Many neuroactive steroids potently and allosterically modulate pentameric ligand-gated ion channels, including GABAₐ receptors (GABAₐRs) and nicotinic acetylcholine receptors (nAChRs). Allopregnanolone and its synthetic analog alfaxalone are GABAₐR-positive allosteric modulators (PAMs), whereas alfaxalone and most neuroactive steroids are nAChR inhibitors. In this report, we used 11β-(p-azidotetrafluorobenzoyloxy)allopregnanolone (F₄N₃Bzoxy-AP), a general anesthetic and photoreactive allopregnanolone analog that is a potent GABAₐR PAM, to characterize steroid-binding sites in the Torpedo α₁βγδ nAChR in its native membrane environment. We found that F₄N₃Bzoxy-AP (IC₅₀ = 31 μM) is 7-fold more potent than alfaxalone in inhibiting binding of the channel blocker [³H]tetracaine, a closed-state selective channel blocker, or of [³H]acetylcholine. Photolabeling identified three distinct [³H]F₄N₃Bzoxy-AP–binding sites in the nAChR transmembrane domain: 1) in the ion channel, identified by photolabeling in the M2 helices of βVal-261 and δVal-269 (position M2–13); 2) at the interface between the αM1 and αM4 helices, identified by photolabeling in αM1 (αCys-222/αLeu-223); and 3) at the lipid–protein interface involving γTrp-453 (M4), a residue photolabeled by small lipophilic probes and promegestone, a steroid nAChR antagonist. Photolabeling in the ion channel and αM1 was higher in the nAChR-desensitized state than in the resting state and inhibitable by promegestone, a steroid nAChR antagonist. This work was supported by National Institutes of Health Grant GM-58448. The authors declare that they have no conflicts of interest with the contents of this article. This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.  

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tion within the M2 ion channel domain that increased channel lifetime also reduces hydrocortisone potency, but there was no evidence for hydrocortisone competition with QX-222, an open channel blocker (16).

nAChR-rich membranes that can be isolated from the Torpedo electric organ provide a unique preparation to use photooptolabeling techniques to identify steroid-binding sites in a muscle-type nAChR in its native membrane environment. Radiolabeled, photoreactive analogs of hydrophobic general anesthetics, including propofol, meprobamal, and etomidate, have been shown to bind to sites in the TMD of these nAChRs, which upon UV irradiation forms a reactive ketyl radical at the 3-position of the steroid A-ring, F4N3Bzoxy-AP reacts by formation of a stabilized nitrene at the a reactive group (30). Where [3H]promegestone photolabeled amino acids within the TMD, identified by photolabeling of tox in the nAChR ion channel. [3H]F4N3Bzoxy-AP also identified conserved positive charges at the N termini of the M2 helices, which provides a first identification of a steroid-binding site in the nAChR ion channel. [3H]F4N3Bzoxy-AP also identified sites accessible from the lipid, one within the a subunit, identified by photolabeling of Cys-222/α-Leu-223 at the interface between the M1 and M4 helices, and a site near the cytoplasmic surface of the TMD, identified by photolabeling of γTrp-453 in γM4, a residue photolabeled by [3H]promegestone.

**Results**

Ligand binding assays

We compared the effects of F4N3Bzoxy-AP and alphaxalone on the equilibrium binding of [3H]ACh and of channel blockers that bind preferentially in the nAChR desensitized state stabilized by agonist ([3H]tetracaine ([3H]TCP), a PCP analog (31)) or in the closed channel state stabilized by the peptide neurotoxin α-bungarotoxin ([3H]tetracaine (32, 33)) (Fig. 2). F4N3Bzoxy-AP or alphaxalone even at 100 μM altered [3H]ACh-specific binding by <5%, in contrast to proadifen, a prototypic-desensitizing, noncompetitive antagonist (34), that increased binding by ~30% with an EC50 of ~1 μM. Neither F4N3Bzoxy-AP nor alphaxalone at concentrations up to 300 μM had any effect on the binding of [3H]tetracaine to the ion channel in the closed state. In contrast, for nAChRs in the desensitized state stabilized by agonist, both F4N3Bzoxy-AP and alphaxalone inhibited [3H]TCP binding, with F4N3Bzoxy-AP (IC50 = 31 ± 4 μM) 7-fold more potent than alphaxalone (IC50 = 209 ± 12 μM).
Steroid-binding sites in an $\alpha\beta\gamma\delta$ nAChR

