling of the SP receptor in rat brain membranes with [^{3}H]SP has been hampered by high nonspecific binding (Hanley et al., 1980) and by the lack of correlation between the relative potency of some peptides to compete with [^{3}H]SP and their relative potency to produce neuronal depolarization. Nevertheless, [^{3}H]SP has been used successfully in labeling the SP receptor in rat brain slices by autoradiography (Quirion et al., 1983a).

We have used [^{3}H]PHY to label the SP receptor in rat brain membranes because PHY and SP are generally considered to act on the same receptor. Cross-desensitization and inactivation of the SP receptor on the guinea pig ileum have indicated that SP and PHY interact with the same receptor (Lee et al., 1982; Lin and Musacchio, 1983). Furthermore, analysis of the binding of [^{3}H]SP and [^{3}H]PHY to rat submaxillary gland membranes demonstrated that both peptides interact with the same binding site (Bahouth et al., 1985). We demonstrate in this paper that [^{3}H]PHY binds with high specificity to rat brain membranes in the presence of appropriate peptidase inhibitors and monovalent and divalent cations. The binding is saturable, reversible, and inhibited competitively by SP and other peptides. The low nonspecific binding of [^{3}H]PHY makes this label one of the ligands of choice to characterize the SP-P receptor in the central nervous system and in peripheral tissues.

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[4] We followed the uniform tachykinin nomenclature recommended by the satellite symposium to the 9th IUPHAR International Congress of Pharmacology on "SP: Metabolism and Biological Actions," which is the following: neurokinin A (NKA), is also known as neurokinin alpha or neuromedin L or substance K (SK). Neurokinin B (NKB) is also known as neurokinin beta or neuromedin K.

Materials and Methods

Materials. Peptides were purchased from Peninsula Laboratories (Belmont, CA). Polyethylenimine (PEI), HEPES, bacitracin, chymostatin, choline chloride, bovine serum albumin (BSA), soybean trypsin inhibitor, trypsin, GDP, GTP, 5′-guanylimidodiphosphate (Gpp(NH)p), *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), and dithiothreitol (DTT) were all obtained from Sigma Chemical Co. (St Louis, MO). GF/B glass fiber filters were purchased from Whatman Ltd. (Maidstone, England). Samples were filtered with the multiple cell harvester (M-24) from Brandel Biomedical Research and Development Labs. (Gaithersburg, MD).

Synthesis, storage, and purity of [^{3}H]PHY. [^{3}H]PHY was prepared from PHY (Cambridge Research Biochemicals, Cambridge, England) by catalytic tritiation of iodinated PHY in which the methionine residue was temporarily protected as the sulfoxide. The product was isolated by semi-preparative high performance liquid chromatography (HPLC) and had a satisfactory amino acid analysis after acidic hydrolysis. The specific activity was 30.4 ± 2.3 Ci mmol^{-1} and the radiochemical purity was 99% inclusive of not more than 1% of the sulfoxide. The purity of [^{3}H]PHY was periodically checked by reverse phase HPLC as described for SP (Huidobro-Toro et al., 1982).

[^{3}H]PHY was diluted to 10 μM with 10 mM acetic acid, and small aliquots were stored in cryotubes in liquid nitrogen. The stock was diluted to 1.5 to 2 μM in 5% ethanol, 1 mM mercaptoethanol and stored at −20°C under nitrogen. Under these conditions, the decomposition of [^{3}H]PHY was less than 5%/ month.

Tissue preparation. Male Sprague-Dawley rats (200 to 250 gm) were killed by decapitation. The brains without the cerebella were homogenized in 50 vol (w/v) of 20 mM HEPES, pH 7.4, at 4°C using a Brinkman Polytron tissue disruptor (speed 8, for 30 sec), and the homogenate was centrifuged at 900 × *g* for 10 min. The low speed pellet was discarded and the supernatant was centrifuged at 34,000 × *g* for 30 min at 4°C. The resulting pellet was resuspended in 11.5 vol of 0.125 M sodium sulfate, 20 mM HEPES (pH 7.4) at 4°C. Chymostatin dissolved in dimethylsulfoxide (DMSO; 20 mg/ml), and bacitracin in water (50 mg/ml) were added 30 min before the initiation of the binding assay. The final concentration of DMSO did not affect the binding of [^{3}H]PHY. All peptidase inhibitors and peptides were stored at −20°C in small aliquots.

[^{3}H]PHY binding assay. The binding was initiated by the addition of 0.25 ml of the membrane preparation to a final volume of 0.5 ml. The final composition of the incubation medium consisted of 0.125 M sodium sulfate, 2.5 mM $MnCl_2$, 20 mM HEPES, pH 7.4, 0.3 mg/ml of bacitracin, and 0.05 mg/ml of chymostatin. The samples were incubated at 20°C in a shaking water bath, and after 30 min they were filtered through Whatman GF/B filters presoaked in 0.05% PEI in water at room temperature for 4 to 6 hr. The filters were washed prior to the sample application with 5 ml of wash buffer (100 mM choline chloride, 0.1% BSA, and 50 mM sodium phosphate, pH 7.4, at 4°C). After the sample application, the filters were rapidly washed three times with 5 ml of the same ice-cold wash buffer. The bound [^{3}H]PHY was determined by placing the filters in vials containing 1 ml of NCS tissue solubilizer (Amersham Corporation, Arlington Heights, IL). The vials were shaken for 30 min, and then 10 ml of toluene scintillation fluid (0.4%, 2,5-diphenyloxazole and 0.02% *p*-bis[2-(5-phenyloxazolyl)]benzene were added. Tritium was determined in a Beckman LS-230 liquid scintillation spectrometer at 30% efficiency. The proteins were determined by the method of Lowry et al. (1951) as modified by Peterson (1977), and the contribution of bacitracin and chymostatin to the total protein was subtracted. All labeled and unlabeled peptides were diluted in BSA-coated polypropylene Eppendorf tubes. For this purpose, the tubes were filled with ice-cold 0.1% ultrapure albumin for 6 hr at 4°C, and the contents were emptied by suction, air dried, and stored at room temperature until use. For saturation experiments, increasing concentrations of [^{3}H]PHY (1 to 30 nM) were added with the nonspecific binding defined as [^{3}H]PHY bound in the presence of 1 μM PHY.

The effect of pretreatment with ions on [^{3}H]PHY binding to rat brain membranes was analyzed as follows. (*a*) To study whether the effects of sodium sulfate on [^{3}H]PHY binding persist after its removal, the membranes were incubated in 0.125 M Na_2SO_4 for 1 hr at 4°C, then centrifuged at 100,000 × *g*, and the binding assay was carried out in the membranes resuspended in 20 mM HEPES (pH 7.4). (*b*) The tissue was incubated in 0.125 M sodium sulfate for 1 hr at 4°C, then centrifuged, and the pellet was resuspended in the same incubation medium.

