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Agonist-Induced Photoincorporation of a *p*-Benzoylphenylalanine Derivative of Substance P into Membrane-Spanning Region 2 of the *Torpedo* Nicotinic Acetylcholine Receptor δ Subunit

M. P. BLANTON, Y.-M. LI, E. R. STIMSON, J. E. MAGGIO, and J. B. COHEN

Department of Neurobiology (M.P.B., J.B.C.) and Department of Biological Chemistry and Molecular Pharmacology (Y.-M.L., E.R.S., J.E.M.), Harvard Medical School, Boston, Massachusetts 02115

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SUMMARY

The neuropeptide substance P acts, at micromolar concentrations, as a noncompetitive antagonist of nicotinic acetylcholine receptors (AChRs) of both neuronal and muscle subtypes. The mechanism of this inhibition has been shown to be most consistent with stabilization of a nonconducting desensitized state of the AChR, via binding to a site distinct from both the agonist site and the high affinity noncompetitive antagonist site. We have used a radioiodinated photoreactive analogue of substance P, containing the amino acid *p*-benzoyl-L-phenylalanine in place of the Phe[8] residue of substance P, to identify the sites of interaction of substance P within the *Torpedo californica* AChR. AChR-rich membrane suspensions were photolabeled in the absence or presence of the agonist carbamylcholine and/or nonradioactive substance P, and incorporation into AChR subunits was assessed by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In the absence of agonist ^{125}I incorporation was detected in each subunit and was insensitive to substance P, whereas in the presence of carbamylcholine there was a 2-fold increase in photoincorporation into the AChR δ subunit that was inhibited by the addition of an excess of substance P. The sites of specific photoincorporation in the δ subunit were initially mapped by use of *Staphylococcus aureus* V8 protease to a 14-kDa fragment extending from δ Ile-192 to Glu-280. Further fragmentation of this 14-kDa fragment with trypsin and *S. aureus* V8 protease established that the sites of specific incorporation were restricted to the region δ Ser-253 to Glu-280, which contains the membrane-spanning region 2 that is known to form the lining of the ion channel. These results establish that in the presence of agonist at least a part of the undecapeptide substance P binds within the ion channel in the desensitized state of the AChR, and it is likely that the binding of substance P to this site is responsible for the action of substance P as a noncompetitive AChR antagonist.

SP is an undecapeptide of the tachykinin neuropeptide family that functions as a neurotransmitter in parts of the central and peripheral nervous systems (reviewed in Refs. 1–3). Many of the biological activities of SP are mediated by a high (nanomolar) affinity receptor of the G protein-coupled receptor superfamily. The primary structure (3) of the SP receptor (also known as the NK-1 receptor) shows the seven transmembrane domains typical of this superfamily. This receptor, which is unevenly distributed throughout the central nervous system and peripheral tissues, does not account for all of the biological activities of the peptide. Pharmacological studies (reviewed in Ref. 4) have demonstrated at least one additional target, the nicotinic AChR.

Inhibition of nicotinic AChR responses by SP at micromolar concentrations has been reported in a variety of neuronal and muscle systems over almost two decades since the initial report of Steinacker and Highstein (5). For example, SP acts as a desensitizing noncompetitive antagonist of acetylcholine in adrenal chromaffin cells (6), in the neuron-like pheochromocytoma cell line PC-12 (7, 8), in the muscle-like cell line BC_3H_1 (9), and with the AChRs from *Torpedo* electroplax (10). Although some of this inhibition of AChRs could theoretically result indirectly (via second messenger systems) (11) from the interaction of SP with its G protein-coupled receptor, several observations suggest that the effects of SP on AChRs are instead due to a direct interaction between the neuropeptide and the cholinergic receptor. Both EC_{50} values and structure/activity relationships for several analogues clearly demonstrate that AChR inhibition by SP is not mediated by any of the known G protein-coupled tachykinin receptors (8–10). Fur-

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ABBREVIATIONS: SP, substance P; AChR, acetylcholine receptor; Phe(pBz), *p*-benzoylphenylalanine; TID, 3-trifluoromethyl-3-(*m*-iodophenyl)-diazirine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; HPLC, high performance liquid chromatography; M1, membrane-spanning region 1; M2, membrane-spanning region 2; PCP, phencyclidine; NK, neurokinin; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl.

thermore, Min *et al.* (4) have reported specific binding of [^{3}H]-SP to AChRs and modulation of that binding by carbamylcholine in a cell-free system.

Analysis of the interactions of SP with AChR-rich membranes isolated from *Torpedo* electric organ provides strong evidence for the existence of a specific binding site on the AChR. SP inhibits carbamylcholine-stimulated $^{22}Na^+$ efflux from these vesicles in a dose-dependent fashion, with an IC_{50} of 3 μM (10), a value similar to that seen for the inhibition of agonist-induced flux into the PC-12 cell line (IC_{50}, 1 μM) (9). SP did not appear to bind to either the agonist site or the high affinity noncompetitive antagonist site (12), because at equilibrium it increased affinity of the AChR for [^{3}H]acetylcholine and for the aromatic amine noncompetitive antagonist [^{3}H]-PCP (in the absence of agonist). Using [^{3}H]SP, it was determined that in the absence of agonist the peptide bound to membranes with low affinity (K_d, ~40 μM) at 6–8 sites/AChR, whereas in the presence of carbamylcholine [^{3}H]SP bound with high affinity (K_d, ~0.6 μM) to ~1 site/AChR (4) in addition to the low affinity sites. These results indicate that SP is a noncompetitive antagonist that binds to a site in the AChR that is distinct from, but coupled allosterically to, the agonist site. The site also is distinct from the site of binding of PCP, a noncompetitive antagonist that binds within the channel (for review, see Ref. 13).

