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APPARENT MOLECULAR WEIGHT OF SUBSTANCE P / NEUROKININ-1 RECEPTORS DETERMINED USING A PHOTOAFFINITY LABELLED PROBE, [(3'-^{125}I) D-Tyr^{0}, (4'-N_3)Phe^{8}, Nle^{11}]-SUBSTANCE P

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A photoreactive derivative, [(3'-^{125}I) D-Tyr^{0}, (4'-N_3)Phe^{8}, Nle^{11}]-substance P (SP) was prepared and iodinated using carrier-free [^{125}I] to determine the apparent molecular weight of one sub-type of neurokinin (NK) receptor, the SP/NK-1 type. The unlabelled analogue competed for [^{3}H]-SP sites with an IC_{50} of 10 nM. The radioactive photoprobe ($K_D \approx 0.17$ nM, B_{max}=15.6 fmol/mg protein) was used to photoaffinity label membranes prepared from rat brain. Autoradiographs revealed that a single band with an apparent molecular weight of 46,000 daltons was specifically labelled. This labelling was inhibited by non-radioactive SP in a concentration-dependent manner (1.0nM-0.1mM) suggesting that the observed labelling represents the SP/NK-1 receptor type.

Substance P (SP)was first described by Von Euler and Gaddum (1) as a potent hypotensive and spasmogenic factor present in equine brain and intestine. This undecapeptide belongs to a family of naturally occuring peptides, known as the tachykinins. Recently, two novel tachykinins have been identified in mammalian brain tissues, namely neurokinin A (NKA) and neurokinin B (NKB) (2,3). These newly described neurokinins and SP share many biological effects, although it is likely that they also possess additional effects of their own suggesting the existence of various classes of neurokinin receptors (4).

Already, recent evidences based on the comparative potencies of series of agonists and antagonists (5-9) and binding data (4, 10-15)

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have supported the existence of three neurokinin receptors namely the neurokinin-1 (NK-1), neurokinin-2 (NK-2) and neurokinin-3 (NK-3) sub-types. However, it is not clear if these various receptor sub-types represent different molecular entities or merely different conformations of the same receptor protein.

As a first step toward that goal, we are currently attempting to determine the apparent molecular weight of the various NK receptors using photoaffinity labelled probes. We report here on the photoaffinity labelling of the SP/NK-1 receptor sites in rat brain membranes using a radioiodinated photoreactive derivative [(3'-^{125}I) D-Tyr^{o}, (4'-N_3)Phe^{8}, Nle^{11}]-SP or N_3-SP. This photoreactive probe competes for [^{3}H]-SP sites with an IC_{50} of 10 nM suggesting interaction with the NK-1 receptor sub-type. Autoradiographs of the SDS-PAGE reveal the specific labelling of a band with an apparent molecular weight of 46,000 daltons. The concentration-dependent inhibition of the labelling of the 46,000 daltons band by SP suggests that the observed labelling represents the SP/NK-1 receptor type.

MATERIALS AND METHODS

Binding assays. Membrane preparations and binding assays were prepared essentially as described previously (16) using 1.4 nM [^{3}H]-SP (45.7 Ci/mmol, New England Nuclear, Boston, MA) in the competition experiments and [^{125}I]-N_3-SP (0.2 nM) in saturation experiments.

Synthesis of N_3-SP. The peptide sequence D-Tyr-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe(4'-NO_2)-Gly-Leu-Nle-NH_2 was built up by stepwise automatic solid phase synthesis using methylbenzhydrylamine resin as anchor (0.8g) according to the standard procedures (17). As synthesis scheme was used the classical Boc-TFA-HF-scheme using the following amino-acid derivatives: Boc-Nle, Boc-Leu, Boc-Gly, Boc-Phe(4'-NO_2), Boc-Phe, Boc-Gln, Boc-Pro, Boc-Lys(Cl_2Z), Boc-Arg(Tos) and Bis-Boc-D-Tyr. After HF-cleavage, the crude product was lyophilized, filtered over LH20 with DMF and finally purified by two prep-HPLC operation on C-18, 30μ support, eluted by 0.1% TFA in a 20% to 60% acetonitrile gradient. The product containing fractions were lyophilized twice from 2N HOAc yielding 192 mg of [D-Tyr^{0}, (4'-NO_2)Phe^{8}, Nle^{11}]SP, FAB-MS: 1539 (M^{+}+1). This precursor was transformed into the azido peptide by already published procedures (18). In brief, 6.0 mg of the nitropeptide were hydrogenated, diazotized at 0^{0}C and treated with NaN_3. The reaction mixture was neutralized with NaOAc, diluted with H_2O and directly applied to a small prep HPLC, eluted with the above gradient. The pure peptide fractions were collected and twice lyophilized which produced 5.0 mg of pure photolabel: [D-Tyr^{0}, (4'-N_3)Phe^{8}, Nle^{11}]SP or N_3-SP.

