A photoreactive analog of allopregnanolone enables identification of steroid-binding sites in a nicotinic acetylcholine receptor

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Many neuroactive steroids potently and allosterically modulate pentameric ligand-gated ion channels, including GABA_A receptors (GABA_ARs) and nicotinic acetylcholine receptors (nAChRs). Allopregnanolone and its synthetic analog alfaxalone are GABA_A-positive allosteric modulators (PAMs), whereas alphaxalone and most neuroactive steroids are nAChR inhibitors. In this report, we used 11β-(p-azidotetrafluorobenzoyloxy)allopregnanolone (F4N3Bzoxy-AP), a general anesthetic and phototoxic allopregnanolone analog that is a potent GABA_A PAM, to characterize steroid-binding sites in the Torpedo α6βγδ nAChR in its native membrane environment. We found that F4N3Bzoxy-AP (IC50 = 31 μM) is 7-fold more potent than alphaxalone in inhibiting binding of the channel blocker [3H]tetraclidine to nAChRs in the desensitized state. At 300 μM, neither steroid inhibited binding of [3H]tetraclaine, 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. These results directly indicate a steroid-binding site in the nAChR ion channel and identify additional steroid-binding sites also occupied by other lipophilic nAChR antagonists.

Many steroids, including endogenous 3α-hydroxy metabolites of progesterone and deoxycorticosterone and synthetic analogs, act as potent general anesthetics, sedatives, anxiolytics, or anticonvulsants (1, 2). They are positive allosteric modulators of muscle and neuronal nicotinic acetylcholine receptors (nAChRs), cation-selective pLGICs (6–8), and negative allosteric modulators of muscle and neuronal nicotinic acetylcholine receptors (nAChRs), cation-selective pLGICs (6–8). The complex effects of steroid PAMs on GABA_A gating and the locations of residues identified by mutational analyses as steroid sensitivity determinants suggest the existence of multiple steroid-binding sites in the transmembrane domain (TMD) of heteromeric GABA_ARs (9, 10). Recent crystal structures of chimeric homopentameric receptors containing GABA_AR α subunit TMDs identify a binding site for steroid PAMs in the TMD at subunit interfaces (11, 12), which is distinct from the intersubunit sites in the extracellular third of the TMD for etomidate, propofol, and barbiturates identified in heteromeric GABA_ARs by photolabeling and mutational analyses (13, 14). A distinct intrasubunit site for pregnenolone sulfate, an inhibitory steroid, was also identified in a chimeric homomeric receptor TMD (11).

For muscle-type nAChRs, single-channel analyses of the acute effects of natural and synthetic steroids indicate a reduction of open channel lifetime (15–17), but it is not known whether this results from steroid binding within the ion channel or indirectly as a consequence of interactions at the lipid interface. As cholesterol is present in high concentrations in synaptic membranes and is important for facilitating agonist-induced conformational transitions, inhibition by steroids may result from perturbation of nAChR–cholesterol interactions (18–20). Mutations of some amino acids in the M4 helices that are exposed at the lipid interface have as large an effect on channel gating as the substitutions at channel-lining residues (21, 22). Also, substitutions at a position in αM4 exposed at the lipid interface reduced hydrocortisone potency (23). A muta-

The abbreviations used are: PAM, positive allosteric modulator; TMD, transmembrane domain; F4N3Bzoxy-AP, 11β-(p-azidotetrafluorobenzoyloxy)allopregnanolone; TID, 3-(trifluoromethyl)-3-(m-iodophenyl)diazirine; R-mTFD-MPAB, R-1-methyl-S-allyl-S-(3-(trifluoromethyl)-diazirinylphenyl) barbituric acid; GABA_A, γ-aminobutyric acid type A receptor; nAChR, nicotinic acetylcholine receptor; Carb, carbamylcholine; PCP, phenycyclidine; TCP, pentycyclidine; ACh, acetylcholine; V8 protease, S. aureus endopeptidase Glu-C; EndoLYS-C, L. enzymogenes endopeptidase Lys-C; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tricine, N-(12-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; PTH, phenylthiohydantoin; rpHPLC, reversed-phase high-performance liquid chromatography; pLGIC, pentameric ligand-gated ion channel.

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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 photoaffinity-labeling 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, mephobarbital, and etomidate, have been shown to bind to sites in the TMD, with the inter- and intra-subunit sites (24, 25).

