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Regional Distribution and Biochemical Properties of ^{125}I-Tyr^8-Substance P Binding Sites in Synaptic Vesicles*

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Summary. 1. Binding of ^{125}I-Tyr^8-substance P (SP) to synaptic vesicles shows an uneven distribution within the brain and the spinal cord. The regional distribution has a positive correlation with the SP-content, except in the hypothalamus.

2. Ca^{2+} and Mg^{2+}-ions (1 and 10 mM) decrease the number of binding sites without alteration of affinity. EDTA and EGTA enhance SP-binding which is interpreted as being due to removal of the inhibitory influence of endogenous Ca^{2+} and Mg^{2+} through chelation with these agents. No significant inhibition of SP binding was observed by Na^+ or K^+ in concentrations below 100 mM.

3. Pretreatment of synaptic vesicles with trypsin or with phospholipase A_2, C and D leads to a total loss of SP binding showing a proteolipid or a joint protein-phospholipid nature of these binding sites. SH groups do not contribute to SP binding since no effect of N-ethylmaleimide and monoiodoacetic acid on SP binding was found.

Key words: Substance P-binding – Synaptic vesicles – Regional distribution.

Introduction

The highest concentrations of substance P (SP) in subcellular fractions were found in synaptosomal preparations (Inouye and Kataoka, 1962; Ryall, 1964). The attachment of SP to synaptic vesicles within the nerve ending was shown by electron microscopy after immunohistochemical staining (Pickel et al., 1977; Pelletier et al., 1977; Cuello et al., 1978). Mayer et al. (1979) found a high affinity binding of ^{125}I-Tyr^8-SP to synaptic vesicles ($K_D = 0.3$ nM). Binding of ^{3}H-SP to crude synaptic membranes (Nakata et al., 1978) and binding of ^{125}I-Tyr^8-SP to synaptic plasma membranes (Saria et al., 1978) revealed kinetic data differing from binding to synaptic vesicles (higher K_D and lower B_{max}-values). SP can be extracted by a total lipid extraction procedure according to Folch et al. (1957); (Lembeck et al., 1978). The ability of phospholipids to bind SP was demonstrated by Lembeck et al. (1979). Mayer et al. (1979) found that binding sites in synaptic vesicle preparations are extractable with petroleum ether:chloroform (2:1). SP could be released from synaptosomal preparations by increased K^+-concentrations as well as by field stimulation (Schenker et al., 1976; Lembeck et al., 1977). K^+-ions have also been shown to release SP from hypothalamic slices (Cuello et al., 1978), from spinal cord in vitro (Otsuka and Konishi, 1976; Gamse et al., 1979) and from cultured sensory ganglia (Mudge et al., 1979). Capsaicin is able to release SP from spinal cord slices (Gamse et al., 1979). The release of SP was found to be Ca^{2+}-dependent in all cases. Since the release from synaptic vesicles could run parallel with dissociation from storage sites, the influence of cations on the binding of ^{125}I-Tyr^8-SP to synaptic vesicles was investigated. Nakata et al. (1978) found that the distribution of ^{3}H-SP binding sites of crude membrane preparations corresponds to the SP content of the investigated brain regions. Therefore the regional distribution of ^{125}I-Tyr^8-SP binding sites in synaptic vesicles was also investigated. Since the SP binding sites are soluble in organic solvents (Mayer et al., 1979) and the existence of a rather high affinity binding between SP and phospholipids ($K_D = 0.1\,\mu$M) was established (Lembeck et al., 1979), the involvement of membrane phospholipids in ^{125}I-Tyr^8-SP binding to synaptic vesicles was investigated. The high specificity of the SP binding sites in synaptic vesicles as well as the very high

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affinity ($K_D = 0.3$ nM) (Mayer et al., 1979) cannot be explained exclusively by a phospholipid binding. Therefore the participation of a protein moiety within the binding site was also investigated.

Methods

1. Preparation of Synaptic Vesicle Membranes. Adult Sprague-Dawley rats of both sexes were killed by a blow on the neck. The brains were removed and placed on ice before further preparations. Dissection of brains into medulla oblongata (MO), midbrain (MB), corpus striatum (ST), cortex (CO), cerebellum (CE) and hypothalamus (HY) was performed according to Glowinski and Iversen (1969). Spinal cord, dorsal and ventral roots were separated.