Iodination and Purification of N_3-SP. The technique used for the iodination of the peptide was described previously (19). Essentially, iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Sigma Chemical Company, St-Louis, MO) in 10 µl of chloroform was pipetted into a 100 µl polyethylene tube and allowed to evaporate. This lead to a plating of 0.5 µg of iodogen onto the wall of the tube. To this were added 20 µg of N_3-SP in 20 µl of potassium phosphate buffer pH 7.5 and 1 mCi of Na[^{125}I] (New England Nuclear). The reaction was stopped by adding 100 µl of KI (1M), 100 µl of Na_2SO_3 (1M) and 300 µl of H_2O and purified on a HPLC apparatus according to a procedure developed in our laboratory (20). The fractions corresponding to the elution volume of the monoiodinated peptide are pooled, diluted with 0.9 % of saline and lyophilized. The radioactive peptide was redissolve with water before using in photoaffinity labelling reaction.

Photoaffinity labelling with radioactive peptide. The synaptosomes were prepared according to a modified method of Whittaker et al (21). Briefly, adult male Sprague-Dawley rats (150-200g; Canadian Breeding Farms, St-Constant, Québec) were decapitated, the brains (minus cerebellum) were removed, put in 0.32 M ice-cold sucrose and homogenized with a glass-teflon homogenizer for 12 strokes. Then, the tissues were centrifuged at 1000g for 10 min. The supernatant was collected and the pellet was resuspended with 0.32 M ice-cold sucrose and recentrifuged at 1000g for 10 min. The supernatant were combined and centrifuged at 12,000g for 20 min. After the centrifugation, the supernatant was discarded and the pellet was washed once with 0.32 M ice-cold sucrose and recentrifuged at 12,000g for 20 min. The pellet fraction which contained the synaptosomes, was used in the photoaffinity labelling reaction. Purified rat brain synaptosomes were incubated for 90 min at room temperature in a 15 ml tube at room temperature containing 1.5 ml of synaptosome preparations in an assay buffer (50 mM Tris, 0.02% (w/v) bovine serum albumin (ICN, Cleveland, Ohio), 0.2 mg/100 ml chymostatin (Sigma), 0.4 mg/100 ml leupeptin (Sigma), 4 mg/100 ml bacitracin (Sigma), 3 mM $MnCl_2$, pH 7.4), 200 µl of [^{125}I]-N_3-SP (≈ 10^7 cpm or 10 fmol) and 200 µl of assay buffer or non-radioactive SP (various concentrations, IAF Biochem, Laval, Québec) to determine specific labelling. The membrane suspensions were then exposed for 15 min to UV light. The photolabelled membranes were washed from unbound peptides three times with 5 ml of assay buffer and centrifuged for 10 min at 50,000g. After washing, the pellet was solubilized for SDS gel electrophoresis in 250 µl of a buffer containing 50 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, 1% (v/v) glycerol, 5% (v/v) 2 β-mercaptoethanol and heat at 95^{0}C for 4 min. All the above chemicals were obtained from Bio-Rad laboratories (Canada) Ltd (Mississauga, Ontario). The solubilized proteins were kept frozen at -20^{0}C until used. SDS-PAGE of the solubilized proteins were performed on slab gels (Bio-Rad) by the method of Laemmli (22) with 12% acrylamide. Protein molecular weight standards (Bio-Rad) used for calibration of SDS-PAGE included: phosphorylase b, 130,000; bovine serum albumin, 75,000; ovalbumin, 50,000; carbonic anhydrase, 39,000; soybean trypsin inhibitor, 27,000 and lysozyme, 17,000.

