Photoaffinity Labeling the Substance P Receptor Using a Derivative of Substance P Containing *p*-Benzoylphenylalanine†

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ABSTRACT: A novel photoreactive substance P (SP) analogue has been synthesized by solid-phase peptide synthesis methodology to incorporate the amino acid *p*-benzoyl-L-phenylalanine [L-Phe(pBz)] in place of the Phe^8 residue of SP. [Phe^8(pBz)]SP was equipotent with SP in competing for SP binding sites on rat submaxillary gland membranes and had potent sialagogic activity in vivo. In the absence of light, the ^{125}I-labeled Bolton–Hunter conjugate of [Phe^8(pBz)]SP bound in a saturable and reversible manner to an apparently homogeneous class of binding sites (B_{max} = 0.2 pmol/mg of membrane protein) with an affinity K_D = 0.4 nM. The binding of ^{125}I-[Phe^8(pBz)]SP was inhibited competitively by various tachykinin peptides and analogues with the appropriate specificity for SP/NK-1 receptors. Upon photolysis, up to 70% of the specifically bound ^{125}I-[Phe^8(pBz)]SP underwent covalent linkage to two polypeptides of M_r = 53 000 and 46 000, identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. Quantitative analysis of the inhibitory effects of SP and related peptides on ^{125}I-[Phe^8(pBz)]SP photoincorporation indicated that the binding sites of the two photolabeled polypeptides have the same peptide specificity, namely, that typical of NK-1-type SP receptors. In addition, the labeling of the two polypeptides was equally sensitive to inhibition by guanyl-5′-yl imidodiphosphate, a nonhydrolyzable analogue of GTP. Further information on the relationship between the two labeled SP binding sites was provided by enzymatic digestion studies: the M_r = 46 000 polypeptide contains N-linked carbohydrates and is derived most likely from the higher molecular weight species by proteolytic nicking. The highly specific and remarkably efficient photolabeling achieved with ^{125}I-[Phe^8(pBz)]SP suggests that this photoaffinity probe will be of considerable value for the characterization of the molecular structure of the SP receptor.

There is considerable evidence to suggest that many of the biological actions of substance P (SP)[1] in peripheral tissues and in brain involve an interaction of the peptide with receptors that have similar pharmacological properties and are distinct from those for the other mammalian tachykinins (Quirion & Dam, 1988; Maggio, 1988; Lee et al., 1986; Pernow, 1983). The biochemical nature of these SP receptors, however, remains to be clarified. Evidence has been obtained by a reconstitution technique (Macdonald & Boyd, 1989) and by kinetic analysis of the effects of guanine nucleotides on SP receptor binding (Luber-Narod et al., 1990) that SP receptors bind SP with high affinity only when coupled to a guanine nucleotide binding protein (G protein). The purification of other G protein linked receptors such as those for epinephrine and acetylcholine has depended on the availability of specific antagonists that bind with high affinity even in the absence of G proteins. These ligands have been used to identify solubilized receptors at various stages of purification and to prepare affinity gels by covalent attachment to inert supports. Since high-affinity antagonists for SP receptors are not available, we sought to develop a photoaffinity ligand that could be used to covalently radiolabel the binding site(s) of SP receptors while in their membrane-bound and thus high-affinity state. Provided conditions for efficient photolabeling could be achieved, a SP receptor photoaffinity ligand would be of considerable value for identifying during subsequent purification steps not only the SP receptor polypeptide but also peptide fragments derived from its binding site.

In this paper we document the effectiveness for photolabeling the SP receptor of a novel photoreactive SP derivative that contains the amino acid *p*-benzoyl-L-phenylalanine [L-Phe(pBz)]. This photolabile amino acid was chosen on the basis of its chemical stability under peptide synthesis conditions and a highly selective photochemistry. Upon low-level ultraviolet light irradiation, the ketone carbonyl of the benzophenone moiety undergoes a $n \rightarrow \pi^*$ transition to give a triplet biradical that has been shown to possess high reactivity for C–H bonds (Breslow, 1980) but low reactivity toward water (Helene, 1972). The value of Phe(pBz) as a photoreactive probe has been demonstrated in previous studies in which synthetic peptides containing Phe(pBz) were used to photolabel in solution purified preparations of calmodulin (Kauer et al., 1986) and also cAMP-dependent protein kinase (Miller & Kaiser, 1988). To test whether this approach could be adapted to photolabel membrane-bound SP receptors, an analogue of SP was synthesized in which the Phe^8 residue in the carboxyl-terminal sequence of SP was replaced by L-Phe(pBz). Evidence is presented indicating that the ^{125}I-labeled Bolton–Hunter conjugate of [Phe^8(pBz)]SP binds to SP/NK-1 receptors in rat submaxillary gland membranes in a reversible and saturable manner with high affinity and appropriate

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[1] Abbreviations: SP, substance P; NKA, neurokinin A (also known as substance K); NKB, neurokinin B; L-Phe(pBz), *p*-benzoyl-L-phenylalanine; ^{125}I-SP and ^{125}I-[Phe^8(pBz)]SP, ^{125}I-labeled Bolton–Hunter conjugate of SP and [Phe^8(pBz)]SP, respectively; G protein, guanine nucleotide regulatory protein; GppNHp, guanyl-5′-yl imidodiphosphate; endo F, endoglycosidase F; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography.

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specificity. Moreover, upon photolysis, up to 70% of the specifically bound ^{125}I-[Phe^8(pBz)]SP becomes covalently attached to the receptor. Thus, this photoreactive probe may prove very useful for characterization of the molecular structure of the SP receptor.