To identify the region(s) of the AChR interacting with SP, we have made use of a novel photoreactive derivative of the peptide that was originally developed to examine the interaction of SP with its NK-1 type receptor (14). An analogue of SP was synthesized in which the Phe^8 residue was replaced with the photolabile amino acid L-Phe(pBz). This photolabile amino acid is advantageous because it is chemically stable under the conditions used for peptide synthesis and because the photolabile benzophenone group exhibits selective photochemistry (15). Upon irradiation in the near-UV range, the ketone carbonyl of the benzophenone moiety undergoes an $n \rightarrow \pi^*$ transition to give a triplet biradical that possesses high reactivity for C-H bonds (16). Importantly, the triplet biradical also exhibits low reactivity towards water, potentially allowing very high yields of photoincorporation (17). Our SP analogue was also synthesized with an added amino-terminal tyrosine residue, so that the photoreactive peptide could be efficiently radioiodinated. ^{125}I-Tyr^0,Phe^8(pBz)-SP binds to the NK-1 receptor with the same (nanomolar) affinity as SP itself[1] and therefore provides a valuable probe for determining the site of the interaction of SP with the NK-1 receptor, as well as with the AChR. Because the L-Phe(pBz) residue and the radioactive ^{125}I tracer are in separate residues of the probe, radiosequence analysis cannot be used to determine the exact amino acid residue(s) of the AChR that are photo-cross-linked to the SP probe.

We report here that the SP analogue ^{125}I-Tyr^0,Phe^8(pBz)-SP photoincorporates into each of the *Torpedo californica* AChR subunits and that in the presence of carbamylcholine there is a 2-fold increase in photoincorporation into the AChR δ subunit that is inhibited by the addition of an excess of SP and is attributed to the labeling of a specific site. The sites of specific photoincorporation in the δ subunit are contained within a proteolytic fragment (δ Ser-253 to Glu-280) containing M2 of the δ subunit, which contributes to the lining of the ion channel. Thus, in the desensitized state of the AChR stabilized by agonist at equilibrium, at least a part of SP binds within the ion channel, and SP interaction with this site is likely to be responsible for its action as a noncompetitive AChR antagonist.

[1] Y.-M. Li, unpublished observations.

Experimental Procedures

Materials. *Staphylococcus aureus* V8 protease was purchased from ICN Biochemicals and *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin from Worthington Biochemical Corp. Genapol C-100 (10%) was purchased from Calbiochem. Prestained low molecular weight gel standards were purchased from GIBCO-BRL. *N*-Glycanase and endoglycosidase H were purchased from Genzyme.

Preparation of Fmoc-Phe(pBz). DL-Phe(pBz) was synthesized as described by Kauer *et al.* (18). Either the racemic DL-Phe(pBz) was resolved into L- and D-Phe(pBz) according to the method of Kauer *et al.* (18) or the racemic mixture was treated directly with Fmoc chloride (Aldrich Chemical Co.) or Fmoc *N*-hydroxysuccinimide ester (Sigma Chemical Co.) to provide the protected amino acid Fmoc-Phe(pBz) for solid-phase synthesis.

Peptide synthesis. Peptide synthesis of the SP analogue Tyr^{0}ArgProLysProGlnGlnPhePhe^8(pBz)GlyLeuMet-NH_2, using a standard Fmoc solid-phase synthetic strategy, was performed by the Harvard Medical School Biopolymer Facility. The crude synthetic peptide received was then purified by HPLC using a Vydac C_{18} (4.6 × 250 mm, 5 μm, 300 Å) reverse phase column with a Waters liquid chromatographic system equipped with a variable wavelength UV detector; elution was with a linear water/acetonitrile gradient (26–56% acetonitrile, 1%/3 min, 1.0 ml/min flow rate) containing 10 mM TFA. The crude racemic peptide Tyr^0,DL-Phe^8(pBz)-SP had two major UV-active (at 254 nm) components of equal intensity (eluting at 33.2% and 33.9% acetonitrile). The L-peptide was identified by elution position. That is, L-Phe^8(pBz)-SP synthesized from resolved L-Phe(pBz) showed only one UV-active peak, which was coincident with the earlier eluting HPLC peak of DL-Phe^8(pBz)-SP [synthesized from DL-Phe(pBz)]. Thus, the first HPLC peak corresponds to L-Phe^8(pBz) and the second peak corresponds to the D-Phe^8(pBz) diastereomer. In addition, Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) (19) formed an adduct with isolated L-Phe(pBz) free amino acid that preceded (in HPLC) that from D-Phe(pBz). Furthermore, when the Tyr^0, L-Phe^8(pBz)-SP and Tyr^0,D-Phe^8(pBz)-SP fractions were tested for NK-1 receptor binding and biological activity, the first eluting fraction, i.e., Tyr^0,L-Phe^8(pBz)-SP, was much more active than the second fraction or D-isomer. By analogy with the greater activity of L-Phe^8-SP, relative to D-Phe^8-SP (20), this further confirms that the first peak is the L-isomer. The L-Phe(pBz) isomer has also been shown by enzymatic digestion to precede the D-Phe(pBz) isomer of other peptides in reverse phase HPLC elution (21).

The isolated Tyr^0,L-Phe^8(pBz)-SP was analyzed for purity and correct structure by amino acid analysis, laser desorption/time of flight mass spectroscopy, and amino-terminal sequence analysis. The peptide was sequentially $Tyr_{0.2}Arg_{0.9}Pro_{0.7}Lys_{1.0}Pro_{0.7}Gln_{1.0}Gln_{1.0}Phe_{1.2}$-Phe-(pBz)-$Gly_{1.0}Leu_{1.0}Met_{0.8}$, with m/z 1616.2 [$(M+1)^+$]. Neither Phe(pBz) nor PTH-Phe(pBz) eluted from the analyzer column under standard conditions. Nevertheless, the high UV extinction coefficient of Phe(pBz) at 254 nm (21×10^3 M^{-1} cm^{-1}) verifies that Phe(pBz) is incorporated.

