Enantiomeric barbiturates bind distinct inter- and intrasubunit binding sites in a nicotinic acetylcholine receptor (nAChR)

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Nicotinic acetylcholine receptors (nAChRs) and γ-aminobutyric acid type A receptors (GABA\(_A\)Rs) are members of the pentameric ligand-gated ion channel superfamily. Drugs acting as positive allosteric modulators of muscle-type α\(_2\)β\(γ\)δ nAChRs, of use in treatment of neuromuscular disorders, have been hard to identify. However, identification of nAChR allosteric modulator binding sites has been facilitated by using drugs developed as photoreactive GABA\(_A\)R modulators. Recently, R-1-methyl-5-allyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid (R-mTFD-MPAB), an anesthetic and GABA\(_A\)R potentiator, has been shown to inhibit Torpedo α\(_2\)β\(γ\)δ nAChRs, binding in the ion channel and to a γ\(^{-}\)α\(^{-}\) subunit interface site similar to its GABA\(_A\)R intersubunit binding site. In contrast, S-1-methyl-5-propyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid (S-mTFD-MPPB) acts as a convulsant and GABA\(_A\)R inhibitor. Photolabeling studies established that S-mTFD-MPPB binds to the same GABA\(_A\)R intersubunit binding site as R-mTFD-MPAB, but with negative rather than positive energetic coupling to GABA binding. We now show that S-mTFD-MPPB binds with the same state (agonist) dependence as R-mTFD-MPAB within the nAChR ion channel, but it does not bind to the intersubunit binding site. Rather, S-mTFD-MPPB binds to intrasubunit sites within the α and δ subunits, photolabeling αVal-218 (αM1), δPhe-232 (δM1), δThr-274 (δM2), and δIle-288 (δM3), Propofol, a general anesthetic that binds to GABA\(_A\)R intersubunit sites, inhibited \(^{3}H\)S-mTFD-MPPB photolabeling of these nAChR intrasubunit binding sites. These results demonstrate that in an nAChR, the subtle difference in structure between S-mTFD-MPPB and R-mTFD-MPAB (chirality; 5-propyl versus 5-allyl) determines selectivity for intra- versus intersubunit sites, in contrast to GABA\(_A\)Rs, where this difference affects state dependence of binding to a common site.

Excitatory nicotinic acetylcholine receptors (nAChRs)\(^2\) and inhibitory γ-aminobutyric acid type A receptors (GABA\(_A\)Rs)

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2 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; PAM, positive allosteric modulator; TMD, transmembrane domain; mTFD-MPPB, 1-methyl-5-propyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid; R-mTFD-MPAB, R-1-methyl-5-allyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid; GABA\(_A\)R, γ-aminobutyric acid type A receptor; Carb, carbachol; PCP, phencyclidine; TCP, tenocyclidine; ACh, acetylcholine; AziPm, 2-isopropyl-5-(3-trifluoromethyl)-3H-diazirin-3-ylphenol; TID, 3-(trifluoromethyl)-3-(m-iodophenyl)diazirine; V8 protease, S. aureus endopeptidase Glu-C; EndoLys-C, L. enzymes endoproteinase Lys-C; TPCP, L-1-tosyl-amido-2-phenylethyl chloromethyl ketone; OPA, o-phthalaldehyde; PTH, phenylthiohydantoin; rpHPLC, reversed-phase high-performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(3hydroxymethyl)ethyl]glycine.
intrasubunit drug binding sites are present in pockets within each subunit helix bundle and intersubunit binding sites in pockets at each subunit interface.

Studies using photoreactive analogs of general anesthetics, including etomidate, propofol, and mephobarbital that act as GABAAR PAMs but as nAChR inhibitors, have identified two homologous classes of intersubunit binding sites in the TMD of GABAARs (Fig. 1A; reviewed in Refs. 18 and 19). In nAChRs, these drugs each bind to sites in the ion channel and to intrasubunit sites within the nAChR subunit helix bundle and/or intersubunit sites at the γ−α− subunit interface, where a photoreactive etomidate analog that acts as a low efficasy PAM also binds (20, 21).

In GABAARs, the photoreactive anesthetic barbiturate R-mTFD-MPPB (Fig. 1B) binds selectively to sites at the γ−α− interface (22, 23). R- and S-mTFD-MPPB act as GABAAR PAMs (22). S-mTFD-MPPB, differing from R-mTFD-MPPB only in terms of chirality and a 5-propyl versus 5-allyl substituent, acts in vivo as a convulsant and as an αβγδ nAChR inhibitor, whereas R-mTFD-MPPB acts as a PAM (24). Direct GABAAR photolabeling with [3H]S-mTFD-MPPB established that it binds to the same sites as R-mTFD-MPAB, but with opposite state dependence; R-mTFD-MPAB binds with highest affinity in the presence of GABA, whereas S-mTFD-MPPB binds preferentially in the presence of the inverse agonist bicuculline (25).

R- and S-mTFD-MPAB act as potent inhibitors of the Torpedo αβγδ muscle-type nAChR, each binding with high affinity to a site in the ion channel in the desensitized state and with R-mTFD-MPAB also binding to a site at the γ−α− interface (26). In this report, we identify the Torpedo nAChR amino acids photolabeled by [3H]S-mTFD-MPPB to determine whether or not it binds to the same sites as R-mTFD-MPAB. We find that both barbiturates bind to the same site in the ion channel. In addition, S-mTFD-MPPB binds in the transmembrane domain to intrasubunit sites within the α and δ subunits, but not to the intersubunit site that binds R-mTFD-MPAB. These results provide the first demonstration of the subtle difference in structure that is sufficient to determine drug selectivity for inter- or intrasubunit sites in a heteromeric nAChR.

Results

Radioligand binding assays

The effect of mTFD-MPPB on the equilibrium binding of [3H]ACh was determined at [3H]ACh concentrations sufficient to occupy ~20% of nAChR-binding sites to allow determination of enhancement or reduction of binding. Similar to R-mTFD-MPAB (26), S- and R-mTFD-MPPB each increased [3H]ACh binding with EC50 values of 6.4 ± 2.3 and 7.6 ± 2.8 μM, respectively (Fig. 2A and Table 1). The ~20% maximal enhancement of binding indicated lower efficacy as a desensitizing agent than for proadifen, a prototypic desensitizing antagonist (27) that in parallel experiments increased binding by 30% (EC50 = 0.95 ± 0.40 μM).

