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Substance P: Characteristics of Binding to Synaptic Vesicles of Rat Brain

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Summary. 1. The binding of substance P (SP) to synaptic vesicles from rat brain was studied by use of the ^{125}I-Tyr^8-analogue of SP.

2. The pH dependence of the binding of both peptides to the lipid extractable fraction of synaptic vesicles was shown to be comparable.

3. The binding of ^{125}I-Tyr^8-SP shows a rate constant of association ($k_1 = 6.6 \times 10^6$ M^{-1} s^{-1}), a rate constant of dissociation ($k_{-1} = 6.4 \times 10^{-4}$ s^{-1}) and gives a K_D of 1×10^{-10} M. K_D derived from equilibrium studies was 3.2×10^{-10} M.

4. The binding of ^{125}I-Tyr^8-SP to lipids of synaptic vesicles was shown to be reversible, saturable and highly specific.

5. The kinetic data suggest one population of binding sites with a maximal number of 0.8 pmol per mg protein of the synaptic vesicle preparation.

6. Unlabeled SP and the (2–11)-, (3–11)- and (4–11)-analogues of SP inhibit the binding of ^{125}I-Tyr^8-SP in a decreasing order in a competitive way when added in excess. Tyr^8-SP and eledoisin did not interfere with the binding of ^{125}I-Tyr^8-SP whereas uperolein and neurotensin caused a partial inhibition. Physalaemin and D-Ala^2-D-Met^5-enkephalin enhance the binding of ^{125}I-Tyr^8-SP in a cooperative way.

Key words: Substance P – Substance P analogues – Enkephalin – Synaptic vesicles.

Introduction

The presence of Substance P (SP) in nerve ending preparations (Lembeck and Holasek, 1960; Inouye and Kataoka, 1962; Ryall, 1964), and in synaptic vesicles (Hökfelt et al., 1977; Cuello et al., 1977; Pickel et al., 1977), as well as its release from synaptosomes (Schenker et al., 1976; Lembeck et al., 1977), from isolated spinal cord (Otsuka and Konishi, 1976) and from hypothalamic slices (Jessel et al., 1976) raised the question of a specific binding in synaptic vesicles of the brain.

SP was shown to be bound to lipids in extracts of nervous tissue (Heizmann et al., 1966). Lembeck et al. (1978) have shown that SP-binding is reversible and "binding sites" consist of phospholipid material. Binding of other biologically active substances to lipids was demonstrated for opiates by Loh et al. (1974) and Abood and Hoss (1975), for serotonin by Johnson et al. (1977a, b, 1978). These authors discussed the binding to lipids predominantly in connection with receptor function. The binding of biologically active peptides to membrane particle fractions was shown for angiotensin (reviewed by Devynck and Meyer, 1978) and for neurotensin by Kitabgi et al. (1977) and Uhl and Snyder (1977). The lipid-binding of SP was interpreted as a possible storage site (Lembeck et al., 1978).

The present experiments were performed to investigate (a) the binding of SP to synaptic vesicles (b) to ensure that ^{125}I-Tyr^8-SP has the same binding characteristics as unlabeled SP and (c) to study the binding kinetics and specificity of ^{125}I-Tyr^8-SP binding to synaptic vesicles.

Methods

A. Preparation of Synaptic Vesicles

Male adult Sprague-Dawley rats (strain OFA-SD-SPF 200–250 g) were killed by a blow on the neck. Brains were removed quickly and chilled immediately. After removal of the cerebellum the remaining tissue was weighed. All further separation procedures were carried out at 4° C.

Synaptic vesicles were prepared by differential centrifugation according to DeRobertis et al. (1964). The tissue was homogenized

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by 4 strokes with a teflon pestle in a glass homogenizer in 0.32 M sucrose to form a 10% (w/v) homogenate. The first centrifugation was performed at 2.000 g for 10 min. The supernatant was re-centrifuged at 10,000 g for 20 min to yield a crude mitochondrial pellet which was lysed by osmotic shock in the 2.5-fold volume (w/v) of tissue weight of destilled water containing 10 μM $CaCl_2$ to prevent membrane coacervation. After cautious homogenization, debris of disrupted nerve endings and large membrane particles were sedimented at 11,000 g for 20 min. The vesicle containing supernatant fluid was centrifuged at 100,000 g for 30 min.