Synaptic vesicles from whole brain or from the above mentioned regions were prepared similarly to the method of De Robertis et al. (1963). All handling was carried out at 4°C. Tissues were homogenized in 0.32 M sucrose to obtain a 10% (w/v) homogenate by 4 passes with a motor driven teflon pestle at 3,000 rpm in a glass homogenizer tube. The homogenate was spun down at 2,000 g for 10 min to sediment crude cell debris and nuclei. The supernatant was centrifuged again at 12,000 g for 20 min. The resulting crude mitochondrial pellet (P_2-pellet) was homogenized in the 2.5-fold volume (original tissue weight) water, containing 10 µM $CaCl_2$. After 20 min incubation at 4°C the samples were recentrifuged at 12,000 g for 20 min. The resulting supernatant was centrifuged for 30 min at 100,000 g. The final pellet resulting from about 10 g brain tissue was suspended and homogenized in 1.0 ml 50 mM Tris-buffered sucrose (0.32 M) of pH 7.2, containing 10 µM $CaCl_2$. This suspension was stored at −20°C and showed no loss of binding properties up to 2 months.

The purity of synaptic vesicle preparations was monitored by marker enzymes. Acetylcholinesterase, measured according to Ellman et al. (1961) was used as marker for plasma membranes; its activity was 11-fold higher in the 12,000 g sediment obtained after osmotic shock than in synaptic vesicles. Mg^{2+}- and Na^+-K^+-stimulated adenosine triphosphatase activities were determined by incubation of ATP-containing buffer solutions (Hosie, 1965) and measurement of the remaining ATP with the firefly enzyme system (Kalbhen and Koch, 1969). The ratio of Na^+-K^+-activated adenosine triphosphatase to Mg^{2+}-activated adenosine triphosphatase was 22 in the plasma membrane containing 12,000 g pellet after osmotic shock and 0.03 in the synaptic vesicle fraction. The enrichment of synaptic vesicles within the used preparations was ascertained by electron microscopy.

2. Preparation and Purification of ^{125}I-Tyr^8-SP. 10 µg Tyr^8-SP were iodinated by 5 mCi $Na^{125}I$ (15 mCi/µg iodine) according to Mroz et al. (1977). Control of stability and specific activity measurements of ^{125}I-Tyr^8-SP were performed as described by Mayer et al. (1979). The specific activity of ^{125}I-Tyr^8-SP was 600 Ci/mmol. Unlabelled I-Tyr^8-SP was prepared identically with the appropriate amount of "cold" NaI.

3. Control of the Integrity of ^{125}I-Tyr^8-SP after Incubation with Synaptic Vesicles. The integrity of the tracer after incubation with synaptic vesicles was monitored by ion exchange chromatography on Sulfopropyl-Sephadex C-25 and by gel filtration on Sephadex G-25 as described previously (Mayer et al., 1979) and by TLC on silica gel 60 with n-butanol:acetic-acid:water (4:1.5:5).

4. Extraction of SP and Radioimmunoassay. SP from brain tissue was extracted according to Chang and Leeman (1970) and measured in a radioimmunoassay according to Mroz et al. (1977).

5. Protein Measurement. Protein was measured according to Lowry et al. (1951). The protein of the organic extract resulting from the termination procedure of the binding assay (Methods 7) was measured after evaporation and resolubilization of the residue in hot 1 N NaOH.

6. Phosphorus Determination. Phosphorus was determined according to Bartlett (1959). The term "phospholipid" refers to phosphorus extracted by chloroform:methanol (2:1).

7. Binding Assay. For a routine binding assay, variable amounts of ^{125}I-Tyr^8-SP were added to synaptic vesicle preparations corresponding to 5 − 10 µg protein in a final volume of 1.0 ml Tris-HCl-buffer, pH 7.2. After incubation for 20 min at 30°C the binding assay was terminated by extraction with petroleum ether:chloroform (2:1), as described previously (Mayer et al., 1979). The radioactivity in the resulting organic phase was regarded to be bound to synaptic vesicle membranes.

