Chapter 4 Identification of the Sites of Incorporation of [^{3}H]Progestin Aryl Azide in the nAChR

ABSTRACT

Progesterone at micromolar concentrations is a noncompetitive antagonist of the *Torpedo* nicotinic acetylcholine receptor (nAChR). To localize the sites of interaction of progesterone with the nAChR, we have used a photoactivatible derivative of progesterone, [^{3}H]progestin aryl azide. At 0.4 μM, [^{3}H]progestin aryl azide photoincorporated into all four subunits of the nAChR. The incorporation was not affected by the presence of other cholinergic drugs. Within the α subunit, ~75% of the incorporation was mapped to a 10 kD fragment containing the fourth transmembrane segment (αM4), known to contribute to the protein-lipid interface. Approximately 10% of the [^{3}H]progestin aryl azide incorporation in the α subunit was in a 20 kD fragment containing the first three transmembrane segments (αM1, αM2, and αM3). Efforts to further localize the incorporation provided evidence that [^{3}H]progestin aryl azide incorporated into the αM4 segment as well as the M4 segments of the other subunits. However, the yields of these segments recovered for sequence analysis, along with the instability of the adducts formed with the β, γ, and δ subunits to purification conditions, precluded the identification of labeled amino acids.

INTRODUCTION

The anesthetic properties of steroids were first reported by Seyle (1941). Since then, some steroids, such as alphaxalone-alphadalone, have been used clinically for inducing and maintaining anesthesia. The action of ligand-gated ion channels is altered by steroids, consistent with the effects of several other general anesthetics. However, while several recent studies have begun to identify specific residues in the $GABA_A$,

glycine, and nACh receptors which affect the potency of other general anesthetics (Wick *et al.*, 1998; Forman, 1997), the site of action of steroids on these receptors is unclear. Although the potentiation of the $GABA_A$ receptor by steroids is not due to non-specific effects on lipid order (Paul and Purdy, 1992), the site of action is distinct from the binding sites of GABA and competitive antagonists, barbiturates, or benzodiazepines. Additionally, mutations within the second (M2) and third (M3) segments that have been shown to affect the potency of several general anesthetics, such as the long chain alcohols, do not affect the potency of steroid anesthetics (Belelli *et al.*, 1997; McGurk *et al.*, 1998). Recent work by Rick *et al.* (1998) using chimeric $GABA_A$ receptors has indicated that N-terminal region of the $GABA_A$ receptor, prior to the M2 segment, confers sensitivity to steroids.

Unlike the $GABA_A$ receptor, steroids act as noncompetitive antagonists (NCAs) on the nAChR. Their method of action on the nAChR, however, appears different than that of other NCAs such as the aromatic amines. When progesterone is applied before agonist, and then washed off, the current is still inhibited (Valera *et al.*, 1992). Hydrocortisone and 11-deoxycortisone cause a decrease in burst duration, indicating that the channel may be able to adopt a closed conformation while still being bound by the steroid (Bouzat and Barrantes, 1993). Additionally, the application of hydrocortisone with QX-222 alters the single channel properties from those seen with QX-222 alone, suggesting that the two drugs bind different sites (Bouzat and Barrantes, 1996). These results suggest that steroids are not traditional channel-blockers, if they even bind in the channel at all.

The high lipophilicity of steroids suggests a possible interaction at the protein-lipid interface. However, electrophysiological experiments with the nAChR have provided evidence that the steroids do not act via the protein-lipid interface. Binding to a

site accessible only from the extracellular side is supported by a decrease in inhibition when hydrocortisone and 11-deoxycortisone are applied intracellularly (Bouzat and Barrantes, 1993). Experiments with a modified progesterone coupled to bovine serum albumin show that even a strongly hydrophilic steroid could still inhibit current, suggesting that the steroid does not need to enter the lipid bilayer to exert its effects (Valera *et al.*, 1992; Ke and Lukas, 1999).