The resulting pellet was resuspended in 0.03 M sodium-phosphate buffered isotonic sucrose pH 7.4, carefully homogenized and stored at −20° C until use. The preparation showed no loss of binding properties up to 4 weeks.

The brain tissue of 10 rats (about 14 g) yielded a total of 5 mg protein in the synaptic vesicle preparation as measured by the method of Lowry et al. (1951).

The amount of bound SP was expressed per mg protein which served as reference for the amount of synaptic vesicles in a sample.

B. Preparation of ^{125}I-Tyr^8-SP

Iodination of the Tyr^8-analogue of SP (Tyr^8-SP) was performed according to Mroz et al. (1977). A sample of 10 μg peptide was iodinated with 5 mCi $Na^{125}I$ by addition of 1 μg chloramine T. The reaction was carried out in 0.3 M sodium phosphate buffer at pH 7.4 and terminated after 30 s by addition of 5 mM carbonate buffer pH 9.9 containing 1 μg/ml bovine serum albumin.

Separation of the monoiodinated peptide from the diiodinated compound and unlabeled Tyr^8-SP was performed by ion exchange chromatography on Sulfopropyl-Sephadex C-25 columns equilibrated in disposable 10 ml glass pipets. Elution was carried out with 20 ml 5 mM and 100 ml 20 mM carbonate buffer pH 9.9. Radioactivity of the fractions was measured, fractions corresponding to monoiodinated Tyr^8-SP were pooled, neutralized with 1.0 N HCl and stored at −20° C. Specific activity of this tracer as measured by radioimmunoassay using different amounts of tracer at known SP concentrations. It was 6×10^5 mCi/mol.

Free ^{125}I increased to 4−6% within two months as measured by thin layer chromatography on silica gel plates developed in chloroform-methanol-water (65:25:4).

C. Degradation of ^{125}I-Tyr^8-SP Exposed to Synaptic Vesicles

Integrity of the tracer during incubation was tested by ion exchange chromatography and gel filtration. In both cases, ^{125}I-Tyr^8-SP at a concentration of 10^{-8} M was incubated with synaptic vesicles equivalent to 50 μg protein at 30° C for 30 min as described under Methods D. Incubation was terminated by sedimentation at 100.000 g for 20 min. Aliquot portions of the supernatant were subjected to the following separation methods:

a) Ion exchange chromatography: Sulfopropyl-Sephadex C-25 was equilibrated by washing with 200 ml 5 mM carbonate buffer pH 9.9 in a 10 ml disposable glass pipet used as a column. Elution was performed with 5, 10 and 20 mM of carbonate buffer pH 9.9. Fractions of 2 ml were collected at a flow rate of 10 ml/h and their radioactivity was measured.

b) Gel filtration: The sample was layered on a Sephadex G-25 fine column (14× 600 mm) equilibrated with 0.4 M pyridine-acetate buffer of pH 5.5. Elution was performed at a flow rate of 30 ml/h. 2 ml fractions were collected and their radioactivity was measured.

D. Binding Assay

The binding was studied according to the method used by Kitabgi et al. (1977) for neurotensin. 20 μl synaptic vesicles corresponding to 12 μg protein were incubated in 1 ml 0.03 M sodium phosphate buffer pH 6.0 at 30° C. 10 μl ^{125}I-Tyr^8-SP were added to a final concentration of 0.25×10^{-9} M in a routine binding assay. At the pH of 6.0, SP was found to be bound to lipids in brain homogenates (Lembeck et al., 1978).