8. pH-Dependence of ^{125}I-Tyr^8-SP-Binding to Synaptic Vesicles. Buffers used for these experiments were: 0.05 M glycine-HCl (pH 2.2, 3.4, and 4.2), 0.03 M phosphate (pH 5.5, 6.0, 6.5, and 7.0) and 0.05 M Tris-HCl (pH 8.2 and 9.0).

9. Influence of Cations on ^{125}I-Tyr^8-SP-Binding. For investigation of the influence of cations the following salts were added to the incubation medium: $CaCl_2$ (1, 3, 6, and 10 mM), $MgCl_2$ (1, 3, 6, and 10 mM), NaCl (10 and 100 mM), and KCl (10 and 100 mM). It was ascertained that altered salt concentrations did not influence the partition of the tracer into the organic phase in absence of synaptic vesicles.

10. Enzymatic Treatment of Synaptic Vesicles. Synaptic vesicles in 50 mM Tris-HCl at pH 7.4 were pretreated for 20 min at 30°C with 0.01 − 1,000 µg of the following enzymes, before the binding assay (Methods 7):

a) Trypsin: The stability of the tracer against the used trypsin preparations was proved by TLC as described under Methods 5.

b) Phospholipase A_2

c) Phospholipase C: The preincubation was performed with 30 mM phosphate buffer since phospholipase C is inhibited by Tris.

d) Phospholipase D: The assay buffer contained 5 mM $CaCl_2$ which is essential for full activity of phospholipase D. This amount of Ca^{2+} was removed by addition of 5 mM EDTA before the binding assay.

11. Influence of SH-Blockers on ^{125}I-Tyr^8-SP-Binding. 0.5 mM N-ethylmaleimide or 5 mM monoiodoacetic acid was added to the incubation medium before the binding assay (Methods 7).

12. Bioassays. Isolated guinea pig ileum: The isolated ileum was suspended in oxygenated (95% O_2, 5% CO_2) Tyrode solution in a bath of 5 ml at 37°C. Synthetic SP was used as standard.

Rabbit blood pressure: The blood pressure of rabbits anaesthetized with sodium pentobarbitone was recorded from the carotid artery with a Condon manometer. Samples were injected into the jugular vein. Synthetic SP was used as standard.

13. Substances. Substance P: Peninsula, San Carlos, CA, USA, Tyr^8-Substance P: Beckman Instruments, Palo Alto, CA, USA, Acetylthiocholine iodide: Serva, Heidelberg, FRG, Adenosine-5′-triphosphate: Fluka AG, Buchs, Switzerland, Firefly-lantern extract: Sigma Chemical Comp., St. Louis, MO, USA, $Na^{125}I$: Radiochemical Centre, Amersham, Great Britain, Bovine serum albumin: Miles Lab. Inc., Kankakee, IL, USA, Trypsin (TPCK-treated): Merck AG, Darmstadt, FRG, Phospholipase A_2: Boehringer, Mannheim, FRG, Phospholipase C: Sigma Chemical Comp., St. Louis, MO, USA, N-ethylmaleimide: Serva, Heidelberg, FRG. All other chemicals were of analytical grade obtained by Merck AG, Darmstadt, FRG.

Antibody for SP was a generous gift from Dr. S. E. Leeman, Harvard Medical School, Boston, MA, USA.

Table 1. Relative biological activity of I-Tyr^8-SP on the isolated guinea pig ileum and on rabbit blood pressure. The concentration of I-Tyr^8-SP was determined in the SP-radioimmunoassay. 100% = activity of synthetic SP. $\bar{x} \pm s_{\bar{x}}$, n as indicated in the Table. For details see Methods

	% activity	n
Guinea pig ileum	100 ± 11	3
Rabbit blood pressure	103 ± 10	5

Results

1. Biological Activity of I-Tyr^8-SP

The biological activity of I-Tyr^8-SP in two bioassays is shown in Table 1.

2. Phospholipid/Protein Ratio of Synaptic Vesicle Preparations

The synaptic vesicle preparation contained 0.73 $\pm$ 0.02 µmol phospholipids per mg protein ($\bar{x} \pm s_{\bar{x}}$, $n = 3$). The petroleum ether:chloroform extract of the synaptic vesicle preparation contained 34% of its phospholipids and 4% of its proteins.