We compared patterns of nAChR subunit photolabeling after irradiation of nAChR-rich membranes at 365 or 254 nm in the absence or presence of an agonist (carbamylcholine (Carb)), PCP, alphaxalone, or the steroid noncompetitive antagonists alphaxalone or promegestone. After photolabeling with $[^3H]$F$_4$N$_3$Bzoxy-AP (3 $\mu$m) and fractionation of membrane polypeptides by SDS-PAGE, the $[^3H]$ incorporation into the nAChR subunits and other membrane polypeptides was assessed by fluorography (Fig. 3A) for qualitative characterization and by liquid scintillation counting of excised subunit gel bands to quantify photoincorporation (Fig. 3, B–D). As seen by fluorography, after irradiation at 365 nm, the nAChR subunits photolabeled most prominently were the $\alpha$ and $\gamma$ subunits, with the most prominently photolabeled gel bands those of 34 and 32 kDa previously identified as the voltage-dependent anion channel and ADP/ATP carrier from contaminating mitochondrial fragments (35). Irradiation at 254 nm resulted in prominent, pharmacologically-specific photolabeling in the nAChR $\alpha$ subunit, with Carb enhancing photolabeling compared with control and PCP inhibiting the Carb-enhanced photolabeling. Quantification of photolabeling by liquid scintillation counting (Fig. 3, B and C) that binds to sites in the ion channel and at the $\gamma$$\alpha$ subunit interface (36). To test the effect of a second steroid antagonist on $[^3H]$F$_4$N$_3$Bzoxy-AP photolabeling at 254 nm, we used promegestone, which inhibits with an IC$_{50}$ of 10 $\mu$m the ACh responses of Torpedo nAChRs expressed in Xenopus oocytes and the binding of the channel blocker $[^3H]$PCP to nAChR-rich membranes in the presence of Carb (Fig. 3D) (26). Carb enhanced nAChR $\alpha$ subunit photolabeling by ~40%, and promegestone at 300 $\mu$m reduced photolabeling to the level seen in the control condition or in the presence of Carb and PCP. Similarly for the $\beta$ and $\delta$ subunits, promegestone inhibited the small enhancement of photolabeling (~20%) seen in the presence of Carb.

$[^3H]$F$_4$N$_3$Bzoxy-AP photolabeling of Torpedo nAChR-rich membranes

Figure 3. $[^3H]$F$_4$N$_3$Bzoxy-AP photoincorporation into Torpedo nAChR-rich membranes. Membrane suspensions equilibrated with $[^3H]$F$_4$N$_3$Bzoxy-AP (3 $\mu$m) were irradiated at 365 nm for 30 min or 254 nm for 2 min in the absence or presence of different cholinergic ligands, and triplicate samples were fractionated by SDS-PAGE. After staining for protein, one gel was prepared for fluorography, and subunit gel bands were excised from the second for $[^3H]$ incorporation into nAChR subunit gel bands after irradiation at 365 nm (B) or 254 nm (C) from the same experiment as the fluorogram and from an independent experiment at 254 nm (D). The average cpm + S.D. are plotted for samples from two gels. Included in C and D are the $p$ values, where statistically significant ($p < 0.05$, one-way ANOVA and Tukey’s multiple comparison test for pairs of labeling conditions (GraphPad Prism 7)). A, left, electrophoretic mobilities are indicated of the nAChR $\alpha$, $\beta$, $\gamma$, and $\delta$ subunits, rapsyn (Rsn), the Na$^+$/K$^+$-ATPase $\alpha$ subunit (Na$_{\alpha}$/K$_{\alpha}$), and the mitochondrial voltage-dependent anion channel (34 kDa) and ADP/ATP carrier (32 kDa).

$[^3H]$F$_4$N$_3$Bzoxy-AP photolabeling in the nAChR ion channel

Pharmacologically-specific photolabeling was most prominent in the $\alpha$ subunit after photolabeling at 254 nm, but we first characterized photolabeling within the M2 channel-forming helices after irradiation at 365 nm, a wavelength that minimizes nonspecific UV-induced protein degradation. Photolabeling was characterized in $\beta$M2 and $\delta$M2, because fragments beginning at their N termini ($\beta$Met-249 and $\delta$Met-257) can be isolated readily (37–39) at ~10-fold higher mass levels than the $\alpha$M2 fragment that requires a more complex purification procedure (40). Trypsin digests of $\beta$ subunits isolated from nAChRs photolabeled with $[^3H]$F$_4$N$_3$Bzoxy-AP in the absence or presence of Carb were fractionated by Tricine gel SDS-PAGE...
and then rpHPLC (Fig. 4A). When the fragment beginning at βMet-249 was sequenced from the major peak of $^3$H (Fig. 4B), the peak of $^3$H release in cycle 13 identified photolabeling of βVal-261 (a channel-lining residue, position M2–13’) in the presence of Carb, whereas labeling in the absence of agonist, if it occurred, was at <10% that level. Similarly, after fractionation of an EndoLys-C digest of photolabeled δ subunit by Tricine-gel SDS-PAGE and then rpHPLC (Fig. 4C), sequence analysis of the major peak of $^3$H established the presence of the fragment beginning at δMet-257 (Fig. 4D). The peak of $^3$H release in cycle 13 in the presence of Carb indicated agonist-dependent photolabeling of δVal-269, the position in δM2 equivalent to βVal-261. In photolabelings with $[^3]$H]F$_4$N$_3$Bzoxy-AP at 254 nm, we also determined that βVal-261 and δVal-269 were photolabeled in an agonist-dependent manner and at similar efficiency as for photolabeling at 365 nm (Table 1).