Preparation of ^{125}I-Tyr^0,Phe^8(pBz)-SP. The radioligand ^{125}I-Tyr^0,Phe^8(pBz)-SP was formed using the general iodination techniques described by Too and Maggio (22). Typically, 10 nmol of dry peptide were dissolved in 50 μl of 0.5 M phosphate buffer, pH 7.5, and vortex-mixed with 1 mCi of $Na^{125}I$ (10 μl; Amersham IMS-30). Chloramine-T (10 μg) was added and the mixture was vortex-mixed for 90 sec before the reaction was quenched with 100 μg of $Na_2S_2O_5$. To separate the peptide from free ^{125}I, the mixture was diluted and acidified with 0.525 ml of 60 mM TFA, with the addition of 25 μl of 2% bovine serum

albumin solution to limit nonspecific adsorption. The mixture was then applied to an activated C_{18} Sep-Pak cartridge (Waters), and the iodide and peptide were eluted from the cartridge with a series of 0.5-ml portions of 10 mM TFA solutions of increasing alcohol (ethanol/methanol, 1:1) content, i.e., 10%, 10%, 20%, 40%, 60%, 80%, 90%, 95%, and 100%. The iodide eluted immediately, whereas the peptide was retained until the alcohol mixture reached about 60%. The peptide fractions (containing both oxidized and reduced methionine), eluting with 60–90% alcohol, were collected and reduced in volume under a nitrogen stream to remove the alcohol. After the addition of 20% (v/v) β-mercaptoethanol, the sample was heated at 90° for 2 hr to reduce methionine sulfoxide. Further purification was achieved by reverse phase HPLC on a Vydac C_{18} column. The eluent was collected in fractions as a function of increasing percentage of acetonitrile in the shallow gradient, and the fractions were counted for radioactivity. The reduced (methionine) monoiodinated (tyrosine) peptide eluted in a peak (at 34.6% acetonitrile) that was later than the original compound (at 33.2% acetonitrile) and the oxidized products but before the reduced diiodinated peptide. The reduced monoiodinated ^{125}I-Tyr^0,Phe^8(pBz)-SP (specific activity, ~2000 Ci/mmol) was collected and protected from oxidation with 0.5% (v/v) β-mercaptoethanol for storage at −20° before use. The radioligand was shown to bind reversibly, saturably, and with high (nanomolar) affinity in SP (NK-1) receptor binding assays.[2]

AChR-enriched membranes. AChR-enriched membranes were isolated from the electric organ of *T. californica* (Marinus Inc., Westchester, CA) according to the procedure of Sobel *et al.* (23), with the modifications described previously (24). The final membrane suspensions in 36% sucrose/0.02% NaN_3 were stored at −80° under argon and contained 1–1.5 nmol of acetylcholine binding sites/mg of protein, as measured by a direct [^{3}H]acetylcholine binding assay (25).

Photoaffinity labeling of AChR-enriched membranes with ^{125}I-Tyr^0,Phe^8(pBz)-SP. *Torpedo* membranes (2 mg/ml, ~1 μM AChR) in *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0) plus 100 mM Tris·HCl, pH 7.0, were incubated with 3 μg/ml chymostatin, 5 μg/ml leupeptin, and 30 μg/ml bacitracin for 15 min before the addition of ^{125}I-Tyr^0,Phe^8(pBz)-SP. ^{125}I-Tyr^0,Phe^8(pBz)-SP was added typically to a final concentration of 1–2 nM (final concentrations of 0.34% acetonitrile, 0.001% TFA, and 0.005% β-mercaptoethanol). The initial suspension was then divided, additional ligands were added, and the final suspension was incubated at room temperature for 30 min. Membrane suspensions were then transferred to the wells of polystyrene tissue culture dishes, and a solution depth of ~3 mm was maintained for both analytical and preparative scale labelings. The polystyrene dishes were irradiated for 8 min on ice using a focused, 100-W, short-arc, mercury lamp, with a filter in place to eliminate light below 310 nm. Membrane suspensions were then pelleted (150,000 × *g*), and the samples were solubilized in sample loading buffer (26) and subjected to SDS-PAGE.

SDS-PAGE and autoradiography. SDS-PAGE was performed as described by Laemmli (26), using either 1.0-mm (analytical scale) or 1.5-mm (preparative scale) 8% polyacrylamide gels with 0.33% bisacrylamide. Polypeptides were visualized by staining with Coomassie Blue R-250 (0.25%, w/v, in 45% methanol, 10% acetic acid) and destaining in 25% methanol, 10% acetic acid. Analytical scale gels were then dried and exposed to Kodak X-OMAT LS film, with a DuPont Cronex intensifying screen, at −80° for various times (3–7 days). For preparative scale gels, bands corresponding to AChR subunits were excised and the protein was isolated from the excised gel pieces using a passive elution protocol similar to that described by Hager and Burgess (27). The excised gel fragments were cut into small pieces and protein was eluted for 4 days in 20 ml of elution buffer (0.1% SDS, 0.5% β-mercaptoethanol, 100 mM NH_4HCO_3, pH 7.8). The eluate was filtered (Whatman no. 1 filters), and the protein was concentrated using either Centriprep-30 or -10 concentrators (Amicon). Excess SDS was removed by acetone precipitation (overnight at −20°).

[2] Y.-M. Li, unpublished observations.

Proteolytic digests of ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled δ subunits. For *S. aureus* V8 protease digestion, acetone-precipitated δ subunits or δ subunit proteolytic fragments were resuspended in 0.1% SDS, 100 mM NH_4HCO_3, pH 7.8, at 1–2 mg/ml protein. V8 protease was added to a total enzyme to substrate ratio of 1:1 (w/w), and the mixture was incubated at room temperature for 3–4 days. For trypsin digestion, acetone-precipitated material was resuspended in a small volume (~50 μl) of buffer (0.1% SDS, 100 mM NH_4HCO_3, pH 7.8). The SDS concentration was then reduced by dilution with buffer without SDS, and Genapol C-100 (Calbiochem) was added, resulting in final concentrations of 0.02% SDS, 0.5% Genapol C-100, and 1–2 mg/ml protein. Trypsin was added to a total enzyme to substrate ratio of 1:1 (w/w), and the mixture was incubated at room temperature for 3–4 days. When δ subunits or δ subunit fragments were treated with either endoglycosidase H or *N*-glycanase, samples were resuspended in the buffer used for trypsin digestion. Both trypsin and V8 protease digests were separated on a Tricine gel system described by Schagger and von Jagow (28). Gels were composed of a 10-cm-long small-pore separating gel (16.5% total acrylamide concentration with 1% bisacrylamide), a 2-cm spacer gel (10% total arcylamide concentration, with 0.03% bisacrylamide), and a 2-cm stacking gel (4% total acrylamide concentration with 0.12% bisacrylamide). Typically, a small aliquot of each digest (~5%) was first resolved on an analytical scale Tricine gel (1.0-mm thick), and the autoradiogram of that dried gel was then used as a template to identify ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled bands when the rest of the digest was resolved on 1.5-mm preparative gels. Labeled bands were then excised and the labeled polypeptides were isolated from the gel pieces by passive elution as described above. The molecular weights of labeled bands were estimated using prestained standards (GIBCO-BRL), i.e., ovalbumin (43,000), carbonic anhydrase (29,000), α-lactoglobulin (18,400), lyzozyme (14,300), bovine trypsin inhibitor (6,200), insulin A chain (3,400), and insulin B chain (2,300).

