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Application of [^{125}I]-[Tyr^{8}]-substance P prepared by the chloramine-T method to receptor-binding experiments after subsequent reduction with mercaptoethanol and purification by reversed-phase liquid chromatography

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Abstract

Radiolabeling of [Tyr^{8}]-substance P ([Tyr^{8}]-SP) with the ^{125}I-isotope was performed by use of the chloramine-T technique. The primary formed radiolabeled product, having been quantitatively converted to the corresponding sulfoxide yielding [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP completely lacked any binding to proteins rich in SP receptor populations. However, after reductive treatment with mercaptoethanol for about 2 h, a complete reconstitution of the Met^{11} thioether structure was observed. The reduced peptide, consisting of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP was separated from its by-products by reversed-phase high-performance liquid chromatography on octadecylsilyl silica gel with 100 m*M* triethyl ammonium formate buffer containing 22% acetonitrile (pH 2.2). The labeled SP derivative prepared by this two-step synthesis was obtained in 73% overall yield related to the [Tyr^{8}]-SP starting material and exhibited a specific activity of $1.9 \cdot 10^{6}$ Ci/M. In contrast to [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP, satisfactory receptor-binding was now observed with the [^{125}I]-[Tyr^{8}]-(Met^{11})-SP derivative.

Keywords: Chloramine-T method; Substance P; [^{125}I]-[Tyr]^{8}-substance P

1. Introduction

The undecapeptide substance P (Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2=SP) is a member of a family of structurally related peptides, called the tachykinins, with the common C-terminal sequence –Phe–Xaa–Gly–Leu–Met–NH_2, Xaa being the only variable amino acid. It was first discovered in 1931 by Von Euler and Gaddum [1] in alcoholic extracts of equine brain and synthesised in 1971 using the solid-phase strategy [2,3]. The peptide is released from a 110 amino acid precursor protein [4] by tryptic cleavage and further processed in biological systems (tissue, body fluids) to smaller fragments exerting various biological and pharmacological actions, by a multitude of enzymatic activities [5–9]. Some of the most salient pharmacological properties of SP include the action on blood vessels (vasodilatation, lowering of blood

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pressure, extravasation), bradycardia, salivation [10,11], bronchoconstriction and mucus hypersecretion [12–16], regulation of extrapyramidal and cognitive functions, pain perception [11,17,18] and its role as an important factor in the modulation of neuroimmunological responses [19].

Considering the widespread pharmacological actions exerted by substance P, it is logical that the development of drugs competing with the parent peptide for receptor-binding sites may play an increasing role for treatment of either CNS or peripheral diseases. Therefore, receptor-binding studies of putative SP receptor antagonists are indispensable in order to provide more insight into the mechanism of the pharmacological and biological actions of SP as well as synthesis of drugs exhibiting powerful SP antagonism.

Marked heterogeneity of SP receptors is reported and at least three types of SP receptors [20–23] are postulated, termed the SP-P (where physalaemin is most potent), SP-E (eledoisin being most potent) and SP-K (where substance K, another mammalian tachykinin is most potent) receptor subtypes. However, due to the fact that large differences exist in the affinity of SP towards these receptor subtypes, it is appropriate to use the more general expression 'tachykinin' receptor. In this paper an optimised strategy of SP receptor-binding to rat brain membranes on the basis of [^{125}I]-[Tyr^{8}]-substance P used as the radiolabel is described. The genuine radiolabel, quantitatively consisting of the corresponding sulfoxide [24,25], was subjected to reductive treatment with mercaptoethanol and purified by reversed-phase HPLC (RP-HPLC) To our knowledge, no attempts have hitherto been undertaken to use ^{125}I-radiolabeled [Tyr^{8}]-SP treated in this way for receptor-binding experiments.

2. Experimental

2.1. Materials

Acetonitrile of HPLC grade was a product of Rathburn (Walkerburn, UK). Water for use in HPLC was prepared with a Milli Q^{TM} reagent water system from Millipore (Bedford, MA, USA) and furthermore doubly distilled for the use in receptor-binding experiments. Chloramine-T, hydrogen peroxide (30%), triethylamine, formic acid (85%), acetic acid, sodium acetate, sodium chloride, potassium chloride, manganese dichloride tetrahydrate, all of analytical grade, were obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane hydrochloride (Tris–HCl), ethylene glycol-bis-(2-aminoethyl)-tetraacetic acid (EGTA) and dimethyl sulfoxide (DMSO) were from Fluka (Neu-Ulm, Germany). [Tyr^{8}]-SP$_{1-11}$ was from Peninsula Laboratories (Belmont, CA, USA) and Na^{125}I (Code No. IMS 30) was purchased as a 0.1 *M* sodium hydroxide solution from Amersham-Buchler (Braunschweig, Germany). Substance P$_{1-11}$ (SP$_{1-11}$), physalaemin, eledoisin and mercaptoethanol were obtained from Sigma (Deisenhofen, Germany). Bacitracin, chymostatin, leupeptin, bovine serum albumin (BSA) and dithiothreitol (DDT), all of research grade, were from Serva (Heidelberg, Germany).