Photoaffinity labeling studies with the steroid noncompetitive antagonist of the nAChR, [^{3}H]promegestone, however, showed incorporation only into the fourth transmembrane segment (M4), in amino acids known to contribute to the protein-lipid interface (Blanton *et al.*, 1999). There was no detectable incorporation of [^{3}H]promegestone into any residues in the M2 segment, which lines the lumen of the channel and has been photoaffinity labeled by several other noncompetitive antagonists. This lack of incorporation, however, may be attributable either to a lack of binding within the channel or the absence of side chains within the lumen of the channel into which the promegestone reactive group can incorporate.

As an alternative to promegestone, Kym *et al.* (1995) synthesized [^{3}H]progestin aryl azide (Figure 4-1) as a photoaffinity probe of the progesterone receptor. With the progesterone receptor, [^{3}H]progestin aryl azide showed photoattachment efficiency of ~60%, while promegestone only showed an efficiency of ~5%. These two steroids utilize different photoreactive groups, one an enone and the other an aryl azide. Additionally, these reactive groups are at opposite ends of the steroid, ring A as opposed to ring D, perhaps allowing reaction with different regions of the receptor.

Here we present studies examining the photolabeling of nAChR-rich membranes by the photoaffinity steroid [^{3}H]progestin aryl azide. This compound incorporated into each nAChR subunit. Within the α subunit, the primary site of labeling was contained

within a ~10 kD fragment which included the M4 segment. However, the low levels of incorporation as well as low mass levels were compounded by the instability of the adducts to HPLC and sequencing conditions, so no information could be obtained regarding the specific side chains labeled.

MATERIALS AND METHODS

Materials

nAChR-enriched membranes were isolated from *Torpedo californica* electric organ according to the method described by Sobel *et al.* (1977), modified as described by Pedersen *et al.* (1986). The final membrane suspensions were stored in 38% sucrose at -80 °C under argon. The membranes used here contained 0.5-1.0 nmol acetylcholine binding sites per milligram of protein. [^{3}H]Progestin aryl azide (16α,17α-[*(S)*-1'-(4-azidophenyl)(ethylenedioxy)]pregn-4-ene-3,20-dione) was a kind gift from Dr. John Katzenellenbogen, synthesized according to the methods described in Kym *et al.* (1995), with a specific activity of 16 Ci/mmol. *S. aureus* V8 protease was from ICN Biomedical Inc, EndoLysC from Boeringher Mannheim, trifluoroacetic acid (TFA) from Pierce. 1-Azidopyrene (1-AP) was purchased from Molecular Probes. 10% Genapol C-100 was from Calbiochem. Tetracaine, progesterone, oxidized glutathione, Tricine, trypsin, and carbamylcholine were from Sigma. EndoglycosidaseH was from Genzyme.

Photoaffinity labeling of nAChR-enriched membranes with [^{3}H]progestin aryl azide

For analytical labeling experiments, freshly thawed *Torpedo* membranes (100 μg per condition) were diluted with TPS and pelleted (15000xg) for 30 minutes. The pellets were resuspended 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; TPS), and [^{3}H]progestin aryl

azide was added. The membranes were split into aliquots, and additional ligands were added to the final concentrations indicated in the figure legends. The final concentration of membranes was approximately 2 mg/ml (~1 μM nAChR), and the [^{3}H]progestin aryl azide was present at 0.4 μM. The presence of 0.1 mM oxidized glutathione (GSSG) as a scavenger during photolysis reduced the incorporation into both nAChR and non-nAChR polypeptides by ~40%, and no further reduction was seen at GSSG concentrations up to 1 mM GSSG. Therefore, after a 1 hour incubation at room temperature, oxidized glutathione (GSSG) was routinely added to a final concentration of 1 mM. Since [^{3}H]progestin aryl azide was reported to absorb strongly at 246 nm (Kym *et al.*, 1993), the suspensions were irradiated at 254 nm (Spectroline EF-16) for 2 minutes in a plastic 96-well plate on ice. Five minute photolysis did not increase the incorporation appreciably. The suspensions were diluted with sample loading buffer and directly submitted to SDS-PAGE.