After 20 min bound ^{125}I-Tyr^8-SP was extracted by addition of 4 ml petroleum ether (bp. 40−60° C)-chloroform (2:1). After thorough mixing using a Branson sonifier the organic phase was separated by centrifugation and its radioactivity was measured in a Packard Auto-Gamma-Tandem scintillation spectrometer (counting efficiency 60%). About 4% of the total amount of added ^{125}I-Tyr^8-SP were recovered in the organic phase. This part is regarded as ^{125}I-Tyr^8-SP specifically bound to lipids of synaptic vesicles and the kinetic data of it were investigated.

The measured radioactivity was corrected for the partition coefficient of ^{125}I-Tyr^8-SP determined by identical incubations without synaptic vesicles. Partition of the tracer into the organic phase without addition of synaptic vesicles was 0.5% at the highest concentration used and was not altered by added peptides.

E. Comparison of pH Dependence of the Binding of SP and ^{125}I-Tyr^8-SP

The binding assay was carried out at 30° C over a pH range between 1.6−5.5. Buffers used were 0.1 M glycine-HCl for pH 1.6−3.6 and 0.03 M phosphate for pH 5.5. The incubation was terminated after 20 min as described under Methods D. Unlabeled SP was first extracted with petroleum ether-chlorofrom as described above. This organic phase was evaporated and re-extracted by addition of 5 ml acetone − 0.1 N HCl (100:3) according to Chang and Leeman (1970). Acetone was removed by evaporation and samples were freeze dried. The residue was dissolved in 1 ml saline containing 0.1% gelatine and 0.5 μg/ml Cialit®. The samples were neutralized by addition of 0.5% $NaHCO_3$ and bioassayed on the isolated guinea pig ileum. The ileum was suspended in an oxygenated Tyrode solution containing 0.1 μg atropine, 1.0 μg mepyramine and 0.1 μg methysergide per ml at 37° C.

F. Materials

Substance P, Tyr^8-Substance P (Beckman Instruments, Palo Alto, Calif., U.S.A.); Deca-, Nona-, and Octa-Substance P (Peninsula, San Carlos, Calif., U.S.A.); Physalaemin (Farmitalia SA., Milano, Italy); Neurotensin (Beckmann Instruments, Palo Alto, Calif., U.S.A.); Eledosin (Schering AG, Berlin, Germany); Bovine serum albumin (Miles Inc., Kankakee, Ill., U.S.A.); D-Ala^2-D-Met^5-Enkephalin (Wellcome Research Labs. Beckenham, Kent, England); Cialit® (2-ethyl-mercury-mercapto)-benzoxazol-5-Na-carbonic acid (Asid GmbH, München, Germany); Methysergid bimaleate (Sandoz AG, Basel, Switzerland); Mepyramine hydrochloride (Specia SA, Paris, France); Atropine sulphate (Merk AG, Darmstadt, Germany); Antibody for Substance P was a generous gift from Dr. S. E. Leeman, Harvard Medical School, Boston, Mass, U.S.A. $Na^{125}I$ (Radiochemical Centre, Amersham, England); All other reagents were of analytical grade.

Results

A. Comparison of the pH Dependence of ^{125}I-Tyr^8-SP and SP Binding (Fig. 1)

The pH dependence of binding of ^{125}I-Tyr^8-SP and of unlabeled SP was found to be comparable. Binding of both peptides was minimal at pH 1.6 and increased

steadily up to pH 5.5. The binding curves were superimposable and suggest that both ligands react with the same or a similar binding site.

B. Stability of ^{125}I-Tyr^8-SP During Incubation with Synaptic Vesicles

^{125}I-Tyr^8-SP was incubated with synaptic vesicles for 30 min and thereafter subjected to ion exchange chromatography. 81% of the radioactivity of samples incubated with vesicles, compared to 79% of non-incubated samples were eluted as intact peptide. The rest of the radioactivity was eluted at the position of free iodine. The identity of this peak with free iodine was confirmed by thin layer chromatography, which showed that the radioactivity of the peak and free iodine cochromatographed. This release of free iodine was shown to occur during ion exchange chromatography since gel filtration at pH 5.5 delivered 98% of total applied radioactivity as intact peptide, when incubated samples were examined. The experiments showed, therefore, that ^{125}I-Tyr^8-SP was not degraded during the incubation with synaptic vesicles.