Experimental Procedures

Materials. Substance P (SP), substance P free acid (SPFA), and neurokinin A (NKA) were from Peninsula Laboratories (San Carlos, CA). [Sar^9,Met(O$_2$)^{11}]-SP, [Nle^{10}]NKA(4–10), and [MePhe^7]NKB were generously provided by D. Regoli (University of Sherbrooke). Endoglycosidase F (endo-β-*N*-acetylglucosaminidase F; EC 3.2.1.96) was obtained from Boehringer Mannheim. All other biochemicals, except where noted otherwise, were obtained from Sigma. Electrophoresis reagents were from Bio-Rad.

Peptide Synthesis. *p*-Benzoyl-L-phenylalanine [L-Phe(pBz)] was prepared as described previously (Kauer et al., 1986). The *tert*-butyloxycarbonyl derivative was prepared with di-*tert*-butyl dicarbonate (Moroder et al., 1976). The peptides were prepared on *p*-methylbenzhydrylamine resin obtained from Peninsula Laboratories (San Carlos, CA). The Boc-amino acids used for synthesis were also obtained from Peninsula and were checked for purity by TLC prior to use. The symmetric anhydride method (Yamashiro & Li, 1975) was used for coupling Boc-Arg(N^g-Tos), Boc-Gly, Boc-Leu, Boc-Lys(N^ϵ-2-ClZ), Boc-Met, Boc-Phe, and Boc-Pro. Boc-Gln and Boc-Phe(pBz) were coupled with the hydroxybenzotriazole coupling method (Blake & Li, 1975). Diisopropylcarbodiimide was used as the coupling agent (Tartar & Gesquiere, 1979) with both methods. The synthesis was carried out in a Beckman 990 peptide synthesizer by protocols similar to those used by Yamashiro and Li (1978), except that Boc-Gln was deprotected with 4 N HCl in dioxane (from Pierce, Rockford, IL) for 20 min (Barany & Merrifield, 1980). The peptides were cleaved from the resin and deprotected by treatment with HF/*p*-cresol/dimethyl sulfide (26:2.4:1) for 60 min at 0 °C (performed by Immuno-Dynamics, La Jolla, CA). Following HF cleavage, the crude product was purified by reversed-phase HPLC using a C_{18} column (Waters) and a 0–60% aqueous acetonitrile gradient containing 0.1% trifluoroacetic acid, at 1.5 mL/min for 60 min, and UV detection at 260 nm. The purity and composition of the peptide were confirmed by analytical HPLC and amino acid analysis: Glx$^{2.11}$(2), Pro$^{1.88}$(2), Gly$^{1.02}$(1), Met$^{0.96}$(1), Leu$^{1.0}$(1), Phe$^{1.1}$(1), Lys$^{0.91}$(1), Arg$^{0.98}$(1). UV spectrophotometric analysis of the peptide (λ_{max} = 262 nm, ϵ = 18 000 M^{-1} cm^{-1}) indicated that the Phe(pBz) residue had remained intact throughout the synthetic and purification procedures. A radioiodinated derivative of [Phe^8(pBz)]SP was prepared by conjugation with ^{125}I-labeled Bolton–Hunter reagent, obtained as the monoiodinated derivative (2200 Ci/mmol) from New England Nuclear. The reaction conditions were those described previously for the ^{125}I-labeled Bolton–Hunter conjugate of SP (Liang & Cascieri, 1981).

Preparation of Rat Submaxillary Gland Membranes. Submaxillary glands from adult Sprague-Dawley rats were used to prepare membranes as described previously (Macdonald & Boyd, 1989). Membranes were also prepared with homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 0.1 mM phenylmethanesulfonyl fluoride) that had been further supplemented with benzamidine (0.1 mM), tosylphenylalanine chloromethyl ketone (0.1 mM), *N*-*p*-tosyl-L-lysine chloromethyl ketone (0.1 mM), leupeptin (5 μg/mL), chymostatin (3 μg/mL), and soybean trypsin inhibitor (5 μg/mL). The presence of these additional protease inhibitors in the homogenization buffer had no significant effect on either the reversible binding or the photolabeling profiles.

Binding Assays. For saturation binding experiments, rat submaxillary gland membranes (50–100 μg of protein) were incubated with ^{125}I-SP or ^{125}I-[Phe^8(pBz)]SP (0.1–5 nM) for 30 min at 22 °C in a total volume of 200 μL of 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$ and 1 mM EGTA, pH 7.4, and supplemented with 200 μg/mL crystalline bovine serum albumin, 3 μg/mL chymostatin, 5 μg/mL leupeptin, and 30 μg/mL bacitracin. For competition binding experiments, membranes were incubated with 0.5 nM ^{125}I-SP or ^{125}I-[Phe^8(pBz)]SP and varying concentrations of peptides for 30 min at 22 °C in Tris buffer (as above). In binding experiments utilizing ^{125}I-[Phe^8(pBz)]SP, the incubation mixtures were protected from the light. For all experiments, nonspecific binding was defined by the addition of 1 μM SP. Binding was terminated by addition of 5 mL of ice-cold 50 mM Tris-HCl–10 mM $MgCl_2$, pH 7.4, and rapid filtering through a glass fiber filter (Whatman GF/C) which had been soaked for >2 h in 0.1% poly(ethylenimine) (Bruns et al., 1987). The incubation tubes and filters were washed with 3 × 5 mL of the same ice-cold buffer. The filters were then measured for radioactivity in a Nuclear Enterprises NE1600 γ-counter at a counting efficiency of 65%.