^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled fragments were further purified by reverse phase HPLC using a Brownlee Aquapore C_4 column (100 × 2.1 mm). Solvent A was 0.08% TFA in water, and solvent B was 0.05% TFA in 60% acetonitrile/40% 2-propanol. The flow rate was maintained at 0.2 ml/min, and 0.5-ml fractions were collected. Before injection, material isolated from excised gel pieces was filtered (Whatman no. 1 filters), the protein was concentrated using a Centricon-3 concentrator (Amicon), and the material was then centrifuged briefly (15,000 rpm for ~10 sec) in a table-top microfuge to sediment any insoluble material. Peptides were eluted with a nonlinear gradient (Waters model 680 gradient controller, curve 7) of 25–100% solvent B in 80 min. The elution of peptides was monitored by the absorbance at 210 nm, and the elution of radioiodinated products was monitored by γ counting of the fractions.

Sequence analysis. Amino-terminal sequence analysis was performed with an Applied Biosystems model 477A protein sequencer, using gas-phase cycles. Pooled HPLC samples were dried by vacuum centrifugation, resuspended in a small volume (~20 μl) of 0.05% SDS solution, and immobilized on chemically modified, glass fiber disks (Beckman Instruments), rather than polybrene-treated filters, to improve the sequencing yields for hydrophobic peptides (29). Approximately 30% of the released PTH-amino acids were separated with an on-line model 120A PTH-amino acid analyzer. Initial yield (I_o) and repetitive yield (R) were calculated by nonlinear, least-squares regression of the observed release (M) for each cycle (n), using the equation $M = I_oR^n$. PTH derivatives of serine, threonine, cysteine, and histidine were omitted from the fit.

Results

To identify possible sites of SP photoincorporation in the AChR, we used photolabeling conditions that would facilitate detection of pharmacologically sensitive incorporation, even though nonspecific interactions of an amphipathic peptide such as SP might also result in substantial photolabeling insensitive

to the presence of nonradioactive SP or cholinergic agonists or antagonists. Thus, AChR-enriched membrane suspensions (2 mg/ml, 1 μM AChR) were incubated with a low concentration (1–2 nM) of the radioiodinated, photoreactive, SP analogue ^{125}I-Tyr^0,Phe^8(pBz)-SP, rather than with the high (greater than micromolar) concentration that would be necessary to fully occupy the AChR binding site. Under these conditions, in the presence of agonist ~0.1% of AChRs would bind SP at its "high affinity" site (4), whereas in the absence of agonist that binding would be reduced by >10-fold. Near-UV irradiation (>310 nm) resulted in photoincorporation of ^{125}I into each of the receptor subunits (Fig. 1), as well as into a number of other polypeptide bands present in the AChR-enriched membrane preparation. Based upon γ counting of excised gel pieces and of isolated subunits, carbamylcholine (3 mM) induced a 2.05 ± 0.1-fold (five experiments) increase in the extent of photoincorporation into the AChR δ subunit (Fig. 1, *lane 2*), and this increased photoincorporation was inhibited by the addition of an excess (100 μM) of SP (Fig. 1, *lane 3*). For the other subunits the presence of carbamylcholine and/or 100 μM SP resulted in no significant (<10%) change in the level of photolabeling. The selective specific photolabeling of the δ subunit in the presence of agonist is consistent with the results of Min *et al.* (4). If the photoaffinity analogue binds to the AChR with affinity similar to that of [^{3}H]SP (4), then the observed level of agonist-sensitive ^{125}I incorporation suggests an efficiency of photoincorporation of ~0.2%, a value 100-fold lower than that observed for ^{125}I incorporation into the NK-1 receptor (14).

To localize the site of increased ("specific") incorporation, δ subunits labeled with ^{125}I-Tyr^0,Phe^8(pBz)-SP in the absence (~130 cpm/μg) or presence (~260 cpm/μg) of carbamylcholine were isolated from a preparative scale labeling (8 mg of membranes for each condition). Labeled δ subunits (~200 μg) were digested exhaustively with *S. aureus* V8 protease, and aliquots (5%) of the digests were resolved on an analytical scale (1.0-mm) Tricine gel. In the autoradiogram of the dried gel, a strongly labeled band migrating with an apparent molecular mass of 14 kDa (δ-V8–14) was seen in the digest of δ subunits labeled in the presence of carbamylcholine (Fig. 2, *lane 2*) but not in its absence (Fig. 2, *lane 1*). In addition, there were radioactive fragments with apparent molecular masses of approximately 4 kDa and 5.5 kDa, with similar levels of photolabeling in the absence and presence of agonist. Although incorporation into the two lower molecular mass bands represents sites not displaceable by SP (i.e., "nonspecific" labeling), δ-V8–14 contained the site in the δ subunit sensitive to agonist

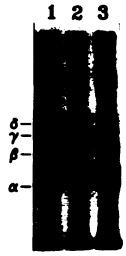

Fig. 1. Photoincorporation of ^{125}I-Tyr^0,Phe^8(pBz)-SP into AChR-enriched membranes. AChR-enriched membranes (2 mg/ml, 1 μM AChR) were incubated with 1 nM ^{125}I-Tyr^0,Phe^8(pBz)-SP and irradiated (>310 nm) for 8 min. Polypeptides were separated by SDS-PAGE, visualized with Coomassie Blue, and subjected to autoradiography. Membrane suspensions contained no other ligands (*lane 1*), 3 mM carbamylcholine (*lane 2*), or 3 mM carbamylcholine and 100 μM SP (*lane 3*).