**Promegestone inhibition of photolabeling in βM2 and δM2**

To examine the effects of promegestone on $[^3]$H]F$_4$N$_3$Bzoxy-AP photolabeling, membranes equilibrated with Carb in the absence or presence of 100 μM promegestone were irradiated at 254 nm, and the subunit fragments beginning at the N termini of βM2 and δM2 were isolated for sequence analysis. For the fragment beginning at βMet-249, promegestone reduced the peak of $^3$H release in cycle 13 (βVal-261) by ~40% (Fig. 5A). For photolabeling within δM2, the fragment beginning at δMet-257 was isolated at a high level (I$_0$ = 310 pmol, both conditions). In the presence of Carb there were peaks of $^3$H release at cycle 13, 16, and 20, consistent with photolabeling of the channel-lining residues δM2–13’, -16’, and -20’ (δVal-257, δLeu-272, and δGln-276) (Fig. 5B). Promegestone reduced photolabeling of δVal-269 by ~50%.

**$[^3]$H]F$_4$N$_3$Bzoxy-AP photolabeling residues in αM1**

Based upon the photoincorporation seen at the subunit level (Fig. 3D), irradiation at 254 nm resulted in pharmacologically-specific photolabeling in the nAChR α subunit. To identify photolabeling in the α subunit, we took advantage of the fact that in gel digestion of the α subunit with V8 protease results in the formation of three large, nonoverlapping subunit fragments that are readily resolved by SDS-PAGE (41, 42), including one of 20 kDa (αV8–20) that begins at αSer-173 and extends through the M1–M2–M3 helices and another of 10 kDa (αV8–10) that begins at αAsn-339 in the cytoplasmic domain and

| Table 1 Efficiency of $[^3]$H]F$_4$N$_3$Bzoxy-AP photolabeling of amino acids in the Torpedo nAChr transmembrane domain |

| Labeled residues | 365 nm control/+Carb | 254 nm control/+Carb |
|------------------|----------------------|----------------------|
| βVal-261 (M2–13’) | <0.1/1.4 ± 0.9 (3) | <0.1/5.0 ± 2.6 (9) |
| δVal-269 (M2–13’) | <0.1/0.4 ± 0.2 (3) | <0.05/0.23 ± 0.08 (5) |
| γTrp-453 (M4) | 7 ± 4 (2)/6 (1) | ND |
| αCys-222 (M1) (αLeu-233)* | ND | 0.7 ± 0.2 (2)/1.7 (3) |

* Because αLeu-223 immediately follows αCys-222, which is photolabeled more efficiently, the efficiency of photolabeling of αLeu-233 cannot be calculated reliably.
Steroid-binding sites in an αβγδ nAChR

extends through the M4 helix. Furthermore, digestion of αV8–20 with EndoLys-C generates fragments readily separated by rpHPLC, one beginning at αHis-186 that contains αM1 and a second beginning at αMet-243, the N terminus of αM2, that extends through αM3 (40). When an EndoLys-C digest of αV8–20 isolated from nAChRs photolabeled at 254 nm in the presence of Carb was fractionated by rpHPLC (Fig. 6A), the major peak of $^3$H eluted at ~70% organic solvent B where the fragment containing αM1 is known to elute (40, 43), with little $^3$H eluting in the more hydrophobic fractions where the fragment containing αM2 elutes. Sequence analysis of fractions containing the peak of $^3$H (Fig. 6B) established the presence of the αHis-186 fragment ($I_0 = 4 \text{ pmol}$), with no release of $^3$H above background in 15 cycles of Edman degradation. Similarly, the fragment beginning at αMet-243 ($I_0 = 1 \text{ pmol}$), which eluted at ~85% organic solvent B, was sequenced without any $^3$H release above background in 15 cycles of Edman degradation that extended to αM2–15’ (Fig. 6C). These results indicated that the $^3$H incorporation in αV8–20 was likely to be within αM1.

To identify amino acids photolabeled in αM1, α subunits were isolated from nAChRs photolabeled at 254 nm in the absence or presence of Carb and in the presence of Carb and promegestone. During sequencing of the fragment beginning at αle–210 ($I_0 = 60 \text{ pmol}$, each condition), isolated by rpHPLC from tryptic digests of nAChRs (36, 43), there was a peak of $^3$H release in cycles 13/14 consistent with photolabeling of αCys-222/αLeu-223 (Fig. 6D). These residues were photolabeled at higher efficiency in the presence of Carb than in the absence (Table 1), and promegestone reduced the agonist-enhanced labeling by ~50$.^\%$. To confirm photolabeling of αCys-222/αLeu-223, a fragment beginning at αGln-208 was generated for sequencing by isolating the labeled αHis-186 fragments from α subunit EndoLys-C digests and treating them with cyanogen bromide to cleave at the C terminus of αMet-207 (43, 44). When the αGln-208 fragments were sequenced (Fig. 6E), the peak of Carb-enhanced $^3$H release in cycle 15 confirmed labeling of αCys-222. Sequence analysis of fragments beginning at αle–210 from an independent labeling experiment (+ Carb and + Carb + PMG) provided additional evidence that αLeu-223 was photolabeled as well as αCys-222, because in this case the peak of $^3$H release was in cycle 14 rather than 13 (Fig. 6F).