Binding data were analyzed by the nonlinear least-squares curve-fitting program LIGAND (Munson & Rodbard, 1980). Biological activity was assessed with the rat sialagogic assay as described by Leeman and Hammerslag (1967). Protein was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Photoaffinity Labeling. Membranes (3–5 mg of protein/mL) were incubated in the dark with 0.5–1.0 nM ^{125}I-[Phe^8(pBz)]SP for 30 min at 22 °C in buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4) supplemented with protease inhibitors (see above) and competing peptides at the concentrations indicated. Following incubation, the samples were diluted 10-fold with ice-cold buffer and centrifuged at 40000*g* for 15 min. The pellets were resuspended in the same volume of ice-cold buffer and recentrifuged. This step was repeated twice. In the initial labeling experiments, aliquots containing 100 μg of protein were removed after each resuspension step and assayed in duplicate by ultrafiltration as described above. These determinations of the total and nonspecific binding indicated that there was only a small (less than 10%) loss of the specific component of ^{125}I-[Phe^8(pBz)]SP binding over the entire washing procedure but a 70%–90% reduction in the nonspecific component. The membrane pellets obtained following the last centrifugation step were resuspended in ice-cold buffer at 1–2 mg of membrane protein/mL and irradiated for 15 min on ice in polystyrene tissue culture dishes at a distance of 6 cm from a 100-W long-wave (365 nm) UV lamp (Blak-Ray). The photolabeled membranes were then obtained by centrifugation (40000*g* for 15 min). In initial experiments to optimize the efficiency of photolabeling, the extent of irreversible labeling was conveniently assessed by ultrafiltration of aliquots of the photolabeled membranes that had been incubated at 22 °C for 30 min at pH 11.0 or at pH 7.6 in the presence of 10 μM GppNHp, conditions that produce rapid dissociation of any ligand that remains reversibly bound following photolysis (Macdonald & Boyd, 1989; Luber-Narod et al., 1990).

SDS–PAGE and Autoradiography. Photolabeled membranes were solubilized for 5 min at 60 °C in sample buffer (2% SDS, 10% glycerol, and 5% β-mercaptoethanol in 25 mM

Tris-HCl, pH 6.8). Samples (50–100 μg of membrane protein) were electrophoresed in polyacrylamide slab gels according to the method of Laemmli (1970). Gels were dried with a Hoefer Scientific Instruments slab gel drier (Model SE540) and exposed at −80 °C to Kodak XAR-5 film with one intensifying screen for various times (8–30 h). The molecular weights of the radiolabeled polypeptides were determined from the following molecular weight standards (Bio-Rad): rabbit muscle phosphorylase *b* (M_r = 97 400), bovine serum albumin (M_r = 66 300), hen egg white ovalbumin (M_r = 42 700), ovine carbonic anhydrase B (M_r = 31 000), soybean trypsin inhibitor (M_r = 21 500), and hen egg white lysozyme (M_r = 14 400).

To document quantitative photoincorporation of ^{125}I-[Phe^8(pBz)]SP into receptor polypeptides, the autoradiographs were aligned with the dried gels, the radiolabeled polypeptides were cut out, and the amount of radioactivity was measured. In the experiments to examine the inhibitory effect of SP and related tachykinin peptides on photolabeling by ^{125}I-[Phe^8-(pBz)]SP, individual lanes on the autoradiographs were analyzed densitometrically on a Hoefer (GS 300) scanning densitometer.

Enzymatic Treatment of Photolabeled Polypeptides. Rat submaxillary gland membranes, photoaffinity labeled as described above, were resuspended at 2 mg of membrane protein/mL in 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA. An equal volume of 2% digitonin prepared in the same buffer was added and after gentle shaking for 60 min at 4 °C, the preparation was centrifuged at 65000*g* for 60 min at 4 °C. The supernatant typically contained about 80% of the radioactivity present in the original membrane pellet and about 20% of the protein. For trypsin treatment, the digitonin extract was diluted 10-fold with buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0) and incubated for 2 h at 37 °C in the absence and presence of TPCK-treated trypsin (Sigma) at a final concentration of 0.01 mg/mL. The digitonin extract was also diluted 10-fold with buffer (100 mM potassium phosphate, 10 mM EDTA, pH 7.6)) and incubated for 18 h at 37 °C in the absence and presence of endoglycosidase F (Boehringer Mannheim) (final concentration = 1 unit/mL). Following the incubations the samples were treated with SDS–PAGE treatment buffer and stored at −20 °C prior to analysis of the enzymatic reaction products by SDS–PAGE and autoradiography.

Results

The binding properties of nonradioactive [Phe^8(pBz)]SP were assessed initially by competitive displacement of the binding of the ^{125}I-labeled Bolton–Hunter reagent conjugate of SP (^{125}I-SP) to rat submaxillary gland membranes. Previous studies (Buck & Burcher, 1985) have established that ^{125}I-SP binds to a single class of high-affinity binding sites on this membrane preparation with a peptide specificity that identifies these sites as SP/NK-1 receptors. As shown in Figure 1, [Phe^8(pBz)]SP competes effectively for the binding sites occupied by ^{125}I-SP. The concentration of [Phe^8(pBz)]SP required to displace half of the specific ^{125}I-SP binding (IC_{50}) was 1.0 ± 0.2 nM, n = 3, a value not significantly different from that found when SP itself was the competing ligand (Figure 1).