For proteolytic mapping of [^{3}H]progestin aryl azide labeled α subunit with *S. aureus* V8 protease (Cleveland *et al.*, 1977; White and Cohen, 1992), labeling was carried out with 400 μg (analytical mapping) or 10 mg (preparative) nAChR-rich *Torpedo* membranes. For analytical mapping, samples were photolyzed in a 24-well plate, while for preparative mapping, the samples were photolyzed in glass dishes (5.5 cm diameter). Following photolysis, the membrane suspensions were pelleted. For analytical mapping, samples were resuspended in 40 μl 50 mM sodium phosphate, pH 7, 1% SDS, and 40 μl of 50 mM sodium phosphate, pH 6, with or without 5 mU endoglycosidase H was added to each. After an overnight incubation, samples were diluted with sample buffer and submitted to SDS-PAGE. For preparative mapping, samples were resuspended in TPS (2 mg/ml) and then labeled further with 1-azidopyrene (1-AP) (Blanton and Cohen, 1994) to ease identification and isolation of subunits and fragments from gels. 1-AP (62.5 mM in

DMSO) was added to a final concentration of 500 μM. After a 90 minute incubation, the samples were photolyzed for 15 minutes on ice using a 365 nm lamp (Spectroline EN-16). Membranes were pelleted (15000xg) for 30 minutes, resuspended in sample buffer, and submitted to SDS-PAGE.

Gel Electrophoresis

Gel electrophoresis was carried out as described in Chapter 3.

Proteolytic digestion

For EndoLysC digestion, acetone precipitated peptides were resuspended in 15 mM Tris, pH 8.1, 0.1% SDS. EndoLysC (1.5 mU in resuspension buffer) was added to a final volume of 100 μl. The digestion was allowed to proceed for 7-9 days before separation of fragments by Tricine SDS-PAGE (Schagger and von Jagow, 1987). For trypsin digestion, acetone precipitated peptides were resuspended in a small volume (40 μl) of 100 mM NH_4CO_3, 0.1% SDS, pH 7.8. Genapol C-100 and trypsin were added, with a final concentration of 0.02% SDS, 0.5% Genapol C-100, and 1:1 (w/w) trypsin. The digestion was allowed to proceed 3-4 days at room temperature prior to separation of the fragments by Tricine SDS-PAGE. Following Tricine SDS-PAGE, bands were excised based on fluorescence and molecular weight, and fragments were isolated as above. Pellets were resuspended in either 40 μl (for sequencing directly) or 200 μl (for HPLC purification) 15 mM Tris pH 8.1, 0.1% SDS.

HPLC purification

Proteolytic fragments from enzymatic digestion of [^{3}H]progestin aryl azide labeled subunits were further purified by reverse-phase HPLC, as described by Blanton and Cohen (1992), using a Brownlee C4 Aquapore column (100 x 2.1 mm; 7 um particle size). Solvent A was 0.08% TFA in water, and solvent B was 0.05% TFA in 60%

acetonitrile/40% 2-propanol. A nonlinear gradient (Waters Model 680 gradient controller, curve No. 7) from 25% to 100% solvent B in 80 minutes was used. The rate of flow was 0.2 ml/min, and 0.5 ml fractions were collected. The elution of peptides was monitored by absorbance at 215 nm, and the fluorescence from 1-AP was detected by fluorescence emission (357 nm excitation, 432 nm emission). Additionally, 50 μL aliquots from the fractions were taken to determine the distribution of ^{3}H by liquid scintillation counting.

Sequence Analysis

Automated amino terminal sequence analysis was performed on an Applied Biosystems Model 477A protein sequencer with an in-line 120A PTH analyzer. When multiple HPLC fractions were combined, solvent was removed by vacuum centrifugation, and samples were resuspended in 40 μl 15 mM Tris pH 8.1, 0.1% SDS. The SDS present in the storage buffer was removed using a pre-wash step. The pre-wash step consisted of a four minute treatment with gas-phase TFA followed by a five minute wash with ethyl acetate (this pre-wash step was not used for samples labeled with [^{3}H]ethidium diazide (Chapter 2) or [^{3}H]3-azioctanol (Chapter 3)). When single HPLC samples (450 μl per fraction) were sequenced, they were directly loaded onto chemically modified glass fiber disks (Beckman) in 20 μl aliquots, allowing the solvent to evaporate at 40°C between loads. Sequencing was performed using gas-phase TFA to minimize possible hydrolysis. After conversion of the released amino acids to PTH amino acids, the suspension was divided into two parts. One portion, approximately one-third, went to the PTH analyzer while the remaining two-thirds was collected for scintillation counting. Yield of PTH amino acids was calculated from peak height compared with standards using the program Model 610A Data Analysis Program Version 1.2.1. Initial yield and repetitive yield were calculated by a nonlinear least squares regression to the equation $M=I_0*R^n$, where M is

the observed release, I_0 is the initial yield, R is the repetitive yield, and n is the cycle number. Derivatives known to have poor recovery (Ser, Arg, Cys, and His) were omitted from the fit.