We also characterized the effects of S- and R-mTFD-MPPB on the binding of cationic channel blockers that bind preferentially to the nAChR ion channel in the desensitized state stabi-
Inter- and intrasubunit nAChR anesthetic binding sites

lized by the agonist Carb ([3H]trenocyclidine ([3H]TCP), a PCP analog (28, 29)) or in the resting, closed channel state stabilized by α-bungarotoxin ([3H]tetracaine (30, 31)). As shown in Fig. 2B and Table 1, in the presence of Carb, S- and R-mTFD-MPPB each fully inhibited [3H]TCP binding with IC50 values of 6.6 ± 0.9 and 3.1 ± 0.3 μM, respectively. In contrast, in the presence of α-bungarotoxin, R-mTFD-MPPB inhibited [3H]tetracaine binding with an IC50 of 408 ± 27 μM, whereas S-mTFD-MPPB even at 300 μM did not inhibit binding (Fig. 2B and Table 1).

These results establish that S- and R-mTFD-MPPB each bind in the ion channel with >100-fold higher affinity in the nAChR-desensitized state than in the resting, closed channel state.

[3H]S-mTFD-MPPB photolabeling of Torpedo nAChR-rich membranes

After irradiation, membrane suspensions were fractionated by SDS-PAGE, and the covalent incorporation of [3H]S-mTFD-MPPB was characterized by fluorography (Fig. 3A) and by liquid scintillation counting of bands excised from the stained gels (Fig. 3B). In the absence of other drugs (control conditions), the nAChR α subunit was labeled most prominently. Photoincorporation into each nAChR subunit was enhanced in the presence of agonist (Carb) compared with control. Tetracaine did not reduce subunit photolabeling in the absence of agonist, but the enhanced nAChR subunit photolabeling in the presence of Carb was reduced in the presence of PCP or R-mTFD-MPPB.

For the nAChR α, β, and γ subunits, PCP or R-mTFD-MPPB reduced photolabeling to levels close to that in the control condition, whereas for the δ subunit, the inhibition was partial. These results suggest that the Carb-enhanced nAChR subunit photolabeling results from [3H]S-mTFD-MPPB photolabeling in the ion channel in the nAChR-desensitized state, with an additional PCP-insensitive binding site within the nAChR δ subunit.

To provide an initial characterization of the location of photolabeled residues within a nAChR subunit, we used in-gel digestion of labeled α subunits to generate four large non-overlapping fragments of 4 kDa (αV8-4, predominately starting from αSer-1), 18 kDa (αV8-18, beginning at αThr-52 and con-

**Table 1**

| Ligands          | [3H]ACh           | [3H]TCP           | [3H]Tetracaine    |
|------------------|------------------|------------------|------------------|
|                  | IC50 μM | Emax % | n | IC50 μM | n | IC50 μM | n |
| S-mTFD-MPPB      | 6.4 ± 2.3 | 118 ± 2 | 4 | 6.6 ± 0.9 | 5 | >1000 | 2 |
| R-mTFD-MPPB      | 7.6 ± 2.8 | 119 ± 1 | 2 | 3.1 ± 0.3 | 2 | 408 ± 27 | 2 |
| Prazosin         | 0.95 ± 0.40 | 130 ± 6 | 4 | ND | ND | ND | ND |

For each independent equilibrium binding assay, binding at each modulator concentration was determined in duplicate, and the specific binding at each concentration was normalized to the total specific binding in the absence of modulator. n, number of independent experiments; ND, not determined.

Figure 2. Effects of S-mTFD-MPPB (△, □), R-mTFD-MPPB (▼, ■) or proadifen (○) on the equilibrium binding to Torpedo nAChR-rich membranes of [3H]ACh (A) and [3H]TCP (+ Carbo) and [3H]tetracaine (+ α-bungarotoxin) (B). Binding assays were performed at 4 °C by centrifugation. Each independent experiment was performed in duplicate, and the data were normalized to the specific binding in the absence of competitor. Pool data (average ± S.D.) are plotted. See Table 1 for the number of independent experiments and the calculated IC50/EC50 values. For all samples, the final ethanol concentration was 1% (v/v), a concentration that reduced [3H]TCP and [3H]tetracaine binding by <10% and enhanced [3H]ACh binding by <10%. The total control/nonspecific binding was as follows: for [3H]ACh, 2,600/60 cpm; for [3H]TCP, 6,200/1,600 cpm; for [3H]tetracaine, 4,300/1180 cpm.

Figure 3. [3H]S-mTFD-MPPB photoincorporation into Torpedo nAChR-rich membranes. [3H]S-mTFD-MPPB (0.9 μM) was photoincorporated in the absence (control, lane 1) or presence of 100 μM tetracaine (lane 2), 1 mM Carb (lane 3), 1 mM Carb and 100 μM PCP (lane 4), or 1 mM Carb and 60 μM R-mTFD-MPPB (lane 5), and aliquots in duplicate were fractionated by SDS-PAGE. After staining the gel with GelCode Blue Safe Protein Stain, one set was prepared for fluorography (Figs. 3A, lane 3, 4, 5), and gel bands were excised from the second for 1H determination (B). The electrophoretic mobilities of the nAChR α, β, γ, and δ subunits, rapyn (Rnl), and the Na+/K+/ATPase α subunit (αNa/K) are indicated on the left of A. C, 1H incorporation in the large nAChR α subunit fragments generated by in-gel digestion of α subunits with V8 protease. Gel bands containing α subunits were isolated by SDS-PAGE from nAChR-rich membranes photolabeled with 1.5 μM [3H]S-mTFD-MPPB in the absence (control) or presence of 1 mM Carb, without or with 100 μM PCP (Carb and Carb/PCP, respectively). The gel bands containing αV8-20, αV8-18, αV8-10, and αV8-4 were excised from the stained mapping gel, and 1H incorporation was determined by liquid scintillation counting.
no peaks of $^3$H release were detected when the fragment beginning at His-186 was isolated by in-gel digestion of α subunits with V8 protease. A. $^3$H elution profiles for EndoLys-C digests of αV8-20 fractionated by rpHPLC. B and C. $^3$H (control (○)), Carb (●), and Carb/PCP (▲) released during sequence analysis of fragments containing αM2 (●) and αM1 (c) from rpHPLC fractions 30–32 and 25–28, respectively. B, when sequencing the fragment beginning at αMet-243 (Ile-9 = 5 (■), 9 (■), and 17 (▲) pmol), the major peak of $^3$H release in cycle 6 indicates photolabeling in the presence Carb of serotonin. For this sample, the efficiencies of photolabeling (cpm/pmol) were 10-fold higher in the presence of Carb than in its absence, and PCP inhibited that photolabeling by 90%. The efficiencies of photolabeling were then treated with CNBr to cleave at αMet-207. C, bottom, when sequencing was continued from αGln-208 (Ile-9 = 10 (■), 16 (▲) pmol), the peak of $^3$H release in cycle 11 was consistent with photolabeling of αVal-218 at efficiencies of 0.2 cpm/pmol (control), 1.6 cpm/pmol (Carb), and 5 cpm/pmol (Carb/PCP). D. $^3$H (Carb (●) and Carb/PCP (▲)) and PTH-derivatives (Carb (■) and Carb/PCP (▲)) released during sequence analysis of a fragment beginning at Val-218 (Ile-9 = 22 pmol, both conditions) isolated by rpHPLC from trypsin digests of α subunits from an independent photolabeling of nACH-rich membranes with 0.4 μM [3H]-mTFD-MPPB in the presence of 0.4 mM Carb and 100 μM PCP. Sequencing filters were treated with OPA at cycle 2 to prevent further sequencing of any fragments not containing a proline in that cycle. The peak of $^3$H release in cycle 9 confirmed photolabeling of αVal-218 at efficiencies of 3 cpm/pmol (Carb) and 12 cpm/pmol (Carb/PCP).