Promegestone, a progestin steroid with intrinsic photolabeling activity (Fig. 1), is a potent inhibitor of Torpedo nAChRs expressed in Xenopus oocytes and of binding of [3H]phencyclidine (PCP), a channel blocker, to nAChR-rich membranes (26). Whereas [3H]promegestone photolabeled amino acids at the lipid interface in the M4 helices, no evidence was found of photolabeling ion channel residue sites. However, promegestone may have bound in the ion channel without efficient photolabeling.

To further characterize steroid-binding sites in the Torpedo nAChR, we now use 11β-(p-azidotetrafluorobenzoyloxy)allopregnanolone (F4N3Bzoxy-AP) (Fig. 1), a photoreactive allopregnanolone derivative that is a potent general anesthetic and GABA_A-positive allosteric modulator (27). In contrast to promegestone, 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 11-position in the steroid C-ring (28, 29). Because a nAChR agonist containing the same photoreactive group photolabeled aliphatic and aromatic amino acid side chains within the ACh-binding sites in the nAChR extracellular domain (30), F4N3Bzoxy-AP should have the capacity to photointegrate into many of the amino acid side chains in the nAChR TMD.

We found that, similar to promegestone, F4N3Bzoxy-AP inhibited binding of a channel blocker in the nAChR-desensitized state. In contrast to promegestone, [3H]F4N3Bzoxy-AP photolabeled residues in the nAChR ion channel in the desensitized state (positions βM2–13 and δM2–13), numbered from the 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 α subunit, identified by photolabeling of αCys-222/αLeu-223 at the interface between the M1 and M4 helices, and a site near the cytoplasmic surface of the TMD, identified by photolabeling of γTryp-453 in γM4, a residue photolabeled by [3H]promegestone.

Results

Radioligand 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 αβγδ 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]F4N3Bzoxy-AP (3 µM) 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. 3B–D). As seen by fluorography, after irradiation at 365 nm, the nAChR subunits photolabeled most prominently were the α and γ 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 (34 kDa) and ADP/ATP carrier (32 kDa).

[3H]F4N3Bzoxy-AP photoincorporation into Torpedo nAChR-rich membranes. Membrane suspensions equilibrated with [3H]F4N3Bzoxy-AP (3 µM) 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 gel for liquid scintillation counting (Fig. 3, left). Irradiation and the binding of the channel blocker [3H]PCP to nAChR-rich membranes (35). Irradiation at 254 nm resulted in prominent, pharmacologically-specific photolabeling in the nAChR ion channel (36).

Photolabeling with [3H]F4N3Bzoxy-AP photoincorporation into nAChR membranes (36). Irradiation at 254 nm resulted in prominent, pharmacologically-specific photolabeling in the nAChR ion channel (36).

Photolabeling with [3H]F4N3Bzoxy-AP photoincorporation into nAChR membranes (36). Irradiation at 254 nm resulted in prominent, pharmacologically-specific photolabeling in the nAChR ion channel (36).
and then rpHPLC (Fig. 4A). When the fragment beginning at βMet-249 was sequenced from the major peak of 3H (Fig. 4B), the peak of 3H 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 3H established the presence of the fragment beginning at δMet-257 (Fig. 4D). The peak of 3H 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 [3H]F₄N₃Bzoxy-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 [3H]F₄N₃Bzoxy-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 3H 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 (I₀ = 310 pmol, both conditions). In the presence of Carb there were peaks of 3H release at cycle 13, 16, and 20, consistent with photolabeling of the channel-lining residues δM2–13’, -16’, and -20’ (δVal-249, δM2–13’, -16’, and -20’; Fig. 5B). Promegestone reduced photolabeling of δVal-269 by ~50%.

Table 1

<table>
<thead>
<tr>
<th>Labeled residues</th>
<th>365 nm control</th>
<th>254 nm control</th>
<th>( \frac{\text{cpm/pmol}}{\text{Carb}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>βVal-261 (M2–13’)</td>
<td>&lt;0.1/1.4 ± 0.9 (3)</td>
<td>&lt;0.1/5.0 ± 2.6 (9)</td>
<td></td>
</tr>
<tr>
<td>δVal-269 (M2–13’)</td>
<td>&lt;0.05/0.4 ± 0.2 (3)</td>
<td>&lt;0.05/0.23 ± 0.08 (5)</td>
<td></td>
</tr>
<tr>
<td>γTrp-453 (M4)</td>
<td>7 ± 4 (2/6) (1)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>αCys-222 (M1); αLeu-223*</td>
<td>ND</td>
<td>0.7 ± 0.2 (2/4) ± 1.7 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*Because αLeu-223 immediately follows αCys-222, which is photolabeled more efficiently, the efficiency of photolabeling of αLeu-223 cannot be calculated reliably.

