# Chapter 2 Identification of the Sites of Incorporation of [$^3$H]Ethidium Diazide in the nAChR


## ABSTRACT

The binding sites of ethidium, a noncompetitive antagonist of the nAChR, have been localized in the *Torpedo* nAChR in the desensitized state by use of a photoactivatible derivative, [$^3$H]ethidium diazide. At 10 μM [$^3$H]ethidium diazide, the incorporation into the α, β, and δ subunit was inhibited by the presence of PCP. Within the α-subunit, the incorporation was mapped to a 20 kD fragment beginning at Ser-173 and containing the first three transmembrane segments, αM1, αM2, and αM3. Further digestion of this fragment generated two fragments with PCP-inhibitable incorporation, one containing αM1 and one containing both αM2 and αM3. Within αM2, specific incorporation was present in αLeu-251 and αSer-252, residues that have been previously shown to line the lumen of the ion channel. Digestion of δ-subunit with *S. aureus* V8 protease generated a 14 kD and a 20 kD fragment, both of which began at Ile-192 and contained PCP-inhibitable labeling. The 14 kD fragment, containing δM1 and δM2, was further digested to generate a 3 kD fragment, containing δM2 alone, with PCP-inhibitable incorporation. Digestion of the 20 kD fragment, which contained δM1, δM2, and δM3, generated two fragments with incorporation, one containing the δM1 segment, and the other containing δM2 and δM3. These results establish that in the desensitized state of the nAChR, the high affinity binding site of ethidium is within the lumen of the ion channel, and that the bound drug is in contact with amino acids from both the M1 and M2 hydrophobic segments.

## RESULTS

### Equilibrium binding of [$^3$H]ethidium to nAChR-rich membranes

To characterize the reversible interactions of ethidium with the nAChR, equilibrium binding of [$^3$H]ethidium to *Torpedo* nAChR-rich membranes was measured in the presence of cholinergic agonists, competitive antagonists, and noncompetitive antagonists. To quantify binding in the desensitized state, [$^3$H]ethidium binding was measured in the presence of 2 mM carbamylcholine in the presence and absence of 100 μM PCP (Figure 2-1A). The binding in the desensitized state was characterized by binding to a high affinity site (Keq ~0.6 μM) as well as a nonspecific component defined by the binding in the presence of PCP, a line with a slope of ~0.08. This high affinity binding was similar to literature values, measured both by the inhibition of [$^3$H]PCP binding (Keq ~0.4-0.6 μM (Herz *et al.*, 1987; Pedersen, 1995)) as well as by the fluorescence of bound ethidium (Keq ~0.3-0.8 μM (Herz *et al.*, 1987; Lurtz *et al.*, 1997)). In the presence of d-tubocurare, [$^3$H]ethidium was bound with a similar high affinity to the same number of sites. There was no evidence of high affinity [$^3$H]ethidium binding in the presence of αBgTx, as the binding in the presence of αBgTx was approximately linear with a slope of ~0.09, similar to the slope in the presence of carbamylcholine and PCP. Since high affinity binding of [$^3$H]ethidium was seen when the agonist site was occupied either by agonist or competitive antagonist, [$^3$H]ethidium must bind to a site distinct from the ACh site. The binding at this site was inhibited by the presence of PCP, indicating that this site was a noncompetitive antagonist site.

[$^3$H]Ethidium bound with an apparent lower affinity and to a greater number of sites in the absence of carbamylcholine than in the presence of carbamylcholine (Figure 2-1B). The binding of [$^3$H]ethidium in the absence of other drugs is expected to be

complex since [$^3$H]ethidium was expected to bind both the two agonist sites as well as the NCA site. The reported affinity for the α-γ agonist site is ~5 μM (Pedersen, 1995), while it is ~100 μM for the α-δ agonist site. Based on the binding reported above, [$^3$H]ethidium binds with low affinity in the resting state. Since [$^3$H]ethidium bound with high affinity in the presence of dTC, which shifts the nAChR state equilibrium toward the desensitized state even when bound to just the α-γ site, the high affinity binding of [$^3$H]ethidium to the NCA site should occur following binding of [$^3$H]ethidium to the α-γ site. Therefore, the binding of [$^3$H]ethidium at the low concentrations was modeled as two-interdependent sites using Equation 2 (see Methods). This model assumed an equal number of available binding sites for the two sites but that the binding to a second site only occurs following binding to the first site. When the binding of [$^3$H]ethidium at low concentrations was fit to the two-interdependent-sites model, the measured $K_{eq}$s were 24 ± 8 μM and 4.2 ±2.3 μM. [$^3$H]Ethidium, therefore is predicted to bind the α-γ site with a $K_{eq1}$ of 24 μM (compared to the reported value of ~5 μM), which would then lead to nAChR desensitization. Once the nAChR was desensitized, [$^3$H]ethidium would bind the NCA site with a $K_{eq2}$ of 5 μM. The number of binding sites for each site was ~280 nM, near that predicted by the binding to the high affinity site in the presence of carbamylcholine ($B_{max}$=214 nM). When the data was fit to a single site model, the $K_{eq}$ was 100 ± 92 μM, higher than expected for binding to at least the first agonist site, and the $B_{max}$ was ~2800 nM, not a realistic prediction. If $B_{max}$ was set to 200 μM, near that predicted by the binding in the presence of carbamylcholine, the $K_{eq}$ was 1.7±2.0e-6. However, the curve was not well fit to the data (Figure 2-1B, dashed line). Alternatively, if the binding was expected to be a two independent sites with different affinities (Equation 3), when $B_{max}$ was set to 200 μM, the two $K_{eq}$s were not similar to those expected from literature values ($K_{eq1}$=7.8±0.2 mM, $K_{eq2}$=8.7±0.9 M), while the curve appeared to fit the data well (not

shown). Shown in Figure 2-1C are the binding curves calculated without the nonspecific binding, including the single and two-interdependent site model fits for the binding in the absence of other drugs. Although these two lines appear similar, the calculated values for $B_{max}$ and $K_{eq}$ for the single site model are not physically realistic.