The relative potencies of SP and various peptide fragments and analogues of SP to compete for ^{125}I-SP binding to salivary gland membranes have been found to correlate well with the relative potencies in stimulating salivation when injected into anesthetized rats (Liang & Cascieri, 1981). Injection of [Phe^8(pBz)]SP was also found to stimulate salivation in a dose-dependent manner. Over the range of 20–100 pmol of peptide injected per 100 g of rat weight, [Phe^8(pBz)]SP and SP were equipotent. An interesting but as yet unexplained result was that, at higher doses, the SP derivative was more potent in stimulating salivary secretion than the parent peptide.

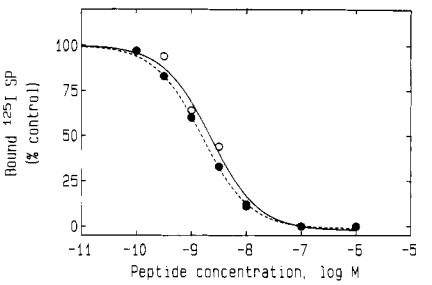

FIGURE 1: Comparison of the concentration dependencies of SP and [Phe^8(pBz)]SP inhibition of ^{125}I-SP binding to rat submaxillary gland membranes. ^{125}I-SP (0.2 nM) was incubated at 22 °C for 30 min with rat submaxillary gland membranes (100 μg/0.2-mL assay volume) in the presence of the specified concentrations of SP (○) and [Phe^8(pBz)]SP (●). Bound ^{125}I-SP was determined in triplicate as described under Experimental Procedures and is given as a percentage of the specific binding measured in the absence of competing peptide. The lines through the experimental points represent the computer-generated best fit to the data of a single representative experiment.

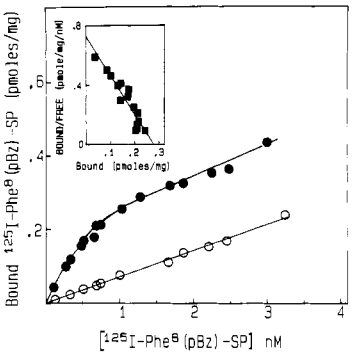

FIGURE 2: Saturation binding isotherm of ^{125}I-[Phe^8(pBz)]SP to rat submaxillary gland membranes. Increasing concentrations of ^{125}I-[Phe^8(pBz)]SP were incubated in the dark with rat submaxillary gland membranes (100 μg of protein/0.1-mL assay volume), and the binding was measured as described under Experimental Procedures. Total binding (●); nonspecific binding (○) in presence of 1 μM SP. Each point is the average of duplicate determinations. Inset: Scatchard analysis of specific binding of ^{125}I-[Phe^8(pBz)]SP (■) (K_D = 0.4 nM, B_{max} = 0.28 pmol/mg). The experiment was repeated three times with similar results.

Binding of ^{125}I-[Phe^8(pBz)]SP to Rat Submaxillary Gland Membranes. A radioiodinated derivative of [Phe^8(pBz)]SP was prepared by conjugation with ^{125}I-labeled Bolton–Hunter reagent, a reagent that has been used previously to radiolabel SP (Liang & Cascieri, 1981) and other tachykinins (Cascieri et al., 1985; Buck et al., 1984). The equilibrium binding of ^{125}I-[Phe^8(pBz)]SP to rat submaxillary gland membranes was attained at 22 °C by 20 min. The binding consisted of a specific, saturable component and a nonspecific component, identified by measuring the binding in the presence of a saturating amount of SP (Figure 2). Scatchard analysis of the saturable component observed in this and two similar binding experiments indicated that ^{125}I-[Phe^8(pBz)]SP bound with high affinity (K_D = 0.44 ± 0.13 nM) to an apparently homogeneous population of binding sites (B_{max} = 240 ± 80 fmol/mg of protein). These equilibrium parameters are the same, within experimental error, as those characterizing the binding of the ^{125}I-labeled Bolton–Hunter conjugate of SP to the same membrane preparation (data not shown).

Competitive binding assays were used to characterize further the peptide specificity of ^{125}I-[Phe^8(pBz)]SP binding to rat

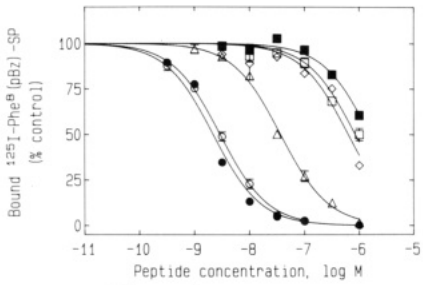

FIGURE 3: Inhibition of ^{125}I-[Phe^8(pBz)]SP binding to rat submaxillary gland membranes by SP and related peptides. A constant concentration of ^{125}I-[Phe^8(pBz)]SP (0.2 nM) was incubated in the dark at 22 °C for 30 min with rat submaxillary gland membranes (50 μg of protein/0.1-mL assay volume) in the presence of increasing concentrations of SP (●), [Sar^9,Met(O$_2$)^{11}]SP (○), NKA (△), [Nle^{10}]NKA(4–10) (□), [MePhe^7]NKB (◇), and SPFA (■). Bound ^{125}I-[Phe^8(pBz)]SP was determined in triplicate as described under Experimental Procedures and is represented as a percentage of the specific binding measured in the absence of competing peptide. The lines through the experimental points represent the computer-generated best fit to the data of a single representative experiment performed three times.