3. Distribution of Specific Binding Sites of ^{125}I-Tyr^8-SP in Synaptic Vesicles and SP-Immunoreactivity in Different Regions of the CNS

Synaptic vesicle binding sites for ^{125}I-Tyr^8-SP were found to have an uneven regional distribution within the brain and the spinal cord. The distribution of binding sites corresponds to the SP concentration, except in the hypothalamus, where the content of SP was considerably higher than the number of binding sites in synaptic vesicles (Fig. 1). Relative fewer binding sites were also found in the striatum (Fig. 1).

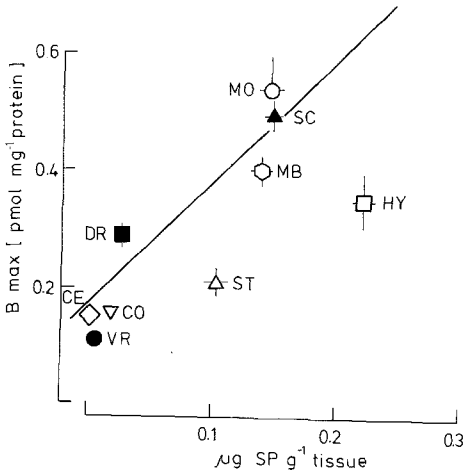

Fig. 1. SP content of different brain regions (*abscissa*) and B_{max} of ^{125}I-Tyr^8-SP binding to synaptic vesicles (*ordinate*). *CO* cortex, *CE* cerebellum, *ST* corpus striatum, *MB* midbrain, *MO* medulla oblongata, *HY* hypothalamus, *SC* spinal cord, *DR* dorsal roots, *VR* ventral roots. Linear regression line was calculated with exclusion of the data of the hypothalamus. Correlation coefficient $r = 0.89$, $\bar{x} \pm s_{\bar{x}}$, n of binding experiments = 4–6. n of SP content = 7–9

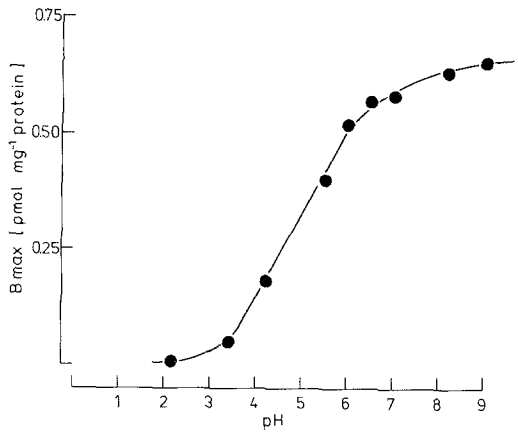

Fig. 2. B_{max} of ^{125}I-Tyr^8-SP binding to synaptic vesicles at different pH. Composition of buffer solutions see Methods 8. Each value represents the mean of triplicate experiments

4. Influence of pH on ^{125}I-Tyr^8-SP-Binding to Synaptic Vesicles

The binding increased with pH, approaching a maximum at pH-values about 8.5 (Fig. 2).

5. Influence of Cations on ^{125}I-Tyr^8-SP-Binding to Synaptic Vesicles

Inhibition of binding by Ca^{2+} and Mg^{2+} was already observed at concentrations of 1 mM. The inhibition by Ca^{2+} and Mg^{2+} increased linearly with logarithmic concentrations of these ions (Fig. 3). Na^+ and K^+ inhibited binding at a concentration of 100 mM but not at a concentration of 10 mM (Fig. 4). Addition of 1 mM EGTA, which is known to react only with Ca^{2+} resulted in a slight increase of the number of binding sites. Addition of 1 mM EDTA, which reacts with Ca^{2+} and Mg^{2+} shows further increase in binding sites (Fig. 4). Scatchard analysis of the binding under increased Ca^{2+} and Mg^{2+} concentrations showed no change in affinity (Fig. 5). This indicated a non competitive antagonism between ^{125}I-Tyr^8-SP and both divalent cations.