2.2. Preparation and pre-purification of [^{125}I]-[Tyr^{8}]-SP

Radioidination of [Tyr^{8}]-SP and pre-purification by ion-exchange chromatography on carboxymethylcellulose CM-52 as well as selection of fractions (showing an optimum ratio of specific to non-specific binding as checked by the affinity of the label to the specific antibody SP 9-3a) used for subsequent reduction with mercaptoethanol, was extensively described in Refs. [24,25] and thus will not be further treated here.

2.3. Treatment of [^{125}I]-labeled [Tyr^{8}]-SP with mercaptoethanol and subsequent oxidation with hydrogen peroxide

To an aliquot of 500 μl of pooled selected fractions of the CM-52 effluent (approx. $11 \cdot 10^{6}$ cpm≅3.3 p*M*) was added 500 μl of 0.25 *M* sodium acetate buffer (pH 4.0) and 100 μl of mercaptoethanol and the mixture heated to 80°C for 2 h. Excessive reducing agent and solvent were evaporated by a gentle stream of nitrogen and the residue reconstituted in 1000 μl of water. For analytical purpose 1000 μl of a sample previously diluted to approximately 800 000 cpm (i.e., ≅240 f*M*) with 0.25 *M* sodium acetate buffer (pH 4.0) was subjected

to HPLC as described below. For subsequent oxidation approximately 350 000 cpm (i.e., ≅105 f*M*) of the purified mercaptoethanol-treated radiolabel in 100 μl of 0.25 *M* sodium acetate buffer (pH 4.0) was reacted with 100 μl of 10 m*M* hydrogen peroxide in 0.25 *M* sodium acetate buffer (pH 4.0) for 30 min at 80°C, diluted with water to a final volume of 1000 μl and subjected to RP-HPLC.

2.4. Chromatographic system

Isocratic RP-HPLC was chosen for separation of the desired component from unwanted by-products. The whole system was composed of a pulse-dampened LC 410 pump obtained from Kontron Analysentechnik (Munich, Germany), an injection valve equipped with a 1000-μl sample loop from Valco Instruments (Schenkon Switzerland) and a type Frac 200 fraction collector purchased from Pharmacia (Freiburg, Germany). A Spherisorb ODS II column (125×4.6 mm I.D., 5 μm particles) obtained from Bischoff Analysentechnik (Leonberg, Germany) was used as the stationary phase. Chromatographic separation was performed with 22% acetonitrile in 100 m*M* triethylammonium formate (TEAF) buffer (apparent pH 2.2[1]). Before separation of the ^{125}I-labeled peptide, two injections each of 5 μg of SP_{1-11} dissolved in 1000 μl of 0.25 *M* sodium acetate buffer (pH 4.0) followed by a blank run with 0.25 *M* sodium acetate buffer (pH 4.0) were made in order to minimise 'silanophilic interactions'. An aliquot (1000 μl) of the reduced peptide was injected into the HPLC system and separated at a flow-rate of 1 ml/min. Fractions (1 ml) were collected and aliquots (100 μl) withdrawn for monitoring of the elution profile by means of a type LB 2104 12-channel radioactivity monitor from Berthold (Wildbad, Germany). Thereafter, fractions attributable to labeled SP with an intact methionine moiety, i.e., fractions 7–9 (see Fig. 2), were pooled and evaporated to dryness in a Speed Vac concentrator obtained from Savant (Hicksville, NY, USA) and stored at −40°C until use.

[1]The term 'apparent' means that pH adjustment is done with the final aqueous–organic solution and not withthe TEAF aqueous phase prior to addition of the organic modifier.