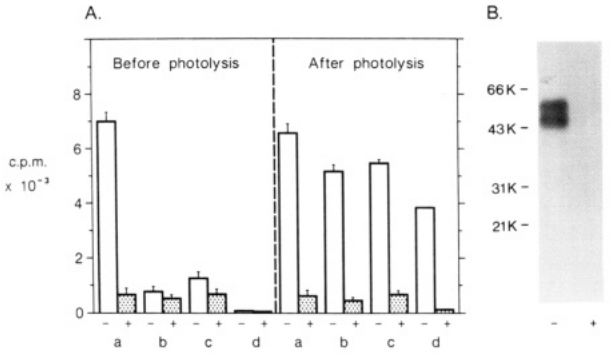

FIGURE 4: Photolabeling of rat submaxillary gland membranes with ^{125}I-[Phe(pBz)]SP. Rat submaxillary gland membranes (1 mg of membrane protein/mL) were equilibrated at 22 °C with ^{125}I-[Phe^8(pBz)]SP (0.3 nM) in the absence (−) and presence (+) of 1 μM SP and washed with ice-cold buffer as described under Experimental Procedures. Membranes were divided, and half was photolyzed at 4 °C for 10 min with 350-nm light while the other half remained in the dark at 4 °C. (Panel A) ^{125}I-[Phe^8(pBz)]SP bound to both the photolyzed and nonphotolyzed membranes was determined in triplicate by ultrafiltration of aliquots (100 μg of membrane protein) that had been treated as follows: (a) no further treatment; (b) warmed to 22 °C and incubated for 30 min at pH 11.0; (c) warmed to 22 °C and incubated for 30 min at pH 7.6 in the presence of 10 μM GppNHp. Membranes (100 μg of membrane protein) were also subjected to SDS–PAGE, and the labeled bands were visualized by autoradiography as described. The region of the gel containing the radiolabeled polypeptides (M_r = 43 000–55 000) was excised and the amount of radioactivity determined (shown in lanes d). (Panel B) Autoradiograph of photolabeled membranes following SDS–PAGE. No labeled bands were detected with the nonphotolyzed membranes.

submaxillary gland membranes (Figure 3). SP competed for the binding sites occupied by ^{125}I-[Phe^8(pBz)]SP with an IC_{50} = 1 nM, a value that is the same as that reported above (see Figure 1) for displacement of ^{125}I-SP binding. Deaminidated SP (SP free acid), which is inactive in various SP/NK-1 bioassays including stimulation of salivation (Liang & Cascieri, 1981), inhibited ^{125}I-[Phe^8(pBz)SP binding only when added at high concentrations (>100 nM). Neurokinin A (NKA),[1] which is a more potent agonist than SP at NKA/NK-2 receptors but is less active at SP/NK-1 receptors, exhibited a >10-fold lower potency than SP in competing for ^{125}I-[Phe^8(pBz)]SP binding. The recent development of selective agonists for the various neurokinin receptor classes (Regoli et al., 1988) permitted further characterization of the binding specificity. Only the selective SP/NK-1 receptor agonist [Sar^9,Met(O$_2$)^{11}]SP, and not [Nle^{10}]NKA(4–10) and [MePhe^7]NKB, selective agonists for NK-2 and NK-3 receptors, respectively, was found to be a potent competitor of ^{125}I-[Phe^8(pBz)]SP binding (Figure 3).

The results of these competition binding experiments, when considered together with the saturation binding experiments, clearly document that, under nonphotolyzing conditions, ^{125}I-[Phe^8(pBz)]SP and ^{125}I-SP bind to the same sites on rat submaxillary gland membranes with a specificity that identifies these sites as SP receptors of the NK-1 type.

Photoaffinity Labeling of SP Receptors in Rat Submaxillary Gland Membranes. The primary goal of this work was to develop a specific and efficient photoaffinity label for SP receptors that would be useful in the purification of the SP receptor and in the identification of the amino acid residues of the receptor involved in peptide recognition. Since it was important to avoid labeling of nonreceptor proteins, nonspecific interactions of the photoligand were reduced prior to photolysis by repeated dilution and centrifugation steps conducted at 4 °C (see Experimental Procedures for details). This procedure, which takes advantage of the slow rate of dissociation at 4 °C of specifically bound ^{125}I-[Phe^8(pBz)]SP, reduced the nonspecific component to less than 10% of the total binding. In contrast to their stability at 4 °C, photoligand–receptor complexes dissociate readily when incubated at 22 °C in buffer (1) at pH 11.00 or (2) at pH 7.6 in the presence of 10 μM GppNHp, so that following a 30-min incubation under either condition a virtually complete loss of the specific binding was observed (Figure 4A). However, as is also shown in Figure 4A, if membranes equilibrated with the photoligand were irradiated at 4 °C for 15 min with 350-nm light and then subjected to alkaline pH or guanine nucleotide exposure, there was only a small loss of specific binding. These results thus showed that a high percentage of the specifically bound ^{125}I-[Phe^8(pBz)]SP had become irreversibly bound during the photolysis step.

The photolyzed membranes were analyzed further by SDS–PAGE and autoradiography. Two major radiolabeled bands at M_r = 53 000 and M_r = 46 000 were readily discernible within a diffuse pattern of labeling (Figure 4B). Removal of the β-mercaptoethanol from the SDS–PAGE sample buffer or the addition of the proteolytic enzyme inhibitors PMSF, TLCK, TPCK, chymostatin, leupeptin, and EDTA had no effect on the labeling profile. Quantitative measurements of the total amount of ^{125}I-[Phe^8(pBz)]SP that was covalently bound to the polypeptides at M_r = 53 000 and M_r = 46 000 indicated that 70 ± 5% (n = 4) of the ^{125}I-[Phe^8(pBz)]SP bound reversibly prior to photolysis had undergone covalent attachment, thus confirming that a high level of photoincorporation had been achieved.