6. Influence of SH-Group Reagents and Enzymes on the Binding Site

Preincubation of the synaptic vesicle preparation with the SH-group reagents N-ethylmaleimide (0.5 mM)

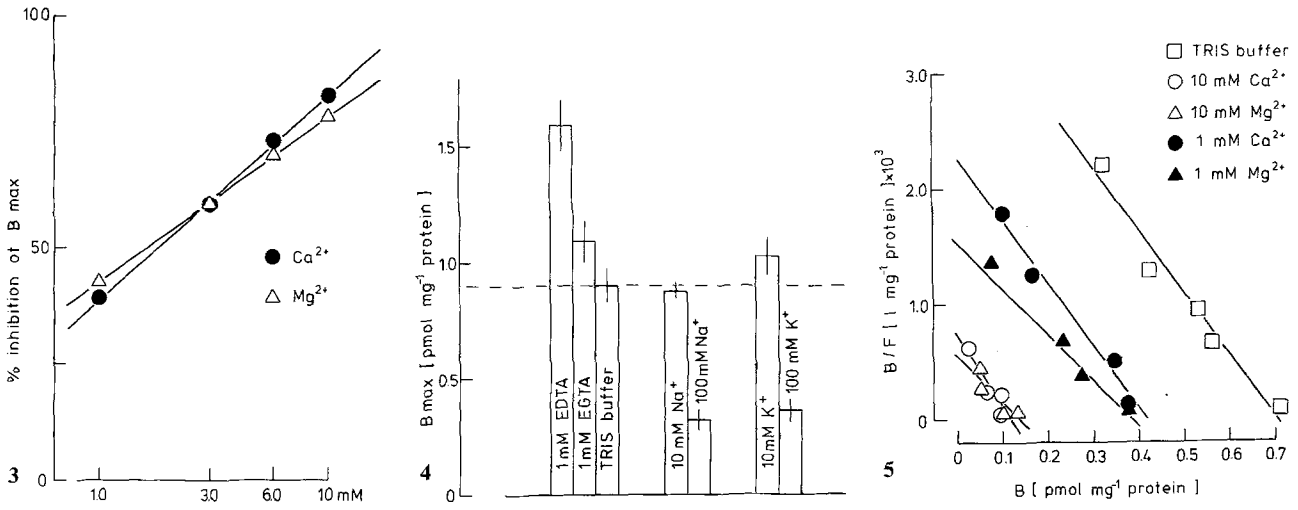

Fig. 3. Percental decrease of B_{max} of ^{125}I-Tyr^8-SP binding to synaptic vesicles (*ordinate*) in presence of different concentrations of Ca^{2+} or Mg^{2+} (*abscissa*). Each value represents the mean of 5 experiments

Fig. 4. Influence of EGTA, EDTA, Na^+ and K^+ in indicated concentrations on B_{max} of ^{125}I-Tyr^8-SP binding to synaptic vesicles. EGTA, EDTA, Na^+ and K^+ in indicated concentrations were added to the Tris-buffer (control) as described in Methods 7. $\bar{x} \pm s_{\bar{x}}$, $n = 5$

Fig. 5. Scatchard plots of ^{125}I-Tyr^8-SP binding to synaptic vesicles in the presence of Ca^{2+} or Mg^{2+} in indicated concentrations and in controls without these cations. Each value represents the mean of triplicate experiments

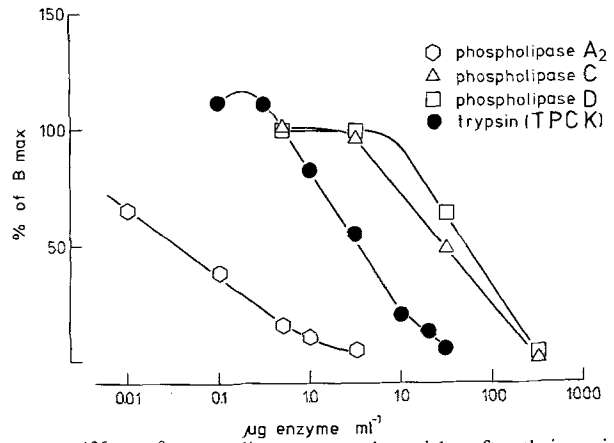

Fig. 6. ^{125}I-Tyr^8-SP binding to synaptic vesicles after their preincubation with enzymes (Methods 10) in concentrations as indicated in the abscissa. 100% = B_{max} after preincubation of synaptic vesicles as described in Methods 10 without addition of enzymes. Each value represents the mean of triplicate experiments

and monoiodoacetic acid (5 mM) did not alter the number of binding sites (B_{max} = 1.08 ± 0.07 pmol per mg protein for N-ethylmaleimide, 0.94 ± 0.05 pmol per mg protein for iodoacetic acid, controls = 1.00 ± 0.06 pmol per mg, n = 3).