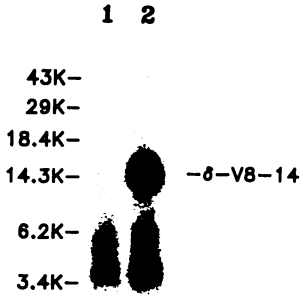

Fig. 2. V8 protease digestions of ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled AChR δ subunits resolved by Tricine SDS-PAGE. AChR δ subunits (~200 μg) isolated from AChR-enriched membranes labeled with ^{125}I-Tyr^0, Phe^8(pBz)-SP in the absence (21,200 cpm) or presence (52,900 cpm) of 3 mM carbamylcholine were digested with *S. aureus* V8 protease, and the digestion products were resolved by Tricine SDS-PAGE followed by autoradiography. Shown is an autoradiogram of a 1.0-mm gel with aliquots (~5%) of the digests of the δ subunits labeled in the absence (*lane 1*) and in the presence (*lane 2*) of 3 mM carbamylcholine. *Numbers to the left*, migration of prestained molecular weight standards.

and nonradioactive SP, i.e., the site of specific photolabeling. In additional experiments, we examined the effects of meproadifen, an aromatic amine noncompetitive antagonist (29), on the agonist-sensitive photolabeling of the δ subunit. Meproadifen at 5 and 150 μM inhibited specific incorporation by 70% and 95%, respectively. All meproadifensensitive photolabeling was restricted to δ-V8–14.

Material contained within the δ-V8–14 band was isolated when the bulk of the V8 digest of the δ subunit was resolved on a preparative scale (1.5-mm) Tricine gel. When the material eluted from the δ-V8–14 band was further purified by reverse phase HPLC (Fig. 3A), the ^{125}I counts eluted in a peak centered at 13 ml (~60% solvent B). HPLC fractions 25–28 were pooled and subjected to amino-terminal amino acid sequence analysis, with the ^{125}I loaded on the amino acid sequencer for the δ-V8–14 sample labeled in the presence of carbamylcholine representing approximately 21% of the counts contained within the undigested δ subunit and 40% of the carbamylcholine-induced incorporation. A single peptide was evident beginning at Ile-192 (initial yield, 94.5 pmol; repetitive yield, 93.5%; 24 cycles sequenced). This sequence was present at a level at least 10-fold higher than that of any other sequence. As expected, ~40% of loaded ^{125}I counts were released in the first cycle of Edman degradation.

To confirm that the Ile-192 peptide was indeed the labeled peptide, we made use of the fact that Asn-208 contains an *N*-linked, high mannose-type carbohydrate that is sensitive to both *N*-glycanase and endoglycosidase H (30).[3] Treatment of the δ subunit (~10 μg) with endoglycosidase H (33 milliunits)

[3] In the sequence analysis of a large (20-kDa) δ subunit fragment beginning at Ile-192 (δ-V8–20), the presence of Asn-208 (cycle 17) is never detected, whereas Asn-200 and Asn-211 are detected. This indicates that Asn-208 is modified in some manner, shifting the mobility of the PTH-amino acid derivative outside of the range detected on the reverse phase HPLC column of the PTH-amino acid analyzer. Treatment of δ-V8–20 with *N*-glycanase (Genzyme), followed by sequence analysis, results in the detection of PTH-aspartic acid in the cycle corresponding to Asn-208. *N*-Glycanase catalyzes the hydrolysis of asparagine-linked oligosaccharides at the β-aspartyl-glycosylamine bond between the innermost *N*-acetylglucosamine and the asparagine residue, leaving an "aspartic acid" residue in the peptide chain (40). Finally, endoglycosidase H treatment shifts the mobility of δ-V8–20 by approximately 2 kDa on a Tricine SDS-PAGE gel a result indicating that Asn-208 contains an *N*-linked high mannose-type carbohydrate (data not shown).

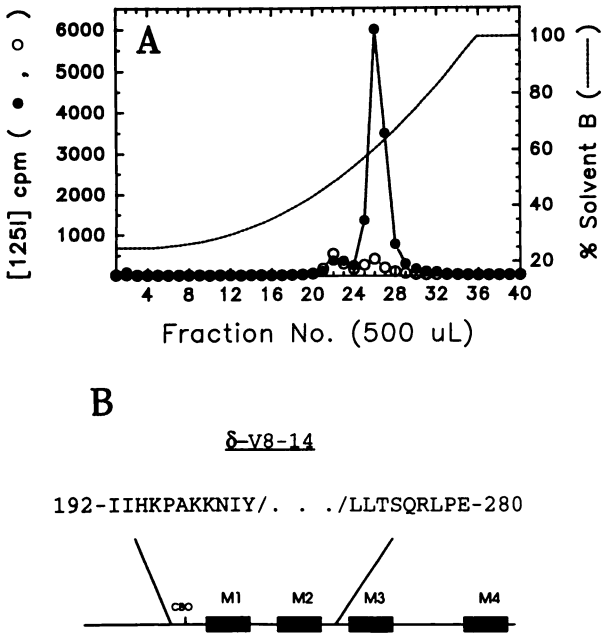

Fig. 3. Reverse phase HPLC purification of the ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled δ-V8–14 fragment. A, The δ subunit fragment δ-V8–14 (Fig. 2) was isolated when the bulk of the material from exhaustive V8 protease digestion of the δ subunit was resolved on a 1.5-mm Tricine (21,200 and 52,900 cpm were loaded on the gel and 2,300 and 14,200 cpm were recovered in the δ-V8–14 bands labeled in the absence and in the presence of carbamylcholine, respectively). The material eluted from the gel was further purified by reverse phase HPLC, as described in Experimental Procedures. Peptides were eluted from the Aquapore C_4 column with a nonlinear gradient of 25–100% solvent B in 80 min (– – –). Shown is the ^{125}I elution profile for δ-V8–14 labeled in the presence of 3 mM carbamylcholine (●) and in the absence of carbamylcholine (○). Based on the amount of recovered ^{125}I counts, HPLC column recoveries were >90%. B, The map of the amino acid sequence of the AChR δ subunit is shown. *Lower*, entire amino acid sequence of the AChR δ subunit. *Black rectangles*, M1, M2, membrane-spanning region 3 (*M3*), and membrane-spanning region 4 (*M4*). *CBO*, *N*-linked glycosylation site at Asn-208. *Upper*, expanded portion showing the location of the δ-V8–14 fragment relative to the entire amino acid sequence of the δ subunit.