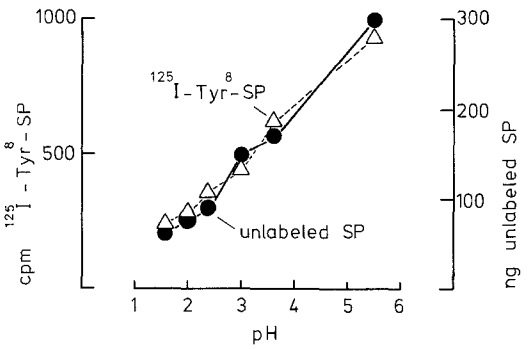

Fig. 1. pH dependence in binding of SP (●) and ^{125}I-Tyr^8-SP (△) to vesicle membranes. 370 nM SP or 0.25 nM ^{125}I-Tyr^8-SP were incubated with samples of synaptic vesicles corresponding to 12 µg protein for 20 min at 30° C. Unlabeled SP was bioassayed on the isolated guinea pig ileum. Radioactivity of ^{125}I-Tyr^8-SP was measured directly. Each point is the mean of triplicate experiments

C. Time Course of Association and Dissociation of ^{125}I-Tyr^8-SP to Synaptic Vesicles

The method used by Kitabgi et al. (1977) for kinetic studies on the binding of neurotensin was adapted for the following experiments. At 30° C and a ^{125}I-Tyr^8-SP concentration of 0.25×10^{-9} M, the specific binding reached equilibrium within 20 min (Fig. 2). Since no degradation of the peptide by exposure to vesicles was observed and only up to 4% of the peptide was bound, it can be assumed that the concentration of the ligand was constant for the time of the experiments. Therefore, the interaction of ^{125}I-Tyr^8-SP with binding sites can be analyzed as a pseudo first-order reaction by the following equation:

$$\ln \frac{[Beq]}{[Beq] - [B]} = ([L]k_1 + k_{-1})\, t$$

in which [Beq] is the amount of bound ^{125}I-Tyr^8-SP at equilibrium, [B] is the amount of bound peptide at a given time t, [L] is the concentration of the labeled ligand; k_1 is the rate constant of association and k_{-1} is the rate constant of dissociation. When

$$\ln \frac{[Beq]}{[Beq] - [B]}$$

was plotted as a function of time, a straight line was obtained (Fig. 2). The slope of this line could be substituted in the equation mentioned above.

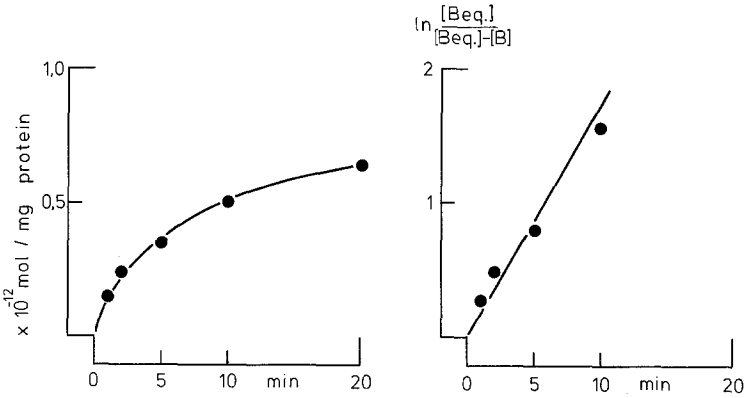

Fig. 2
Time course of binding of ^{125}I-Tyr^8-SP to synaptic vesicles. Left: 0.25 nM ^{125}I-Tyr^8-SP was incubated at 30° C in samples of synaptic vesicles corresponding to 12 µg/ml protein. Binding was measured at the times indicated in the abscissa. Right: The kinetic of ^{125}I-Tyr^8-SP binding was linearized according to the equation of a pseudo first-order reaction as explained in the text. Each point is the mean of triplicate experiments