Specificity and Guanine Nucleotide Sensitivity of Photoaffinity Labeling. The various peptides used to establish the specificity of the reversible binding of the photoligand were also used to characterize in further detail the specificity of photolabeling of the M_r = 53 000 and M_r = 46 000 polypeptides (Figure 5). SP inhibited the photolabeling of both polypeptides in a concentration-dependent manner (Figure 5, lanes 1–6). Densitometric scanning of the individual lanes on the autoradiograph indicated that the labeling at M_r = 53 000 and 46 000 was equally sensitive to SP inhibition ($IC_{50} \sim 1$ nM). The specific agonist of SP/NK-1 receptors, [Sar^9,Met(O$_2$)^{11}]SP, was also found to be a potent inhibitor of photolabeling with inhibition of each of the labeled bands exhibiting an $IC_{50} \sim 6$ nM. On the other hand, the specific NK-2 and NK-3 receptor agonists, [Nle^{10}]NKA(4–10) and

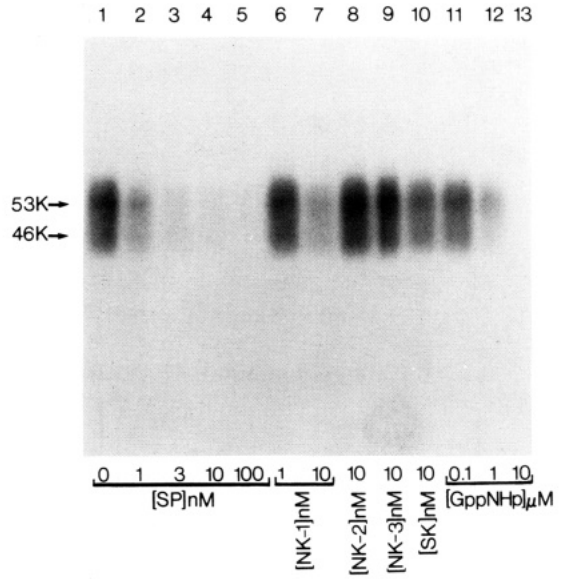

FIGURE 5: Peptide specificity and guanine nucleotide sensitivity of ^{125}I-[Phe^8(pBz)]SP photoincorporation into rat submaxillary gland membranes. Membranes were photoaffinity labeled with ^{125}I-[Phe^8(pBz)]SP alone (lane 1) or in the presence of the specified concentrations of competing tachykinin peptides (lanes 2–10) or GppNHp (lanes 11–13) as described under Experimental Procedures. Samples were subjected to SDS–PAGE and autoradiography. The amount of labeling at M_r = 53 000 and M_r = 46 000 was assessed by densitometric scanning of the individual lanes of the autoradiogram (see text for details). The results are representative of at least three similar experiments. Abbreviations: NK-1, [Sar^9,Met(O$_2$)^{11}]SP; NK-2, [Nle^{10}]NKA(4–10); NK-3, [MePhe^7]NKB; SK, substance K (neurokinin A).

[MePhe^7]NKB, respectively, when added at a concentration of 10 nM, had no detectable effect on the photolabeling of either the M_r = 53 000 or M_r = 46 000 polypeptides, while the same concentration of substance K/neurokinin A reduced the photolabeling of both polypeptides by less than 40%. These results establish that the photolabeled SP binding sites of M_r = 53 000 and M_r = 46 000 have the same peptide specificity, namely, that characteristic of SP/NK-1 receptors.

Previously we (Macdonald & Boyd, 1989; Luber-Narod et al., 1990) and others (Lee et al., 1983; Bahouth & Musacchio, 1985) have shown that the binding of radiolabeled SP to rat salivary gland membranes is inhibited in the presence of low concentrations of the nonhydrolyzable GTP analogue GppNHp. Photolabeling of the membranes by ^{125}I-[Phe^8-(pBz)]SP was also inhibited by GppNHp with the labeling at M_r = 53 000 and M_r = 46 000 being equally susceptible to inhibition by the nucleotide; the IC$_{50}$ was ~120 nM in each case (Figure 5, lanes 11–13).

Enzymatic Studies of the Photolabeled Polypeptides. In photolabeling experiments using four different membrane preparations, the labeled polypeptides at M_r = 53 000 and M_r = 46 000 were always detected, although the relative amounts were somewhat variable from preparation to preparation. The presence of two polypeptides with SP receptor binding properties could result from the presence of two distinct SP receptors, a single SP receptor with differing amounts of attached carbohydrate residues, or simply could be the result of proteolytic degradation of the larger M_r = 53 000 polypeptide to a smaller fragment, both containing the same carbohydrate moieties.

The photolabeled membranes were solubilized with digitonin and then incubated with endoglycosidase F, which hydrolyzes N-linked carbohydrate residues, and also with the endoprotease trypsin (Figure 6). Following endoglycosidase F treatment,