**Photoincorporation of [$^3$H]ethidium diazide into nAChR-rich membranes**

Initial photoincorporation experiments were designed to determine the general aspects of [$^3$H]ethidium photoincorporation as well as the effects of agonists and NCAs on the incorporation. nAChR-rich membranes (2 mg/ml) were equilibrated with 10 μM [$^3$H]ethidium diazide in the presence and absence of 2 mM carbamylcholine or 100 μM PCP. These experiments were carried out in the presence of 10 mM oxidized glutathione (GSSG) as a scavenger (Gallagher and Cohen, 1999). Initially, incorporation of $^3$H was determined following SDS-PAGE by fluorography. In the presence of carbamylcholine, [$^3$H]ethidium diazide incorporated into all subunits of the nAChR as well as into rapsyn, a 43 kD protein associated with the cytoplasmic aspect of the nAChR, and the α-subunit of $Na^+/K^+$ ATPase, a 90 kD polypeptide from a contaminating membrane fraction (Figure 2-2). The incorporation in the α, β, and δ subunits was reduced in nAChR equilibrated with PCP.

The effect of oxidized glutathione (GSSG) as a scavenger was tested over a range of concentrations. In the presence of carbamylcholine, increasing GSSG concentrations reduced the incorporation in all polypeptides, both in the presence and absence of PCP (Figure 2-3). The incorporation in non-nAChR polypeptides, those running at 37 and 90 kD (corresponding to calectrin and the α-subunit of $Na^+/K^+$ ATPase, respectively), was similar in the two conditions at all concentrations of GSSG. The absolute amount of PCP-inhibitable labeling in the α, β, and δ subunits was constant across the range of

GSSG conditions tested, indicating that GSSG reduced the nonspecific, but not the specific, incorporation into the polypeptides.

In further experiments in the presence of 10 mM GSSG, the effects of carbamylcholine and PCP as well as several other drugs were studied by quantification of $^{3}H$ from bands excised from a gel (Figure 2-4). Bands containing the nAChR subunits, as well as non-nAChR polypeptides were excised. The presence of cholinergic agonists and antagonists did not affect the incorporation into two bands containing non-nAChR polypeptides, a band at 43 kD, containing the protein rapsyn, as well as one at 58 kD, which contained syntrophin, a protein of the dystrophin complex (Carr *et al.*, 1989). For membranes equilibrated with carbamylcholine, the incorporation of [$^{3}H$]ethidium diazide in the $\alpha$ and $\delta$ subunits was reduced by 40% by the presence of PCP, while in the $\beta$-subunit, the incorporation was reduced 20%. As was seen for [$^{3}H$]ethidium binding to *Torpedo* nAChR membranes, nAChR subunit labeling by [$^{3}H$]ethidium diazide in the presence of $\alpha$BgTx was similar to that in the presence of carbamylcholine and PCP in all subunits. The incorporation in the presence of dTC was similar to that seen with carbamylcholine, consistent with the binding affinity of [$^{3}H$]ethidium in the presence of these drugs. For the nAChR $\beta$ and $\delta$ subunits, the incorporation in the absence of other drugs was less than that in the presence of carbamylcholine, while the incorporation in $\alpha$ was similar in both conditions. Based upon the total pmols of nAChR loaded in each gel lane and the observed $^{3}H$ in the subunit gel slice, the total $^{3}H$ incorporation in $\alpha$ and $\delta$ in the presence of carbamylcholine was approximately 0.04 mol [$^{3}H$]ethidium diazide/mol subunit, while the incorporation in the presence of both carbamylcholine and PCP was approximately 0.02 mol/mol.

**Mapping the [$^3$H]ethidium diazide incorporation in nAChR α-subunit with *S. aureus* V8 protease**

To further characterize the site of incorporation, the α-subunit was subjected to proteolysis with *S. aureus* V8 protease in a mapping gel (Figure 2-5). 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–αGly-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). The autoradiogram shows that the major site of incorporation was in the αV8-20 fragment, and that incorporation was inhibited by PCP. Based on counting excised gel fragments, ~75% of the $^3$H incorporated into the α-subunit labeled in the absence of PCP was in αV8-20, and that incorporation was reduced by ~60% in fragments from nAChR equilibrated with PCP.

**Localization of [$^3$H]ethidium diazide incorporation into the α-subunit**

To further characterize the incorporation in the α-subunit, 10 mg of nAChR membranes were labeled in the presence of 10 mM GSSG and 2 mM carbamylcholine and the absence (denoted as +/-) or presence (+/+) of PCP. These membranes were also labeled with 1-azidopyrene (1-AP) (Blanton and Cohen, 1994) 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. αV8-20 was identified by fluorescence and mobility, excised, eluted, and concentrated.