RESULTS

Photoincorporation of [^{3}H]progestin aryl azide into nAChR-rich membranes

Initial experiments were designed to characterize the general pattern of [^{3}H]progestin aryl azide photoincorporation as well as to test the sensitivity of the incorporation to various drugs. Membranes (2 mg of protein/mL) were equilibrated with 0.4 μM [^{3}H]progestin aryl azide in the presence and absence of several drugs. After irradiation for 2 minutes at 254 nm, the pattern of incorporation was assessed by SDS-PAGE followed by fluorography. As is evident in the fluorograph of the 8% polyacrylamide gel (Figure 4-2), [^{3}H]progestin aryl azide photoincorporated into all four subunits of the nAChR as well as into the α-subunit of Na^+/K^+ ATPase. Incorporation was greatest in the α and γ subunits and was present to a lesser extent in β and δ. The presence of carbamylcholine had no effect on the incorporation. Additionally, the presence or absence of 10 μM or 100 μM progesterone was without effect. Proadifen, an aromatic amine nAChR NCA, at 100 μM also showed no inhibition of [^{3}H]progestin aryl azide incorporation. 100 μM Tetracaine appeared to have reduced the incorporation in all polypeptide bands, though that effect is most likely due to the UV absorbance of tetracaine itself. Based upon scintillation counting of excised gel bands, ~30 cpm/pmol was incorporated in the α subunit. This incorporation, with ~0.3% of the α-subunits labeled, is similar to the levels of labeling by promegestone (~0.1% of α-subunits labeled) at similar concentrations (Blanton, 1999).

Mapping the [^{3}H]progestin aryl azide incorporation in nAChR α subunit with V8 protease

The distribution of [^{3}H]3-azioctanol incorporation within the α-subunit was examined by digestion of the labeled subunit with *S. aureus* V8 protease V8 protease cleavage in the gel generates 4 large fragments, αV8-20, αV8-18, αV8-10, and αV8-4, named according to their apparent molecular weights (Pedersen *et al.*, 1986). αV8-20 (Ser-173–Glu-338) contains the αM1, αM2 and αM3 transmembrane segments, as well as a portion of the N-terminal extracellular segment. The αM4 transmembrane segment is within αV8-10 (Asn-339–Gly-437). αV8-18 (Val-46–Glu-172) contains a glycosylation sensitive to EndoglycosidaseH, and when membranes are treated with EndoglycosidaseH, the deglycosylated fragment now runs at ~12 kD (αV8-12). When the incorporation of [^{3}H]progestin aryl azide was mapped using *S. aureus* V8 protease, incorporation was primarily within the αV8-10 fragment, as seen in the fluorogram (Figure 4-3). Based upon liquid scintillation counting of excised gel bands, in the absence of other drugs, ~75% of the incorporated ^{3}H was in αV8-10, ~10% in αV8-20, and ~6% in αV8-18.

Localization of [^{3}H]progestin aryl azide incorporation within αV8-10

To further characterize the incorporation of [^{3}H]progestin aryl azide in the αV8-10 fragment, 10 mg of nAChR membranes were labeled with [^{3}H]progestin aryl azide in the presence of 1 mM GSSG. These membranes were also labeled with 1-azidopyrene (1-AP) (Blanton and Cohen, 1992) for ease of identifying subunits and fragments following SDS-PAGE, as described in Methods. Following separation of subunits by SDS-PAGE, the α-subunit was transferred to the well of a mapping gel for digestion with V8 protease. Proteolytic fragments were identified after electrophoresis by illumination

at 365 nm to detect 1-AP incorporation. αV8-10 was identified by fluorescence and mobility, excised, eluted, and concentrated.