$[^3H]_N_7, B_2A_2O_2-AP$ photolabeling in M4 and M3 helices

The nAChR M4 helices are most broadly exposed to lipid, with amino acids from the M1 and M3 helices also exposed at the lipid interface (42, 45). Photolabeling studies with $[^3H]_promege$stone, which forms a reactive ketyl radical upon irradiation at 312 nm, identified photolabeling of residues in αM4 (αCys-412 and αCys-418), βM4 (βTyr-441 and βCys-447), and γM4 (γCys-451 and γTrp-453) that are exposed at the nAChR–lipid interface and also photolabeled by small hydrophobic probes (26). Any $[^3H]_promege$stone photolabeling of residues in βM2 or δM2, if it occurred, was at <10$^\%$ the efficiency of any of the photolabeled cysteines. To identify photolabeling in αM4, we isolated by rpHPLC the fragment beginning at αTyr-401, the αM4 N terminus, from trypsin digests of αV8–10 isolated from nAChRs photolabeled at 254 and at 365 nm. No peaks of $^3$H release above background were detected during sequence analyses of these fragments through 20 cycles of Edman degradation (Fig. 7, A and B), which indicated that labeling, if it occurred, was at <0.3 cpm/pmol, i.e. at <5$^\%$ the efficiency of labeling of αCys-222 but possibly at the labeling efficiency of δVal-269 (δM2–13’).

Photolabeling in γM4 was characterized by a strategy similar to that used for αM4. The γ-subunits were digested in gel with V8-protease to produce fragments of ~14 kDa (γV8–14) beginning at γLeu-373 and γlle-413, and EndoLys-C digests of those fragments were fractionated by rpHPLC. For nAChRs photolabeled at 365 nm, sequencing of the fragment beginning at γAla-450, the N terminus of γM4, revealed a peak of $^3$H release in cycle 4, corresponding to photolabeling of γTrp-453 in the absence or presence of Carb at a photolabeling efficiency 4-fold higher than that of βVal-261 (βM2–13’) (Fig. 7C and Table 1). In contrast, for nAChRs irradiated at 254 nm, photolabeling of γTrp-453, if it occurred, was at <10$^\%$ the efficiency of βVal-261. This preferential photolabeling at 365 nm compared with 254 nm of γTrp-453, the only Trp in the nAChR TMD, may be the source of the increased γ subunit labeling seen after irradiation at 365 nm compared with 254 nm (Fig. 3).

Multiple lipid-exposed residues in the nAChR M3 helices have been photolabeled by small hydrophobic photoprobes (39, 42), whereas other residues in γM3 contributing to a binding pocket at the γ-α subunit interface have been photolabeled by...
photoreactive etomidate and barbiturate analogs (36, 46, 47). For nAChRs photolabeled at 365 or 254 nm in the presence of Carb, we characterized [3H]F4N3Bzoxy-AP photolabeling in /H9253M3 and /H9254M3 by sequencing for 30 cycles of Edman degradation fragments that begin at /H9253Thr-276 and /H9254Thr-281 and extend through the M3 helices. The protocol used (47) allowed efficient recovery of the fragments (I0 = 30–90 pmol), but at either labeling wavelength, there were no peaks of 3H release above background, which indicated that any labeling, if it occurred, was at <0.3 cpm/pmol.

\[^{3}H\text{F}_4\text{N}_3\text{Bzoxy-AP photolabeling in the } \delta \text{ subunit helix bundle}\]

No photolabeling was detected of the residues in \( \delta \text{M1} (\delta \text{Thr-228} \text{ and } \delta \text{Phe-232}), \delta \text{M2 (} \delta \text{M2–18}), \text{ or the } \delta \text{M2–M3 loop (} \delta \text{Ile-288) within the intrasubunit-binding site near the extracellular end of the } \delta \text{ subunit helix bundles that are photolabeled in an agonist-dependent manner by general anesthetics (halothane and photoreactive analogs of etomidate and propofol (39, 47, 48)), a convulsant barbiturate (43), and small hydrophobic nAChR inhibitors (38, 49). Any photolabeling of } \delta \text{M2–18, if it occurred, was at less than 5}\% \text{ the level of } \delta \text{M2–13 (Fig. 5B), and based upon sequencing the fragment beginning at } \delta \text{Phe-208 (I0 = 120 pmol), any labeling of } \delta \text{Phe-232 or } \delta \text{Cys-236 was at less than 10}\% \text{ the level of labeling of } \alpha \text{Cys-222 and } \alpha \text{Leu-223 that was enhanced in the presence of Carb compared with control, and promegestone reduced the Carb-enhanced labeling.}