Figure 4. Identification of [3H]-mTFD-MPPB photolabeled amino acids within αM1 and αM2. nACH-rich membranes were photolabeled with 0.4 μM [3H]-mTFD-MPPB in the absence of other drugs (control; ○), or in the presence of 1 mM Carb (●), or 1 mM Carb and 100 μM PCP (▲), and αV8-20 was isolated by in-gel digestion of α subunits with V8 protease. A. $^3$H elution profiles for EndoLys-C digests of αV8-20 fractionated by rpHPLC. B and C. $^3$H (control (○)), Carb (●), and Carb/PCP (▲) released during sequence analysis of fragments containing αM2 (●) and αM1 (c) from rpHPLC fractions 30–32 and 25–28, respectively. B, when sequencing the fragment beginning at αMet-243 (Ile-9 = 5 (■), 9 (■), and 17 (▲) pmol), the major peak of $^3$H release in cycle 6 indicates photolabeling in the presence Carb of serotonin. For this sample, the efficiencies of photolabeling (cpm/pmol) were 10-fold higher in the presence of Carb than in its absence, and PCP inhibited that photolabeling by 90%. The efficiencies of photolabeling were then treated with CNBr to cleave at αMet-207. C, bottom, when sequencing was continued from αGln-208 (Ile-9 = 10 (■), 16 (▲) pmol), the peak of $^3$H release in cycle 11 was consistent with photolabeling of αVal-218 at efficiencies of 0.2 cpm/pmol (control), 1.6 cpm/pmol (Carb), and 5 cpm/pmol (Carb/PCP). Sequencing filters were treated with OPA at cycle 2 to prevent further sequencing of any fragments not containing a proline in that cycle. The peak of $^3$H release in cycle 9 confirmed photolabeling of αVal-218 at efficiencies of 3 cpm/pmol (Carb) and 12 cpm/pmol (Carb/PCP).

Inter- and intrasubunit nACH anesthetic binding sites

[3H]-mTFD-MPPB photolabels residues in αM2 and αM1.

To identify photolabeled amino acids within αV8-20, this fragment was isolated from nACHRs photolabeled on a preparative scale in three conditions (control, Carb, and Carb/PCP). When EndoLys-C digests of αV8-20 were fractionated by rpHPLC (Fig. 4A), there were peaks of $^3$H eluting at ~60 and ~80% organic solvent, where fragments beginning at αHis-186 and extending through αM1 and at αMet-243, the N terminus of αM2, respectively, are known to elute (33). $^3$H within both peaks was increased in the presence of agonist, but PCP strongly reduced labeling only in the more hydrophobic peak.

For nACHRs labeled in the presence of Carb, sequence analysis of the fragment beginning at αMet-243 revealed a major peak of $^3$H release in cycles 5 and 6 with additional peaks in cycles 9 and 13, consistent with photolabeling of αLeu-247, αSer-248, αLeu-251, and αVal-255 at positions M2′-5′, M2′-6′, M2′-9′, and M2′-13′ that line the lumen of the ion channel (Fig. 4B). The efficiencies of photolabeling (cpm/pmol) at M2′-5′ and -6′ were 10-fold higher in the presence of Carb than in its absence, and PCP inhibited that photolabeling by ~90%, whereas both the state dependence and PCP sensitivity were reduced at M2′-9′ (Table 2).

Sequence analysis of the fragment beginning at αHis-186 from nACHRs photolabeled in the absence of agonist revealed no peaks of $^3$H release during 15 cycles of Edman degradation, which included the core aromatics αTyr-190 and αTyr-198 of ACh binding site segment C (top panel in Fig. 4C). To identify labeling within αM1, the filter was then treated with CNBr to cleave at αMet-207 before M1. Sequencing through αM1 then identified a single peak of $^3$H release in cycle 11, consistent with photolabeling of αVal-218, but only for the sample from nACHRs photolabeled in the presence of Carb and PCP (bottom panel in Fig. 4C and Table 3).

To confirm [3H]-mTFD-MPPB photolabeling of αVal-218 in αM1, fragments beginning at αLeu-210 were isolated for sequence analysis by rpHPLC from trypsin digests of α subunit (26) from an independent photolabeling experiment in the presence of Carb or Carb plus PCP (Fig. 4D). The peak of $^3$H release in cycle 9 confirmed photolabeling of αVal-218 at ~3-fold higher efficiency in the presence of Carb and PCP than
in the presence of Carb alone (Table 3). There was no evidence of photolabeling of α-Leu-231, the residue in αM1 at the γ2-α2 interface photolabeled by [3H]-mTFD-MPPB (26). In Torpedo nAChR structural models based either upon cryo-electron microscopy analyses of Torpedo nAChR-rich membrane tubular crystals (34) or X-ray structure of expressed, purified (α4)(β2)3 human nAChR (16), α-Val-218 projects into the α subunit helix bundle pocket (see “Discussion”).

Table 2
[3H]S-mTFD-MPPB photolabels δ subunit residues in the ion channel and in the helix bundle pocket

The photolabeling efficiency (cpm/pmol of PTH derivative) for each residue was calculated from the observed 3H release, the initial peptide mass, and repetitive yield as described under “Experimental procedures.” For n = 2, the average efficiencies ± S.D. are tabulated.