In order to localize the incorporation of [$^3$H]ethidium diazide in αV8-20, αV8-20 was further digested with EndoLysC, which is known to generate a ~10 kD fragment which begins at αMet-243, the N-terminus of the αM2 segment, and presumed to continue through αM3 (Pedersen *et al.*, 1992). There are only three other lysines in αV8-20, two lysines near the beginning of αV8-20 (αLys-185 and αLys-179) and one prior to αM3 (αLys-276). No cleavage has been reported at αLys-276 (Pedersen *et al.*, 1992; Gallagher and Cohen, 1999), and Figure 2-6A shows the expected cleavage products. When the digest was fractionated by HPLC, two hydrophobic peaks, one centered at fraction 29 (~69% organic) and one at fraction 34 (~93% organic), were present (Figure 2-6B). Additional $^3$H was not retained on the column, accounting for ~20% of the total $^3$H eluted from the column. Since similar levels of $^3$H were present in the flow-through when intact αV8-20 was purified by HPLC (Figure 2-6B, inset), this $^3$H was not associated with a hydrophilic digestion fragment, but rather resulted from covalent adducts which were unstable to HPLC conditions or $^3$H noncovalently associated with αV8-20.

Sequence analysis of fraction 34 of the EndoLysC digest of αV8-20 labeled both in the absence and presence of PCP showed a single sequence beginning at αMet-243 (+/-: $I_0$=109 pmol; +/+: $I_0$=159 pmol) (Figure 2-7A). No other sequences were detected with more than 5% of the mass of that sequence. Based on the loaded $^3$H and the mass present, ~40 cpm were incorporated per pmol fragment labeled with [$^3$H]ethidium diazide in the absence of PCP. This incorporation was reduced by ~50% for samples labeled in the presence of PCP. While only low levels of $^3$H release were seen, the fragment labeled

in the absence of PCP showed $^{3}H$ release reproducibly in cycles 9 and 10, corresponding to αLeu-251 and αSer-252. Additionally, release in cycle 15 was seen, though not reproducibly (compare upper and lower panels of Figure 2-7A). [$^{3}H$]Ethidium diazide incorporated into αLeu-251 at 0.5 cpm/pmol (~0.0009 mol [$^{3}H$]ethidium diazide/mol αLeu-251). $^{3}H$ was also released in the first cycles, accounting for ~2% of the loaded $^{3}H$; this release was likely due either to the removal of peptide poorly absorbed onto the glass filter or instability of the covalent adduct to sequencing conditions and was similar to that seen during sequence analysis of fragments photolabeled by a different probe (Blanton *et al.*, 1999).

Sequence analysis of fraction 29 (Figure 2-7B) revealed the presence of two α-subunit fragments, one beginning at αHis-186 (+/-: $I_0$=128 pmol; +/+: $I_0$=208 pmol), and a secondary sequence beginning at αAsp-180 (+/-: $I_0$=18 pmol; +/+: $I_0$=39 pmol). Both of these fragments must have contained the αM1 segment since there is no other lysine between αHis-186 and αLys-242, prior to αM2. No additional sequences with more than 5% of the mass of the primary sequence were seen. Based on the $^{3}H$ loaded and the mass levels of the two fragments, ~40 cpm of $^{3}H$/pmol was incorporated into the fragments in the absence of PCP. This incorporation was reduced by ~60% by the presence of PCP during photolysis. Sequence analysis of this fraction from both labeling conditions showed no release of $^{3}H$ after 25 cycles of Edman degradation, except for the progressively declining release in the first three cycles. The region sequenced contained part of the ACh site (α190-200) but ends prior to M1. The lack of release could either be due to instability of the covalent adduct or labeling at a residue further than 25 amino acids from the N-terminus. For example, if the incorporation were in the αM1 segment, which begins at α211, 25 residues from the N-terminus of this fragment, no release would have been seen.

**Localization of [$^3$H]ethidium diazide incorporation into the δ-subunit**

In gel digestion of nAChR δ-subunit with V8 protease is known to create two fragments with N-termini of δIle-192. One fragment, of approximately 20 kD (δV8-20), contains the δM1, δM2, and δM3 segments (Blanton and Cohen, 1994). The second fragment, of ~14 kD (δV8-14), ends at δGlu-262 (Blanton *et al.*, 1994) and, therefore, contains the δM1 and δM2 segments, but not δM3. When the sites of [$^3$H]ethidium diazide incorporation in the δ-subunit were mapped on an analytical scale by in gel digestion with V8 protease, PCP inhibitable labeling was seen in bands centered at ~ 20 kD and ~12 kD (Figure 2-8A).

To identify the sites of incorporation of [$^3$H]ethidium diazide in the nAChR δ-subunit, 10 mg of nAChR-rich membranes were photolabeled with [$^3$H]ethidium diazide in the absence or presence of PCP followed by labeling with 1-AP. The subunits were separated by SDS-PAGE, and the δ-subunit was excised and digested with *S. aureus* V8 protease in a mapping gel. After digestion of δ-subunit by V8 protease in the mapping gel, the mobility of digestion products was identified by 1-AP fluorescence. Two fluorescent bands, with estimated mobility near 14 kD (δV8-14) and 20 kD (δV8-20) were excised, eluted, and resuspended, as were other fluorescent bands and non-fluorescent regions of the gel. The two noted fluorescent bands contained ~20% and ~30% of the total eluted $^3$H, respectively, as well as the greatest PCP-inhibitable incorporation. Aliquots from these two bands were run on a mapping gel without V8 protease to clearly determine the molecular weight (Figure 2-8B). While all lanes contained similar $^3$H in the dye front, the specifically labeled material from the δV8-14 band ran near 14 kD and that from the δV8-20 band ran near 20 kD. The material in these two bands was used in subsequent digestions.