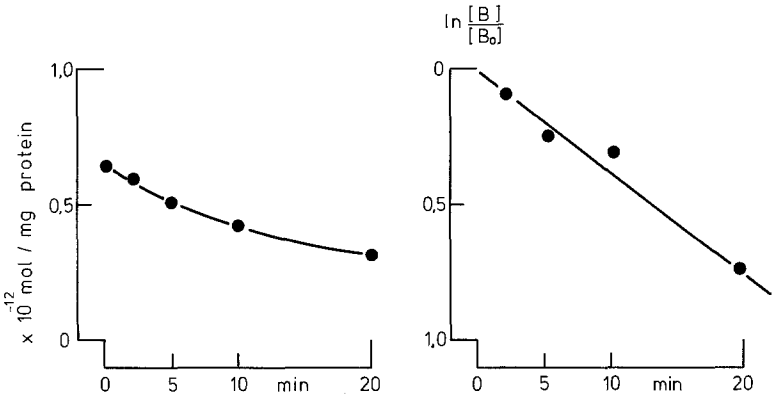

Fig. 3
Time course of dissociation of ^{125}I-Tyr^8-SP. Left: 0.25 nM ^{125}I-Tyr^8-SP was first incubated for 20 min at 30° C in samples of synaptic vesicles corresponding to 12 µg protein per ml. Then unlabeled SP to a final concentration of 10^{-5} M was added. ^{125}I-Tyr^8-SP which remained bound after addition (time 0 at figure) of unlabeled SP was measured at time intervals indicated in the abscissa. Right: Dissociation was linearized to a first order kinetic assuming that reassociation of ^{125}I-Tyr^8-SP with synaptic vesicles was inhibited by the excess of unlabeled SP. Each point is the mean of a triplicate experiment

The value of k_{-1} was determined by plotting dissociation against time according to the equation

$$\ln\left(\frac{[B]}{[B_0]}\right) = -k_{-1}\, t$$

(Fig. 3). $[B_0]$ is the amount of the bound ligand at addition of unlabeled SP.

Dissociation of ^{125}I-Tyr^8-SP from synaptic vesicles was made measurable by addition of unlabeled SP (final concentration 10^{-5} M) after equilibrium. A k_{-1} of 6.4×10^{-4} s^{-1} was calculated from the slope of the linear regression. This value substituted in the first equation results in a k_1 of 6.6×10^6 M^{-1}s^{-1}. These rate constants give a dissociation constant $K_D = \frac{k_{-1}}{k_1}$ of 1.0×10^{-10} M.

D. Studies on Binding of ^{125}I-Tyr^8-SP at Equilibrium (Fig. 4)

Increasing amounts of ^{125}I-Tyr^8-SP were added to samples of synaptic vesicles corresponding to 12 µg/ml protein and incubated for 20 min at 30° C.

The binding was found to be a saturable reaction. Scatchard analysis (Scatchard, 1949) fo the data resulted in a straight line indicating only one population of binding sites. Intercept at the abscissa showed 0.8×10^{-12} mol binding sites per mg protein.

The negative reciprocal slope shows a $K_D = 3.2 \times 10^{-10}$ M, which is in the same order of magnitude as the K_D derived from time course experiments under results C.

E. Inhibition of ^{125}I-Tyr^8-SP Binding by SP and Its C-Terminal Fragments (Fig. 5)

Addition of unlabeled SP inhibited the binding of ^{125}I-Tyr^8-SP in proportion to its concentration within a range of 10^{-8} M to 10^{-6} M ($K_i = 1.7 \times 10^{-7}$ M).