Table 3
Pharmacological specificity of [3H]S-mTFD-MPPB photolabeling of Torpedo nAChR amino acids within intrasubunit binding pockets in the α and δ subunit helix bundle pockets (cpm/pmol of PTH derivative)

The photolabeling efficiency (cpm/pmol of PTH derivative) for each residue was calculated from the observed 3H release, the initial peptide mass, and repetitive yield as described under “Experimental procedures.” For Experiment 1, single samples were sequenced to determine photolabeling efficiency in the absence of Carb (Econtrol), and two independent experiments were carried out in the presence of Carb to determine photolabeling efficiencies in the absence (Econtrol) and presence of carb (Ecarb). To take into account the differences in Econtrol between experiments, the effect of Carb was quantified as the ratio Ecarb/Econtrol for each paired experiment. For Experiment 2, two αM1 and δM2 samples and single samples for the δ subunit fragments were sequenced. The effect of propofol was quantified as the ratio E propofol/Ecarb for each sample. Averages ± S.D. were tabulated when two samples were sequenced.
elutes, and a minor peak of $^3$H eluted at ~60% organic where the fragment beginning at $\delta$Phe-206 before $\delta$M1 elutes. Sequence analysis of the fragment beginning at $\delta$Met-257 (Fig. 5B) showed that for nAChRs labeled in the presence of Carb, there was a major peak of $^3$H release in cycle 9 with smaller peaks of release in cycles 6, 13, 17, and 18, indicating primary photolabeling at $\delta$M2-9' in the ion channel with lower level photolabeling of channel lining residues at $\delta$M2-6', 13', and 17' as well as labeling of $\delta$M2–18' ($\delta$Thr-274) that contributes to the $\delta$ subunit helix bundle. Photolabeling of M2-6' and M2-9' was at 5–10-fold higher efficiency in the desensitized state (Carb) than in the absence of agonist, whereas PCP in the presence of Carb inhibited photolabeling at M2-6' and M2-9' by ~90 and ~70%, respectively, with little, if any, inhibition of photolabeling at M2-13' and M2-18' (Tables 2 and 3).

Sequence analysis of the fragment beginning at $\delta$Phe-206 (Fig. 5C) revealed a single major peak of $^3$H release at cycle 27, consistent with photolabeling of $\delta$Phe-232 in S1M, for the sample from nAChRs labeled in the presence of agonist. That residue was photolabeled at >10-fold higher efficiency in the presence of Carb than in its absence, and PCP in the presence of Carb slightly enhanced rather than inhibited photolabeling (Table 3).

**Propofol inhibits intrasubunit binding site photolabeling**

Propofol, a widely used intravenous anesthetic and GABA$_A$R PAM, binds to intersubunit binding sites in GABA$_A$Rs (37). In Torpedo nAChRs, propofol acts as a desensitizing negative allosteric modulator and, based upon inhibition of $^3$H[AziPm photolabeling, it binds in the $\delta$ subunit helix bundle pocket and also within the ion channel (35). To determine whether propofol also bound in the $\alpha$ subunit helix bundle pocket, we examined the effects of propofol on $[^3]$H$S$-mTBD-MPPB photolabeling in the presence of Carb with PCP included to enhance $[^3]$H$S$-mTBD-MPPB photolabeling $\alpha$M1 and minimize photolabeling in the ion channel. As shown in Fig. 6A and Table 3, sequence analysis through $\alpha$M1 established that propofol inhibited photolabeling of $\alpha$Val-218 in the presence of Carb and PCP. Similarly, sequence analysis of photolabeling in $\delta$M1 and $\delta$M2 (Fig. 6B and C) established that propofol also inhibited photolabeling in the $\delta$ helix bundle pocket of $\delta$Phe-232 and $\delta$Thr-274 (Table 3) as well as in the ion channel ($\delta$M2-9', -13', and -17'). Consistent with the results of Fig. 5B, no photolabeling of $\delta$M2-6' was seen in the presence of PCP. These results indicate that in Torpedo nAChRs, S-mTBD-MPPB binds to sites within the $\alpha$ and $\delta$ subunit helix bundle pockets, and propofol inhibits binding at both sites.

$[^3]$H$S$-mTBD-MPPB photolabeling in $\beta$M2 and $\gamma$M2

To extend the characterization of photolabeling in the M2 ion channel domain, we also sequenced fragments beginning at the N termini of $\beta$M2 and $\gamma$M2, fragments beginning at $\beta$Met-249 that can be isolated from subunit trypsin digests by SDS-PAGE and rpHPLC (38) and at $\gamma$Cys-252 that can be isolated by rpHPLC from EndoLys-C digests of subunit fragments produced by in-gel digestion with V8 protease fragment (26, 39). Sequencing through $\beta$M2 from nAChRs photolabeled in the presence of agonist (Fig. 7A) revealed major peaks of $^3$H release in cycles 6 and 9, with additional peaks in cycles 13 and 17, consistent with photolabeling ion channel residues $\delta$M2-6', -9', -13', and -17'. Sequencing through $\gamma$M2 (Fig. 7B) revealed a major peak of $^3$H release in cycle 6 with an additional peak in cycle 9. As seen for photolabeling in $\alpha$M2 and $\delta$M2, labeling efficiency at M2-6' was increased by ~7-fold in the presence of agonist compared with the absence, and PCP strongly inhibited photolabeling at M2-6' and -9', but not at M2-13' or M2-17' (Table 2).

**Photolabeling in the M3 helices**

Inspection of nAChR structural models allows identification of residues in M3 helices that are predicted to be exposed to lipid, to intersubunit interfaces, or to the intrasubunit helix bundles pocket. $[^3]$H$R$-mTBD-MPAB photolabeled residues in $\gamma$M3 (Figs. Met-299) and $\alpha$M1 ($\alpha$Lue-231) that contribute to a binding pocket at the $\gamma'$–$\alpha'$ interface (26). Photolabeling of

Figure 6. Propofol inhibits $[^3]$H$S$-mTBD-MPPB photolabeling in $\alpha$M1 ($\alpha$Val-218), $\delta$M1 ($\delta$Phe-232), and $\delta$M2. Fragments containing $\alpha$M1 (A), $\delta$M1 (B), and $\delta$M2 (C) were isolated for sequence analysis from nAChR-rich membranes photolabeled with 0.4 $\mu$M $[^3]$H$S$-mTBD-MPPB in the presence of 1 mM Carb + 100 $\mu$M PCP (\(\nabla\)) or 1 mM Carb + 100 $\mu$M PCP + 100 $\mu$M propofol (\(\bigcirc\)). Shown are $[^3]$H$\gamma$Val, $[^3]$H$\delta$Val, and PTH-derivatives (\(\nabla\), \(\bigcirc\)) released during sequence analysis of fragments beginning at allele-210 (A, $\upsilon_0 = 50$ pmol, each condition, sequencing filters treated with OPA at cycle 2), $\delta$Phe-206 (B, $\upsilon_0 = 45$ pmol, each condition), and $\delta$Met-257 (C, $\upsilon_0 = 100$ pmol, each condition). The peaks of $^3$H release in cycles 9 (A) and 27 (B) indicated photolabeling of $\alpha$Val-218 at 1.7 cpm/pmol (Carb/PCP) and 0.3 cpm/pmol (Carb/PCP/propofol) and of $\delta$Phe-232 at 2 cpm/pmol (Carb/PCP) and 0.8 cpm/pmol (Carb/PCP/propofol). C, the peaks of $^3$H release at cycles 9, 13, 17, and 18 and indicated photolabeling of ion channel residues $\delta$M2-9', -13', and -17' and of $\delta$Thr-274 in the $\delta$ helix bundle, with propofol inhibiting photolabeling by 60–80%.
γMet-299 was state-independent and insensitive to PCP, but it was inhibited in a concentration-dependent manner by R-mTfD-MPAB. In contrast, the hydrophobic probe [125I]TID, which also contains the trifluoromethylphenyl diazirine reactive group, photolabeled γPhe-292, γLeu-296, and γAsn-300, residues exposed at the lipid interface, and the corresponding residues in βM3 and δM3 (40). To determine whether [3H]S-mTfD-MPPB photolabeled γMet-299 or other residues in γM3, we isolated and sequenced a fragment beginning at Met-299 or other residues in γM3. In the presence of Carb and Carb plus PCP (Fig. 8), the major peaks of [3H] release in cycles 6 and 9 (Carb) indicate photolabeling of γM3 (35), but not by [3H]S-mTfD-MPPB (26, 35, 40). Although present, sequencing of those fragments was prevented by treatment of the sequencing filters with OPA in cycle 6, which blocks further sequencing of peptides not containing a proline at that cycle (44, 54).