Digestion of nAChR δ-subunit with EndoLysC generates a 10 kD fragment beginning at δMet-257, the N-terminus of M2, and containing the M3 segment, with no cleavage observed after δLys-290, at the N-terminus of M3 (Gallagher and Cohen, 1999). We used EndoLysC digestion of δV8-14 and δV8-20 to map the site(s) of [$^3$H]ethidium diazide incorporation into the δ-subunit. Within δV8-14 there are two sites of possible EndoLysC cleavage, δLys-205 and δLys-256. An additional lysine, at δLys-224, precedes a proline, so no cleavage is expected at this site. Therefore, three fragments can be generated by EndoLysC digestion of δV8-14 (Figure 2-9A), one beginning at δIle-192, with a mobility of ~1.5 kD; one beginning at δPhe-206, containing a glycosylation site, with a mobility of ~10 kD; and one beginning at δMet-257 and continuing through δGlu-280, with a mobility of ~3 kD. Sequence analysis of aliquots of the digest of δV8-14 labeled with [$^3$H]ethidium diazide revealed the presence of two fragments, one beginning at δMet-257 (+/-: $I_0$=3 pmol; +/+: $I_0$=14 pmol) and one beginning at δPhe-206 (+/-: $I_0$=15 pmol; +/+: $I_0$=9 pmol). Additionally, a fragment beginning at δIle-192 was expected but was not visible, possibly from loss during sequencing due to the small size of the fragment. The total digest, as well as undigested δV8-14, was separated by a Tricine SDS-PAGE gel. As shown in Figure 2-9B, for the digest of δV8-14 labeled in the absence of PCP, the primary peak of $^3$H was present in a band at ~3 kD, and this band contained PCP-inhibitable incorporation. The presence of PCP-inhibitable incorporation in the ~3 kD band indicated that [$^3$H]ethidium diazide photoincorporated into the δM2 segment in a PCP-dependent manner. In addition, the broad band of $^3$H between 10 kD and 14 kD, also containing PCP-inhibitable incorporation, is shifted relative to undigested δV8-14 (lower panel) consistent with the presence of the ~10 kD fragment beginning at δPhe-206 and ending prior to δM2, as well as possible partially digested products.

Digestion of δV8-20 with EndoLysC is expected to generate 3 fragments, with N-termini similar to those generated by digestion of δV8-14 with EndoLysC. Cleavage of the lysine at position δLys-290, between the δM2 and δM3 segments, has not been reported. Therefore, the fragments generated should be similar to those created by EndoLysC digestion of δV8-14, with the fragment beginning at δMet-257 containing both the δM2 and δM3 segments (Figure 2-10A). The δV8-20 fragment labeled with [$^3$H]ethidium diazide was digested with EndoLysC and fractionated by HPLC. PCP-inhibitable incorporation was present in hydrophobic fractions centered at fraction 27 (62% organic) and fraction 31 (78% organic) as well as in the flow through. Since $^3$H was also present in the flow through when intact δV8-20 was repurified by HPLC (Figure 2-10, inset), as seen with αV8-20, that $^3$H probably corresponded to incorporation unstable to the acidic HPLC conditions, and accounted for ~20% of the eluted $^3$H.

Sequencing of fraction 31 from the HPLC of both the sample labeled in the absence of PCP as well as that labeled in the presence of PCP showed the presence of two sequences, one beginning at δMet-257 (+/-: $I_0$=19 pmol; +/+: $I_0$=6 pmol) as well as one beginning at δPhe-206 (+/-: $I_0$=27 pmol; +/+: $I_0$=15 pmol) (Figure 2-11). No $^3$H release was detected in 25 cycles of Edman degradation, other than the release in the first cycles, accounting for ~3% of the loaded $^3$H.

Sequence analysis of fraction 27 of the HPLC of EndoLysC-digested δV8-20 labeled with [$^3$H]ethidium diazide in the absence of PCP showed a primary sequence beginning at δPhe-206 ($I_0$=53 pmol) with a minor sequence beginning at δMet-257 ($I_0$=4 pmol), present at less than 10% the mass level of the sequence beginning at δPhe-206. As with fraction 31, no release of $^3$H above background was seen during 25 cycles of sequence analysis (not shown). Since undigested δV8-20 eluted in fraction 31, the sequence beginning at δPhe-206 in fraction 31 of the HPLC of EndoLysC-digested δV8-

20 should contain the M1, M2, and M3 segments. The sequence beginning at δPhe-206 in fraction 27, however, must have been cleaved at the Lys-256, prior to M2. While the $^{3}$H release profile provided no information about the site of [$^{3}$H]ethidium diazide incorporation in the labeled fragments, the presence of PCP-inhibitable incorporation in this HPLC peak indicated that specific incorporation was present within the fragment beginning at δPhe-206, containing M1 without M2.

## DISCUSSION

The data presented in this chapter localized the specific photoincorporation of [$^{3}$H]ethidium diazide in the nAChR α and δ subunit to both the M2 segment and a fragment containing M1. Previous studies of the incorporation of aromatic amine NCAs into the *Torpedo* nAChR in the desensitized state have shown incorporation into only the M2 segment (Giraudat *et al.*, 1986; Giraudat *et al.*, 1987; Revah *et al.*, 1990; Hucho *et al.*, 1986). Additionally, the results reported here have established that, within the α-subunit, αLeu-252 (αLeu-9') and αSer-253 (αSer-10'), specifically, were labeled by the [$^{3}$H]ethidium diazide. These residues line the lumen of the ion channel (Akabas *et al.*, 1994), demonstrating that the high affinity binding site of ethidium is within the nAChR ion channel.