Preparation of subcellular fractions. Rat brain membranes were prepared by the method of De Robertis et al. (1962). Brain without the cerebellum was homogenized in 10 vol of 0.32 M sucrose using a Teflon-glass homogenizer. The homogenate was centrifuged for 15 min at 1,000 × *g* and the nuclear pellet (P_1) was washed once with 0.32 M sucrose. The resulting supernatant was centrifuged at 17,000 × *g* for 20 min to yield the crude mitochondrial pellet (P_2), that was washed once by resuspending in 0.32 M sucrose and centrifuging at 17,000 × *g* for 15 min. The crude P_2 was osmotically shocked in 5 vol of 5 mM HEPES, pH 8.4, for 1 hr at 4°C, then centrifuged at 100,000 × *g* for 40 min. The pellet was resuspended in 0.32 M sucrose and applied to a discontinous sucrose gradient consisting of successive 6-ml layers of 0.4, 0.6, 0.8, 1.0, and 1.2 M sucrose. After centrifugation at 63,580 × *g* for 90 min in a Beckman SW-27 rotor, each band was separated, diluted with 5 mM HEPES, and centrifuged at 100,000 × *g* for 30 min. The supernatant of the crude mitochondrial pellet was centrifuged at 100,000 × *g* for 60 min to obtain the microsomal pellet (P_3). All of the fractions were resuspended in 10 vol of 0.125 M sodium sulfate, 20 mM HEPES (pH 7.4) and assayed for specific [^{3}H]PHY binding.

Calculation and data preparation. The data from each saturation with [^{3}H]PHY and competition experiment with either PHY or the other unlabeled peptides were entered in the appropriate subroutine of the Equilibrium Binding Data Analysis program, EBDA (McPherson, 1983). This program is designed to process raw data from radioligand binding experiments into a form suitable for use by the nonlinear least square fitting program, SCAFIT (Munson and Rodbard, 1980), which was used for the analysis of saturation and competition data. The kinetic data were analyzed by the computer program ESTRIP (Brown and Manno, 1978). The EBDA, SCAFIT, and ESTRIP programs were adapted to the IBM-PC computer and supplied by G. A. McPherson, Victorian College of Pharmacy (381 Royal Pde, Parkville, Victoria 3052, Australia).

Results

General Characteristics of [^{3}H]PHY binding

Inhibition of [^{3}H]PHY degradation. Addition of bacitracin (0.3 mg/ml) and chymostatin (0.05 mg/ml) resulted in a 94% recovery of the [^{3}H]PHY after 30 min of incubation with tissue at 20°C as determined by HPLC. This is in contrast with rat submaxillary gland membranes which, in addition, required sequential pretreatment with 0.1 mM TLCK-TPCK to block the degradation of [^{3}H]SP and [^{3}H]PHY.

Effect of homogenization in sucrose or HEPES. We compared the specific binding in the tissue homogenized in 20 mM HEPES (pH 7.4) or 0.32 M sucrose, 20 mM HEPES (pH 7.4), and the specific binding was 29.9 and 16.0 fmol/mg of protein, respectively. Therefore, subsequent binding experiments were carried out by homogenizing the brain in 20 mM HEPES as outlined under "Materials and Methods."

Effect of temperature. The specific binding of [^{3}H]PHY to rat brain membranes is temperature sensitive. A time course analysis (in the absence of Mn^{2+}) revealed a more rapid initial binding at 37°C than

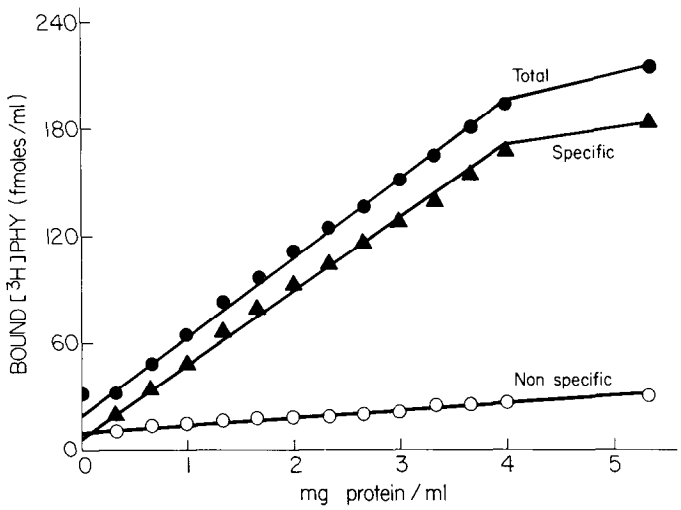

Figure 1. [^{3}H]PHY binding at various tissue concentrations. Different concentrations of rat brain membranes, prepared as described under "Materials and Methods," were incubated with 4 nM [^{3}H]PHY in 0.125 M Na_2SO_4, 2.5 mM $MnCl_2$, 20 mM HEPES (pH 7.4) for 30 min at 20°C. Nonspecific binding (O) was determined by co-incubating the radioactive ligand with 1 μM PHY. Specific [^{3}H]PHY bound (▲) was calculated by subtracting nonspecific binding from total binding (●). The values are the mean of triplicate determinations of an experiment replicated twice.

TABLE I

Effect of trypsin on specific [^{3}H]PHY binding

The 900 × *g* supernatant of the rat brain homogenate was preincubated for 30 min at 20°C with or without different concentrations of trypsin and/or soybean trypsin inhibitor as indicated. Soybean trypsin inhibitor was added to the trypsin-containing samples after 30 min, and the incubation was continued for 5 min. The samples were then cooled on ice and centrifuged at 34,000 × *g* for 30 min. The pellets were resuspended in the assay buffer and membrane aliquots were assayed for [^{3}H]PHY binding. Values are the mean of triplicate samples of a single experiment replicated once.

Trypsin (μg/ml)	Trypsin Inhibitor (μg/ml)	[^{3}H]PHY Bound (% of control)
30		26.8
30	90	44.7
100		20.8
100	300	51.7
300		12.3
300	600	48.8

at 20°C. However, after 30 min of incubation, the binding of [^{3}H]PHY was 16.1 fmol/mg of protein at 20°C and comparatively less at 37°C (66%). The specific binding in the tissue incubated at 4°C for 30 min was only 25% of that at 20°C. Specific [^{3}H]PHY binding was abolished when the tissue was preincubated at 70°C for 5 min. It is noteworthy that the nonspecific binding increased markedly with the heat treatment.

Effect of pH. The pH profile for specific [^{3}H]PHY binding at 20°C demonstrated that the binding was maximal between pH 8.0 and 9.0. A gradual decrease was observed above pH 9.0 and a sharp decrease was observed below pH 8.0. However, we decided to carry out the binding assay at the physiological pH of 7.4.

Tissue linearity of [^{3}H]PHY binding. The total and the specific binding of [^{3}H]PHY increased linearly with protein concentrations up to 4.0 mg/ml (Fig. 1). Under these conditions, the [^{3}H]PHY specific binding was about 85% of the total binding observed, and 3.2 mg/ml of protein was routinely used.