αV8-10 labeled with [^{3}H]progestin aryl azide was digested with trypsin, and the fragments were separated by HPLC (Figure 4-4A). A peak of ^{3}H eluted between fractions 34-37 (~84% organic), with little ^{3}H in the flow through, indicating that [^{3}H]progestin aryl azide incorporation in αM4 is stable to HPLC conditions. A tryptic fragment of αV8-10 beginning at αTyr-401 which was labeled with [^{3}H]promegestone was reported to have eluted at ~82% organic (Blanton *et al.*, 1999). Fractions 34-36 were pooled, dried, resuspended in 40 µl buffer, and subjected to sequence analysis. Since SDS was present in the resuspension buffer, the filter was pre-treated with TFA and washed with ethyl acetate to remove SDS. The ethyl acetate wash was assayed for ^{3}H, and contained ~20% of the ^{3}H originally present in the 3 HPLC fractions. Sequence analysis of these fractions (Figure 4-4B) following pooling, drying, and resuspension showed the presence of three sequences, beginning at αTyr-401 (I_o=27 pmol), at the N-terminus of αM4; αSer-388 (I_o=25 pmol), which also contained the αM4 segment; and αMet-243 (I_o=10 pmol), which is the N-terminus of αM2. The presence of the sequence beginning at αMet-243 was due to accidental contamination of the αV8-10 sample with αV8-20. While low levels of release appeared consistent through triplicate counting of cycles 17 and 23, ~10 cpm above background, no release was evident when similar fractions from a separate labeling experiment were sequenced. This second experiment contained only two sequences, one beginning at αTyr-401 (15 pmol) and one beginning at αSer-388 (20 pmol). However, the sequence which showed low levels of ^{3}H in cycle 17 and 23 of release had ~2-fold more mass of αTyr-401, and additionally there was more ^{3}H within the fractions pooled (~6000 cpm vs. ~3500 cpm).

Localization of [^{3}H]progestin aryl azide incorporation in the nAChR β subunit

A ~5 kD proteolytic fragment of β which contains the βM4 segment can be generated by a trypsin digestion of the β-subunit (Blanton *et al.*, 1998a). β-subunit labeled with [^{3}H]progestin aryl azide was therefore digested with trypsin, and the fragments were separated by Tricine SDS-PAGE. After elution, the fragments were either sequenced directly, as was done with those from the first labeling experiment, or further separated by HPLC before sequence analysis, in the second labeling. When samples were sequenced directly following gel elution, the band spanning ~5-7 kD contained four fragments containing βM4 (total I_o=167 pmol) as well as a sequence beginning at βSer-126 (I_o=44 pmol). No ^{3}H release above background was seen, though only 2000 cpm was loaded on the filter. Since these samples contained SDS, pretreatment of the sequencing filter with TFA was necessary prior to sequencing. During this treatment, ~15% of the loaded ^{3}H was removed.

As an alternative approach, from a separate labeling experiment, the tryptic digest of β-subunit was fractionated by Tricine SDS-PAGE, and then the eluted fragments were further purified by HPLC. Upon HPLC purification of the band containing fragments of ~4-8 kD (band 2), ~50% of the ^{3}H eluted in the flow-through (Figure 4-5), indicating instability of incorporation to the conditions of HPLC. Hydrophobic fractions which contained ^{3}H were loaded directly onto sequencing filters, avoiding the pre-wash step, and subjected to Edman degradation. The results are summarized in Table 4-1. Sequence analysis of fraction 31 (~78% organic), containing ~540 cpm, revealed the presence of two fragments, one beginning at βLys-216 (15 pmol), the N-terminus of the βM1 segment, and one beginning at βAsp-427 (7 pmol), the N-terminus of the βM4 segment. No ^{3}H release above background was evident in 20 cycles, with the ^{3}H in the first cycle ~20 cpm (not shown). Sequence analysis of fraction 34 (~93% organic), containing ~560

cpm, revealed the presence of a fragment beginning at βMet-249 (18 pmol), the N-terminus of the βM2 segment. When Band 1 was purified by HPLC, ~30% of the ^{3}H was present in the flow-through. Fractions 22, 25, 26, and 28 were sequenced (summarized in Table 4-1) and showed sequences attributable to both the nAChR β subunit and several contaminating fragments (from nAChR δ-subunit as well as the β-subunit of Na^{+}/K^{+} ATPase).