The pharmacology of the photoincorporation of [$^{3}$H]ethidium diazide well reflects the pharmacology of binding, and was similar to that reported by Witzemann and Raftery (1978). Their data also indicated that, in the desensitized state, the photoincorporation in the α and δ subunits was inhibited by the presence of an aromatic amine noncompetitive antagonist. The dependence of subunit photoincorporation on cholinergic ligands reported here, however, was in apparent contrast to that reported by Pedersen (1995) using fluorescence to detect levels of incorporation in subunits after SDS-PAGE. In that

report, the incorporation of ethidium diazide in α and γ subunits was reduced by ~80% by the presence of carbamylcholine, while the addition of PCP produced little or no further change. A likely explanation for that result is that, surprisingly, in subunits resolved by SDS-PAGE the covalent adduct formed within the ACh binding site remains fluorescent, while the adduct formed in the channel in the α and δ subunits is nonfluorescent.

To localize the incorporation in the nAChR to specific fragments of the protein, the α and δ subunits were digested first with V8 protease to generate a fragment containing the first three transmembrane segments, and that fragment was then isolated and digested with EndoLysC. In both subunits, two fragments were generated that contained PCP-inhibitable incorporation, one spanning a portion of the extracellular domain through the M1 segment, the other containing the M2 and M3 domains. In the α-subunit, sequence analysis of the fragment containing M2 and M3 established PCP-inhibitable incorporation of [$^3$H]ethidium diazide in αLeu-252 (αLeu-9') and αSer-253 (αSer-10'). Thus, in the desensitized nAChR, the high affinity binding site of ethidium is contained at least in part within the M2 ion channel domain.

Since the site of [$^3$H]ethidium diazide incorporation in the δ fragment containing δM2 and δM3 was not evident by sequence analysis, the presence of incorporation into the δM2 fragment was determined by alternative means. The δV8-14 fragment, spanning from δIle-192 to δGlu-280, contained a portion of the extracellular segment as well as the δM1 and δM2 segments, but not the δM3 segment. Digestion of this fragment by EndoLysC was expected to generate a 3 kD fragment consisting solely of the δM2 segment. Separation of the digestion products on an SDS-PAGE gel resulted in a 3 kD band with associated $^3$H. Therefore, the M2 segment of δ-subunit was labeled with [$^3$H]ethidium diazide in a PCP-dependent manner. Unfortunately, the specific residues in δM2 labeled with [$^3$H]ethidium diazide could not be determined by sequence analysis.

Although many other NCAs which have incorporated into the M2 segments label homologous positions in several subunits (White and Cohen, 1992; Giraudat *et al.*, 1986; Giraudat *et al.*, 1987; Revah *et al.*, 1990; Hucho *et al.*, 1986; Gallagher and Cohen, 1999), sequence analysis failed to provide any evidence of reaction of [$^3$H]ethidium diazide with either δLeu-266 or δSer-267, the homologs to the positions labeled by [$^3$H]ethidium diazide in α. However, due to the low mass levels of the δ-subunit fragment observed during sequencing (19 pmol in fraction 31) (Figure 2-11), incorporation at δLeu-266 at similar levels to αLeu-252 would only have resulted in 6 cpm of release, well below the detection limits of this method.

αLeu-252 and αSer-253 are not necessarily the primary or only sites of labeling in αM2. Approximately 20% of the $^3$H incorporated into αV8-20 was not stably incorporated under the acidic HPLC conditions. Additionally, some of the $^3$H incorporation was labile to the acid treatment during sequence analysis, as seen by the $^3$H release detected in the first cycles of sequence analysis. Acid sensitivity was clearly demonstrated when samples were pretreated with TFA for 4 minutes, followed by a wash with ethyl acetate for 5 minutes to remove excess SDS from the filter prior to sequence analysis. During this treatment, between 10-50% of the $^3$H loaded with a fragment labeled with [$^3$H]ethidium diazide was released (this prewash was not used in any of the sequences reported here). While as much as 0.15 mol [$^3$H]ethidium diazide was incorporated/mol of sequence beginning at αMet-243, based on loaded $^3$H and the mass present, αLeu-252 was only labeled at ~0.0008 mol [$^3$H]ethidium diazide/mol residue. Therefore the discrepancy between the $^3$H incorporated in the fragment and that detected in αLeu-252 was due to either greater incorporation originally at αLeu-252 and αSer-253 or at another site, but this incorporation was labile to the conditions of sequence analysis. This discrepancy is even more evident with the δ-subunit. Although clear evidence of

incorporation of [$^3$H]ethidium diazide within the δM2 segment was shown, no $^3$H release was seen in any cycles of sequence analysis through this segment. The adducts formed between the [$^3$H]ethidium diazide and the δM2 residues, therefore, must have been labile to the HPLC and sequencing conditions.

This difficulty in localization of the incorporation in the subunits resulted from the sensitivity of the incorporation to the HPLC and sequencing conditions. This sensitivity could be due to the formation of acid-labile adducts upon photolysis. The photoactivatible group used in these experiments, the aryl azide, can undergo an intermolecular rearrangement after photolysis to a ketenimine azepine (Bayley, 1983). The adducts formed with the azepine are predicted to be sensitive to acid and may account for the loss of $^3$H during HPLC and sequencing.

Another problem with the rearrangement product of the aryl azide is that it is more stable than the initial nitrene. This compound is expected to react preferentially with nucleophiles, such as cysteine. This selective reactivity may pose a problem when the binding site contains no cysteines, as was the case for the M2 segments. However, [$^3$H]ethidium diazide successfully photoincorporated into the M2 segments of α and δ, and, particularly, into αLeu-252 and αSer-253.

The binding of ethidium within the M2 channel domain is inconsistent with the site of ethidium binding predicted for the *Torpedo* nAChR from the results of fluorescence resonance energy transfer (FRET). The results of FRET have been interpreted to indicate that, within the desensitized state, the high affinity ethidium binding site is ~50 Å from the lipid head groups, within the extracellular domain, well above the transmembrane domain of the nAChR. The discrepancy is likely to reflect the complexity of distance determination by FRET. In order to calculate distances using FRET, either the donor or acceptor molecules must be able to rotate freely (Clegg, 1995).