Effect of trypsin. Trypsin treatment of rat brain membranes resulted in a 75% loss of specific [^{3}H]PHY binding. These effects were dose dependent, and they were partially antagonized by the soybean trypsin inhibitor (Table I).

Effect of sulfhydryl reagents. Preincubation of the membranes with NEM or PCMB resulted in a dose-dependent loss of PHY-binding sites with an IC_{50} of 1.0 and 0.15 nM, respectively (Fig. 2). The action of NEM was more pronounced when incubated with the membranes at 37°C than at 4°C. The inhibitory action of NEM on [^{3}H]PHY binding could be prevented by co-incubation with reduced glutathione or DTT but could not be reversed either by washing or by an additional incubation with DTT.

Subcellular localization of [^{3}H]PHY binding sites

Subcellular fractions prepared by differential and sucrose density gradient centrifugation as described under "Materials and Methods" were tested for [^{3}H]PHY binding. Initial experiments demonstrated that the "nuclear" fraction (1,000 × *g* sediment) contained very low specific activity binding (5.4 fmol/mg of protein), and the "mitochondrial" fraction (17,000 × *g* sediment), which contains the synaptosomes, had the highest specific activity, 35.5 fmol/mg. To determine with more precision the site of the [^{3}H]PHY binding in the 17,000 × *g* sediment, this fraction was further fractioned as described under "Materials and Methods." A significant amount of [^{3}H]PHY binding was observed in all of the fractions, but the highest specific activity and binding were found in the "microsomal" fraction followed by the synaptosomal membrane fractions (Table II).

Kinetics of [^{3}H]PHY binding

The association rate of [^{3}H]PHY to rat brain membranes suspended in 0.125 M Na_2SO_4, 2.5 mM $MnCl_2$, 20 mM HEPES (pH 7.4)

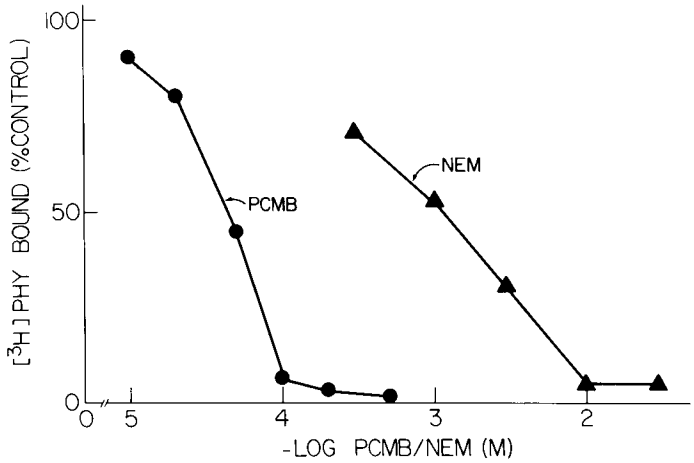

Figure 2. Effect of sulfhydryl reagents on [^{3}H]PHY binding. Rat brain membranes were incubated in 20 mM HEPES (pH 7.4) with increasing concentrations of either NEM (30 min, 37°C) or PCMB (15 min, 30°C). Equimolar amounts of DTT were added to the NEM and the corresponding control samples. All of the samples were cooled immediately on ice and centrifuged at 34,000 × *g* for 30 min. After washing three times, the membranes were suspended in the incubation buffer and assayed for [^{3}H]PHY binding. The data are given as percentage of binding of control membranes. Values are the mean of triplicate determinations of an individual experiment which was replicated once.

TABLE II

Subcellular distribution of [^{3}H]PHY binding in rat brain

Subcellular fractions were prepared as described under "Materials and Methods," and the specific binding of [^{3}H]PHY (3.65 nM) was determined in each fraction. Nonspecific binding was determined in the presence of 1 μM PHY. Results are the mean of triplicate determinations of a single experiment.

Sucrose Fractions (0.32 M)	Specific Binding	
	fmol/mg of protein	*fmol/gm of wet weight*[a]
Nuclear (1,000 × *g* pellet, P_1)	18.9 (58)[b]	141.6
Lysed P_2 subfractions		
Synaptosomal membranes I[c]	29.0 (67)	109.2
Synaptosomal membranes II[d]	33.4 (78)	41.8
Disrupted synaptosomes[e]	16.7 (67)	16.8
Mitochondria[f]	6.9 (48)	17.4
Microsomal (100,000 × *g* pellet P_3)	47.5 (86)	234.6

[a] [^{3}H]PHY specific binding per gram of brain wet weight.
[b] Data in parentheses are the percentage of specific [^{3}H]PHY bound.
[c] Sucrose concentration, 0.6 to 0.8 M.
[d] Sucrose concentration, 0.8 to 1.0 M.
[e] Sucrose concentration, 1.0 to 1.2 M.
[f] The 63,580 × *g* pellet from the lysed P_2 fraction.

is shown in Figure 3*A*. The specific binding reached equilibrium between 20 and 30 min. Under these conditions, the nonspecific binding was already maximal at 2 min. Since the total binding of [^{3}H]PHY was less than 5% of the total radiolabeled ligand added, and no degradation of the peptide occurred within this period, the data obtained from the specific binding were linearized according to the pseudo-first order reaction (Kitabgi et al., 1977). A plot of $ln\ (B_{eq}/B_{eq}-B)$ versus time, where B is the amount of specific [^{3}H]PHY bound at time t, and B_{eq} is the amount bound at equilibrium, is represented in Figure 3*A*, *inset*. From these results, the K_{obs}, which is the observed forward rate constant of the pseudo-first-order reaction, was 0.107 min^{-1}.

Specific binding was reversible, because rapid dissociation of the ligand was observed when 1 μM unlabeled PHY was added after equilibrium had been reached (Fig. 3*B*). The dissociation data were linearized by plotting $ln\ B/B_o$ versus time. The slope, which is the dissociation rate constant (K_{-1}), was 9.94 × 10^{-3} sec^{-1}. From the