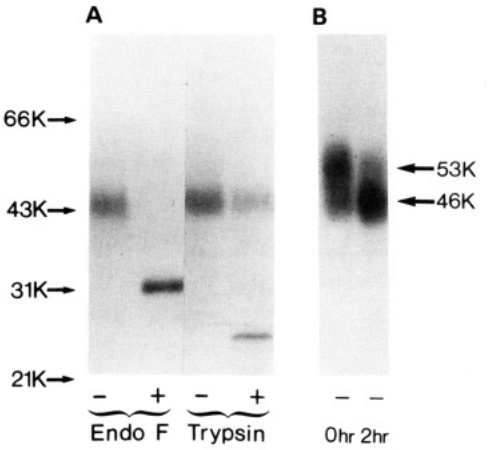

FIGURE 6: Endoglycosidase F and trypsin treatment of photoaffinity labeled SP receptor polypeptides. (A) Rat submaxillary gland membranes, photoaffinity labeled with ^{125}I-[Phe^8(pBz)]SP and solubilized with 1% digitonin, were incubated in the absence (–) and presence (+) of 1 unit/mL endo F and also in the absence (–) and presence (+) of 0.01 mg/mL trypsin as detailed under Experimental Procedures. Following the incubation period, samples were resolved by SDS–PAGE and visualized by autoradiography. (B) Equivalent aliquots of the solubilized labeled membrane preparatin were either treated with SDS–PAGE treatment buffer immediately following the solubilization steps and stored at –20 °C prior to electrophoresis (0 h) or incubated at 37 °C for 2 h and then prepared for SDS–PAGE (2 h). Both experiments were repeated twice with similar results.

a single narrow radiolabeled band of M_r = 34 000 was detected. Incubation of the solubilized polypeptides with trypsin yielded a major tryptic cleavage product at M_r = 28 000. The mobility of this radiolabeled tryptic fragment was not increased further by subsequent endoglycosidase F treatment (data not shown), an indication that the peptide sequence that contains the site(s) for attachment of N-linked carbohydrates had been removed by digestion with trypsin. In addition to an increase in mobility, the photolabeled bands were observed to be more discrete following treatment with either enzyme. The diffuse labeling observed prior to enzymatic treatment thus appears to be due to the presence of the N-linked carbohydrate residues.

It is also important to note that when the solubilized photolabeled membranes were incubated in buffer without added enzyme, there was a marked decrease in the labeling at M_r = 53 000 and an increase at M_r = 46 000 together with the appearance of a small amount of radiolabeling at M_r ~ 42 000 (Figure 4A, control lanes, and Figure 4B).

Although more detailed and quantitative experiments will be necessary to establish the precise relationships between the various radiolabeled polypeptides, the simplest interpretation of the data currently available is that both the M_r = 46 000 and M_r = 53 000 polypeptides contain N-linked carbohydrates and that the lower molecular weight glycoprotein is derived from the higher molecular weight species, probably by proteolytic nicking, although a partial loss of carbohydrate residues by the action of an endogenous glycolytic enzyme cannot be ruled out.

Discussion

We describe here the successful use of a novel photoreactive analogue of SP, ^{125}I-[Phe^8(pBz)]SP, for irreversibly labeling SP receptors in crude membrane preparations obtained from rat submaxillary glands.

The carboxyl-terminal region of SP was selected for introduction of the photoreactive amino acid L-Phe(pBz) to optimize the possibility that the photolabel group would be in close proximity to amino acid residues comprising the peptide

binding site of the SP receptor. The importance of the C-terminal region of SP for interaction with the binding site of the SP receptor has been established by the demonstration that C-terminal fragments of six amino acids or larger are capable of interacting with the SP receptor, while even shorter C-terminal fragments and all N-terminal fragments have little or no affinity [for a review, see Maggio (1988)]. A further consideration in the design of the photoaffinity probe was the finding that, despite its location in the region critical for binding, Phe^8 can be structurally modified without causing a marked decrease in affinity (Lee et al., 1983; Viger et al., 1983). The results of the present study demonstrate that replacement of Phe^8 by its *p*-benzoyl derivative, Phe(pBz), is in fact well tolerated, with the binding affinity and biological activity being maintained. ^{125}I-[Phe^8(pBz)]SP binds with high affinity to sites on rat submaxillary membranes that possess a specificity typical of NK-1-type SP receptors and, most importantly, upon UV irradiation is photoincorporated with remarkedly high efficiency into two major radiolabeled polypeptides of M_r = 53 000 and M_r = 46 000.

The observation of two photolabeled SP binding sites differing in molecular size contrasts with the conclusion of previous reversible binding studies that rat submaxillary gland contains a single population of SP binding sites (Lee et al., 1983; Buck & Burcher, 1985). Saturation analysis of ^{125}I-[Phe^8(pBz)]SP binding under reversible conditions also suggests that binding is to an apparently homogeneous population of high-affinity sites. The results of the reversible binding and photolabeling experiments are nevertheless reconcilable provided the two photolabeled sites exhibit the same or at least very similar binding properties. The photolabeling protection experiments (Figure 6) which provide a means of analyzing independently the binding properties of the M_r = 53 000 and M_r = 46 000 polypeptides indicate that this is indeed the case.

The notion of a heterogeneity in substance P binding sites, a GTP-sensitive site and a GTP-insensitive site, had been introduced earlier by Lee et al. (1983). However, in a recent study of association–dissociation rates of SP binding in the presence of guanine nucleotides (Luber-Narod et al., 1990), it was found that while guanine nucleotide inhibition was not a simple monophasic kinetic phenonomen, all high-affinity SP binding was in fact susceptible to the effects of the nucleotide. In this context it is of interest that photolabeling of the M_r = 53 000 and the M_r = 46 000 polypeptides is equally sensitive to inhibition by GppNHp. Furthermore, since guanine nucleotide inhibition of SP binding has been shown to be due to the ability of guanine nucleotides to decrease the affinity of SP receptors for agonists (Luber-Narod et al., 1990) via an interaction with a GTP-binding regulatory protein that is necessary for the expression of high-affinity agonist binding (Macdonald & Boyd, 1989), it seems reasonable to conclude that both polypeptides are photolabeled when coupled to a G protein.