Discussion

In this report, we use \( \text{F}_4\text{N}_3\text{Bzoxy-AP, a photoreactive analog of alphaxalone and allopreganolone that is a potent general anesthetic and GABA}_A\text{R PAM (27), to identify the locations of steroid-binding sites in the } \text{Torpedo nAChR in its native membrane environment. Many endogenous (glucocorticoids and progesterone) and synthetic (alphaxalone and promegestone) steroids act as noncompetitive antagonists of } \text{Torpedo and vertebrate skeletal muscle and neuronal nAChRs, but it is uncertain whether they act from the lipid interface or from sites within the nAChR (6, 8). Promegestone, which potently inhibited } \text{Torpedo nAChR responses and the binding of a desensitized state selective channel blocker, photolabeled amino acids...} \)
Steroid-binding sites in an \( \alpha \beta \gamma \delta \) nAChR

The efficiency of labeling of photolabeled amino acids in a homology model of the TMD. Based upon computational docking (Fig. 8), the photolabeling in the ion channel of the M2–13 residues, the residues that contribute to the lumen of the channel are the same in the closed, open, and desensitized states (50). The hydrophobic side chains of M2–13, along with those at M2–9, contribute to a hydrophobic plug preventing ion permeation in the closed channel state (37, 51). Small charged (tetracaine) and uncharged (TID, benzophenone, and Azipm) drugs bind at that level preferentially in the absence of agonist (33, 37, 39, 49). Similar to \( F_2 N_B \) Bzoxy-AP, bulkier photoreactive etomidate and barbiturate analogs bind near M2–13 in the channel with higher affinity in the desensitized state (36, 43, 47).

Because more limited quantities of fragments containing \( \alpha M2 \) or \( \gamma M2 \) could be isolated for sequencing than \( \beta M2 \) or \( \delta M2 \), it remains possible that there is also unidentified labeling in those fragments at the same efficiency as \( \delta Val-269 \). The large quantities of \( \beta M2 \) and \( \delta M2 \) fragments allowed identification of agonist-dependent photolabeling in \( \beta M2 \) and \( \delta M2 \), even though it comprised a smaller fraction of subunit labeling than that seen for the \( \alpha \) subunit (Fig. 3). There was, however, agonist-enhanced PCP-inhibitable \( \alpha \) subunit labeling in nAChRs photolabeled at 254 nm, with the agonist-enhanced labeling inhibitable by 300 \( \mu M \) promegestone. At the amino acid level, promegestone at 100 \( \mu M \) reduced photolabeling of \( \beta Val-261 \) and \( \delta Val-269 \) by ~50%. Because promegestone is more potent than \( F_2 N_B \) Bzoxy-AP as an inhibitor of \([H]TCP binding \) (26), ~90% inhibition would be predicted if the two steroids bound in a mutually exclusive fashion. However, even PCP and chlorpromazine, two positively charged drugs, can bind simultaneously at different levels in the ion channel (52). Further studies are necessary to determine whether the inhibition of photolabeling is allosteric rather than competitive.

\[ F_2 N_B \] Bzoxy-AP binding at the nAChR–lipid interface

The photolabeling of \( \gamma Trp-453 \) in \( \gamma M4 \) provides evidence that \( F_2 N_B \) Bzoxy-AP interacts with the same region of \( \gamma M4 \) near the cytoplasmic end of the TMD as promegestone and a tricyclic aromatic probe, diazofluorene, which photolabel the same residue (26, 53), and TID, which photolabels \( \alpha Cys-451 \) (42). However, it was surprising not to find labeling of any positions in \( \alpha M4 \), which is photolabeled at \( \alpha Cys-412 \) and other residues by promegestone, diazofluorene, TID, and many general anesthetics (Fig. 8C). The lack of labeling of \( \alpha Cys-412 \) cannot result from an inability of \( F_2 N_B \) Bzoxy-AP to form a stable adduct with cysteines, because \( \alpha Cys-222 \) in \( \alpha M1 \) was one of the residues labeled most efficiently. One explanation for this unexpected result is that when \( F_2 N_B \) Bzoxy-AP binds in proximity to \( \alpha M4 \), positions M2–13’ and M2–20’ and between the \( \alpha \) subunit M1 and M4 helices, extending from \( \alpha Cys-222 \) to the extracellular end of TMD.

Steroid binding in the ion channel

The agonist-enhanced photolabeling of \( \beta Val-261 \) and \( \delta Val-269 \) provides direct evidence that a steroid can bind in proximity to position M2–13’ in the ion channel. Based upon the \([H]TCP- \) and \([H]tetracaine-binding assays, \( F_2 N_B \) Bzoxy-AP binds in the ion channel in the desensitized state \( (IC_{50} = 30 \, \mu M) \) with ~10-fold higher affinity than in the closed channel state. As the structural changes in the ion channel associated with channel gating involve only small twists or tilts of the M2 helix, the residues that contribute to the lumen of the channel are the same in the closed, open, and desensitized states (50). The hydrophobic side chains of M2–13, along with those at M2–9, contribute to a hydrophobic plug preventing ion permeation in the closed channel state (37, 51). Small charged (tetracaine) and uncharged (TID, benzophenone, and Azipm) drugs bind at that level preferentially in the absence of agonist (33, 37, 39, 49). Similar to \( F_2 N_B \) Bzoxy-AP, bulkier photoreactive etomidate and barbiturate analogs bind near M2–13 in the channel with higher affinity in the desensitized state (36, 43, 47).