[3H]F₄N₃Bzoxy-AP photolabeling of amino acids in the Torpedo nAChR transmembrane domain

The photolabeling efficiency (cpm/pmol of the PTH-derivative) for each residue was calculated from the observed 3H release, the initial peptide mass (I₀), and repetitive yield (R) as described under “Experimental procedures.” Tabulated values are the mean ± S.D., with the number of sequencing runs indicated in parentheses. With the exception of γTrp-453, the photolabeling efficiencies were determined from two (365 nm; αCys-222 at 254 nm) or three (254 nm; M2–13’) independent preparative photolabelings. ND means not determined.
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 3H eluted at ~70% organic solvent B where the fragment containing αM1 is known to elute (40, 43), with little 3H eluting in the more hydrophobic fractions where the fragment containing αM2 elutes. Sequence analysis of fractions containing the peak of 3H (Fig. 6B) established the presence of the αHis-186 fragment (I0 = 4 pmol), with no release of 3H above background in 15 cycles of Edman degradation. Similarly, the fragment beginning at αMet-243 (I0 = 1 pmol), which eluted at ~85% organic solvent B, was sequenced without any 3H release above background in 15 cycles of Edman degradation that extended to αM2–15’ (Fig. 6C). These results indicated that the 3H 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 (I0 = 60 pmol, each condition), isolated by rpHPLC from tryptic digests of nAChRs (36, 43), there was a peak of 3H 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 3H 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 3H release was in cycle 14 rather than 13 (Fig. 6F).

([3H]F4N3Bzoxy-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]promegestone, 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]promegestone 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 3H 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 βVal-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 γLeu-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 3H 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 photoprobe (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 aM3 and aM4 subunits by sequencing for 30 cycles of Edman degradation of fragments that begin at Thr-276 and Thr-281 and extend through the M3 helices. The protocol used (47) allowed efficient recovery of the fragments (0.3–90 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 less than 0.3 cpm/pmol.

[3H]F4N3Bzoxy-AP photolabeling in the δ subunit helix bundle

No photolabeling was detected of the residues in δM1 (δTyr-228 and δPhe-232), δM2 (δM2–18), or δM2–M3 loop (δile-288) within the intrasubunit-binding site near the extracellular end of the δ subunit helix bundle 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 δM2–18, if it occurred, was at less than 5% the level of δM2–13 (Fig. 5B), and based upon sequencing the fragment beginning at δPhe-208 (l0 = 120 pmol), any labeling of δPhe-232 or δCys-236 was at less than 10% the level of labeling of aM2–13 or δCys-232.

Discussion

In this report, we use F4N3Bzoxy-AP, a photoreactive analog of alphaxalone and allopreganolone that is a potent general anesthetic and GABAAR PAM (27), to identify the locations of steroid-binding sites in the Torpedo nAChR in its native membrane environment. Many endogenous (glucocorticoids and progesterone) and synthetic (alphaxalone and promegestone) steroids act as noncompetitive antagonists of 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 Torpedo nAChR responses and the binding of a desensitized state selective channel blocker, photolabeled amino acids...
Steroid-binding sites in an αβγδ nAChR

The photolabeling of βVal-261 and δVal-269 provides direct evidence that a steroid can bind in proximity to position M2–13’ in the ion channel. Based upon the [3H]TCP- and [3H]tetracontaine-binding assays, F4N3Bzoxy-AP binds in the ion channel in the desensitized state (IC50 = 30 μM) 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 helices, 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 F4N3Bzoxy-AP, bulkier photoreactive etomidate and barbitalloy 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 αM2 or γM2 could be isolated for sequencing than βM2 or δM2, it remains possible that there is also unidentified labeling in those fragments at the same efficiency as δVal-269. The large quantities of βM2 and δM2 fragments allowed identification of agonist-dependent photolabeling in βM2 and δM2, even though it comprised a smaller fraction of subunit labeling than that seen for the α subunit (Fig. 3). There was, however, agonist-enhanced PCP-inhibitable α subunit labeling in nAChRs photolabeled at 254 nm, with the agonist-enhanced labeling inhibitable by 300 μM promegestone. At the amino acid level, promegestone at 100 μM reduced photolabeling of βVal-261 and δVal-269 by ~50%. Because promegestone is more potent than F4N3Bzoxy-AP as an inhibitor of [3H]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.