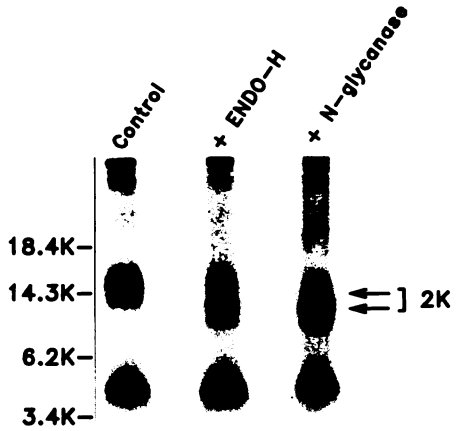

Fig. 4. V8 protease digestions of endoglycosidase-treated ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled δ subunits resolved by Tricine SDS-PAGE. Aliquots of δ subunits (~10 μg, 2700 cpm) labeled in the presence of 3 mM carbamylcholine were incubated in the absence (*Control*) or in the presence of either endoglycosidase H (*ENDO-H*) (33 milliunits) or *N*-glycanase (3 units) for 4 days and then digested with V8 protease. The digestion products were resolved by Tricine SDS-PAGE, followed by autoradiography. Shown is the autoradiogram of a 1.0-mm thick gel. *Numbers to the left*, migration of prestained molecular weight standards. *Arrows*, approximately 2-kDa shift in the mobility of δ-V8–14 produced by either endoglycosidase H or *N*-glycanase treatment.

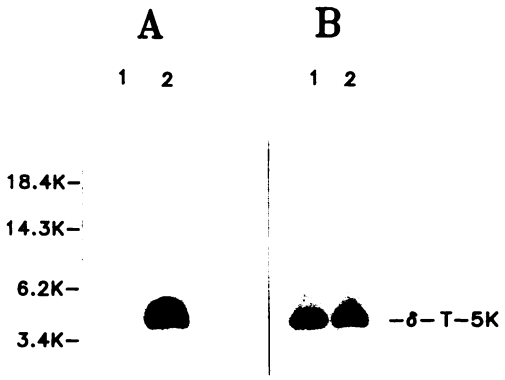

Fig. 5. Trypsin digestions of the ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled δ-V8–14 fragment resolved by Tricine SDS-PAGE. A, The ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled, AChR δ subunit fragment δ-V8–14 (~40 μg) produced by V8 protease digestion was digested with trypsin, and the digestion products were resolved by Tricine SDS-PAGE, followed by autoradiography. Shown is an autoradiogram of a 1.0-mm-thick gel with aliquots (~5%) of the digests of δ-V8–14 labeled in the absence (940 cpm) (*lane 1*) and in the presence (2870 cpm) (*lane 2*) of 3 mM carbamylcholine. *Numbers to the left*, migration of prestained molecular weight standards. B, Aliquots of δ-V8–14 (~3 μg) labeled in the presence of 3 mM carbamylcholine were incubated in the absence (*lane 1*) or in the presence (*lane 2*) of 3 units (~0.12 μg) of *N*-glycanase for 4 days and then digested with trypsin. Shown is the autoradiogram of a 1.0-mm thick gel.

or *N*-glycanase (3 units), followed by digestion with V8 protease, resulted in an approximately 2-kDa increase in the mobility of ^{125}I-labeled δ-V8–14 (Fig. 4). A similar shift of mobility was seen when δ-V8–14 (~4 μg) was deglycosylated with *N*-glycanase (data not shown). Because the labeled δ-V8–14 peptide contains a simple *N*-linked carbohydrate (~2 kDa) and 1 mol of ^{125}I-Tyr^0,Phe^8(pBz)-SP (1.74 kDa), the mass of the δ-V8–14 subunit fragment is predicted to be ~10 kDa, and the peptide with its amino terminus at Ile-192 is predicted to terminate at Glu-280 (Ile-192 to Glu-280; calculated molecular weight, 10,158). This peptide contains M1 and M2 and the intervening sequence, as well as an approximately 30-amino acid stretch preceding the amino terminus of M1 (Fig. 3B). Cleavage at the sites preceding (Glu-255, before the M2 segment) or following (Glu-332, after the membrane-spanning region 3 segment) the Glu-280 site would generate peptides with calculated molecular weights of 7,428 and 15,948, respectively.

To further localize the site of incorporation in the δ subunit, δ-V8–14 was digested with trypsin. When aliquots of the digests were resolved on an analytical scale (1.0-mm) Tricine gel, the labeled material migrated as a single band with an apparent molecular mass of 5 kDa (δ-T-5K) (Fig. 5A). Treatment of δ-V8–14 (~3 μg) with *N*-glycanase (3 units) before digestion with trypsin did not produce an increase in the mobility of the δ-T-5K band (Fig. 5B, *lane 2*). This result indicates that the site of ^{125}I-Tyr^0,Phe^8(pBz)-SP incorporation is not in a tryptic fragment containing Asn-208 (i.e., Phe-206 to Arg-223). The material contained within δ-T-5K was isolated when the bulk of the tryptic digest of δ-V8–14 was resolved on a 1.5-mm-thick Tricine gel. When the material eluted from the δ-T-5K band was further purified by reverse phase HPLC (Fig. 6A), the majority of ^{125}I counts eluted in a peak centered at 11.5 ml (~51% solvent B). In addition, although only a single band was evident in the autoradiogram of the analytical Tricine gel of