Preincubation of synaptic vesicles with trypsin led to a total destruction of binding sites. The lowest concentrations of 0.1 µg/ml trypsin caused a slight augmentation of the binding, higher concentrations greatly decreased binding. Preincubation with phospholipase A_2, phospholipase C and phospholipase D abolished binding (Fig. 6).

Discussion

^{125}I-Tyr^8-SP seems to be qualified for investigation of SP binding sites, because its biological activity in two bioassays is comparable to the activity of synthetic SP.

The high purity of the synaptic vesicle preparation was shown by the high ratio of 22 between Mg^{2+} and Na^+-K^+-activated adenosine triphosphatase. This value is in good agreement with Nagy et al. (1976), who found comparable data with synaptic vesicle preparations of high purity. The presence of only 9% of the acetylcholinesterase activity of lysed P_2-pellets in synaptic vesicles also confirms the purity of the synaptic vesicle preparation used. Electron microscopy of this preparation showed mainly synaptic vesicles of a diameter ranging from 20–80 nm besides few other membrane debris. The phospholipid content of the synaptic vesicle preparation of 0.73 ± 0.02 µmol per mg protein is in good agreement with the values for membrane preparations by Eichberg et al. (1964), Whittaker (1966), Breckenridge et al. (1973) and Nagy et al. (1976). This suggests the mainly membrane origin of the measured protein in our preparation. The phospholipid and protein content of the petroleum ether : chloroform extract indicates the possibility of the extraction of a proteolipid complex which might act as binding site for ^{125}I-Tyr^8-SP.

The first characterization of SP binding sites was made by its lipid moiety. Binding of SP to phosphatidyl serine and phosphatidyl ethanolamine in lipid extracts of brain tissue and in vitro has been demonstrated

(Lembeck et al., 1978, 1979). It was also shown that ^{125}I-Tyr^8-SP could be extracted with an unpolar solvent (petroleum ether:chloroform 2:1) after incubation of synaptic vesicles with ^{125}I-Tyr^8-SP which can only be explained by the removal of ^{125}I-Tyr^8-SP together with an unpolar binding site. This extract contained phospholipids as well as protein, so that lipids, proteolipids or hydrophobic proteins have to be considered as possible binding sites. Possible lipid participation in receptor binding was also assumed for opiates (Loh et al., 1974; Hoss and Smiley, 1977; Loh et al., 1978; Abood and Hoss, 1975) and for acetylcholine (De Robertis et al., 1970; Donnelan et al., 1975; Llorente de Carlin, and De Robertis, 1976; Sobel and Changeux, 1977; Taylor, 1978). Therefore, a possible lipid involvement in ^{125}I-Tyr^8-SP binding can be considered. The affinity of the binding of ^{125}I-Tyr^8-SP to synaptic vesicles ($K_D = 0.3$ nM, Mayer et al., 1979) was much higher than to phospholipids in vitro ($K_D = 0.1$ μM, Lembeck et al., 1979); this suggests the involvement of other binding components besides phospholipids. The pH-dependence of the binding of ^{125}I-Tyr^8-SP to synaptic vesicles differs also from the binding of SP to pure phospholipids in vitro; whereas the association of SP to phospholipids was increased to a maximum within the narrow range of only two pH units (Lembeck et al., 1979), the association of ^{125}I-Tyr^8-SP to synaptic vesicles covers the wide range between pH 3 and 7, suggesting the involvement of more than one ionic binding function.

The involvement of lipids in SP-binding sites is substantiated by the finding that phospholipases reduce SP-binding to synaptic vesicles. Loss of one fatty acid (by phospholipase A_2), splitting the phosphoglycerol ester (by phospholipase C) and removal of serine, ethanolamine or choline (by phospholipase D) caused a loss of SP-binding. These results indicate that intact phospholipids are essential for SP-binding. Phospholipase A_2 pretreatment also abolishes the binding of opiate agonists (Pasternak and Snyder, 1975). This effect was interpreted by Lin and Simon (1978) as a destruction of the membrane environment, which stabilizes the conformation of the opiate receptor. The same assumption can be made for SP-binding sites.