F4N3Bzoxy-AP binding at the nAChR–lipid interface

The photolabeling of γTrp-453 in γM4 provides evidence that F4N3Bzoxy-AP interacts with the same region of γM4 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 γCys-451 (42). However, it was surprising not to find labeling of any positions in αM4, which is photolabeled at αCys-412 and other residues by promegestone, diazofluorene, TID, and many general anesthetics (Fig. 8C). The lack of labeling of αCys-412 cannot result from an inability of F4N3Bzoxy-AP to form a stable adduct with cysteines, because αCys-222 in αM1 was one of the residues labeled most efficiently. One explanation for this unexpected result is that when F4N3Bzoxy-AP binds in proximity to αM4,
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 F₄N₃Bzoxo-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 F₄N₃Bzoxo-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 [³H]R-mTFD-MPPB (36); and (iii) the amino acids (cyan) in αM1 (αLeu-231) and γM3 (γMet-299) photolabeled by the anesthetic barbiturate [³H]R-mTFD-MPAB (36); and (iv) the amino acids (orange) in αM4, αM1 (αPhe-227/αLeu-228), and γM3 (γPhe-292, γLeu-296, and γAsn-300) photolabeled by [¹²⁵I]TID, which also labels αThr-422 and αVal-425 (orange) but not γTrp-453 or αHis-408 (42); (ii) αVal-218 (green) in αM1, photolabeled by a convulsant barbiturate [³H]R-mTFD-MPPB (43); (iii) the amino acids (cyan) in αM1 (αLeu-231) and γM3 (γMet-299) photolabeled by the anesthetic barbiturate [³H]R-mTFD-MPAB (36); and (iv) the amino acids (orange) in αM4, αM1 (αPhe-227/αLeu-228), and γM3 (γPhe-292, γLeu-296, and γAsn-300) photolabeled by [¹²⁵I]TID, which also labels αCys-222/αLeu-223 (42). C and D, locations of F₄N₃Bzoxo-AP (molecular volume = 448 Å³) docked in the binding sites are shown as Connolly surface representations of the volumes defined by the 10 most energetically favorable binding poses (ion channel, volume = 942 Å³ and αM1/αM4, volume = 596 Å³).

Figure 8. F₄N₃Bzoxo-AP-binding sites in the Torpedo nAChR. A, T. californica nAChR homology model was constructed based on the T. marmorata nAChR structure (PDB code 2BG9 (45)), with modifications to correct for the previously identified error in assignment of amino acids in the M2 and M3 helices (see under “Computational docking”). A, side view of the nAChR extracellular and transmembrane domains (α, yellow; β, brown; γ, green; δ, cyan). B, view of the nAChR TMD from the base of the extracellular domain, C, side view from the lipid of the γ-α subunit interface. D, side view of the ion channel with an α subunit omitted for visualization of photolabeled residues. The amino acids photolabeled by [³H]F₄N₃Bzoxo-AP are shown in stick representation in the ion channel (β and D, magenta), in αM1 (β and C, αCys-222/αLeu-223 (red)), and in γM4 (β and C, γTrp-453, black)). Also included in stick representation in C are: (i) the amino acids in αM4 and γM4 (purple) photolabeled by [³H]promegestone, which also photolabels γTrp-453 (26), and by [¹²⁵I]TID, which also labels αThr-422 and αVal-425 (orange) but not γTrp-453 or αHis-408 (42); (ii) αVal-218 (green) in αM1, photolabeled by a convulsant barbiturate [³H]R-mTFD-MPPB (43); (iii) the amino acids (cyan) in αM1 (αLeu-231) and γM3 (γMet-299) photolabeled by the anesthetic barbiturate [³H]R-mTFD-MPAB (36); and (iv) the amino acids (orange) in αM4, αM1 (αPhe-227/αLeu-228), and γM3 (γPhe-292, γLeu-296, and γAsn-300) photolabeled by [¹²⁵I]TID, which also labels αCys-222/αLeu-223 (42). C and D, locations of F₄N₃Bzoxo-AP (molecular volume = 448 Å³) docked in the binding sites are shown as Connolly surface representations of the volumes defined by the 10 most energetically favorable binding poses (ion channel, volume = 942 Å³ and αM1/αM4, volume = 596 Å³).

Steroid-binding sites in an αβγδnACHR
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. F$_2$N$_3$Bzoxo-AP and [3H]F$_2$N$_3$Bzoxo-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; 4 nM radioligand, and 0.5 mM diisopropylphosphofluoridate to inhibit acetylcholinesterase; for [3H]TCP and [3H]tetracaine binding, membranes were equilibrated with 0.4 mM CaCl$_2$ overnight (41, 42) were recovered from independent experiments were combined for analysis.