Localization of [^{3}H]progestin aryl azide incorporation in the nAChR γ-subunit

A ~5 kD peptide containing γM4 can be generated by digestion of the γ-subunit with trypsin (Blanton *et al.*, 1999). γ-subunit labeled with [^{3}H]progestin aryl azide was therefore digested with trypsin, and the fragments were separated by Tricine SDS-PAGE. Two bands containing ^{3}H were excised from the Tricine gel based upon molecular weight. Upon HPLC purification of the band containing fragments of ~3-6 kD (band 2), ~50% of the ^{3}H eluted in the flow-through (Figure 4-6A), indicating instability of incorporation to the conditions of HPLC. Hydrophobic fractions which contained ^{3}H were loaded directly onto sequencing filters, avoiding the pre-wash step, and subjected to Edman degradation. The results are summarized in Table 4-1. Sequence analysis of fraction 31 (~78% organic), containing ~610 cpm, revealed the presence of two fragments, one beginning at γVal-466 (7 pmol), the N-terminus of the γM4 segment, and one beginning at γLys-218 (2 pmol), the N-terminus of the γM1 segment. No ^{3}H release above background was evident in 20 cycles. When Band 1, which contained fragments ~1-3 kD, was purified by HPLC, ~50% of the ^{3}H eluted in the flow-through (Figure 4-6B). Fractions 25 and 26 were sequenced (summarized in Table 4-1), and contamination by δ-subunit was evident. Again, no ^{3}H release above background was evident from either of these sequences.

Localization of [^{3}H]progestin aryl azide incorporation in the nAChR δ-subunit

EndoLysC digest of δ-subunit generates a fragment beginning at δMet-257, the N-terminus of δM2, which runs on a Tricine gel between 6 and 10 kD (Gallagher and Cohen, 1999). δ-subunit labeled with [^{3}H]progestin aryl azide was therefore digested with EndoLysC, and the fragments were separated by Tricine SDS-PAGE. A band containing fragments of ~3-10 kD was excised, and the eluted fragments were further purified by reverse-phase HPLC (Figure 4-7). Approximately ~20% of the ^{3}H eluted in the flow-through. Hydrophobic fractions which contained ^{3}H were loaded directly onto sequencing filters, avoiding the pre-wash step, and subjected to Edman degradation. The results are summarized in Table 4-1. Sequence analysis of fraction 31 (~80% organic), containing ~470 cpm, showed the presence of only one sequence, that beginning at δMet-257 (17 pmol), the N-terminus of δM2. Fraction 28 (~66% organic), containing ~410 cpm, contained two sequences, one beginning at δSer-421 (15 pmol) and one beginning at δAsn-437 (11 pmol), both of which contain the δM4 segment. Sequence analysis of fraction 26, containing ~600 cpm, showed the presence of two sequences, one beginning at δPhe-206 (88 pmol) and one beginning at δAsn-200 (102 pmol), both of which should contain the δM1 segment. The separation of these fragments showed the high resolution of the reverse-phase HPLC conditions for these hydrophobic fragments. These fractions were only sequenced for 6 cycles, since no release was expected from such low levels of loaded ^{3}H, and ~20 cpm was present in the first cycle of each.

DISCUSSION

[^{3}H]Progestin aryl azide photoincorporated into all four subunits of the nAChR. Within the α subunit, ~75% of the incorporation was within a fragment containing αM4. Incorporation in the other subunits was not clearly mapped, although there was evidence

for incorporation in the M4 segments, as well as possible incorporation in other transmembrane segments.

The incorporation of [^{3}H]progestin aryl azide at the subunit level was not altered by any of the cholinergic drugs tested. Inhibition at a specific site, however, could have been masked by nonspecific incorporation elsewhere. For example, if the site were in the αV8-20 fragment, which contained only ~10% of the incorporation, a decrease in incorporation would have been undetectable in the intact subunit. An apparent decrease in the incorporation in αV8-20 was observed in the presence of carbamylcholine, with an increase in the incorporation in αV8-18, ~2-fold, though this study was performed only once (Figure 4-3). Further information on the sites of incorporation in these fragments is lacking.