All three residues were also photolabeled in an agonist-dependent manner by [125I]TID (41, 42) and by the photoreactive propofol analog [3H]AzipeM (35), but not by [3H]R-mTfD-MPAB (26).

[3H]S-mTfD-MPPB labeling in αM4

To further examine photolabeling at the lipid interface, we also sequenced the fragment beginning at αTyr-401, which contains αM4 (Fig. 9). Within αM4, the single major peak of [3H] release in cycle 12 indicated photolabeling of αCys-412 at similar efficiency in the absence or presence of Carb or Carb plus PCP. αCys-412 is the residue in αM4 labeled most efficiently by [125I]TID, [3H]AzipeM, and [3H]R-mTfD-MPAB (26, 35, 40). Similar to [3H]R-mTfD-MPAB, the efficiency of [3H]S-mTfD-MPPB photolabeling of αCys-412 (~60 pmol/μmol), although state-independent, was similar to that seen for the ion channel residues labeled most efficiently in the nAChR-desensitized state.
Discussion

S-mTFD-MPPB, a convulsant in vivo, acts as an inhibitor of αβγ GABA<sub>R</sub>Rs, whereas R-mTFD-MPAB, which differs only by chirality and the presence of a 5-allyl rather than 5-propyl substituent, acts as an anesthetic in vivo and as an αβγ GABA<sub>R</sub> PAM (22, 24). In α1β3γ2 GABA<sub>AR</sub>Rs, photoaffinity labeling studies showed that S-mTFD-MPPB and R-mTFD-MPAB bind to the same binding site in the TMD at the γ<sup>+</sup>−β<sup>−</sup> subunit interface, but with the opposite state dependence and in different orientations (23, 25). S-mTFD-MPPB binds preferentially in the presence of bicuculline, an inverse agonist, whereas R-mTFD-MPAB binds preferentially in the presence of GABA. In a muscle-type nAChR, S-mTFD-MPAB acts as an inhibitor, binding to sites in the TMD in the ion channel and at the γ<sup>−</sup>−α<sup>−</sup> subunit interface (26).

To determine whether S-mTFD-MPPB and R-mTFD-MPAB also bind to the same binding sites in a nAChR, in this report, we used radioligand binding assays and photoaffinity labeling to identify binding sites in the Torpedo nAChR for S-mTFD-MPPB. We found that S-mTFD-MPPB binds to the same site in the nAChR ion channel in the desensitized state as R-mTFD-MPAB and with similar high affinity. However, our results establish that S-mTFD-MPPB does not bind to the intersubunit site that binds R-mTFD-MPAB. Rather, S-mTFD-MPPB binds to homologous intrasubunit sites in the α and δ subunits in pockets formed by each subunit’s bundle of transmembrane helices. Furthermore, propofol, but not the positively charged channel blocker PCP, inhibits binding of S-mTFD-MPPB to those intrasubunit sites. Whereas anesthetics, including halothane, propofol, and the photoreactive propofol analog Azipm, have been shown previously to bind in a state-dependent manner within the δ subunit helix bundle pocket (35, 43), this is the first time, to our knowledge, that anesthetics or other drugs have been found to bind within the α subunit intrasubunit site.

A comparison of S-mTFD-MPPB and R-mTFD-MPAB actions and binding sites in Torpedo nAChR and α1β3γ2 GABA<sub>AR</sub> is shown in Table 4. The locations of the amino acids photolabeled by [3H]-S-mTFD-MPPB that define the ion channel and intrasubunit binding sites are shown in Fig. 10 in a Torpedo californica nAChR homology model based upon the recently determined structure of an expressed human (α4)<sub>3</sub>(β2)3 nAChR (16). Also shown in Fig. 10 (B−E) are the most energetically favorable binding poses predicted by computational docking for S-mTFD-MPPB in each of the binding sites.

**Table 4**

Comparison of interactions of R-mTFD-MPAB and S-mTFD-MPPB with Torpedo α1βγδ nAChR and α1β3γ2 GABA<sub>AR</sub>

| Drugs          | Activity | Binding sites | State dependence | Activity | Binding sites | State dependence |
|----------------|----------|---------------|-------------------|----------|---------------|-------------------|
| R-mTFD-MPAB    | Inhibitor| Ion channel   | Desensitized      | Enhancer | Intersubunit (α<sup>−</sup>−β<sup>−</sup> and γ<sup>−</sup>−β<sup>−</sup>) | Desensitized (+ GABA) |
| S-mTFD-MPPB    | Inhibitor| Ion channel   | No                | Inhibitor| Intersubunit (γ<sup>−</sup>−β<sup>−</sup>) | Resting (+ bicuculline) |
|                |          | Intersubunit  | Desensitized      |          |               |                    |
|                |          | (α and δ)     |                   |          |               |                    |

Figure 9. [3H]-S-mTFD-MPPB photolabels αCys-412 in αM4. [3H] (control (○), Carb (●), and Carb/PCP (▲)) and PTH-derivatives (control (□), Carb (■), and Carb/PCP (△)) released during sequencing are shown for the fragment beginning at αM4-401 (61 ng [16 pmol], and 8 ng [7 pmol]) isolated by rHPLC from a tryptic digest of αM4-10 from the photolabeling experiment of Fig. 5. The peak of [3H] release at cycle 12 indicates photolabeling of αCys-412 at efficiencies of 50 cpm/pmol (control and Carb) and 70 cpm/pmol (Carb/PCP).