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the photoreactive azide incorporated at the steroid 11 position is oriented toward the lipid rather than the nAChR.

In the nAChR structure, the photolabeled residues in αM1 (αCys-222/αLeu-223) are predicted to be accessible from the lipid, and computational docking predicts that F4N3Bzoxy-AP will intercalate between αM1 and αM4 in the outer half of the TMD (Fig. 8C). This is the location where cholesterol is predicted to be enriched in the outer leaflet of the lipid bilayer, based upon cryo-EM analyses of lipid distribution in the tubular vesicles formed from Torpedo nAChR-rich membranes (20). As interactions between the M4 and M1/M3 helices are important for channel function (19), our results indicate that binding of F4N3Bzoxy-AP or other steroids at this site can perturb cholesterol–nAChR interactions important for nAChR conformational transitions. Photolabeling of αCys-222/αLeu-223 was state-dependent, as evidenced by enhanced labeling in the presence of agonist, a result also seen for a convulsant barbiturate (43). Consistent with this, mutations of αVal-218, αPro-221, and αCys-222 indicate that structural changes in this region are important determinants of nAChR gating and desensitization (54, 55). The location of this intrasubunit-binding site for steroids between αM1 and αM4 identified by F4N3Bzoxy-AP is similar to a binding site for steroids identified by photolabeling in a prokaryotic GLIC with a cation-selective ion channel, the homomeric Gloeobacter ligand-gated ion channel (GLIC) (56). In contrast, in a homomeric chimeric receptor containing a GABA_A subunit TMD, a binding site for pregnenolone sulfate, an inhibitory steroid, was identified between the M4 and M3 helices near the cytoplasmic end of the TMD (11).
Steroid-binding sites in an αβγδ nAChR

Experimental procedures

Materials

nAChR-rich membranes, containing 0.8–1.1 nmol of [3H]ACh-binding sites per mg of protein, were isolated from Torpedo californica electric organs (Aquatic Research Consultants, San Pedro, CA) as described (57) and stored in 38% (w/v) sucrose at −80 °C until use. F₃N₃Bzoxy-AP and [3H]F₄N₃ Bzoxy-AP (45 Ci/mmol) were synthesized previously (27). [3H]Acetycholine (ACh, 30 Ci/mmol) was synthesized by PerkinElmer Life Sciences. [3H]Tetracaine (30 Ci/mmol) was from Sibtech (Newington, CT). Proadifen, Carb, PCP, and promegestone were prepared at 60 mM in saline buffer (in mM: 250 NaCl, 5 KCl, 3 CaCl₂, 2 MgCl₂, and 5 sodium phosphate, pH 7.0) as described (43). Binding assays were performed at the following final concentrations: for [3H]ACh, 40 nM ACh-binding sites, 4 mM radioligand, and 0.5 mM diisopropylphosphofluoridate to inhibit acetylcholinesterase; for [3H]TCP and [3H]tetracaine, 500 nM ACh-binding sites and 10 nM radioligand. For [3H]TCP binding, membranes were equilibrated with the agonist Carb (1 mM) to stabilize nAChRs in the desensitized state. For [3H]tetracaine binding, membranes were equilibrated with 5 μM α-bungarotoxin to stabilize nAChRs in the resting state. Nonspecific binding of [3H]ACh, [3H]TCP, or [3H]tetracaine was determined in the presence of 100 μM Carb, PCP, or tetracaine, respectively. Stock solutions of F₃N₃Bzoxy-AP, alphaxalone, and promegestone were prepared at 60 μM in methanol, and all samples contained methanol at a final concentration of 0.5% (v/v). For each experiment, f₀, the specifically bound [3H]cpm_total − cpm_nonspecific, was normalized to f₀ for the specifically bound [3H] in the absence of competitor. Individual experiments were carried out with duplicate samples, and data from independent experiments were combined for analysis. The concentration-dependent inhibition of [3H]TCP binding was fit to the equation, fₐ/f₀ = 1/(1 + x/IC₅₀), where IC₅₀ is the total ligand concentration producing the half-maximal inhibition of binding. The numbers of independent experiments are noted in the figure legends.