2.5. Solutions for preparation of rat brain homogenates and receptor-binding experiments

1. Buffer 1: 50 m*M* Tris–HCl, 100 m*M* sodium chloride and 5 m*M* potassium chloride adjusted to pH 7.4
2. Buffer 2: 50 m*M* Tris–HCl, 300 m*M* potassium chloride and 10 m*M* EGTA adjusted to pH 7.4
3. Buffer 3: 50 m*M* Tris–HCl adjusted to pH 7.4
4. Buffer 4: buffer 3 containing 1 m*M* DTT adjusted to pH 7.4
5. Assay buffer: 50 m*M* Tris–HCl containing 3 m*M* manganese dichloride tetrahydrate, 1 m*M* DTT, 4 μ*M* chymostatin, 8 μ*M* BSA, 8 μ*M* leupeptin and 30 μ*M* bacitracin adjusted to pH 7.4. The whole solution was exhaustively deaerated with nitrogen.

The following concentration ranges of SP_{1-11}, eledoisin and physalaemin were used: 10^{-11}–10^{-7} *M* (SP_{1-11}), $3\cdot10^{-11}$–$3\cdot10^{-7}$ *M* (physalaemin), 10^{-9}–10^{-5} *M* (eledoisin). All dilutions were prepared in assay buffer.

2.6. Receptor-binding of mercaptoethanol-treated [^{125}I]-[Tyr^{8}]-SP

2.6.1. Preparation of membranes from whole rat brain

Male Whistar rats (strain Chbb Thom) were decapitated, brains rapidly removed and immersed in ice-cold buffer 1. After homogenisation under ice-cooling with 6 strokes at 1200 rpm in a Heidolph potter (Heidolph, Nürnberg, Germany), the homogenate was transferred to a centrifuge tube. The potter was rinsed with buffer 1 in order to achieve high recovery and the obtained suspension added to the homogenate which was centrifuged for 15 min at 25 000 *g* in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Munich, Germany) and the supernatant decanted. The pellet was resuspended in ice-cold buffer 2, transferred into a potter, manually homogenised, kept on ice for about 30 min and centrifuged as described above. The pellet was resuspended two times in buffer 3 and centrifuged as described above. The resulting pellet containing

membrane proteins was weighed and diluted 30-fold (w/v) with buffer 4. Aliquots (5 ml) were immediately frozen and stored in liquid nitrogen. Thawing of the frozen samples for the use in receptor-binding experiments was rapidly performed with tap water and vortexing.

2.6.2. Receptor-binding experiments

Total binding at each concentration level of non-radioactive SP_{1-11}, physalaemin and eledoisin to rat brain membrane proteins was performed by addition of 100 μl of freshly mercaptoethanol reduced [^{125}I]-[Tyr^{8}]-SP dissolved in assay buffer (approx. 70 000 cpm≅20 f*M*) and 500 μl assay buffer to 200 μl rat brain homogenate prepared as described above. Non-specific binding was determined by addition of 100 μl of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP, 100 μl of SP_{1-11} (2 μ*M*) and 400 μl assay buffer to 200 μl homogenate. For binding/displacement experiments 100 μl radiolabeled SP, 100 μl of each concentration of unlabeled peptide, 200 μl rat brain homogenate and 400 μl assay buffer were incubated in triplicate for 60 min at 20±0.5°C in polypropylene tubes. After centrifuging for 4 min at 4000 *g* at 0–5°C, the supernatants were decanted to waste and the pellets measured in a type LB 2104 12-channel γ-counter.

3. Results

In accordance with earlier investigations [24,25], [^{125}I]-[Tyr^{8}]-(Met^{11})-SP containing an intact thioether structure was prepared in 91% yield starting from pre-purified [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP obtained by the chloramine-T method and subsequent reduction with mercaptoethanol. A specific activity of $1.9 \cdot 10^{6}$ Ci/M was calculated. Optimum yield of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP was achieved after a reaction time of 2 h at 80°C as depicted in Fig. 1 which shows the increase of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP vs. the decrease of [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP as a function of reaction time. However, extension to