Further information on the relationship of these two receptor polypeptides was provided by enzymatic digestion studies. The results obtained suggest that both contain N-linked carbohydrate residues and that the M_r = 53 000 glycoprotein can be converted to the lower molecular weight glycoprotein M_r = 46 000, most likely by an endogenous proteolytic enzyme. Interestingly, several other G protein linked receptors including β-adrenergic and D_2 dopamine receptors (Benovic et al., 1984; Amlaiky & caron, 1986) have also been found to be highly susceptible to proteolytic degradation. Inclusion of multiple classes of specific protease inhibitors shown to be effective in preventing degradation of these receptors did not, however, prevent radiolabeling of the lower molecular weight protein. At the present time, we do not know whether both the intact and proteolytically degraded forms of the SP/NK-1 receptor exist in vivo or whether proteolytic degradation of the receptor occurs during membrane preparation due to an inappropriate choice of protease inhibitors and/or the high proteolytic activity of salivary gland tissue.

While further study will be necessary before it can be established that the M_r = 53 000 polypeptide alone represents the functional SP receptor, we can nevertheless conclude that this polypeptide contains the sites for both SP binding and interaction with G proteins. On the basis of the change in mobility observed following N-deglycosylation and assuming covalent attachment of a single photoligand molecule per receptor, the molecular weight of the deglycosylated but otherwise intact polypeptide backbone of the SP receptor is estimated to be about 40 000. This estimate is similar to the value predicted by nucleotide sequence analysis of a cDNA clone for the rat SP receptor (Yokota et al., 1989; Hershey & Krause, 1990).

Dam et al. (1987) using a photoreactive SP analogue in which *p*-azidophenylalanine was substituted for Phe^8 have demonstrated specific photolabeling of a single polypeptide, M_r = 46 000, in a rat brain membrane preparation. Since the use of protease inhibitors during membrane preparation was not indicated, it is possible that this polypeptide corresponds to the putative proteolytic fragment at M_r = 46 000 labeled with ^{125}I-[Phe^8(pBz)]SP in rat submaxillary gland membranes. Furthermore, in preliminary experiments in which rat brain membranes were prepared and photolabeled as described here for rat submaxillary gland membranes, a similar radiolabeling pattern to that reported here was observed.[2] Another important difference concerns the efficiency of photolabeling; the extent of photoincoporation achieved with ^{125}I-[Phe^8(pBz)]SP is at least 10-fold greater than with the corresponding azido SP analogue. Since both photoaffinity ligands bind with similar high affinities to SP receptors and the photoreactive groups have been introduced at the same location, i.e., in place of Phe^8 of SP, the higher level of photoincorporation by ^{125}I-[Phe^8(pBz)]SP is most likely a reflection of the more favorable photochemistry of the benzophenone group. Upon irradiation with UV light the ketone carbonyl undergoes a n $\rightarrow \pi^*$ transition to give a triplet biradical that is reactive to C–H bonds but, unlike nitrenes, is stable in water (Helene, 1972). An additional advantage provided by using L-Phe(pBz) as a photoreactive probe is the stability of benzophenones to peptide synthesis conditions. Because of the limited chemical stability of aryl azides, it was necessary, in order to obtain the azido SP analogue, to synthesize first a peptide containing *p*-nitrophenylalanine which then had to be transformed to the azido peptide by multiple chemical steps (Dam et al., 1987).

Previously, synthetic peptides incorporating Phe(pBz) have been used to photolabel in solution the peptide binding sites of purified preparations of calmodulin (Kauer et al., 1986) and of cAMP-dependent protein kinase (Miller & Kaiser, 1988). The present work extends the use of this photoreactive amino acid to the identification of SP receptors in a crude membrane preparation. The remarkably efficient photolabeling achieved coupled with the relative ease of the synthetic procedures suggests that the photolabeling procedure developed here for SP receptors may be generally applicable to other peptide receptors, including those for the other mammalian tackykinins NKA and NKB.

[2] C. F. White and N. D. Boyd, unpublished observation.

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Effects of Phospholipids on the Function of $(Ca^{2+}-Mg^{2+})$-ATPase†

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ABSTRACT: The ATPase activity for the $(Ca^{2+}-Mg^{2+})$-ATPase purified from rabbit skeletal muscle sarcoplasmic reticulum is lower when reconstituted into bilayers of dimyristoleoylphosphatidylcholine [(C14:1)PC] than when it is reconstituted into dioleoylphosphatidylcholine [(C18:1)PC]. The rate of formation of phosphoenzyme on addition of ATP is slower for (C14:1)PC-ATPase than for the native ATPase or (C18:1)PC-ATPase. The reduction in rate of phosphoenzyme formation is attributed to a reduction in the rate of a conformational change on the ATPase following binding of ATP but before phosphorylation. The level of phosphoenzyme formed from P_i is also less for (C14:1)PC-ATPase than for (C18:1)PC-ATPase. At steady state at pH 6.0 in the presence of ATP Ca^{2+} is released from (C18:1)PC-ATPase into the medium, but not from (C14:1)PC-ATPase. These effects of (C14:1)PC on the ATPase are reversed by addition of androstenol to a 1:1 molar ratio with (C14:1)PC. The results are interpreted in terms of a kinetic model for the ATPase.

Membrane proteins function in an environment defined in part by the phospholipid component of the biological membrane. It is known that the phospholipid composition of all biological membranes is complex, but it is not known whether or not this complexity is required for the proper function of the membrane [see Lee (1988)]. One approach to the problem is to purify membrane proteins and reconstitute them into phospholipid bilayers of defined composition, so allowing a

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