RESULTS AND DISCUSSION

First, the affinity of the photoreactive N_3-SP analogue for SP/NK-1 receptor sites was determined using [^{3}H]-SP as ligand. N_3-SP inhibited 50% (IC_{50}) of specific [^{3}H]-SP binding at 10 nM in rat brain membranes (n=2, each in triplicate). Thus, it appears that N_3-SP possesses suffi-

cient affinity to be use as ligand in binding assays. This is supported by direct binding assays using [^{125}I]-N_3-SP labelled as described above. As shown in Fig. 1, [^{125}I]-N_3-SP binds with high affinity (K_D≈0.17nM) to single class of receptor sites in rat brain membrane preparations (B_{max}=15.6 fmol/mg protein). These results are comparable to previously reported data using other SP related probes (10,12,15). Thus, it appears that [^{125}I]-N_3-SP can be used as a suitable ligand to characterize SP/NK-1 receptor sites in rat brain membranes preparations.

In subsequent experiments, [^{125}I]-N_3-SP was used as a photoaffinity labelling probe to further characterize SP/NK-1 receptor sites present in rat brain membrane preparations. After exposition to UV light for 15 min, membrane proteins were solubilized, reduced and submitted to SDS-PAGE analysis. As shown in Fig. 2, the resulting autoradiograph (lane A) reveals that [^{125}I]-N_3-SP is covalently bound to a high molecular weight component. One major band is observed corresponding to an apparent molecular weight of 46,000 daltons. The

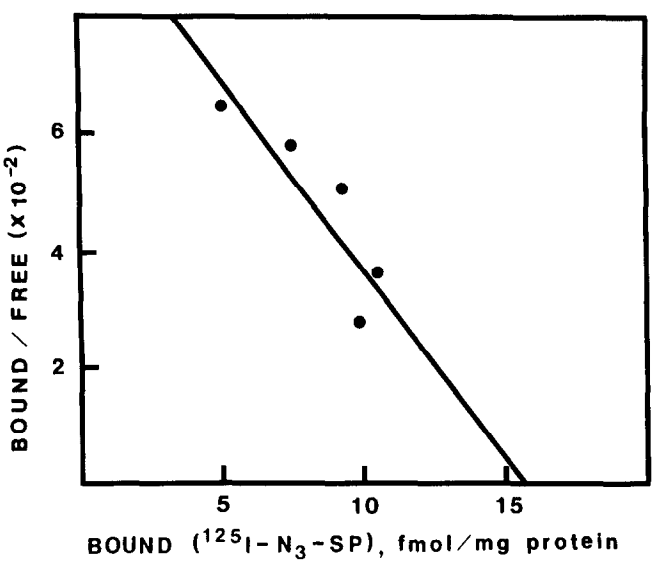
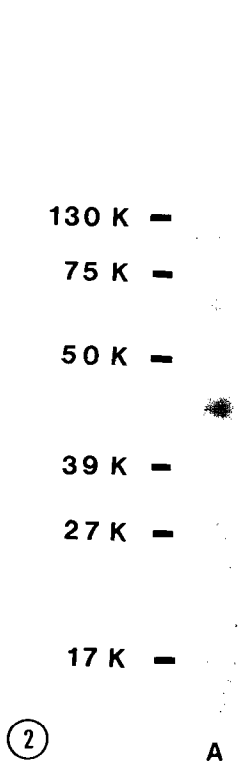


Figure 1. Scatchard analysis of the binding of [^{125}I]-N_3-SP to rat brain membrane preparations. Specific binding was determined as the difference in radioactivity bound in the presence and absence of 1.0 µM unlabelled SP. Specific binding represented between 60-90% of total binding depending on the concentrations of ligand. Binding data were analyzed using a computerized program.