[3H]F₄N₃Bzoxy-AP photolabeling and gel electrophoresis

Conditions for photolabeling were identified by characterizing the absorption and photolysis characteristics of F₃N₃ Bzoxy-AP in methanol. At the absorption maximum of 264 nm, the F₃N₃Bzoxy-AP extinction coefficient was 17,550 ± 450 M⁻¹ cm⁻¹. Photolysis with a 254-nm lamp (Spectronics EF160C) caused a progressive shift of the maximum absorption from 264 to 285 nm with a t₁/₂ of 20 s, associated with the formation of a broad secondary peak with an absorption maximum of ~340 nm. Photolysis with a 365-nm lamp (Spectronics EN-280L) decreased the absorption maximum with a t₁/₂ of 17 min. Based upon these results, nAChR-rich membranes equilibrated with [3H]F₄N₃Bzoxy-AP were irradiated for 2 and 30 min with the 254- and 365-nm lamps, respectively. Before irradiation, Torpedo nAChR-rich membranes (2 μM ACh sites; 2.5 mg of protein/ml in Torpedo saline buffer supplemented with 1 mM oxidized GSH as an aqueous scavenger) were incubated at 4 °C with [3H]F₄N₃Bzoxy-AP for 30 min and then for 30 min in the absence or presence of other ligands. nAChRs were photolabeled on analytical or preparative scales using 0.1 or 10 mg of protein per condition, respectively. After photolabeling, membrane polypeptides were resolved by Tris-glycine SDS-PAGE on gels composed of 8% polyacrylamide, 0.33% bisacrylamide, and membrane polypeptides were visualized with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific). Invitrogen SeeBlue Plus2 pre-stained standards were used as molecular mass markers.

For analytical photolabelings (3 μM [3H]F₄N₃Bzoxy-AP), samples were run in triplicate, with stained subunit bands excised from two sets for [3H] quantification by liquid scintillation counting, and the third set was analyzed by fluorography using Amplify (GE Healthcare). For identification of photolabeled amino acids, nAChR-rich membranes were photolabeled on a preparative scale in five experiments with [3H]F₄N₃ Bzoxy-AP at 0.4–0.8 μM, using three different membrane purifications: two with irradiation at 365 nm in the absence and presence of Carb, and three with irradiation at 254 nm (+ Carb; − Carb/+ Carb/+ Carb + 100 μM promegestone; + Carb/+ Carb + 100 μM promegestone). For the preparative photolabelings, bands containing the nAChR α, β, γ, and δ subunits were excised from the stained gels, and subunits were eluted passively for at least 3 days at room temperature in a buffer containing (in mM): 100 NH₄HCO₃, 2.5 mL-DTT, 0.1% SDS, pH 8.4. Thereafter, eluted materials were concentrated to a final volume of ~400 μl by centrifugal filtration at room temperature using Vivaspin 15 M, 5000 concentrators (Vivasience, Stonehouse, UK), precipitated by 75% acetone overnight at −20 °C, and resuspended in digestion buffer (in mM: 15 Tris, 0.5 EDTA, 0.1% SDS, pH 8.1). For most preparative photolabelings, 25% of α and γ subunit gel bands were eluted directly, and 75% of those gel bands were subjected to in-gel digestion with V8 protease (200 μg) in 15% polyacrylamide, 0.76% bisacrylamide mapping gels. The resultant subunit fragments (αV8–20, αV8–10, γV8–24, and γV8–14) (41, 42) were recovered from gel bands by passive elution, concentrated, precipitated, and resuspended in digestion buffer.

Proteolytic digestions

All enzymatic digestions were performed at room temperature. After photolabelings on a preparative scale, ~50% of eluted α and β subunits and 100% of αV8–10, each in digestion buffer, were diluted 5-fold with 50 mM NH₄HCO₃, pH 8.1, supplemented with 0.5% Genapol to reduce the SDS concentration to 0.02%, and then digested with 200 μg of TPCK-treated trypsin in the presence of 0.4 mM CaCl₂, overnight (β subunit) or for...
3 days ($\alpha$ subunit and $\alpha$V8–10). $\alpha$V8–20, $\gamma$V8–24, and $\gamma$V8–14 and aliquots of $\alpha$ and $\delta$ subunits (~50%) were digested using 0.3–0.5 units of EndoLys-C for 2 weeks. To characterize photo-labeling in the M3 helices (49), aliquots of $\beta$ (40%), $\gamma$ (25%), and $\delta$ (40%) subunits were digested with 200 $\mu$g of V8 protease for 3 days in digestion buffer. Small-pore Tricine SDS-polyacrylamide gels (16.5% T, 6% C) (38, 58) were used to fractionate the trypsin and EndoLys-C digests of $\beta$ and $\delta$ subunits, respectively. The subunit fragments recovered from those Tricine gel bands by passive elution and the other subunit digests were then fractionated by rpHPLC.

**rpHPLC and sequence analysis**

rpHPLC was performed as described (47) using an Agilent 1100 binary rpHPLC system, a Brownlee Aquapore BU-300 column, and a mobile phase containing the aqueous solvent A (0.08% TFA in distilled water) and organic solvent B (3:2 acetonitrile/isopropyl alcohol supplemented with 0.05% TFA). Proteolytic digests were eluted at a flow rate of 0.2 ml/min using a nonlinear gradient, with organic solvent B increasing from 25 to 100% over 90 min with fractions collected every 2.5 min, and $^3$H elution was determined by liquid scintillation counting on 10% of each fraction.