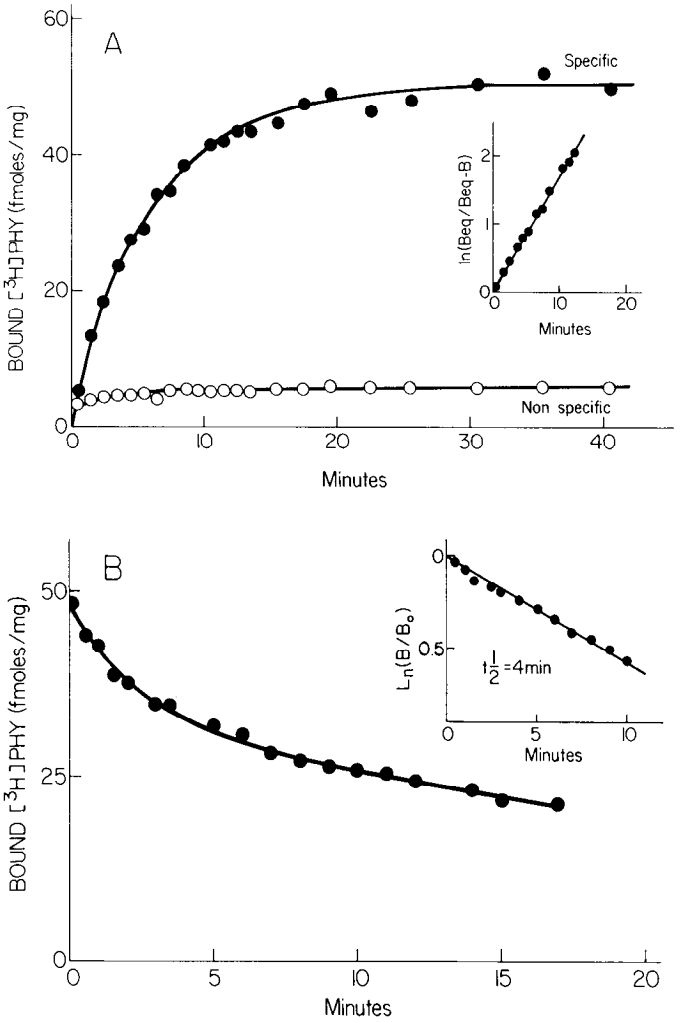

Figure 3. Kinetic analysis of [^{3}H]PHY binding to rat brain membranes. *A*, Association time course of specific [^{3}H]PHY binding. Rat brain membranes in 0.125 M Na_2SO_4, 2.5 mM $MnCl_2$, 20 mM HEPES (pH 7.4) were incubated with 4 nM [^{3}H]PHY in a constantly stirred polypropylene beaker at 20°C. Aliquots (0.5 ml) were filtered through Whatman GF/B filters at the times indicated on the *abscissa* to determine total binding. Nonspecific binding was also determined at each interval in a simultaneous experiment in which unlabeled PHY (1 μM) was added at the beginning of the experiment. Specific binding was calculated by subtracting the nonspecific binding from the total binding. *Inset*, Linearization of the time course curve using the pseudo-first-order equation for association. The experiment was replicated once. *B*, Dissociation of specific [^{3}H]PHY binding. [^{3}H]PHY (4 nM) was incubated with the membrane preparations at 20°C in a constantly stirred polypropylene beaker. After 30 min, an aliquot (0.5 ml) was filtered through Whatman GF/B filter and total [^{3}H]PHY bound (B_o) was determined. Unlabeled PHY (1 μM) in a negligible volume was added to initiate dissociation. The [^{3}H]PHY that remained specifically bound (B) was measured by filtration at the times indicated on the *abscissa*. The nonspecific binding, which has been subtracted from each *point*, was determined at each interval in a simultaneous experiment in which unlabeled PHY (1 μM) was added at the beginning of the incubation. *Inset*, Linearization of the dissociation curve by the first-order rate equation for calculation of the dissociation rate constant. The experiment was replicated once.

slope of the pseudo-first order association reaction and K_{-1}, the association rate constant (K_{+1}) was calculated to be 2.42×10^7 M^{-1} sec^{-1}. Using the equation $K_D = K_{-1}/K_{+1}$, an equilibrium dissociation constant of 4.41 nM was obtained which is in good agreement with the K_D of 3.66 ± 0.36 nM determined by saturation Scatchard analysis in the presence of 2.5 mM $MnCl_2$.

Equilibrium binding studies

Increasing concentrations of [^{3}H]PHY (0.4 to 30 nM) were added to rat brain membranes suspended in 0.125 M Na_2SO_4, 20 mM HEPES, pH 7.4 (without $MnCl_2$). The specific binding was saturable and the nonspecific binding increased linearly with the added concentration of the labeled ligand. The half-maximal [^{3}H]PHY specific binding was achieved at about 5.4 nM [^{3}H]PHY (Fig. 4*A*).

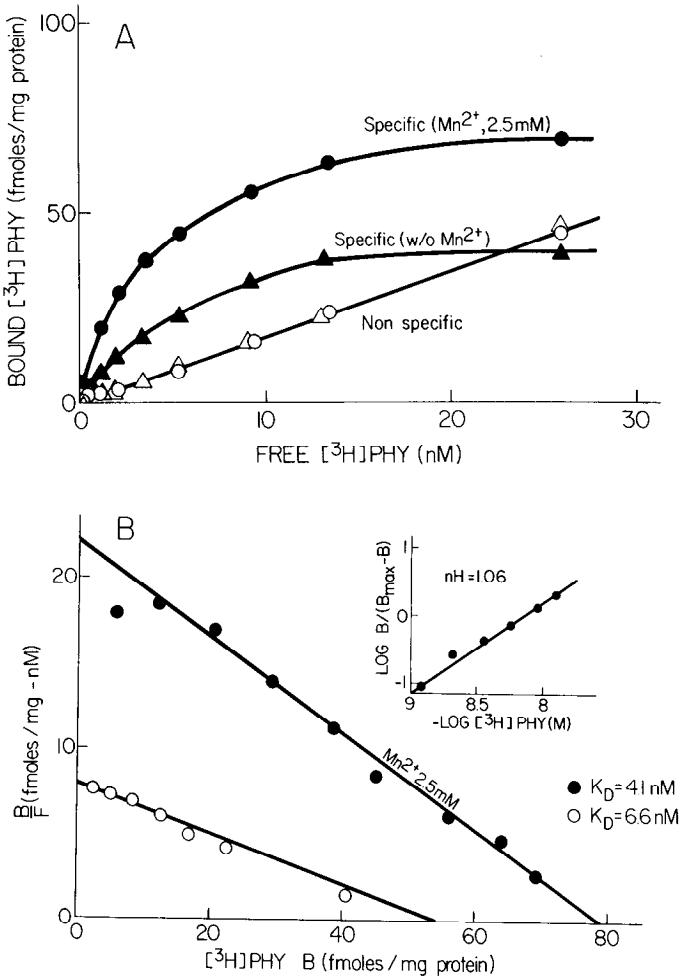

Figure 4. Saturation of [^{3}H]PHY binding sites in rat brain membranes in the absence or presence of $MnCl_2$. *A*, Rat brain membranes (2.5 mg of protein/ml) suspended in 0.125 M Na_2SO_4, 20 mM HEPES (pH 7.4) were incubated with increasing concentrations of [^{3}H]PHY (0.4 to 39 nM). Specific binding in the absence (▲) or presence (●) of 2.5 mM $MnCl_2$ represents the difference between the total and the nonspecific [^{3}H]PHY binding (△, ○). *B*, Scatchard analysis of specific [^{3}H]PHY binding of a single experiment calculated by the weighed least square fitting program LIGAND. The averages of four replicate experiments were the following: $K_D = 6.3 \pm 0.7$ nM and $B_{max} = 55.3 \pm 1.6$ fmol/mg of protein in the absence of $MnCl_2$, and $K_D = 3.7 \pm 0.4$ nM and $B_{max} = 74.2 \pm 1.2$ fmol/mg of protein in the presence of 2.5 mM $MnCl_2$. *Inset*, Hill plot in the presence of $MnCl_2$. The Hill coefficient in the absence of divalent cations was also unity.