Radioligand binding assays

Centrifugation equilibrium binding assays of [3H]ACh, [3H]TCP, and [3H]tetracaine to Torpedo nAChR-rich membranes were performed in Torpedo saline buffer (in mM: 250 NaCl, 5 KCl, 3 CaCl$_2$, 2 MgCl$_2$, 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 disopropylphosphofluoridate to inhibit acetylcholinesterase; for [3H]TCP and [3H]tetracaine, 500 nM ACh-binding sites and 10 mM radioli- gand. 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 defined in the presence of 100 μM Carb, PCP, or tetracaine, respectively. Stock solutions of F$_2$N$_3$Bzoxo-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, $f_o$, the specifically bound [3H] (cpm$_{total}$ – cpm$_{nonspecific}$), was normalized to $f_o$ 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. A concentration-dependent inhibition of [3H]TCP binding was fit to the equation, $f_x/f_o = 1/((1 + x/IC_{50})$, where IC$_{50}$ is the total ligand concentration producing the half-maximal inhibition of binding. The numbers of independent experiments are noted in the figure legends.

[3H]F$_2$N$_3$Bzoxo-AP photolabeling and gel electrophoresis

Conditions for photolabeling were identified by characterizing the absorption and photolysis characteristics of F$_2$N$_3$ Bzoxo-AP in methanol. At the absorption maximum of 264 nm, the F$_2$N$_3$Bzoxo-AP extinction coefficient was 17,550 ± 450 M$^{-1}$ cm$^{-1}$. Photolysis with a 254-nm lamp (Spectronics EF160C) produced a progressive shift of the maximum absorption from 264 to 285 nm with a $t_{1/2}$ 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 $t_{1/2}$ of 17 min. Based upon these results, nAChR-rich membranes equilibrated with [3H]F$_2$N$_3$Bzoxo-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]F$_2$N$_3$Bzoxo-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 GelCodeTM 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]F$_2$N$_3$Bzoxo-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 photola- beled amino acids, nAChR-rich membranes were photolabeled on a preparative scale in five experiments with [3H]F$_2$N$_3$ Bzoxo-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 NH$_4$HCO$_3$, 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 (Vivascience, 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) on 15% polyacrylamide, 0.76% bisacrylamide mapping gels. The resultant subunit fragments (αV8–20, αV8–10, γV8–24, and γV8–14) 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 NH$_4$HCO$_3$, 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 CaCl$_2$, overnight (β subunit) or for
3 days (α subunit and αV8–10). αV8–20, γV8–24, and γV8–14 and aliquots of α and δ 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 β (40%), γ (25%), and δ (40%) subunits were digested with 200 μg 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 β and δ 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 3H elution was determined by liquid scintillation counting of 10% of each fraction.

With the exception of rpHPLC fractions containing αM4, which were loaded onto polyvinylidene difluoride membrane filters at room temperature using Applied Biosystems ProSorb® sample preparation cartridges, all rpHPLC fractions containing 3H-labeled peptides were drop-loaded at 45 °C onto Applied Biosystems Micro TFA glass fiber filters. After loading on filters, samples were treated with Biobrene 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 [3H]F₄N₃Bzoxo-AP photo-labeling in αM1, samples containing the fragment beginning at αHis-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 αMet-207 before αM1.

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 3H 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. \( I_o \), 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 \( I_e = I_i R^x \), where \( I_i \) 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 \( (2 \text{cpm}_x - \text{cpm}_(x−1))/I_0 R^x \), where \( \text{cpm}_x \) is the 3H release in cpm at cycle \( x \). When 1/6 of the material was used for PTH-derivative determination, \( E(x) = ((\text{cpm}_x - \text{cpm}_(x−1))/(5 I_0 R^x) \).

**Computational docking**

A T. californica nAChR homology model was constructed based on the cryo-EM–derived structure of Torpedo marmorata nAChR (PDB code 2BG9 (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 preserved between species, the nonconserved residues in each subunit (3, 17, and 11 in α, β, and δ, respectively; in γ, 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 αThr-237–αSer-302 of the sequence with αGlu-241–αHis-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).

\( F_{N3}Bzoxo-AP \) was docked using 12-Å radius binding-site spheres centered as follows: 1) in the ion channel at the level of βVal-261 and δVal-269; and 2) at the lipid–α subunit interface at the level of αCys-222 and αLeu-223. Each sphere was seeded with 12 distributed replicas of \( F_{N3}Bzoxo-AP \) 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 αM1 and αM4 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 \( F_{N3}Bzoxo-AP \) was docked in these sites of a T. californica homology nAChR model (43) based upon the crystal structure of an (α4)₂(β2)₃ nAChR (Protein Data Bank entry 5KXI (63).)

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