With the exception of rpHPLC fractions containing $\alpha$M4, which were loaded onto polyvinylidene difluoride membrane filters at room temperature using Applied Biosystems ProSorb$^\text{TM}$ sample preparation cartridges, all rpHPLC fractions containing $^3$H-labeled peptides were drop-loaded at 45 °C onto Applied Biosystems Micro TFA glass fiber filters. After loading on filters, samples were treated with BioBreane Plus to stabilize the peptides and then subjected to Edman degradation sequencing on an Applied Biosystems Procise 492 protein sequencer. For some samples, as detailed in the figure legends, sequencing was interrupted at a predetermined cycle, and the filter was treated with o-phthalaldehyde to prevent further sequencing of any peptides not containing a proline at that cycle (57, 59). As one method to characterize $[^3$H]$\text{F}_n\text{N}_3\text{Bzoxy-AP}$ photo labeling in $\alpha$M1, samples containing the fragment beginning at $\alpha$His-186 were sequenced for 18 cycles, and filters were then treated with cyanogen bromide as described (44, 60) to cleave at the C-terminal side of $\alpha$Met–207 before $\alpha$M1.

During N-terminal sequencing, either 1/6 or 2/3 of the material from each cycle of Edman degradation was used for phenylthiohydantoin (PTH)-derivative determination, and 5/6 (or 1/3) was collected to measure $^3$H release by scintillation counting. The amount of PTH-derivative released in a given sequencing cycle (in picomoles) was determined by comparing the peak height of the derivative in the chromatogram to the height of its standard peak ($I_0$), the initial amount of the peptide in the sample (in picomoles), was determined from the amounts of PTH-derivative in each cycle by using SigmaPlot 11 to the equation $I_0 = I_x R$, where $I_x$ is the background-subtracted mass of the amino acid at cycle $x$, and $R$ is the average repetitive yield of the Edman degradation. For samples containing multiple fragments, only PTH-derivatives unique to the fragment of interest were used in the fit, and amino acid derivatives whose amounts cannot be estimated accurately (His, Trp, Ser, Arg, and Cys) were omitted from the fits. When 2/3 of the material was used for PTH-derivative determination, $E(x)$, the photolabeling efficiency (cpm/pmol) of an amino acid residue in cycle $x$, was calculated according to the equation $(2(\text{cpm}_x - \text{cpm}_{(x-1)}))/ (I_0 R^0)$, where $\text{cpm}_x$ is the $^3$H release in cpm at cycle $x$. When 1/6 of the material was used for PTH-derivative determination, $E(x) = ((\text{cpm}_x - \text{cpm}_{(x-1)}))/(5 I_0 R^0)$.

**Computational docking**

A T. californica nAChR homology model was constructed based on the cryo-EM-derived structure of Torpedo marmorata nAChR (PDB code 2BC9 (45) using the Create Homology Model tool in Discovery Studio 2017 (Accelrys Inc., San Diego), with modifications introduced to correct for the previously identified error in assignment of amino acids in the M2 and M3 helices (61, 62). To preserve the side-chain orientations of residues conserved between species, the nonconserved residues in each subunit (3, 17, and 11 in $\alpha$, $\beta$, and $\delta$, respectively; in $\gamma$, an added N-terminal Glu and eight residues) were mutated individually using the Build and Edit Protein Tool. To correct the placement of the M2 and M3 residues, the M1–M2 loops were shortened by four residues and the Create Homology Model tool was used to align $\alpha$Thr–237–$\alpha$Ser–302 of the sequence with $\alpha$Glu–241–$\alpha$His–306 from the structure, along with the equivalent alignments for the other subunits. The full structural model was minimized using CHARMM with the Generalized Born Implicit Membrane solvent model for 12 cycles (Smart Minimized method) to detect inappropriate residue placements and to reduce high-energy interactions (final energy, $-66,779$ kcal/mol).

$\text{F}_n\text{N}_3\text{Bzoxy-AP}$ was docked using 12-Å radius binding-site spheres centered as follows: 1) in the ion channel at the level of $\beta$Val–261 and $\delta$Val–269; and 2) at the lipid–$\alpha$ subunit interface at the level of $\alpha$Cys–222 and $\alpha$Leu–223. Each sphere was seeded with 12 distributed replicas of $\text{F}_n\text{N}_3\text{Bzoxy-AP}$ with the CDOCKER module used to generate 40 molecular dynamics-induced alterations for each replica, and then each altered structure was configured into 30 different starting orientations. The docking results for binding in the ion channel and at the lipid–protein interface between the $\alpha$M1 and $\alpha$M4 helices are shown as Connolly surface representations defined by a 1.4-Å diameter probe of the ensemble of 12 solutions with the lowest CDOCKER interaction energies. Similar docking results were obtained when $\text{F}_n\text{N}_3\text{Bzoxy-AP}$ was docked in these sites of a T. californica homology nAChR model (43) based upon the crystal structure of an $(\alpha4)_2(\beta2)_3$ nAChR (Protein Data Bank entry 5KXI (63).

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