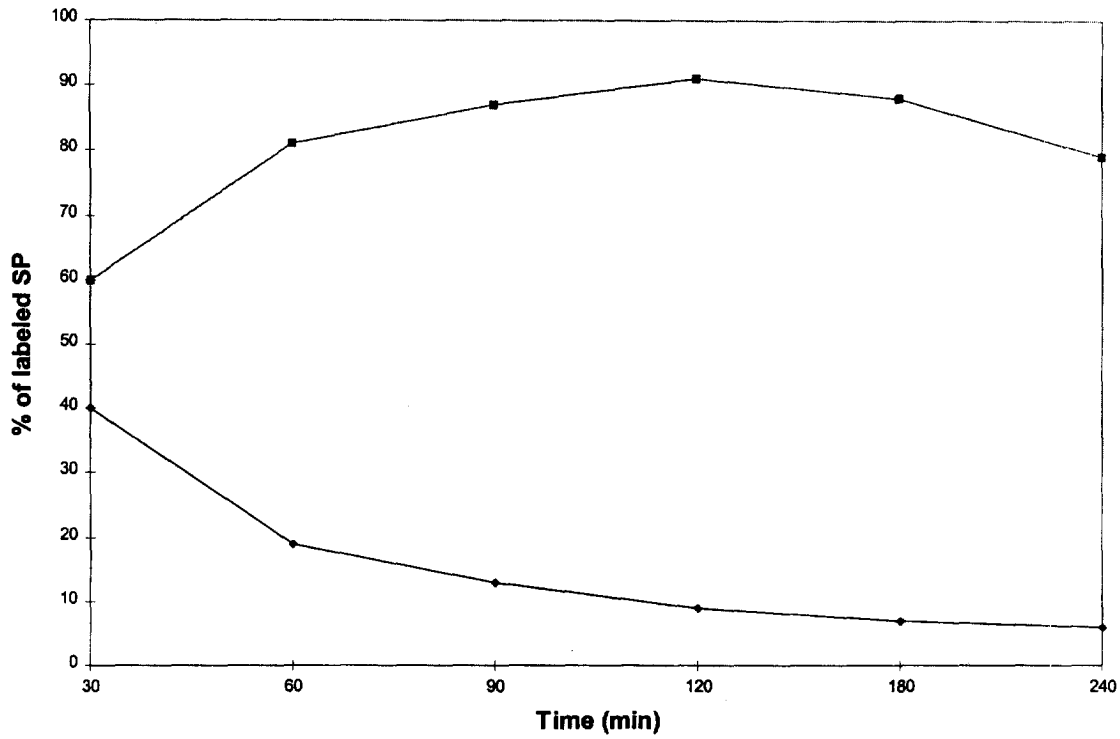

Fig. 1. Relative amounts (%) of [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP (lower curve) and [^{125}I]-[Tyr^{8}]-(Met^{11})-SP (upper curve) after treatment of [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP with mercaptoethanol in dependence of the reaction time as evaluated from the HPLC elution profiles.

about 4 h afforded substantial degradation of the radiolabel. Additional peaks shifted to either higher or lower retention times compared with the target compound were observed and the yield decreased to about 80% (Fig. 1). The HPLC profile of freshly ^{125}I-radiolabeled [Tyr^{8}]-SP after subsequent reduction with mercaptoethanol under optimum conditions is depicted in Fig. 2. Taking into account the reproducible yields of about 80% ^{125}I incorporation, an overall recovery of about 73% of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP was achieved after reductive treatment. Recovery of radioactive material subjected to RP-HPLC was >95% which indicates that residual silanol groups have been sufficiently shielded by the previous treatment with non-radioactive SP_{1-11}. Complete conversion of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP to its sulfoxidised analogue [^{125}I]-[Tyr^{8}]-(Met^{11}→O)-SP was effected by treatment with 10 m*M* hydrogen peroxide at 80°C as depicted in Fig. 3. In addition, the use of a volatile buffer system, TEAF, allowed dissolution of the radiolabel in the assay system without contaminants attributable to residual amounts of buffer ingredients of the HPLC separation step.

Possible degradation of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP by autoradiolysis during storage from a few days up to some weeks, was checked by HPLC and the labeled peptide proved to be sufficiently stable for about three months when used in receptor-binding experiments.

Receptor-binding experiments performed with the radiopeptide prepared in this manner using membrane preparations rich in SP receptor populations, such as those obtained from whole rat brain homogenates, revealed satisfactory binding characteristics. Binding of mercaptoethanol-treated [^{125}I]-[Tyr^{8}]-SP to rat brain membrane preparations was time-dependent and saturable and reached maximum values at about 60 min as shown in Fig. 4. Under the chosen assay conditions about 5% of the radiolabel was bound (approx. 3500 cpm≅1 f*M* vs. approx. 70 000 cpm≅f*M* of total radioactivity applied) and non-specific binding accounts for only about 15% of total binding, i.e., approx. 500 vs. 3500 cpm, respectively. [^{125}I]-[Tyr^{8}]-(Met^{11})-SP was competitively displaced from its receptor protein with increasing concentrations of either SP_{1-11} or the two amphibian tachykinins physalaemin and eledoisin as shown in