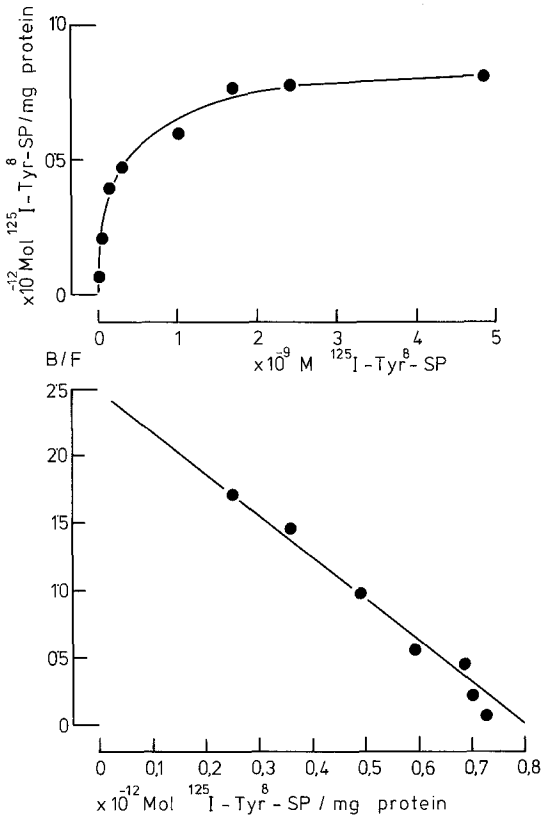

Fig. 4. Binding of ^{125}I-Tyr^8-SP as a function of its concentration. Upper part: The binding (ordinate) was measured after incubation of vesicle samples corresponding to 12 µg/ml protein with increasing concentrations of ^{125}I-Tyr^8-SP (abscissa) for 20 min at 30° C. Lower part: Scatchard plot of the data. Each point respresents the mean of duplicate or triplicate estimations

Affinity to binding sites of the (2–11)-decapeptide ($K_i = 2.8 \times 10^{-6}$ M), the (3–11)-nonapeptide ($K_i = 7.8 \times 10^{-6}$ M) and the (4–11)-octapeptide ($K_i = 8.8 \times 10^{-6}$ M) is progressively lower. K_i was calculated according to the equation

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}$$

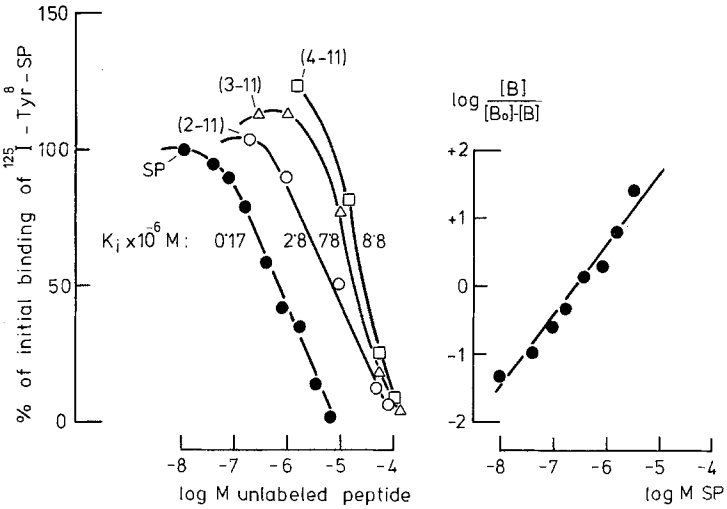

Fig. 5
Left: Inhibition of the binding of 10^{-9} M ^{125}I-Tyr^8-SP by unlabeled SP (●), (2−11)-decapeptide (○), (3−11)-nonapeptide (△) and (4−11)-octapeptide (□). Binding (ordinate) is expressed as the percentage of initial binding of ^{125}I-Tyr^8-SP in the absence of the unlabeled peptides (abscissa). Right: Hill plot of competition for ^{125}I-Tyr^8-SP binding sites by unlabeled SP. [B] is the amount of ^{125}I-Tyr^8-SP bound. [B_0] is the maximal number of binding sites. Each point is the mean of duplicate or triplicate experiments