Scatchard plots were linear, indicating a single class of binding sites. The K_D and the B_{max} were 6.3 ± 0.73 nM and 55.3 ± 1.64 fmol/mg of protein, respectively (Fig. 4*B*). The Hill plot of the data had a slope $n_H = 1.03 \pm 0.12$, and the Hofstee plots were linear, indicating that the ligand was bound to a single population of noninteracting sites.

Effect of monovalent cations

Incubation of rat brain membranes in 20 mM HEPES with 2.6 nM [^{3}H]PHY without the addition of other ions resulted in negligible specific [^{3}H]PHY binding: 1.1 fmol/mg of protein. The specifically bound [^{3}H]PHY increased dramatically when monovalent cations were included in the incubation medium (Table III). The binding of [^{3}H]PHY in the presence of monovalent cations is dependent on the ionic strength (μ) of the ions present, not on the ions used. This was

TABLE III

Specific [^{3}H]PHY binding to rat brain membranes in different ions with 20 mM HEPES

Solutions containing the different salts and 20 mM HEPES were adjusted to pH 7.4. Specific [^{3}H]PHY binding was determined using 3.9 nM [^{3}H]PHY. Data represent the mean ± SD of triplicate determinations of individual experiments. The binding in 0.3 M sucrose, 20 mM HEPES, or 20 mM HEPES (pH 7.4) was 1.1 ± 0.15 fmol/mg of protein.

Ions with 20 mM HEPES	Specific [^{3}H]PHY Bound (fmol/mg of protein)	
	$\mu = 0.3$[a]	$\mu = 0.6$[b]
Experiment 1		
NaCl	14.6 ± 0.5	10.1 ± 0.6[c]
Na_2SO_4	16.8 ± 0.2	18.9 ± 0.3
KCl	13.8 ± 1.1	9.6 ± 0.4[c]
K_2SO_4	17.3 ± 0.9	19.8 ± 0.3
NH_4Cl	11.2 ± 0.2	5.3 ± 0.7[c]
$(NH_4)_2SO_4$	14.3 ± 0.5	13.9 ± 0.7
LiCl	11.5 ± 0.7	6.8 ± 0.5[c]
Li_2SO_4	18.6 ± 0.6	17.2 ± 0.7
Experiment 2		
NaCl	19.0 ± 0.5	15.9 ± 0.7
Na_2SO_4	20.1 ± 0.1	19.2 ± 0.2
HCOONa	22.5 ± 0.7	20.4 ± 0.5
CH_3-COONa	23.4 ± 0.5	21.7 ± 0.8

[a] The concentration of chlorides, acetate, and formate salts was 0.3 M, and that of sulfate salts was 0.1 M at $\mu = 0.3$.

[b] The concentration of chlorides, acetate, and formate salts was 0.6 M, and that of sulfate salts was 0.2 M at $\mu = 0.6$.

[c] $p < 0.01$ compared to [^{3}H]PHY binding at $\mu = 0.3$.

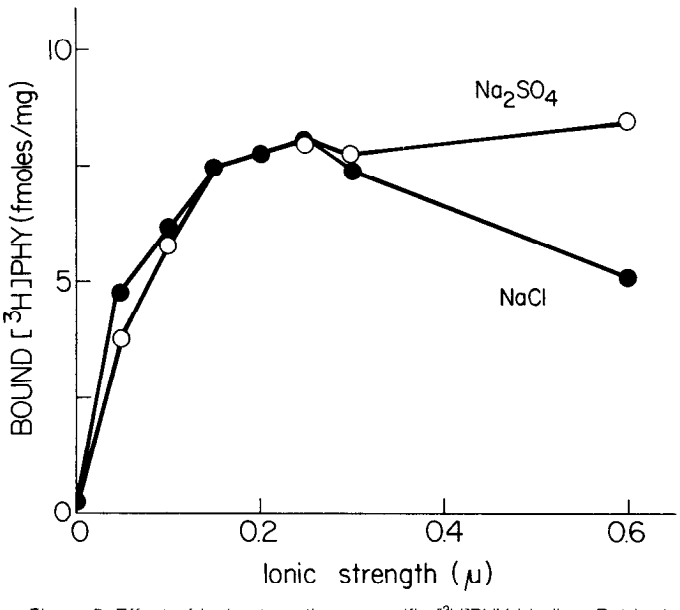

Figure 5. Effect of ionic strength on specific [^{3}H]PHY binding. Rat brain without cerebellum was homogenized in 40 vol of 20 mM HEPES and centrifuged at 34,000 × *g* for 20 min. The pellet was resuspended in 10 vol of 20 mM HEPES, pH 7.4, incubated with TLCK and TPCK (10^{-4} M) for 15 min at 20°C, cooled for 10 min on ice, and centrifuged at 34,000 × *g* for 20 min. The resulting pellet was resuspended in 5 vol of 20 mM HEPES. Aliquots of the membrane preparations were diluted to 10 vol with different concentrations of NaCl and Na_2SO_4. Specific [^{3}H]PHY binding was determined by incubating the membrane preparations with 2.6 nM [^{3}H]PHY for 30 min at 20°C in the presence of different concentrations of salts. Binding of control samples (0 μ) was measured in 20 mM HEPES, pH 7.4. The results shown are representatives of two such experiments performed in triplicate.

established by determining the specific binding of [^{3}H]PHY in different molar concentrations of NaCl and Na_2SO_4 (0.025 to 0.6 M). Optimum binding was observed at 0.1 M Na_2SO_4 ($\mu = 0.3$) and 0.3 M NaCl ($\mu = 0.3$, Fig. 5). Binding assays were also performed using other monovalent cation salts in concentrations that provide an ionic strength of either 0.3 or 0.6. Several salts were studied as described in Table III. Specific [^{3}H]PHY binding in both ionic strengths was comparable among the different sulfates and organic anion salts. However, a decrease in the specific binding of [^{3}H]PHY was observed when the ionic strengths of NaCl, LiCl, KCl, and NH_4Cl were increased from 0.3 to 0.6. This effect is unique to chloride ions, as shown in Table III. Thus, we concluded that when the concentration of chloride is higher than 0.3 M, the specific binding of [^{3}H]PHY is inhibited. Subsequent studies on [^{3}H]PHY binding were carried out by suspending the membranes in 0.125 M sodium sulfate, 20 mM HEPES (pH 7.4) with peptidase inhibitors, and with or without manganese. It is noteworthy that minimal specific binding was observed in either 0.3 M sucrose or 20 mM HEPES.