However, ethidium, when bound to the noncompetitive antagonist site, is likely to be highly constrained, as evidenced by the enhanced fluorescence lifetime of bound ethidium as well as the polarization values, showing a lack of rotational mobility (Herz *et al.*, 1987). Additionally, the lipid environment near the channel has restricted mobility (Marsh and Barrantes, 1978; Marsh *et al.*, 1981) and shows selectivity for the nature of the lipid in the inner annulus (Ellena *et al.*, 1983). Thus it is likely that the lipid probe used in the distance determination is unable to rotate freely or that it may not be localized to the inner annulus of lipids surrounding the nAChR.

Although originally proposed to bind far from the other aromatic amine NCAs, [$^3$H]ethidium diazide incorporated into both the M1 and M2 segments, near the other aromatic amine NCAs. In the desensitized state, other aromatic amine NCA, such as [$^3$H]CPZ and [$^3$H]TPP, labeled residues at the 6' ring (Giraudat *et al.*, 1986; Hucho *et al.*, 1986), while [$^3$H]meproadifen mustard incorporated into 20' (Pedersen and Cohen, 1990). [$^3$H]Quinacrine azide, on the other hand, labeled the M1 segment in the open state (DiPaola *et al.*, 1990). The structural relation between the M1 and M2 segments is not currently known, but the proximity of these segments to the ethidium binding site indicates that both contribute to the channel pore.

Can the sites of incorporation in the αM2 segment tell us anything about the possible sites of incorporation into the M1 segment? The photoreactive probe used in this study, [$^3$H]ethidium diazide, has two reactive groups, ~9 Å apart (Figure 2-12). If [$^3$H]ethidium diazide labeled M1 and M2 from a single binding site, then the site of labeling in M1 should be ~9 Å from the sites in M2, αLeu-252 and αSer-253. αLeu-252 and αSer-253 are predicted to be near the center of the bilayer, facing the channel pore. The diameter of an α-helix is 5 Å, and the distance between two residues on the same face of a helix is ~5 Å. Therefore, it is unlikely that [$^3$H]ethidium diazide

photoincorporated into the residues implicated in the binding of quinacrine, residues at the most extracellular region of the M1 segment. However, if the M1 segment is α helical, it is possible that the site of incorporation is approximately mid-way down the length of the segment, possibly at αVal-218, which has been shown to contribute to channel formation by modification of this position by a water-soluble probe (Akabas and Karlin, 1995). Knowledge of the sites of incorporation of [$^3$H]ethidium diazide into the M1 segments will clarify the contribution of the M1 segments to the nAChR ion channel.

## INTRODUCTION

The aromatic amines are the most studied structural class of the diverse group of compounds that act as noncompetitive antagonists (NCAs) on the nAChR. Affinity labeling and electrophysiology experiments show that many of these drugs bind in the ion pore, which is lined by the α-helical M2 segments from each subunit. In the desensitized state, chlorpromazine (CPZ) and triphenylphosphonium (TPP) label amino acids at position 6' (based on numbering the residues of the M2 segment from a conserved lysine at the intracellular end of the helix) in all of the subunits, and mutagenesis of these positions in both *Torpedo* and mouse nAChR has also shown that these residues affect the block by QX-222 (Giraudat *et al.*, 1986; Giraudat *et al.*, 1987; Revah *et al.*, 1990; Hucho *et al.*, 1986; Charnet *et al.*, 1990; Leonard *et al.*, 1988). In the desensitized state, [$^3$H]meproadifen mustard incorporates into the most extracellular end of αM2, position 20' (Pedersen and Cohen, 1990). In the open state, [$^3$H]quinacrine azide labels the extracellular end of αM1 (DiPaola *et al.*, 1990; Karlin, 1991), and mutational studies have also shown that substitutions in this region affect the potency of quinacrine as a NCA (Tamamizu *et al.*, 1995). All of the residues that have been labeled by these NCAs are accessible to water soluble modification agents, consistent with these residues forming a portion of the channel (Akabas and Karlin, 1995). Additionally, all of these compounds bind competitively with [$^3$H]phencyclidine (PCP) or [$^3$H]histrionicotoxin (HTX), two other aromatic amine NCAs, consistent with a common site of action.

Although known primarily for its ability to intercalate into DNA, the fluorescent compound ethidium is another aromatic amine NCA of the nAChR, which binds competitively with PCP and HTX. The affinity of ethidium for the nAChR in the desensitized state has been determined, with a $K_I$ of 0.4-0.6 μM based on inhibition of [$^3$H]PCP binding (Herz *et al.*, 1987; Pedersen, 1995), or a $K_I$ of 0.3-0.8 μM based on

inhibition by PCP of the fluorescence of bound ethidium (Herz *et al.*, 1987; Herz *et al*, 1989; Lurtz *et al.*, 1997). In contrast to the high affinity binding to the nAChR in the desensitized state, in the presence of α-bungarotoxin (αBgTx), ethidium inhibits [$^3$H]PCP binding with a $K_I$ of 1 mM (Herz *et al.*, 1987). Ethidium also binds the agonist site, with an affinity for the α-γ site of ~5 μM and for the α-δ site of ~100 μM (Pedersen, 1995).