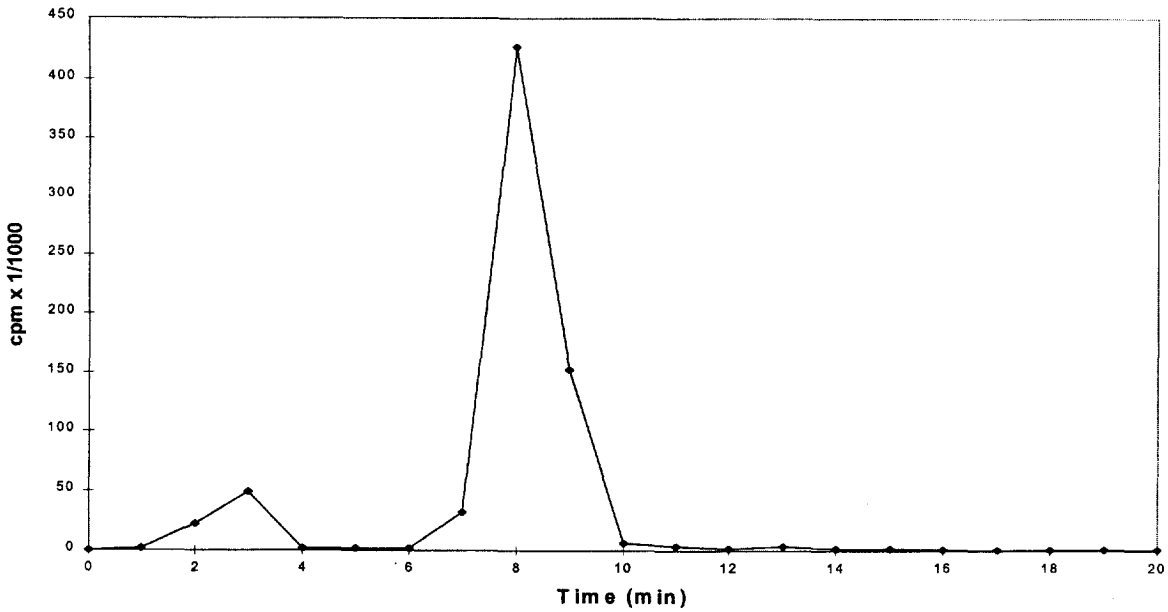

Fig. 2. HPLC profile of ^{125}I-labeled [Tyr^{8}]-SP after reduction with mercaptoethanol for 2 h and purified by RP-HPLC on a Spherisorb ODS II column (125×4.6 mm I.D., 5 μm particles); Fractions 7–9 were pooled and evaporated to dryness for subsequent receptor-binding investigations; chromatographic conditions see Section 2.6.