Since in 20 mM HEPES there was no significant binding (Table III), we investigated the effect of pretreatment with ions on the binding of [^{3}H]PHY and found that if the membranes were preincubated in high ionic strength medium, there was binding in 20 mM HEPES (see "Materials and Methods," condition *a*: $K_D = 6.6$ nM and $B_{max} = 57$ fmol/mg of protein), but not as much as if the membranes were resuspended in sodium sulfate (condition *b*: $K_D = 4.8$ nM and $B_{max} = 75$ fmol/mg of protein) or if they were directly analyzed in the high ionic strength media ($K_D = 3.2$ nM and $B_{max} = 84$ fmol/mg of protein).

Effect of divalent cations

Divalent cations produced a marked increase on binding of [^{3}H]PHY. Manganese was the most potent cation and, at 2 to 5 mM, produced a 65% ($p < 0.01$) increase of specific binding without changing the nonspecific binding (Fig. 4*A*). Addition of magnesium or calcium elicited a 30 to 40% ($p < 0.01$) increase in specific binding at 2 to 5 mM concentration.

Scatchard analysis of the saturation with [^{3}H]PHY in the presence of 2.5 mM $MnCl_2$ (average of four experiments) demonstrated about a 35% increase in the B_{max} to 74.2 ± 1.23 fmol/mg and a decrease in the K_D from 6.3 ± 0.73 nM to 3.66 ± 0.36 nM (Fig. 4*B*). Since the addition of manganese resulted in optimal [^{3}H]PHY binding, subsequent binding studies were carried out in the presence of 0.125 M sodium sulfate, 2.5 mM $MnCl_2$, 20 mM HEPES (pH 7.4).

Effect of guanine nucleotides

The effect of guanine nucleotides on the binding of [^{3}H]PHY to rat brain membranes was studied in the absence or presence of 2.5 mM $MnCl_2$. In the absence of $MnCl_2$, GTP, GDP, and Gpp(NH)p were equally potent in inhibiting the binding of [^{3}H]PHY. The maximal inhibition produced was 25 to 30% ($p < 0.01$) at 1 mM, which was the highest concentration tested. In a typical experiment, 4.8 nM [^{3}H]PHY resulted in specific binding of 1520 cpm/ml, and 1 mM Gpp(NH)p reduced the specifically bound [^{3}H]PHY to 1100 cpm/ml (Fig. 6). In contrast, in the presence of 2.5 mM Mn^{2+} the specifically bound [^{3}H]PHY increased to 3000 cpm/ml, and the addition of Gpp(NH)p markedly reduced the binding in a concentration-dependent manner with an IC_{50} of 3 μM. No reliable estimate of the IC_{50} of other guanine nucleotides could be obtained due to their rapid degradation in the presence of the divalent cation. The addition of Gpp(NH)p inhibited the binding of [^{3}H]PHY in a dose-dependent manner. The maximal inhibition obtained was identical, either in the absence or in the presence of Mn^{2+} (Fig. 6). Similar effects were observed with GTP and GDP but not with the adenine nucleotides, which required much higher concentrations to elicit partial effects.

Inhibition of specific [^{3}H]PHY binding by related peptides

The peptides studied inhibited [^{3}H]PHY specific binding in a competitive manner. SP, PHY, and [Tyr^8]-SP were equipotent in

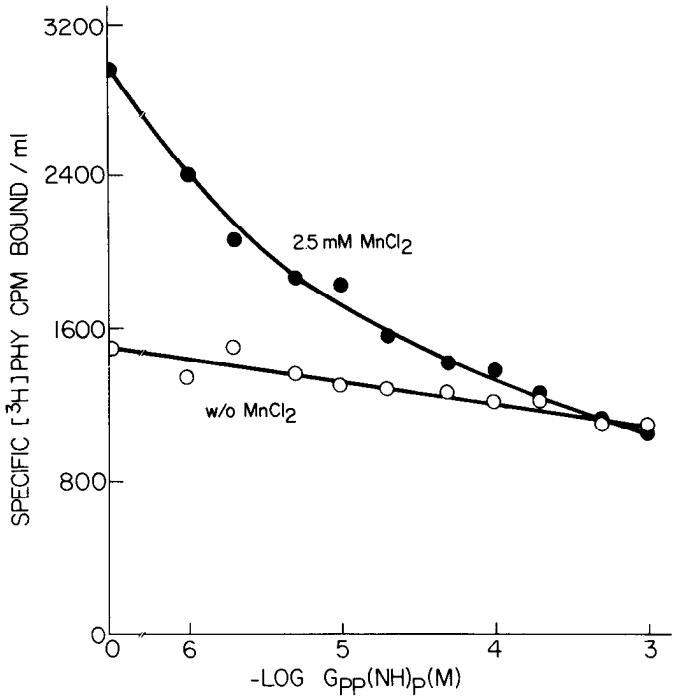

Figure 6. Inhibition of [^{3}H]PHY binding to rat brain membranes by Gpp(NH)p in the absence and presence of 2.5 mM $MnCl_2$. Rat brain membranes suspended in 0.125 M Na_2SO_4, 20 mM HEPES (pH 7.4) were incubated with [^{3}H]PHY (4.7 nM) and with increasing concentrations of Gpp(NH)p as indicated on the *abscissa*. Specific [^{3}H]PHY bound (cpm/ml) in the presence (●) or absence (○) of 2.5 mM $MnCl_2$ represents the mean of triplicate determinations of an experiment which was replicated twice.

TABLE IV

Inhibition of [^{3}H]PHY binding to rat brain membranes by several tachykinins and structurally related peptides

Inhibition of the binding of [^{3}H]PHY (3.0 to 4.2 nM) by increasing concentrations of unlabeled peptides. Values are the mean ± SEM of two or three separate experiments, each in triplicate. The estimates of the IC_{50}, K_I, and Hill slope were obtained with the EBDA computer program (McPherson, 1983) after four to six iterations.

Peptide	IC_{50} (nM)[a]	K_I (nM)[b]	Hill Slope	Relative Potency[c]
PHY	5.6 ± 0.2	2.69	0.96 ± 0.3	1.0
SP	5.9 ± 0.6	2.73	0.98 ± 0.04	0.985
[Tyr^8]-SP	5.7 ± 0.4	2.82	0.97 ± 0.07	0.950
SP(2-11)	17.2 ± 1.6	7.78	0.96 ± 0.06	0.346
SP(3-11)	31.3 ± 3.1	16.2	0.84 ± 0.14	0.166
SP(4-11)	198 ± 27	94.4	0.98 ± 0.08	0.028
[pGlu^1]-SP(5-11)	442 ± 36	199	1.11 ± 0.07	0.014
[pGlu^1]-SP(6-11)	696 ± 74	301	1.16 ± 0.07	0.009
Eledoisin	1,720 ± 492	838	0.93 ± 0.07	0.003
Kassinin	1,328 ± 164	621	0.82 ± 0.06	0.004
Neurokinin A	2,066 ± 134	967	0.96 ± 0.04	0.003
SP(7-11)	>30,000	ND[d]	ND	ND

[a] Concentration of competing ligand required to inhibit the specific binding by 50%.