Since the binding of opiate agonists and antagonists to receptors is greatly influenced by cations (Pert and Snyder, 1974), they were used also for the characterization of the SP-binding. While 10 mM Na^+ decrease opiate agonist binding to about 60% (Pert and Snyder, 1974), this concentration had no influence on ^{125}I-Tyr^8-SP-binding. 100 mM Na^+ led only to a 60% decrease of ^{125}I-Tyr^8-SP binding. At this Na^+-concentration the inhibition of opiate receptor binding is approximately a maximum.

K^+ inhibits ^{125}I-Tyr^8-SP binding in the same concentrations as Na^+, whereas opiate receptor binding was shown to be unaffected by K^+ (Pert and Snyder, 1974).

Opiate agonist binding was shown to be increased by Mg^{2+} above 1 mM, whereas Ca^{2+} at this concentration had no effect (Pasternak et al., 1975b). Ca^{2+} and Mg^{2+} each reduced ^{125}I-Tyr^8-SP binding in a concentration dependent fashion. Removal of endogenous Ca^{2+} and Mg^{2+} by EGTA and EDTA increased binding. Ca^{2+} even in its membrane bound form (Hoss et al., 1979) seems therefore to interfere with SP-binding sites. The ^{125}I-Tyr^8-SP binding to synaptic vesicles was inhibited by the concentrations of Ca^{2+} and Mg^{2+} which inhibited SP binding to phospholipids in vitro (Lembeck et al., 1979). This also suggests the contribution of lipids in binding. Non competitive interaction of Ca^{2+} and Mg^{2+} with SP-binding sites can be derived from Scatchard plots.

Speculations about the SP-binding sites have to be concerned either with storage or attachement to a pharmacologically relevant receptor. Since SP has been visualized in synaptic vesicles (Pickel et al., 1977) our present results have to be mainly concerned with a storage site from which SP can be released by e.g. increased Ca^{2+} concentrations. This interpretation of the SP binding is also supported by the positive correlation between the number of binding sites of SP in the synaptic vesicle fraction and the SP content in 5 out of 6 brain regions, in the spinal cord, in dorsal and in ventral roots (Fig. 1). The hypothalamus contains few SP positive fibers in the eminentia mediana besides numerous SP-immunoreactive cell bodies (Hökfelt et al., 1978). This could be the reason why the hypothalamus contains less synaptic vesicle binding sites than expected from the SP content. Also the striatum, where relatively fewer binding sites could be observed (Fig. 1) contains mainly cell bodies of neurones which project to the substantia nigra (Jessell et al., 1978). A correlation was also found between the distribution of enkephalin and opiate receptors in the pituitary gland (Duka et al., 1978) and in the telencephalon (Atweh and Kuhar, 1977). A correlation between the distribution of neurotensin and its specific binding sites was found by Lazarus et al. (1977).

A further characterization of the SP binding was made by demonstrating a protein involvement. Preincubation with trypsin, which concentration dependently reduced the ^{125}I-Tyr^8-SP binding to synaptic vesicles in a similar way was found for opiate agonist binding (Pasternak and Snyder, 1975). In contrast to opiate receptors which are affected by SH-group blockers (Pasternak et al., 1975a; Simon and Hiller, 1978), no such effect could be seen on the binding of

^{125}I-Tyr^{8}-SP to synaptic vesicles, showing the absence of SH-groups at the active site.

The involvement of a protein besides phospholipids was already assumed from a much higher affinity of ^{125}I-Tyr^{8}-SP to synaptic vesicles than that of SP to phospholipid solutions. Incubation with trypsin resulted in a complete loss of the ^{125}I-Tyr^{8}-SP binding to the synaptic vesicle preparations and substantiates the participation of the protein in the binding.

In summary the conclusion is reached that structures in or on synaptic vesicles which include protein and phospholipids may be involved in storage as well as in release mechanisms of SP which could be influenced by divalent cations.

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