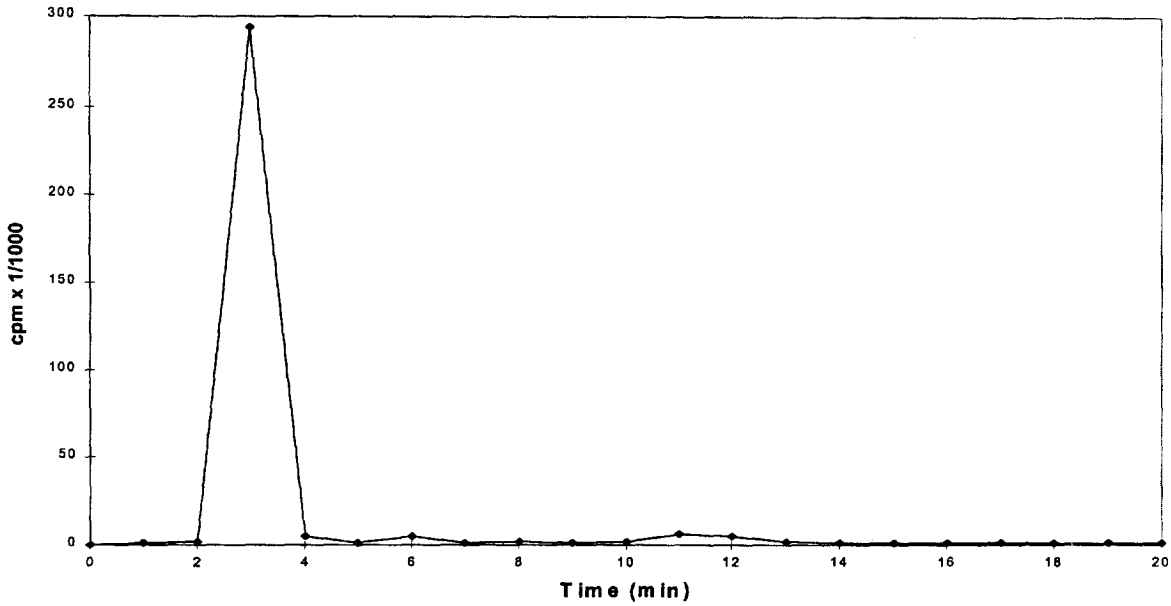

Fig. 3. HPLC profile of mercaptoethanol reduced ^{125}I-labeled [Tyr^{8}]-SP purified by RP-HPLC and re-oxidised with hydrogen peroxide on a Spherisorb ODS II column (125×4.6 mm I.D., 5 μm particles); chromatographic conditions see Section 2.4.

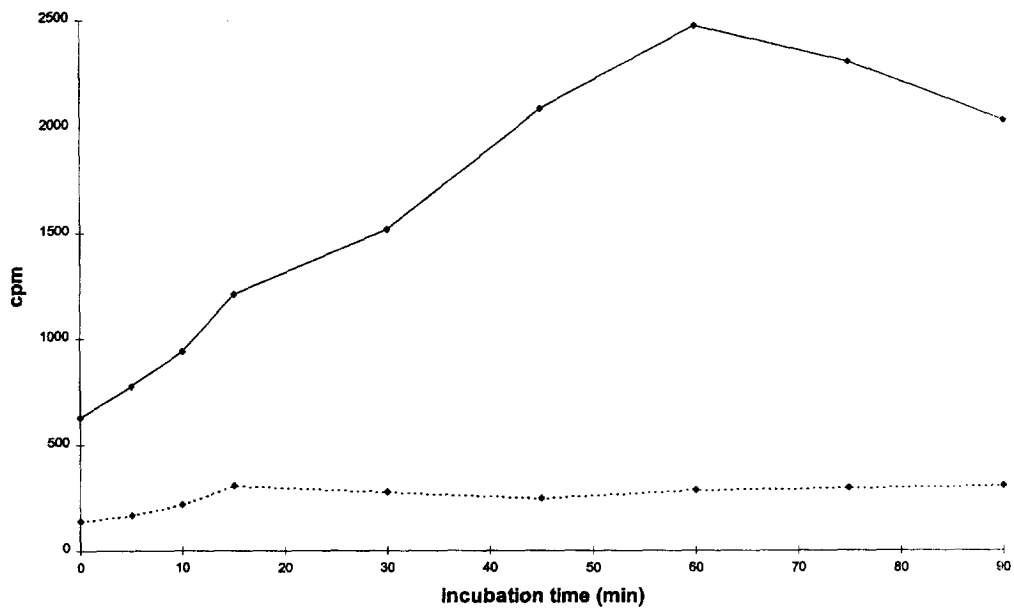

Fig. 4. Dependence of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP binding to whole rat brain homogenates on incubation time. Dotted line (lower curve), non-specific binding.