[b] The inhibition constant (K_I) calculated by the EBDA program as described by Cheng and Prusoff (1973).

[c] Ratio of PHY K_I divided by the K_I of the competing peptide.

[d] ND, not determined.

inhibiting the [^{3}H]PHY binding, with an identical IC_{50} (Fig. 7, Table IV). The affinities of SP(2-11), SP(3-11), and SP(4-11) decreased in inverse proportion to their length. Eledoisin (the SP-E prototype ligand), kassinin, and neurokinin A (NKA) were less than 1% as potent as PHY, whereas, SP(7-11) was inactive. The final estimates of the IC_{50}, K_I, n_H, and the rank order of potencies of different peptides calculated from the pooled data of several experiments are shown in Table IV.

TABLE V

Regional distribution of specific [^{3}H]PHY binding in rat brain membranes

Rat brain regions were dissected and homogenized in 40 vol of 20 mM HEPES (pH 7.4). Specific [^{3}H]PHY binding was measured in 0.125 M Na_2SO_4, 20 mM HEPES, pH 7.4 (without $MnCl_2$), using 5.8 nM [^{3}H]PHY. Values represent the mean of triplicate determinations ± SD of a single experiment at two different tissue concentrations.

Region	Specific [^{3}H]PHY Bound (fmol/mg of protein)
Cerebellum	3.6 ± 0.2
Medulla oblongata	21.7 ± 2.2
Hypothalamus	36.8 ± 2.5
Striatum	34.9 ± 1.4
Midbrain	25.3 ± 0.3
Hippocampus	30.3 ± 2.5
Cortex	23.8 ± 0.6
Olfactory bulb	73.3 ± 1.1

Regional distribution of [^{3}H]PHY binding

A study of different brain regions demonstrated that the binding sites for [^{3}H]PHY were unevenly distributed (Table V). The specific binding was highest in the olfactory bulb and lowest in the cerebellum. The densities of PHY-binding sites in medulla oblongata, hypothalamus, striatum, midbrain, hippocampus, and the cortex were similar.

Scatchard analysis for the binding of [^{3}H]PHY to the brain without cerebellum (in 0.125 M Na_2SO_4, 20 mM HEPES without $MnCl_2$) demonstrated a B_{max} = 55.3 ± 1.6 fmol/mg, with an affinity constant K_D = 6.3 ± 0.73 nM, respectively. The olfactory bulb had a B_{max} = 102 fmol/mg and a K_D = 3.2 nM. When brain membranes were incubated in the presence of Mn^{2+} ions, there was an apparent increase in both the receptor number and affinity, with a B_{max} = 74.2 ± 1.23 fmol/mg and K_D = 3.66 ± 0.36 nM, respectively.

Discussion

We used [^{3}H]PHY to study the SP receptor in rat central nervous system because we have previously demonstrated that this ligand interacts specifically with the SP-P receptor of rat salivary gland membranes (Bahouth et al., 1985). The binding of [^{3}H]PHY is pH dependent and temperature sensitive. Heating or preincubation of the membranes with trypsin led to complete inhibition of specific binding, suggesting that the binding site is proteinaceous in nature. The binding was also inhibited by preincubation with NEM and PCMB, indicating that it is also dependent on sulfhydryl groups. [^{3}H]PHY binding to rat brain membranes suspended in low ionic strength media was negligible and increased by 10- to 20-fold in the presence of salts. This effect was not due to any particular ion but depended on the ionic strength (μ) of the salts. Maximal binding occurred at μ between 0.3 and 0.4 and was maintained at even higher ionic concentrations. Cascieri and Liang (1983) also reported that the binding of [^{125}I-Bolton Hunter]-SP to rat cortex membranes was minimal in 50 mM Tris-HCl. Unlike binding to rat brain membranes, [^{3}H]PHY binding to rat submaxillary gland was significant in low and high ionic strength media (Bahouth et al., 1985). The reason for this disparity in the binding characteristics of membranes from two different organs is not clear. Another characteristic effect of the monovalent ions is that chloride-containing salts inhibited the binding when their concentration exceeded 0.3 M. This inhibitory effect was unique to chloride ions but is probably not physiologically significant, since it occurs only at about twice its humoral concentration.

The addition of divalent cations had a dual effect on the specific binding of [^{3}H]PHY. When 2.5 mM $MnCl_2$ was added, the affinity of

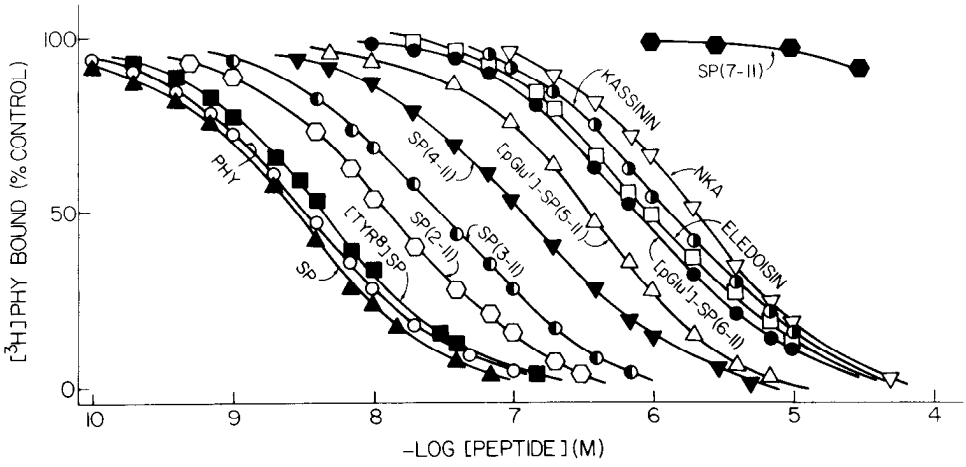

Figure 7. Inhibition of [^{3}H]PHY binding by unlabeled PHY, SP, and structurally related peptides. [^{3}H]PHY was displaced by increasing concentrations of PHY, SP, and its related peptides. The incubation conditions were as described under "Materials and Methods." Results are expressed as the percentage of the specific binding of [^{3}H]PHY, in the absence of unlabeled peptide. All *points* represent the mean of triplicate determinations of a single experiment. The experiments were repeated two or three times, and the results of the analysis of the pooled data, which were calculated with the EBDA program (McPherson, 1983), are summarized in Table IV.

[^{3}H]PHY increased almost 2-fold, from K_D = 6.6 nM to 3.6 nM. In addition, the B_{max} of [^{3}H]PHY increased by 35% (from 55.3 to 74.2 fmol/mg of protein). This effect was not due to the increase in the amount of proteins deposited on the filter as judged by the Lowry assay (Lowry et al., 1951). Cascieri and Liang (1983) previously reported that the addition of 5 mM $MnCl_2$ to rat brain cortex membranes increased the binding of [^{125}I-Bolton Hunter]-SP by 4- to 28-fold. In contrast to the effect of divalent cations on the increase in number of sites and affinity of central nervous system membranes, the divalent cations produced only a modest increase in the binding of [^{3}H]SP and [^{3}H]PHY to rat submaxillary gland membranes, by increasing the B_{max} by 20 to 40% without altering the affinity of the SP receptor (Lee et al., 1983; Bahouth et al., 1985; Bahouth and Musacchio, 1985).