The fluorescence of ethidium has made it an attractive probe in fluorescence quench and fluorescence resonance energy transfer (FRET) studies of the dimensions of the *Torpedo* nAChR. Initial reports, such as the measured distance between the agonist site and the ethidium NCA site, were consistent with the binding site of ethidium in the channel (Herz *et al.*, 1989). However, subsequent work using a fluorescent membrane probe, $C_{12}$-Texas Red, to measure the distance between the membrane surface and the HTX-sensitive ethidium binding site placed ethidium ~50 Å from the lipid head groups. This result suggests that the high affinity ethidium binding site in the *Torpedo* nAChR in the desensitized state is near the most extracellular portion of the nAChR (Johnson and Nuss, 1994).

Another experimental approach to localizing the binding site of ethidium, using photoaffinity probes, has also been attempted. Early work by Witzemann and Raferty (1978) showed that photoincorporation of [$^3$H]ethidium diazide, a photoactivatible derivative of ethidium, into the α and δ subunits of the *Torpedo* nAChR in the desensitized state was inhibitable by HTX. Later, Pedersen (1995) assayed the incorporation of nonradioactive ethidium diazide, as well as two ethidium monoazides, into nAChR by the fluorescence of subunits in the SDS-PAGE gel. In the absence of agonist, the most prominent incorporation into the nAChR was in the α and γ subunits. This incorporation was reduced by ~80% by the presence of carbamylcholine, but the addition of NCAs in the presence of carbamylcholine decreased the photoincorporation

little, if at all. The lack of NCA inhibitable labeling is unexpected since the ethidium analogs competitively inhibit the binding of [$^3$H]PCP similarly to ethidium. Therefore, the derivatives either can not photoincorporate into the NCA site or, once incorporated, their fluorescence is not detectable. Based on the work by Witzemann and Raferty (1978), it is most likely that the ethidium azides incorporate into the NCA, but the adducts are no longer fluorescent.

Here we utilize [$^3$H]ethidium diazide to localize the NCA binding site of ethidium. The photoincorporation of [$^3$H]ethidium diazide into the subunits of the *Torpedo* nAChR reflected the general pharmacology of [$^3$H]ethidium binding. For nAChR equilibrated with carbamylcholine, in the desensitized state, the addition of PCP reduced the incorporation into the $\alpha$ and $\delta$ subunits. [$^3$H]Ethidium diazide photoincorporated into the M2 segments of the $\alpha$ and $\delta$ subunits, as well as into fragments containing the M1 segment of the $\alpha$ and $\delta$ subunits, indicating a binding site in the channel pore. These results lead us to conclude that the high affinity ethidium binding site in the nAChR in the desensitized state is within the channel domain of the nAChR and not in the extracellular domain.

## 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.* (1992). The final membrane suspensions were stored in 38% sucrose at -80 °C under argon. The membranes used here contained 0.5-2.0 nmol acetylcholine binding sites per milligram of protein. [$^3$H]Ethidium (specific activity: 1.15 Ci/mmol) and [$^3$H]ethidium diazide (specific activity: 0.61 Ci/mmol) were a kind gift from Dr.

Steen Pedersen, synthesized according to the methods described in Pedersen (1995) and Lurtz *et al.* (1997). Azidopyrene (1-AP) was from Molecular Probes. Oxidized glutathione, carbamylcholine, nonradioactive ethidium, and Tricine were from Sigma. *S. aureus* V8 protease was from ICN Biomedical, and Endoproteinase-Lys-C (EndoLysC) was from Boeringher Mannheim. Phencyclidine (PCP) was purchased from Alltech Associates. d-Tubocurare was from Sigma, and α-bungarotoxin (αBgTx) was from Biotoxins. EndoglycosidaseH was from Genzyme.

**Equilibrium Binding Assay**

nAChR-rich membranes (~900 nM sites, not determine simultaneously with [$^3$H]ethidium binding assays) 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) were incubated for two hours at room temperature with varying concentrations of [$^3$H]ethidium in the presence or absence of other cholinergic drugs. After incubation, the membrane suspensions (90 μl) were filtered through 13 mm glass fiber filters (Schleicher & Schuell #32) which were pretreated with 1% Prosil (Lancaster) to reduce nonspecific binding. The filters were washed with 2 ml TPS and dried under heat lamps. The free concentration (F) was determined by scintillation counting of an aliquot of the filtrate, while the bound concentration (B) was determined from the filters. The data were fit to Equation 1, where $B_{max}$ is the maximum number of binding sites, $K_{eq}$ is the concentration of [$^3$H]ethidium at which half of the available sites are bound, and $B_{ns}$ is the nonspecific binding. $B_{ns}$ was determined from a linear fit of the binding in the presence of carbamylcholine and PCP. The fit was calculated using SigmaPlot from duplicate samples.

**Equation 1.** $$B = \frac{B_{max} \times F}{K_{eq} + F} + B_{ns} \times F$$

The binding in the absence of other drugs was also fit to a two-interdependent site model

$$R + D_1 + D_2 \xleftrightarrow{K_{eq1}} RD_1 + D_2 \xleftrightarrow{K_{eq2}} RD_1D_2$$

where the binding to the receptor (R) of the first drug, $D_1$, is determined by $K_{eq1}$, while the second drug, $D_2$, binds the receptor with $K_{eq2}$, but only after the binding of the first drug. In this case, the two drugs were assumed to be the same compound, and they were assumed to bind to an equal number of sites on the receptor. The two-interdependent site model was fit to Equation 2, where $K_{eq1}$ and $K_{eq2}$ were the concentration of [$^3$H]ethidium at which half of each site is bound by [$^3$H]ethidium. The number of available binding sites for each site was assumed to be equivalent.