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mTFD-MPAB photolabeled αLeu-231, with any photolabeling of αVal-218, if it occurred, at <5% the efficiency of αLeu-231 photolabeling (26). αLeu-231 contributes to a binding pocket in the γ′–α– interface (Fig. 10D), where [3H]R-mTFD-MPAB also photolabels γMet-299 at 10-fold higher efficiency than αLeu-231. [3H]S-mTFD-MPPB photolabeling of γMet-299 or other residues in γM3, if it occurs, is at <25% the efficiency of αVal-218. In addition to R-mTFD-MPAB, photoreactive analogs of etomidate also bind to this intersubunit site (44, 45). [14C]Halothane (43) and a bupropion analog ([125I]SADU-3-72 (46)) each photolabel αTyr-213 at the extracellular end of αM1. Although within 9 Å of αVal-218, in current structural models, αTyr-213 projects more toward the γ′–α– interface than into the intrasubunit pocket (Fig. 10D). Early studies with
S-mTFD-MPPB and propofol bind within the δ subunit helix bundle pocket

As seen for other nAChR negative allosteric modulators, including [14C]halothane (43), [125I]TID (36, 41), and the photoreactive analogs ([3H]TFD-etalomide and [3H]Azipm (35, 45)), [3H]-S-mTFD-MPPB photolabeled in an agonist-dependent manner residues contributing to the δ subunit helix bundle pocket (Fig. 10E). [125I]TID photolabeling was greatly enhanced in the nAChR open channel and transient desensitized states compared with the equilibrium desensitized state (36, 42), and further studies using rapid-mixing and freeze-quench techniques will be necessary to determine whether [3H]S-mTFD-MPPB has a similar state dependence. Propofol inhibition of [3H]-S-mTFD-MPPB photolabeling of these residues is consistent with its previously reported inhibition of [3H]Azipm photolabeling (35). In contrast to the enhanced [3H]-S-mTFD-MPPB photolabeling of αVal-218 in the presence of PCP, little, if any, enhancement was seen for photolabeling in the δ subunit helix bundle. That PCP did not enhance δ subunit photolabeling serves as a control that the enhanced photolabeling seen at αVal-218 results from positive allosteric coupling between PCP binding in the channel and the α intrasubunit site and is not simply due to an increase in the free [3H]-S-mTFD-MPPB concentration resulting from its displacement by PCP from the ion channel.

S-mTFD-MPPB binding in the nAChR ion channel

Barbiturates of diverse structures act as state-dependent inhibitors of Torpedo nAChR (47), and they probably bind to sites in the ion channel because they fully inhibit binding of channel blockers (48, 49). Our photolabeling results establish that S-mTFD-MPPB binds to the same region in the ion channel as R-mTFD-MPAB (26) and with the same >10-fold selectivity for the desensitized state compared with the resting, closed channel state. Both barbiturates photolabel residues at M2-6’ and M2-9’ most efficiently and also photolabel M2-13’ and M2-17’. Consistent with the location in the agonist-stabilized desensitized state of the high affinity PCP binding site near the cytoplasmic end of the ion channel (50) and the capacity of PCP and uncharged anesthetics to bind simultaneously in the channel, PCP fully inhibited photolabeling at the level of M2-6’ but did not inhibit labeling at the level of M2-13’ or M2-17’. Whereas S-mTFD-MPPB and R-mTFD-MPAB, which are N-methylated, bind in the ion channel preferentially in the desensitized state, many barbiturates lacking the N-methyl group bind preferentially in the resting, closed channel state (49). In recently solved crystal structures of the cationic prokaryotic nAChR homolog GLIC in a locally closed state, the barbiturate binding site in the ion channel has also been localized to the level of M2-2’ to M2-9’ (51).

S-mTFD-MPPB and R-mTFD-MPAB each bind with higher affinity to a site in the ion channel than to intra- or intersubunit sites, and it is binding to the ion channel site that most likely produces nAChR desensitization and inhibition for nAChRs equilibrated with either drug. However, further studies defining the kinetics of inhibition and kinetics of binding to these different classes of sites will be necessary to determine whether either or both barbiturates act primarily as an open channel blocker upon transient exposure to drug and agonist. Electrophysiological and time-resolved photolabeling studies have shown that TID binding to the δ subunit intrasubunit site contributes to inhibition upon initial exposure and that binding in the ion channel occurs more slowly (36).

Computational docking calculations

Based upon calculated CDOCKER interaction energies, S-mTFD-MPPB is predicted to bind to the α (−49 kcal/mol) and δ (−40 kcal/mol) subunit intrasubunit sites and with lower affinity in the ion channel (−37 kcal/mol at the cytoplasmic end centered near M2-2’, −28 kcal/mol at the level of M2-6’ and -9’). However, the results of these calculations also predict that R-mTFD-MPAB binds with similar affinity as S-mTFD-MPPB to the intrasubunit sites that it does not photolabel and that S-mTFD-MPPB binds with similar affinity as R-mTFD-MPAB (−30 kcal/mol) to the γ′–α′ site that it does not photolabel. Potentially, the use of improved lipid-embedded nAChR structural models and docking algorithms in conjunction with molecular dynamics simulations may facilitate computational predictions consistent with the experimental evidence that S-mTFD-MPPB binds to intrasubunit sites, whereas R-mTFD-MPAB binds to an intersubunit site.

Functional significance of intrasubunit and intersubunit binding sites in muscle-type nAChR

There is great interest in developing PAMs for muscle-type nAChRs that could be of use in the treatment of ALS, myasthenia gravis, and other neuromuscular disorders. However, this has proven challenging, as most general anesthetics that act as GABA_A-R PAMs (21) and many neuronal nAChR PAMs act as potent α_2βδ nAChR channel blockers (11, 12). For Torpedo