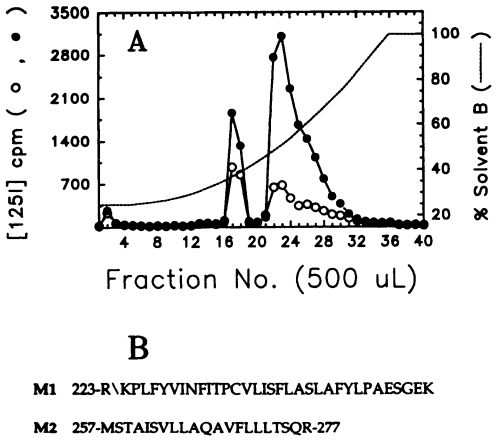

Fig. 6. Reverse phase HPLC purification of the ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled δ-T-5K fragment. A, The δ-T-5K fragment (Fig. 5) was isolated when the bulk of the material from an exhaustive tryptic digestion of the δ subunit fragment δ-V8–14 was resolved on a 1.5-mm thick gel. For δ-V8–14 isolated from AChRs labeled in the presence of carbamylcholine 42,108 cpm were loaded on the gel and 19,500 cpm were recovered in the δ-T-5K band, whereas for the digest of δ-V8–14 labeled in the absence of carbamylcholine 13,740 cpm were loaded and 6,500 cpm were recovered in the δ-T-5K band. The material eluted from the gel pieces was further purified by HPLC as described (see Experimental Procedures and the legend to Fig. 3). Shown is the ^{125}I elution profile for δ-T-5K labeled in the presence of 3 mM carbamylcholine (●) and in the absence of carbamylcholine (○). B, The amino acid sequences of the tryptic fragments identified in the amino-terminal amino acid sequence analysis of δ-T-5K (HPLC fractions 22–24) are shown. The hydrophobic segment M1 is contained within the tryptic fragments beginning at Arg-223 and Lys-224, and M2 is contained within the tryptic fragment beginning at Met-257.

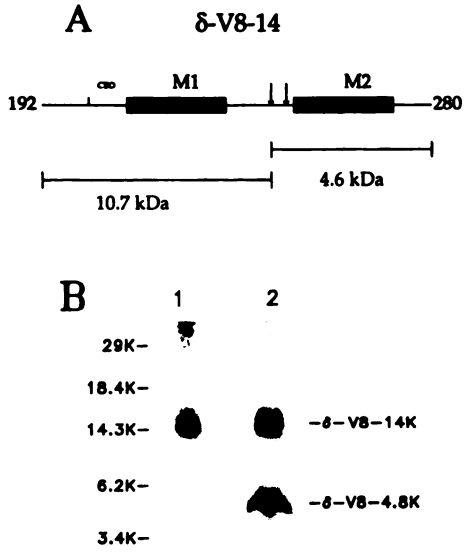

Fig. 7. V8 protease digestions of the ^{125}I-Tyr^0,Phe^8(pBz)-SP-labeled δ-V8–14 fragment resolved by Tricine SDS-PAGE. A, A map of the amino acid sequence of the AChR δ subunit contained within the V8 protease fragment δ-V8–14 (Ile-192 to Glu-280) is shown. *Black rectangles*, M1 and M2. *Arrows*, locations of the two potential sites of V8 protease cleavage (Glu-252 and Glu-255). The resulting V8 protease cleavage fragments are indicated, with their calculated molecular masses. The molecular masses include the mass of 1 mol of ^{125}I-Tyr^0,Phe^8(pBz)-SP (~1.7 kDa) and the fragment beginning at Ile-192 includes the mass of the *N*-linked carbohydrate at Asn-208 (~2 kDa). B, The δ-V8–14 fragment isolated by preparative SDS-PAGE and reverse phase HPLC (Fig. 3) was digested with V8 protease and the digestion products were resolved by Tricine SDS-PAGE, followed by autoradiography. Shown is an autoradiogram of a 1.0-mm thick gel. The V8 digest of δ-V8–14 (*lane 2*) is shown with an undigested sample (*lane 1*) of aliquots (4 μg, 1300 cpm) of δ-V8–14 isolated from AChRs labeled in the presence of 3 mM carbamylcholine. *Numbers to the left*, migration of prestained molecular weight standards.

the δ-V8–14 tryptic digest (Fig. 5A), the HPLC profile exhibited a second, smaller, peak of ^{125}I counts, which eluted at 8.5 ml (~37% solvent B). Because ^{125}I-Tyr^0,Phe^8(pBz)-SP itself (in 0.1% SDS) eluted from the C_4 reverse phase column at the latter solvent concentration, this peak may represent ^{125}I-Tyr^0,Phe^8(pBz)-SP peptide no longer cross-linked to a peptide fragment of the AChR δ subunit. When HPLC fractions 22–24 were pooled and subjected to amino-terminal sequence analysis, three peptides were identified, one beginning before M2 at Met-257 (initial yield, 9.2 pmol; repetitive yield, 93%; 20 sequencing cycles) and two beginning before M1 at Arg-223 (initial yield, 8.9 pmol; repetitive yield, 98%) and at Lys-224 (initial yield, 2.9 pmol; repetitive yield, 96%). Although the HPLC elution profile of δ-T-5K matched that previously observed for the elution of [^{125}I]TID-labeled tryptic fragments containing M2 (31), the presence of peptides containing either the M1 or M2 hydrophobic segment resulted in ambiguity regarding which of the two segments was labeled.

To determine whether the site of ^{125}I-Tyr^0,Phe^8(pBz)-SP incorporation was in M1 or M2 of the δ subunit, δ-V8–14 was further digested with V8 protease. There are only two glutamate residues (Glu-252 and Glu-255) within the stretch of the primary sequence defined by δ-V8–14 (Ile-192 to Glu-280). Cleavage by V8 protease at either of these two, closely spaced, glutamate residues would generate two peptides with substantially different masses (Fig. 7A). The larger molecular mass fragment (Ile-192 to about Glu-252) includes the *N*-linked carbohydrate at Asn-208, contains the M1 hydrophobic segment, and, if labeled, would have a calculated molecular mass of ~10.7 kDa. The smaller fragment (approximately Ser-253 to Glu-280) contains M2 and, if labeled, would have a calculated molecular mass of ~4.6 kDa. When an aliquot of the V8 protease digest of δ-V8–14 was resolved on an analytical scale (1.0-mm) Tricine gel, the labeled material migrated as two bands (Fig. 7B, *lane 2*). One of the bands migrated with exactly the same mobility as undigested δ-V8–14 (Fig. 7B, *lane 1*), and the other migrated with an apparent molecular mass of 4.8 kDa (δ-V8–4.8) (Fig. 7B, *lane 2*). When the radioactive samples eluted from the excised bands were rechromatographed as in Fig. 3, ^{125}I-δ-V8–4.8 eluted in a peak at 47% solvent B (fractions 21–23), whereas δ-V8–14 eluted, as before, at 60% solvent B (fractions 25–27). Based on these results, the site of ^{125}I-Tyr^0,Phe^8(pBz)-SP incorporation resides within the stretch of the δ subunit primary sequence (approximately Ser-253 to Glu-280) that contains almost exclusively M2.