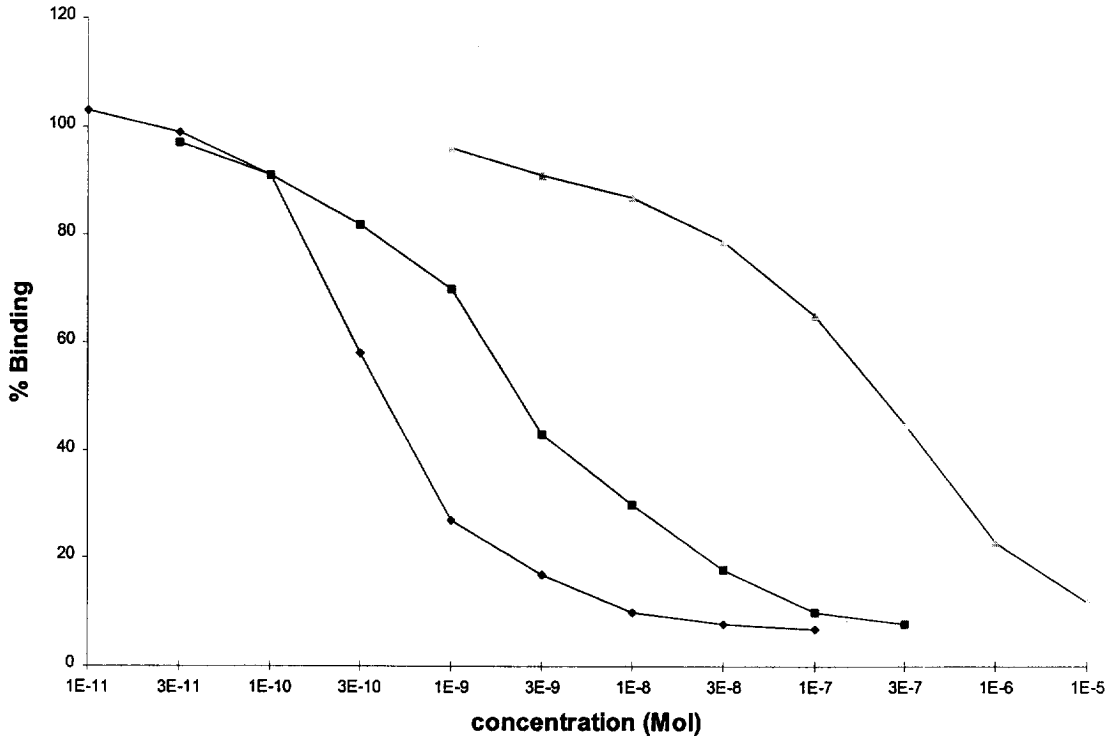

Fig. 5. Displacement curves of mercaptoethanol reduced ^{125}I-labeled [Tyr^{8}]-SP by the unlabeled tachykinins SP_{1-11} (♦), physalaemin (■) and eledoisin (△) in the concentration range of 10^{-11}–10^{-5} M. Non-specific binding was determined by addition of 2 μ*M* SP_{1-11}; receptor-binding conditions see Experimental.

Fig. 5. All three tachykinins effected about 90% displacement of [Tyr^{8}]-(Met^{11})-SP. The concentration–displacement curves of either physalaemin or eledoisin are markedly shifted to higher concentrations as can be seen from the peptide amounts effecting 50% inhibition of [^{125}I]-[Tyr^{8}]-(Met^{11})-SP receptor-binding (IC_{50} values) of about $5\cdot10^{-10}$ *M* for SP_{1-11}, $2.5\cdot10^{-9}$ *M* for physalaemin and $2.5\cdot10^{-7}$ *M* for eledoisin. The coefficients of variation (C.V.) for the different concentrations of SP_{1-11}, physalaemin and eledoisin ranged from 4.7–25.0% (SP_{1-11}, $n^{2}=5$), 5.6–17.9% (physalaemin, $n=13$) and 3.6–14.9% (eledoisin, $n=9$). In contrast to [^{125}I]-[Tyr^{8}]-SP, which was subjected to reductive treatment, the [^{125}I]-[Tyr^{8}]-SP derivative directly obtained from the labeling procedure shows only negligible binding to the membrane proteins not substantially differing from the non-specific interactions (results not shown).

4. Discussion

Preparation of [^{125}I]-[Tyr^{8}]-SP by means of the chloramine-T technique still plays an only minor role in receptor-binding applications, which can be mainly ascribed to the failure of the radiopeptide to bind to SP receptor proteins. Indeed, only negligible binding of a radiolabel prepared in this manner was observed after ion-exchange purification without mercaptoethanol treatment, which however, increased dramatically after subsequent reduction with mercaptoethanol. This lack of binding is probably caused by complete oxidation of the Met^{11} thioether structure in [^{125}I]-[Tyr^{8}]-SP when chloramine-T was used as the oxidative agent [24,25]. For this reason, additional procedures, which either do not effect

[2] *n* corresponds to the number of determinations at each concentration level of competing unlabeled peptide.

oxidative conversion of Met^{11} or subsequent reductive treatment of the radiolabel are required.

Alternative procedures for preparation of radiolabeled SP preserving the intact thioether moiety include the preparation of so-called ^{125}I-Bolton–Hunter SP [26–30] and tritiated SP derivatives [31–33]. Other radiolabeled members of the tachykinin family meeting identical requirements, such as ^{3}H-[Tyr^{8}]-physalaemin [34] and ^{125}I-Bolton-Hunter eledoisin [35] have also been successfully used as radiolabeled antigens for SP receptor-binding investigations. Nevertheless, preparation of the ^{125}I-Bolton–Hunter SP derivative provides substantially lower yields of labeled peptide [36,37] compared with mercaptoethanol-reduced [^{125}I]-[Tyr^{8}]-SP and tritiated labels often suffer from very low specific activities and high prices. However, oxidation of Met residues in peptides under the conditions of radioiodination with chloramine-T is a well-known side reaction reported in a multitude of papers [26,28,38–49], which also takes place during incorporation of ^{125}I into [Tyr^{8}]-SP and quantitative conversion of the Met^{11} residue to the corresponding sulfoxide is observed [24,25]. The problems encountered in procedures used for the preparation of radioiodinated SP analogues is described in more detail in a recently published review article [37].