**Equation 2.** $$B = B_{max} \times F \times \frac{K_{eq1} + 2F}{K_{eq1}K_{eq2} + K_{eq2}F + F^2} + B_{ns} \times F$$

A third model, of two independent sites, was also used to fit the binding of [$^3$H]ethidium in the absence of other ligands. This model utilized the same variables as Equation 2.

**Equation 3** $$B = B_{max} \times F \times \left( \frac{1}{K_{eq1} + F} + \frac{1}{K_{eq2} + F} \right)$$

**Photoaffinity labeling of nAChR-enriched membranes with [$^3$H]ethidium diazide**

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 TPS, and [$^3$H]ethidium diazide 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]ethidium diazide was present at 10 μM. After a 1 hour incubation at room temperature, oxidized glutathione (GSSG) was typically added to a final concentration of 10 mM. The suspensions were irradiated at 254 nm (Spectroline EF-16) for 30 seconds in a plastic 96-well plate on ice. Photolysis for an additional 90 seconds 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]ethidium diazide labeled α-subunit with *S. aureus* V8 protease (Cleveland *et al.*, 1977; White and Cohen, 1992), labeling was carried out with 800 μg (analytical mapping) or 10 mg (preparative) nAChR-rich membranes. For analytical mapping, samples were photolyzed in a 24-well plate while, for preparative mapping, the samples were photolyzed in two glass crystallization dishes per condition (3 mm inner diameter). Following photolysis, the membrane suspensions were pelleted. For analytical mapping, samples were resuspended in 80 μl 50 mM sodium phosphate, pH 7, 1% SDS. Samples were divided in half, 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) following photolysis and pelleting. The samples where 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**

SDS-PAGE was performed as described by Laemmli (1970), modified as described by Pedersen *et al.* (1986). For analytical gels, the polypeptides were resolved on a 1 mm thick 8% acrylamide gel, visualized by staining with Coomassie Blue (0.25% w/v in 45% methanol and 10% acetic acid). For autoradiography, the gels were impregnated with fluor (Amplify, Amersham), dried, and exposed at -80 °C to Kodak X-OMAT film for various times (6-8 weeks). Additionally, incorporation of $^{3}H$ into individual polypeptides was quantified by scintillation counting of excised gel slices, as described in Middleton and Cohen (1991). For analytical V8 mapping gels, following electrophoresis, the gel was briefly stained with Coomassie Blue and destained to allow visualization of the subunits. The subunits were then excised and placed directly into individual wells of a 1.5 mm mapping gel, composed of a 5 cm, 4.5% acrylamide stacking gel, and a 15 cm, 15% acrylamide separating gel. Into each well was added 1:1 gram subunit:gram *S. aureus* V8 protease in overlay buffer (5% sucrose, 125 mM Tris-HCl, 0.1% SDS, pH 6.8). The gel was run at 150V for two hours, then the current was turned off for one hour. The gel was then run at constant current overnight until the dye front reached the end of the gel. The gel was stained, and the $^{3}H$ was quantified by liquid scintillation. For preparative labelings, the polypeptides were resolved on a 1.5 mm thick, 8% acrylamide gel. The α and δ subunits were identified in the 8% gels by 1-AP fluorescence, excised, and loaded directly onto the 1.5 mm mapping gels. The α-subunit proteolytic fragment of ~20 kD (αV8-20) was identified by fluorescence and excised. Fluorescent bands near ~14 kD (δV8-14) and ~20 kD (δV8-20), as well as areas containing no fluorescence, were excised from the V8 mapping gel of δ-subunit. The excised proteolytic fragments were isolated by passive elution into 0.1 M $Na_2CO_4$, 0.1% SDS (Blanton and Cohen, 1994; Hager and Burgess, 1980). The eluate was filtered (Whatman No.1) and concentrated

using Millipore 5K concentrators. To remove excess SDS, acetone was added to the concentrate, and, following incubation at –20 °C overnight, the peptides were pelleted.

**EndoLysC digest**

For EndoLysC digestion, acetone precipitated peptides isolated from the mapping gels were resuspended in 15 mM Tris, pH 8.1, 0.1% SDS. EndoLysC (1.5 mU resuspension buffer) was added to a final volume of 100 µl. The digestion was allowed to proceed for 7-9 days before either purification of fragments by HPLC or separation of fragments by Tricine SDS-PAGE (Schagger and von Jagow, 1987). $^{3}H$ in the Tricine gel was quantified by cutting the gel into 2 mm sections throughout the length of the gel. The gel pieces were solubilized, and the $^{3}H$ was counted as described for SDS-PAGE gels.

**HPLC purification**

Proteolytic fragments from enzymatic digestion of [$^{3}H$]ethidium diazide labeled subunits were further purified by reverse-phase HPLC, as described by Blanton and Cohen (1994), 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, aliquots from the fractions were taken to determine the presence of radiation 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. HPLC samples (450 µl) were directly loaded onto chemically modified glass fiber disks (Beckman) in 20 µl aliquots, allowing the solvent to evaporate at 40 °C between loads. Samples were loaded directly, as opposed to removing solvent by vacuum centrifugation and resuspending in buffer containing SDS, to avoid the pre-wash step necessary when samples containing SDS are loaded onto the sequencer. The pre-wash step consisted of a four minute treatment with gas-phase TFA followed by a five minute wash with ethyl acetate. When samples labeled with [$^3$H]ethidium diazide were subjected to this treatment, up to 50% of the loaded $^3$H was removed during this wash step. 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. The samples were counted for five minutes, in triplicate at least, and the results were averaged to generate reported errors. Yield of PTH amino acids, along with background-subtracted levels, 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 using SigmaPlot. Derivatives known to have poor recovery (Ser, Arg, Cys, and His) were omitted from the fit.