Discussion

We conclude from the studies presented here that, when AChRs are equilibrated with agonist and are therefore in the desensitized state, the radioiodinated photoreactive SP analogue ^{125}I-Tyr^0,Phe^8(pBz)-SP is specifically incorporated into the δ subunit fragment δ Ser-253 to Glu-280, which contains the M2 hydrophobic segment and a few flanking amino acids. Because the analogue is labeled with ^{125}I in the amino-terminal amino acid of the photoaffinity label, this probe cannot be used to identify the δ subunit amino acid that is the site of photo-

labeling. Nevertheless, it is clear that the photoreactive Phe(pBz) in position 8 of the peptide probe must have access to at least the amino-terminal (extracellular) end of the M2 segment. In view of the large body of evidence associating the M2 segment of each subunit with the lumen of the ion channel and the binding site for many noncompetitive antagonists (reviewed in Ref. 13), it is likely that the interaction of SP with this region is also responsible for the inhibitory effects of SP on agonist-induced channel activation. Because ^{125}I-Tyr^{0}, Phe^{8}(pBz)-SP cannot be used to determine by radiochemical sequencing the particular amino acid(s) within M2 that are photolabeled, additional studies will be required to determine whether amino acids lining the channel contribute to the binding site, which we consider most likely, or whether the SP photoaffinity label reacts with amino acids on the face of the M2 helix oriented away from the lumen of the channel.

A channel-binding role for SP is consistent with the results of Min *et al.* (4), who previously determined that in the presence of agonist there was ~1 high affinity SP binding site/receptor. In addition, the simplest interpretation of the effects of SP (which is positively charged at pH 7) on the binding of [^{3}H]-acetylcholine is that the peptide stabilizes the desensitized state of the receptor (12), an effect consistent with a channel site of action, because most positively charged, noncompetitive antagonists that bind within the channel bind with highest affinity to the desensitized state of the AChR. Previously it was thought that SP did not bind to the high affinity noncompetitive antagonist site in the channel, because in the absence of agonist SP actually potentiated the binding of [^{3}H]PCP (12, 32), whereas in the presence of agonist SP inhibited the binding of [^{3}H]PCP, although not in a purely competitive fashion. An allosteric interaction with [^{3}H]PCP may, however, be reconciled with SP binding within the channel if the two compounds bind to different regions of the channel. Recent studies (33, 34) with the novel, uncharged, noncompetitive antagonist, photoaffinity reagent TID provide clear evidence that a single ligand can bind to different regions of the ion channel, depending upon the conformational state of the AChR, and that at least charged and uncharged noncompetitive antagonists can bind simultaneously within the channel. In the absence of agonist, [^{125}I]TID photolabels the aliphatic residues at positions 9 and 13 of each M2 segment, whereas nonradioactive TID has no effect on the binding of [^{3}H]PCP and is an allosteric inhibitor of the binding of [^{3}H]histrionicotoxin, another positively charged, noncompetitive antagonist that itself binds competitively with [^{3}H]PCP. In contrast, in the presence of agonist [^{125}I]TID is photoincorporated specifically into the serines at positions 2 and 6 of the M2 segments, whereas nonradioactive TID acts as a potent competitive inhibitor of [^{3}H]PCP binding and an allosteric inhibitor of [^{3}H]histrionicotoxin binding.

SP binding within the channel domain could also account for the equipotency of SP as an inhibitor of neuronal and muscle-type AChRs, despite their overall structural differences. In general, there appear to be greater differences between muscle-type and neuronal AChRs in the potencies and selectivities of agonists and competitive antagonists, compared with noncompetitive antagonists (35–37). In studies with adrenal chromaffin (neuronal) AChRs, Boyd *et al.* (38) reported an agonist-induced increase in the photoincorporation of ^{125}I-Bolton Hunter-Phe^{8}(pBz)-SP into AChR subunits of 59 and 51 kDa. For $\alpha 3\beta 4$ neuronal AChRs expressed in *Xenopus* oocytes, Stafford *et al.* (39) found that a single mutation (Phe-255 to valine) in M2 of the $\beta 4$ subunit decreased by a factor of 6 the potency of SP as an antagonist (K_i of 3.3–20 μM).

In the studies presented here, where the initial characterization of the photolabeling was carried out at the level of intact AChR subunits, the agonist-induced increase in ^{125}I-Tyr^{0}, Phe^{8}(pBz)-SP incorporation was restricted to the δ subunit. This suggests that the SP analogue is very specifically oriented within the AChR ion channel, especially in view of the high degree of conservation among the M2 sequences in the subunits and the selective photochemistry of the benzophenone triplet biradical that allows reaction with unactivated C-H bonds (15). However, additional studies will be necessary to determine whether there is also agonist-sensitive photolabeling of the M2 segments of other subunits. In studies of [^{125}I]TID photolabeling of AChRs in the presence of agonist (34), specific photolabeling was not detected at the level of intact subunits, but specific photolabeling was revealed when M2 segments were isolated from the remaining subunit regions that contained substantial nonspecific photolabeling. Because the L-Phe(pBz) residue and the radioactive ^{125}I tracer are in separate residues of the probe, radiosequence analysis cannot be used to determine the exact amino acid residues that are labeled in M2. We plan to develop a radioactively tagged L-Phe(pBz) in the future, to determine the labeled residues in M2 and to prepare SP analogues in which the radioactively tagged L-Phe(pBz) amino acid is substituted at other positions, to more closely map how the peptide interacts with the ion channel.

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Send reprint requests to: J. B. Cohen, Department of Neurobiology, Harvard Medical School, 220 Longwood Ave., Boston, MA 02115.