The incorporation in αV8-10 was within the αM4 segment. This incorporation was stable to the conditions of HPLC, and, after trypsin digestion of αV8-10, radioactivity was revealed to be within a hydrophobic fragment beginning at the N-terminus of αM4. In one experiment sequence analysis of this fragment resulted in very low levels of release in cycles 17 and 23, approximately 10 cpm, although this release was not seen in a second experiment which, unfortunately, contained lower levels of mass and radioactivity. Unlike [^{3}H]progestin aryl azide, the sequence analysis of this same fragment labeled with [^{3}H]promegestone showed ~300 cpm release in cycle 13, αCys-412 (Blanton, 1999). However, 129 pmol of the sequence beginning at αTyr-401 was present, and 44000 cpm was loaded. Here, 6000 cpm were present in the HPLC fractions which were pooled, and two sequences containing αM4 were present, one beginning at αTyr-401 (27 pmol) and one beginning at Ser-388 (25 pmol). Therefore, under the conditions seen here, the release from the sequence beginning at αTyr-401 labeled by [^{3}H]promegestone would have only showed ~25 cpm of release. This level of release

would have been detectable had [^{3}H]progestin aryl azide photoincorporated into αCys-412. Additionally, the release from the fragment labeled by [^{3}H]progestin aryl azide was in cycles 17 and 23, which, due to loss in the repetitive yield, would have even lower levels of release if labeled at similar levels as αCys-412 was by [^{3}H]promegestone. Although ~20% of the radioactivity loaded on the sequencer filter was released during prewash, perhaps due to loss from a single site, the lack of release in cycle 13, and the presence in cycles 17 and 23, may indicate that the steroids bind in a single orientation and that the photoreactive group on ring A may be able to access αCys-412 while the photoreactive group on ring D only accesses residues closer to the center of the lipid bilayer.

Within the other subunits, incorporation within the M4 segments is also likely. Sequence analysis of HPLC fractions showed the presence of fragments containing the M4 segments within fractions containing ^{3}H. However, in the case of β and γM4 segments, other fragments were also present, and no release of ^{3}H was seen. Additionally, unlike the incorporation of [^{3}H]progestin aryl azide in αM4, the incorporation in the β-, γ- and δ-subunits was unstable to the conditions of HPLC. Aryl azides are known to rearrange to ketenimine azepines, and photoadducts formed with this group are expected to be labile under acidic conditions, like those used for HPLC. Additional evidence of instability to acid came from the loss of ^{3}H during the pre-wash of the sequencing filter.

In addition to incorporation in the M4 segments, it is likely that there was incorporation within other transmembrane segments of β and δ. In HPLC fraction 26 from band 1 of β, the only nAChR sequence present was δM2. The size of this fragment, ~2-4 kD, indicated that this fragment contained the δM2 segment without the δM3 segment. Additionally, a fragment containing the δM2 and δM3 segments was the only

sequence present in fraction 31 from the HPLC purification of band 2 of δ. Similarly, fraction 34 from the HPLC of β band 2 showed only the sequence beginning at βMet-249, the N-terminus of βM2, and this fragment should contain both the βM2 and βM3 segments, based on the molecular weight. If the ^{3}H in these HPLC fractions were attributable to these fragments containing the M1 and M2 sequences, then they should contain similar levels of incorporation to the β and δM4 segments.

The loss of ^{3}H during HPLC and pre-wash treatment and the low mass levels precluded the identification of residues into which [^{3}H]progestin aryl azide might have photoincorporated. In order to identify particular amino acids labeled by [^{3}H]progestin aryl azide, further studies would require labeling at higher concentrations of [^{3}H]progestin aryl azide, and identification would depend upon efficient isolation of labeled fragments. Alternatively, studies with another steroid containing a photoactivatible group which photoincorporates into the nAChR with higher efficiency and forms bonds upon photoincorporation which are more stable to the conditions of HPLC and sequencing, such as a diazirine or benzophenone, could help define the site of action of neurosteroids on the nAChR.