Figure 10. S-mTFD-MPPB binding sites in the Torpedo nAChR. A. T. californica nAChR homology model was constructed based on the crystal structure of human α(�)d(ኔ), nAChR (Protein Data Bank entry 5XKI (16)). A, side view of the nAChR extracellular and transmembrane domains (α (yellow), β (brown), γ (green), and δ (light blue) with nicotine (red Connolly surface) in the ACh-binding sites and the ion channel in blue. B, a view of the nAChR TMD from the base of the extracellular domain. C, the binding site in the ion channel. D and E, views from the lipid of the γ′–α′ subunit interface (D) and the δ subunit TMD (E), at a tilt angle optimizing visualization of the α and δ subunit helix bundle pockets. The amino acids photolabeled by [3H]-mTFD-MPPB are shown in stick representation in the ion channel (B and C, pink), in the α subunit helix bundle (B and D; αVal-218 (red)), and in the δ subunit helix bundle (B and E; 8βPhe-232 (red), 8Thr-274 (yellow), and 8Leu-288 (green)). In C–E, the locations of S-mTFD-MPPB (molecular volume = 269 Å³) docked in the binding sites are shown in stick representations (carbon (gray), hydrogen (white), oxygen (red), nitrogen (blue), and fluorine (cyan)) in the most favorable binding mode and/or as Connolly surface representations of the volumes defined by the ensemble of the 10 most energetically favorable binding poses. Also highlighted in D and E are the amino acids photolabeled by [3H]-mTFD-MPAB (magenta), 8βLeu-231, and 8γMet-299 (26) at the γ′–α interface, by [14C]halothane (red, αγTr-213, and 8Thr-228 (43)) in the helix bundle pockets, and by [125I]TID (orange, αCys-222, αLeu-223, αPhe-227, αLeu-228, 8βPhe-292, 8γLeu-296, and γAsn-300) at the lipid interface. F, subunit sequence alignment for the M1–M3 region, with the same color coding of amino acids as shown in B–E to identify photolabeled residues.
nAChRs, studies with photoreactive anesthetics led to the identification of drugs that bind with low affinity to the ion channel, including TDBzl-epitomate, a low efficacy PAM that binds to the γ→α′ intersubunit site (44) and TFD-epitomate, a potent inhibitor that binds to that intersubunit site and to the δ subunit helix bundle pocket (45). The identification of S-mTFD-MPPB as a drug binding to the α subunit intersubunit pocket will facilitate the identification of other drugs binding potentially with higher affinity and selectivity to that site.

Experimental procedures

Materials

_Torpedo _nAChR-rich membranes_, isolated from _T. californica_ electric organs (Aquatic Research Consultants, San Pedro, CA) as described (52), contained ~1 nmol of [3H]ACh binding sites/mg of protein, as determined by an ultracentrifugation assay. R- and S-mTFD-MPPB, R-mTFD-MPAB, and S-mTFD-MPPB (49.9 Ci/mmole) were synthesized previously (22, 24, 25). Dipsopropylphosphofluoridate, tetracaine, Carc, PCP, propofol, pradifen, and CNBr were from Sigma-Aldrich. [3H]ACh (30 Ci/mmole) was synthesized by esterification of choline and [3H]acetic anhydride. [3H]TCP (58 Ci/mmol) and [3H]tetracaine (30 Ci/mmole) were obtained from PerkinElmer Life Sciences and Sibtech (Newington, CT), respectively. _Staphylococcus aureus_ endopeptidase Glu-C (V8 protease) was from MP Biomedicals (Solon, OH), t-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was from _S._ aureus and [3H]tetracaine (30 Ci/mmole) was synthesized by esterification of choline and [3H]acetic anhydride. [3H]TCP (58 Ci/mmole) and [3H]tetracaine (30 Ci/mmole) were obtained from PerkinElmer Life Sciences and Sibtech (Newington, CT), respectively.

_Radioligand binding assays_

Equilibrium binding of [3H]ACh, [3H]TCP, or [3H]tetracaine to _Torpedo_ nAChR-rich membranes in _Torpedo_ physiological saline buffer (250 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, and 5 mM sodium phosphate, pH 7.0) was determined by centrifugation as described (45). In brief, membrane suspensions were pre-equilibrated with radioligand for 30 min on ice and then incubated with various concentrations of non-radioactive drugs for 1 h at 4 °C before centrifugation at 18,000 × g for 45 min. After removal of the supernatants, membrane pellets were resuspended in 200 μl of 10% SDS overnight, with pellet and supernatant [3H] determined by liquid scintillation counting. For [3H]ACh binding, membrane suspensions (40 nm ACh-binding sites) were pretreated with 0.5 mM diisopropylphosphofluoridate for 15 min to inhibit acetylcholinesterase activity before incubation with 4 nM [3H]ACh. [3H]TCP and [3H]tetracaine binding was determined with membrane suspensions containing 500 nM ACh-binding sites and 10 nM radioligand. For [3H]TCP binding, nAChRs were stabilized in the desensitized state by preincubation for 30 min with the agonist Carb at 1 mM. For [3H]tetracaine, membranes were preincubated with the competitive antagonist α-bungarotoxin at 5 μM to stabilize nAChRs in the resting, closed channel state. Nonspecific binding of [3H]ACh, [3H]TCP, or [3H]tetracaine was determined in the presence of 100 μM Carb, PCP, or tetracaine, respectively.

For each radioligand, f₀, the specifically bound [3H] (cpm_total – cpm_nonspecific) in the presence of competitor at concentration x, was normalized to f₀ the specifically bound [3H] in the absence of competitor. The concentration-dependent enhancement of [3H]ACh binding was fit using non-linear least squares regression (SigmaPlot version 11) to the equation, f₀/f₀ = 100 + Eₘₐₓ/(1 + IC₅₀/x), and inhibition of [3H]TCP or [3H]tetracaine binding was fit to the equation, f₀/f₀ = 100/(1 + x/IC₅₀), where EC₅₀ and IC₅₀ are the ligand concentrations producing half-maximal enhancement or inhibition, and Eₘₐₓ is the maximal enhancement of [3H]ACh binding.

[3H]-mTFD-MPPB photolabeling and gel electrophoresis

[3H]-mTFD-MPPB photolabeling of _Torpedo_ nAChR-rich membranes (0.9 nmol of ACh-binding sites/mg of protein; 2 mg of protein/ml in _Torpedo_ physiological saline buffer supplemented with 1 mM oxidized glutathione as an aqueous scavenger) was performed at 4 °C on analytical or preparative scales using 0.1 or 10 mg of protein per condition, respectively. After incubation with [3H]-mTFD-MPPB (0.4–0.9 μM) for 30 min and an additional 30-min incubation in the absence or presence of other ligands, the membranes on ice were irradiated for 30 min using a 365-nm UV lamp (model EN-280L, Spectronics Corp., Westbury, NJ) at a distance of <2 cm. After photolabeling, membrane polypeptides were resolved by Tris-glycine SDS-PAGE on gels composed of 8% polyacrylamide, 0.33% bisacrylamide and visualized with GelCode™ Blue Safe Protein Stain (ThermoFisher). For analytical photolabelings, duplicate samples were separated by SDS-PAGE, with stained subunit bands excised from one set for ³H quantification by liquid scintillation counting and the other set analyzed by fluorography using Amplify (GE Healthcare). For preparative photolabelings, bands containing the nAChR α, β, γ, and δ subunits were excised from the stained gels, and material was eluted passively for 3 days at room temperature in elution buffer (100 mM NH₄HCO₃, 2.5 mM Dl-dithiothreitol, 0.1% SDS, pH 8.4). Eluted samples were filtered, concentrated to a final volume of <400 μl by centrifugal filtration using Vivaspin 15 M, 5000 concentrators (Vivasience, Stonehouse, UK), precipitated by 75% acetone overnight at −20 °C, and resuspended in digestion buffer (15 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 8.1). For most preparative photolabelings, only 25% of the α and γ subunit gel bands were eluted, with 75% of those gel bands used for in-gel digestion with V8 protease (100 μg) on 15% polyacrylamide, 0.76% bisacrylamide mapping gels (32, 40). The resultant subunit fragments (αV8-20, αV8-10, γV8-24, and γV8-14) were recovered from gel bands by passive elution, concentrated, and resuspended in digestion buffer. In addition, α subunits from _Torpedo_ nAChR-rich membranes (0.5 mg of protein) photolabeled on an analytical scale with 1.5 μM [3H]-mTFD-MPPB were digested in gel by V8 protease (5 μg), with ³H distribution in the fragments determined by liquid scintillation counting.