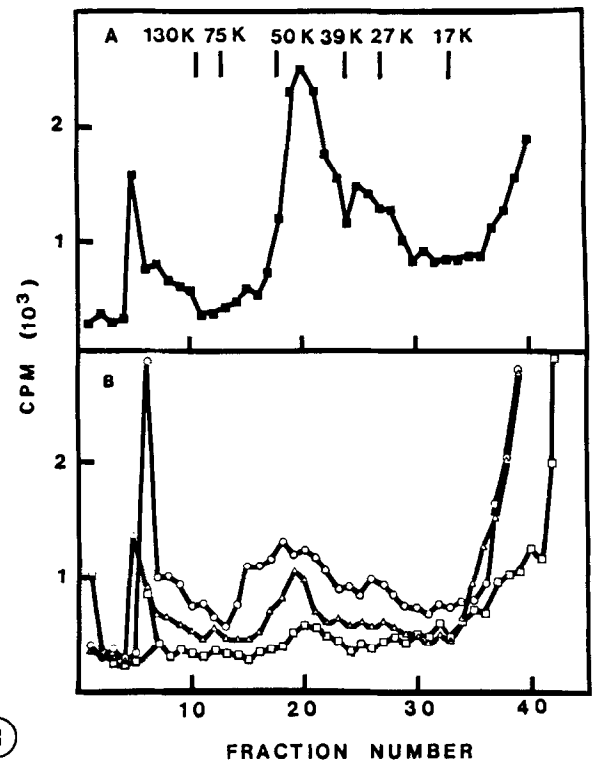


Figure 2. Autoradiographs of electrophoretic separation of [^{125}I]-N_3-SP photolabelled membranes on SDS-PAGE. Rat brain synaptosomes incubated with [^{125}I]-N_3-SP alone (lane A) or in the presence of 100 µM unlabelled SP (lane B). Gels were dried and autoradiographied (LKB Ultrofilm) for 6 weeks. The molecular weight standards were: phosphorylase b, 130,000; bovine serum albumin, 75,000; ovalbumin, 50,000; carbonic anhydrase 39,000; soybean trypsin inhibitor, 27,000 and lysozyme, 17,000.

Figure 3. Electrophoresis of radioactive photolabelled membranes on SDS -PAGE. Slab gels were cut into 2.5 mm slices, the radioactivity of each one was counted using a LKB gamma counter. Radioactive profile of solubilized membranes labelled with [^{125}I]-N_3-SP in the presence of UV light (A). Radioactive membranes in the presence of UV light and (□)0.1 mM SP, (△) 0.1 µM SP or (○)1 nM SP (B). The high activity at the beginning and the end of the gel is due to the stacking gel and the free [^{125}I]-N_3-SP respectively.

specificity of the labelling is demonstrated by the disappearance of this band in presence of 0.1 mM unlabelled SP (Fig. 2, lane B) or in the absence of UV light irradiation (not shown).

Furthermore, the dose-dependent inhibition of the labelling of the 46,000 daltons band observed in presence of increasing concentrations of SP strongly support the specific and selective nature of the labelling (Fig. 3). Fig. 3A shows the radioactive profile of the solubilized membranes labelled with [^{125}I]-N_3-SP following irradiation with UV

light. One major peak of radioactivity corresponding to a molecular weight of 46,000 daltons is observed. This labelling is inhibited by SP in a concentration-dependent manner since the amount of radioactivity recovered following UV irradiation is much lower in the presence of increasing concentrations of unlabelled SP (Fig. 3B). This suggests that the observed band at 46,000 daltons is specifically labelled by the photoaffinity probe [^{125}I]-N_3-SP and is likely represents the SP/NK-1 receptor type.

Very little is currently known on the molecular nature of the various neurokinin receptor sub-types. We presented here some evidences using photoaffinity labelling that the apparent molecular weight of the NK-1 receptor type is in the range of 46,000 daltons. This is similar to molecular weights reported for other peptide brain receptors such as neurotensin (23), cholecystokinin (24) and galanin (25). Moreover, Segawa et al (26) have presented preliminary evidence suggesting that NK-1 receptor sites solubilized from bovine brain tissues using the detergent CHAPS possesses a molecular weight in the range of 55,000 daltons. However, Payan et al (27), using a cross-linking method have reported two apparent molecular weights of 53,000 and 33,000 daltons for the SP binding sites present in human IM-9 lymphoblasts. These differences are most likely related to the various tissues used in these experiments as well as the different approaches utilized to label and solubilize membrane proteins.

Molecular weight identification and solubilization of the SP/NK-1 receptor site should permit further characterization and purification of the receptor. We are currently studying various other photoaffinity labelling probes that could eventually be used as ligands for the NK-2 and NK-3 receptor classes. The development of such tools would be most useful to determine if the various NK receptor sub-types represent distinct molecular entities or different conformations of the same receptor protein.

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