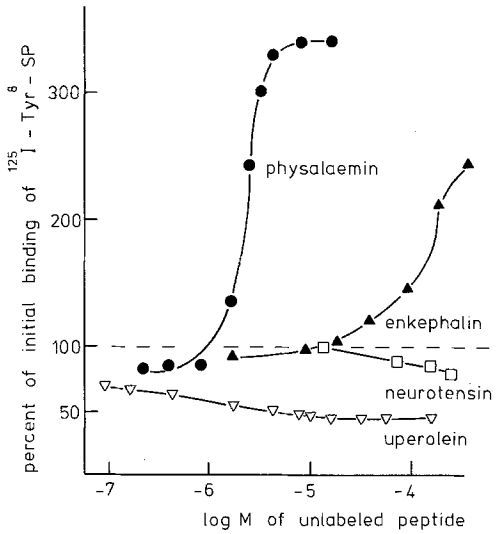

Fig. 6. Interaction of peptides with ^{125}I-Tyr^8-SP binding. Physalaemin (●); D-Ala^2-D-Met^5-enkephalin (▲), uperolein (▽), neurotensin (□). Binding (ordinate) is expressed as the percentage of ^{125}I-Tyr^8-SP binding without addition of unlabeled peptides. Each point is the mean of duplicate or triplicate experiments

where IC_{50} is the inhibitor concentration which displaces 50% of the bound ligand. IC_{50} was determined graphically. The displacement reaction between ^{125}I-Tyr^8-SP and unlabeled SP showed a straight line. Homotropic positive cooperativity was seen when it was plotted according to the Hill equation (Mahler and Cordes, 1971) resulting in a Hill coefficient of 1.33. The displacement curve of the (2−11)-decapeptide was fairly parallel to that of SP. The displacement curves caused by the (3−11)-nonapeptide and by the (4−11)-octapeptide were different. They show enhanced binding of ^{125}I-Tyr^8-SP at low concentrations and a progressive binding inhibition at higher concentrations.

F. Interaction with Other Peptides (Fig. 6)

Tyr^8-SP and eledoisin showed no displacement of ^{125}I-Tyr^8-SP from binding sites up to a concentration of 10^{-4} M. This result indicates high specificity of ^{125}I-Tyr^8-SP-binding. Uperolein, another tachykinin with similar biological activity as SP, showed only 50% inhibition with a completely different slope. Neurotensin inhibited the binding only partially at high concentrations.

Physalaemin showed an augmentation of binding sites for SP. A similar result was obtained by addition of D-Ala^2-D-Met^5-enkephalin.

Discussion

Lembeck et al. (1978) showed a pH-dependent and reversible binding of SP to phospholipids of the brain. Therefore lipid extraction seemed to be a justified procedure to estimate the amount of SP bound to synaptic vesicles. This extraction method has the advantage of eliminating all unspecific binding, because the bulk of the proteins which might interfere with the specific binding reaction was precipitated or remained in the aqueous phase.

The amount of binding of substance P was expressed per mg protein, in order to have a reference for the amount of vesicle membranes.

The experiments were performed with ^{125}I-Tyr^8-SP and it had first to be established that this labeled peptide binds in an identical mode as unlabeled SP. The usefulness of iodinated peptides for investigations on binding kinetics was demonstrated by Glossmann et al. (1974) in the case of angiotensin and by Miller et al. (1978) in the case of enkephalins.

The evaluation of pH-dependence of binding of ^{125}I-Tyr^8-SP and SP itself to synaptic vesicles showed close similarity between both ligands, which suggests

that both peptides react with the same binding site (Fig. 1). Addition of unlabeled SP in excess inhibited the binding of ^{125}I-Tyr^8-SP whereas Tyr^8-SP did not. This result indicates also the identity of the binding site for the radioactive ligand and SP. The complete absence of displacing potency of Tyr^8-SP shows a high degree of specificity of the binding site for SP. Exchanging the Phe^8 residue by Tyr^8 in the SP molecule changes the binding affinity, whereas adding the I atom to the phenolic group of Tyr^8 seems to restore the structure and the binding affinity.