The receptor density that we have observed in this study is similar to that reported by Perrone et al. (1983) for [^{3}H]SP binding to rat brain membranes (B_{max} = 86.5 fmol/mg). However, our B_{max} is substantially higher than that reported for [^{3}H]SP binding to rat brain membranes (Hanley et al., 1980) and slices (Quirion et al., 1983a) or [^{125}I-Bolton Hunter]-SP binding to rat brain synaptosomes (Viger et al., 1983). This discrepancy is probably due to several factors. One is probably related to the way in which we processed the tissue: we discarded the 900 × *g* sediment which contains few receptors but a high concentration of proteins, whereas in the work with tissue slices (Quirion et al., 1983a), the B_{max} has to be calculated for the total proteins. The addition of 2.5 mM $MnCl_2$, which Viger et al. (1983) did not use, is another factor that further contributes to increase the value of the B_{max} as determined in our laboratory.

The affinity of [^{3}H]PHY determined by saturation Scatchard analysis was 3.6 nM and that determined by kinetic analysis was 4.4 nM. These values are similar to those previously reported by others: we calculated the K_i of PHY in published studies using the equation of Cheng and Prusoff (1973) and found that the K_i of PHY in competing with [^{3}H]SP bound to rat brain membranes and slices was 3.5 nM (Perrone et al., 1983; Quirion et al., 1983a) and 2 nM in competing with [^{125}I-Bolton Hunter]-SP bound to rat brain synaptosomes (Viger et al., 1983). The affinity of PHY determined by several pooled competition experiments was 2.7 nM (Table IV). It is noteworthy that the affinity of [^{3}H]PHY in rat submaxillary gland is 2.7 nM (Bahouth et al., 1985), quite similar to the results obtained in brain.

The present study was conducted in a high ionic strength medium (μ = 0.3), much higher than all of the previous studies that deal with labeling of the SP receptor in rat brain. We have previously demonstrated that the affinity of [^{3}H]SP decreases from 0.15 nM in low ionic strength to 2.8 nM in high ionic strength medium, whereas [^{3}H]PHY has a constant affinity (Bahouth et al., 1985). In consequence, the relative potency of PHY to inhibit [^{3}H]SP binding increases in high ionic strength.

PHY and SP were the most potent competitors against [^{3}H]PHY binding with a similar IC_{50} of 5.6 and 5.9 nM, respectively ($p > 0.05$). The relative potency of different SP fragments to compete with [^{3}H]PHY was: SP > SP(2-11) > SP(3-11) > SP(4-11) > [pGlu^1]-SP(5-11) > [pGlu^1]-SP(6-11) >> SP(7-11). These results are in agreement with those of Quirion et al. (1983b), who reported that PHY is at least as potent as SP in competing with [^{125}I]PHY in rat brain sections in Krebs buffer. Other binding studies are generally in agreement with ours except for the potency of PHY. PHY has been reported to be consistently weaker than SP in competing with [^{125}I-Bolton Hunter]-SP bound to brain membranes (Cascieri and Liang, 1983), with [^{3}H]SP bound to rat brain slices (Quirion et al., 1983a), and with [^{125}I-Bolton Hunter]-SP bound to mouse mesencephalic cells in primary culture (Beaujouan et al., 1982). The affinity profile of the different tachykinins and SP analogues in competing with [^{3}H]PHY indicate that this label interacts with the SP-P receptor and that this binding site is similar to the SP-P receptor in rat submaxillary gland membranes labeled with [^{3}H]SP and [^{3}H]PHY (Lee et al., 1983; Bahouth et al., 1985).

Manganese doubled the specifically bound [^{3}H]PHY. However, in the presence of 1 mM Gpp(NH)p, binding of [^{3}H]PHY was the same regardless of the presence or absence of Mn^{2+}. These observations suggest that guanine nucleotides block the effect of divalent cations. Washing the membranes by centrifugation three times restored the stimulation of binding by Mn^{2+} in the Gpp(NH)p-pretreated membranes, indicating that the guanine nucleotide effects are reversible. This also indicates that the binding sites of the guanine nucleotides and divalent cations are not dissociated from the PHY-binding site during the washing procedure. If the SP receptor is protected from inactivation with a high concentration of PHY, NEM blocks the effect of both divalent cations and guanine nucleotides (Musacchio et al., 1984).

We found that guanine nucleotides inhibited the binding of [^{3}H]PHY by only 50 to 60%. This is much less than what was reported by Cascieri and Liang (1983), who showed that Gpp(NH)p and GTP produced a complete inhibition of [^{125}I-Bolton Hunter]-SP binding to rat brain membranes suspended in low ionic strength divalent cation-containing medium (50 mM Tris-HCl, 5 mM $MnCl_2$). This discrepancy could be related to the different experimental conditions: we used, in addition to the divalent cations, high ionic strength media, which increase binding in a guanine nucleotide-insensitive manner, while the divalent cation-dependent binding is sensitive to guanine nucleotides.

The effects of divalent cations and guanine nucleotides on [^{3}H]PHY binding suggest that both agents may act on a GTP-binding regulatory protein as is the case of adenylate cyclase-coupled receptors (Rodbell, 1980). However, the experiments of Lee et al. (1983) indicate that the SP receptor is not coupled to adenylate cyclase, at least in the rat salivary glands. Mantyh et al. (1984) recently reported that SP receptors in the central nervous system

are intimately related to the inositol phospholipid hydrolysis. These considerations indicate that the central nervous system SP-P receptors coupled to inositol phospholipid hydrolysis may be associated to a GTP-binding regulatory protein.

Regional distribution of [^{3}H]PHY-binding sites in rat brain indicates that these sites are present in all major brain areas. The highest concentration of binding sites was in the olfactory bulb, in agreement with the autoradiographic distribution studies of [^{3}H]SP-binding sites in rat central nervous system (Quirion et al., 1983a). In other areas, moderate [^{3}H]PHY binding is observed with a rank order hypothalamus > striatum > hippocampus > midbrain > cortex > medulla > cerebellum. Viger et al. (1983) also reported an uneven distribution of [^{125}I-Bolton Hunter]-SP receptors in rat brain, with a greater receptor density in hypothalamus and the lowest in cerebellum.

This study demonstrates that [^{3}H]PHY labels a physiologically relevant SP receptor in rat brain membranes. The nonspecific binding accounts for only 15% of the total binding, providing an excellent signal-to-noise ratio for the biochemical characterization of SP receptors in the central nervous system. This study shows that the binding of [^{3}H]PHY to rat brain membranes requires both monovalent and divalent cations.

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