In contrast to the use of the native (untreated) ^{125}I-radiolabel, which did not bind to rat brain membrane proteins, the mercaptoethanol-reduced peptide exhibits binding comparable with that achieved by use of the corresponding ^{125}I-Bolton–Hunter analogue. This observation suggests, that preservation of an intact Met^{11}-residue within the peptide sequence is an indispensable prerequisite for peptide-receptor interactions. For this reason, it may be concluded that the presence of $Met^{11}\rightarrow O$ leads to strong polar (e.g., hydrogen bonding) interactions, which markedly prevent a suitable conformation of labeled SP with its complementary structural segment, i.e., its binding site, on the membrane protein. Surprisingly, binding of [^{125}I]-[Tyr^{8}]-SP, prepared by the chloramine-T technique and not subjected to reductive treatment, to dispersed pancreatic acinar cells from the guinea pig and to synaptic vesicles of rat brain, respectively, was reported by Sjödin et al. [50] and Mayer et al. [51], respectively. However in contrast, Michelot et al. [26] preferred the use of ^{125}I-Bolton–Hunter-SP compared with chloramine-T prepared [^{125}I]-[Tyr^{8}]-SP due to the unsatisfactory biological activity of the latter derivative, as also reported by Liang and Cascieri [28]. However, unlike the situation on the membrane protein, substantial binding of SP to its antibody is still achieved with [^{125}I]-[Tyr^{8}]-($Met^{11}\rightarrow O$)-SP directly obtained from radioiodination with chloramine-T. Nevertheless, as shown earlier [24], sensitivity of an SP radioimmunoassay is markedly lower compared with the mercaptoethanol-treated radiopeptide.

Reduced [^{125}I]-[Tyr^{8}]-SP is extensively displaced from its receptor located on rat brain membranes with increasing concentrations of SP_{1-11} as well as physalaemin and eledoisin. Nevertheless, the individual displacement curves for the two amphibian tachkinins are markedly shifted to higher IC_{50} values, being about $2.5\cdot10^{-9}$ M for physalaemin and $2.5\cdot10^{-7}$ M for eledoisin vs. about $5\cdot10^{-10}$ M for SP_{1-11} (Fig. 5). The marked shift of IC_{50} values between SP_{1-11} and eledoisin is in accordance with the findings of Payan et al. [52], Viger et al. [30] and Mohini et al. [27], having also measured an about 10^{3}-fold higher concentration of eledoisin compared with SP in rat brain homogenates.

Taken together, the observation of different affinity of the three tachykinins with respect to their displacement properties on membrane preparations from whole rat brain towards [^{125}I]-[Tyr^{8}]-(Met^{11})-SP, are in accordance with the existence of different SP receptors as evidenced by pharmacological investigations [20–23]. However, it is not the aim of this study to discuss the specific features of tachykinin receptor-binding and, for this reason, the interested reader should refer to the huge amount of special literature published in this area.

5. Conclusions

The receptor-binding investigations performed with ^{125}I-labeled [Tyr^{8}]-SP clearly revealed that only the product subjected to reductive treatment with mercaptoethanol was bound to a membrane preparation obtained from whole rat brain. Competitive displacement of the radiolabel by increasing concentrations of unlabeled SP undecapeptide as well as the amphibian tachykinins physalaemin and eledoisin

was observed. The two-step synthesis of the desired radiolabeled compound [^{125}I]-[Tyr^{8}]-(Met^{11})-SP afforded an overall yield of 73%. This value is substantially higher than the yield obtained with the one-step procedure during preparation of the corresponding ^{125}I-Bolton–Hunter SP derivative. In the latter case a coupling yield of only about 50% is achieved. Therefore, the described labeling procedure with ^{125}I and subsequent reduction with mercaptoethanol offers a rapid and reliable alternative to competing methods providing either high overall yield of target peptide or stability towards autoradiolysis as well as satisfactory binding to rat brain membrane homogenates.

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