When K_D was calculated from the rate constants ($K_D = 0.1$ nM), it was in the same order of magnitude as when determined directly by equilibrium experiments. Therefore the kinetic rate constants seem to be correct.

The affinity of ^{125}I-Tyr^8-SP with a K_D of 0.3 nM as derived from equilibrium data is fairly high, suggesting the occupancy of the binding site by SP at expected concentrations within the nerve ending (Cuello et al., 1977). The affinity is even higher than observed in neurotensin binding (K_D = 2 nM) to synaptic membranes by Kitabgi et al. (1977), and in enkephalin binding (K_D = 1.8 and 5.8 nM) to crude synaptic membrane preparations by Simantov et al. (1978). Nakata et al. (1978) recently reported a specific binding of ^{3}H-SP to a crude synaptic membrane preparation; their K_D of 3 nM was found to be 10 times higher as that in our study. This difference may be explained by the different membrane preparations or by the short incubation of 1 min at 0° C used by Nakata et al. (1978); our experiments indicate that only 8 % of the amount of peptide which is bound at equilibrium time of 20 min is bound within 1 min. The time of 20 min necessary for saturation in our experiments is in good agreement with the equilibrium time found by Kitabgi et al. (1977) for neurotensin and by Simantov et al. (1978) for enkephalin.

The number of binding sites of 0.8×10^{-12} mol per mg protein is in the same order of magnitude as the concentration of SP in synaptic vesicles (Cuello et al., 1977). Nakata et al. (1978) found only 10 % of these binding sites for SP when expressed per mg protein.

Interference of the binding of ^{125}I-Tyr^8-SP with unlabeled SP, which displaces ^{125}I-Tyr^8-SP when added in excess ($K_i = 1.7 \times 10^{-7}$ M) was observed. This displacement reaction showed positive cooperativity when a Hill plot was performed with these data. The Hill coefficient of 1.33 suggests a slight positive cooperativity in binding, which indicates the presence of more than one binding function within the binding site. Similar observations were made by Davis et al. (1977), who investigated ^{3}H-morphine-and ^{3}H-naloxone-binding with the displacement method. The Hill coefficient indicating theoretically the number of binding functions or protomeres, was not a whole number, which might be caused by membrane perturbation during preparation and storage of vesicles. The displacement of ^{125}I-Tyr^8-SP by the (2–11)-SP-analog results in a higher K_i of 2.8×10^{-6} M. This indicates the significance of the cationic function of Arg^1 for binding. The (3–11) and (4–11) SP analogues show an even higher K_i of 7.8 resp. 8.8×10^{-6} M. Their addition in low amounts to the incubation shows a slight enhancement of the bindings of ^{125}I-Tyr^8-SP, which could be also explained by cooperativity.

An unusual interference with the binding of ^{125}I-Tyr^8-SP was shown with physalaemin and enkephalin. Whereas physalaemin below 10^{-6} M shows an insignificant decrease in binding, higher amounts of physalaemin result in a steep increase of the ^{125}I-Tyr^8-SP binding up to more than 300 % at 10^{-5} M. An increase of binding of ^{125}I-Tyr^8-SP to about 200 % was also found by adding enkephalin in concentrations higher than 10^{-4} M. The physiological significance of this phenomenon remains, however, to be established, in spite of the presence of SP and enkephalin in close vicinity in several regions of the central nervous system (Cuello et al., 1978).

This study shows that SP is bound to synaptic vesicles, where it has been localised immunohistochemically (Pickel et al., 1977). The binding of SP in this study refers to lipid-extractable SP, i.e. to SP bound to lipids as found earlier in brain tissue (Lembeck et al., 1978). This binding of SP to lipids shows characteristics similar to that of other biologically active compounds for which a binding to lipids has been demonstrated (opiates: Loh et al., 1974, 1978; Abood and Hoss, 1975; serotonin: Johnson et al., 1977a, b, 1978). The binding site may represent the storage or the receptor site which cannot, however, be differentiated by the present results.

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