_Proteolytic digestions_

All enzymatic digestions were performed at room temperature. From preparative photolabelings, ~50% of eluted α and β subunits and 100% of αV8-10 in digestion buffer were diluted 5-fold with 50 mM NH₄HCO₃ (pH 8.1) containing 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 2–3 days (α subunit and αV8-10), αV8-20, γV8-24, γV8-14, and 60% of δ subunit in digestion buffer were digested with 0.5 units of EndoLys-C for 2–3 days in digestion buffer. Small pore Tricine SDS-PAGE gels (16.5% T, 6% C) (36, 53) were used to fractionate the β and δ subunits after digestion in solution with trypsin and EndoLys-C, respectively. The resultant fragments were recovered from Tricine gel bands by passive elution and concentrated by centrifugation for the further purification by rpHPLC.

**rpHPLC and sequence analyses**

rpHPLC was performed as described (45) using an Agilent 1100 binary rpHPLC system, a Brownlee Aquapore BU-300 column, and a mobile phase consisting of aqueous solvent A (0.08% trifluoroacetic acid) and organic solvent B (3:2 acetonitrile/isopropyl alcohol and 0.05% trifluoroacetic acid). Material (0.08% trifluoroacetic acid) and organic solvent B (3:2 acetonitrile/isopropyl alcohol and 0.05% trifluoroacetic acid) was eluted at a flow rate of 0.2 ml/min using a non-linear gradient with solvent B increasing from 25 to 100% over 90 min. Fractions were collected every 2.5 min, and ³H was determined by counting 10%.

rpHPLC fractions containing αM1, αM4, or δM1 were loaded onto PVDF membrane filters using Applied Biosystems ProSorb™ sample preparation cartridges. All other rpHPLC fractions containing ³H-labeled peptides were drop-loaded onto the Applied Biosystems Micro TFA glass fiber filters at 45 °C. Samples were treated with Biobrene Plus after loading to stabilize the peptides on the filters and then sequenced on an Applied Biosystems Procise 492 protein sequencer. For certain samples, sequencing was interrupted at predetermined cycles to treat the filter with OPA to prevent further sequencing of other peptides not containing a proline at that cycle (52, 54). To facilitate identification of [³H]S-mTFD-MPPB photolabeling in αM1, samples containing the fragment beginning at αHis-186 were sequenced for 15 cycles, and filters were then treated with CNBr as described (17, 55) to cleave at the C-terminal side of αMet-207 before αM1.

During N-terminal sequencing, two-thirds of the material was injected into an rpHPLC system for quantifying the amino acid at each cycle, and one-third of the sample was collected for determination of ³H release. The masses of released phenylthiohydantoin (PTH)-amino acid derivatives were fit versus cycles of Edman degradation (SigmaPlot version 11) according to the equation, \( f_x = I_x \times R \), where \( f_x \) is the mass of amino acid on cycle \( x \), \( I_x \) is the initial mass of the peptide, and \( R \) is the repetitive yield of Edman degradation. The labeling efficiencies (cpm/pmol) of residues photolabeled by [³H]S-mTFD-MPPB were calculated based on the expression, \( (2 \times (\text{cpm}_{x} - \text{cpm}_{x-1}))/I_{x} \times R \).

**Computational docking**

A homology model of the T. californica nAChR was constructed based on the recently solved (3.9 Å) crystal structure of a neuronal (α4‡β2)₃ nAChR (Protein Data Bank entry 5KXI (16)) using the Create Homology Model tool in Discovery Studio 2017 (Biovia). Torpedo α sequences were substituted for the two α4 subunits, and the three β2 subunits were replaced with Torpedo β, γ, and δ subunits in their known positions relative to the α subunits (i.e. clockwise αβαγ when viewed from the extracellular side). The α4 to α substitution required a single-residue insert in loop C of the agonist binding site (Torpedo αThr-196). To align the Torpedo β, γ, and δ subunits with β2, the following adjustments were made. (i) For β and γ subunits, β2Pro-14 and β2Ser-15 were deleted. (ii) Insertions were made between β2Glu-165 and β2Val-166 of 10 (β), 8 (γ), or 12 (δ) residues in the structurally undefined agonist site loop F region. Because the structures of these large inserts are unknown, these inserts were removed from the model before docking. (iii) Four residue insertions were made in γ and δ subunit loop C regions between β2Asp-192 and β2Asp-193. (iv) A single residue was inserted for γ in the M1-M2 loop between β2Cys-237 and β2Gly-238. Nicotine molecules bound to the two agonist sites in the αβ2 structure were retained in the homology model. The model was placed in a membrane force field, and the entire model was minimized to −85,106 kcal/mol.

Initial attempts to dock S-mTFD-MPPB to a pocket between the M1, M2, and M3 helices at the extracellular end of the α or δ transmembrane helix bundles were unsuccessful. To create a pocket in this region, S-mTFD-MPPB was placed within the helix bundle between M1, M2, and M3 adjacent to the labeled residues (for both α and δ subunits), and the model was minimized to −107,840 and −84,853 kcal/mol for α and δ subunits, respectively. A binding site sphere of 12-Å radius was placed around the minimized S-mTFD-MPPB, and the sphere was seeded with 12 copies each of S-mTFD-MPPB and R-mTFD-MPAB. Each seeded molecule was subjected to 40 molecular dynamics-induced alterations, and each altered structure was rotated/translated into 40 different starting orientations. Each sampling was minimized with the residues within the sphere, the final interaction energy was determined, and the lowest-energy solutions were collected along with the predicted orientations of the bound molecules. S-mTFD-MPPB and R-mTFD-MPAB were also docked using 12-Å binding site spheres in the pocket at the γ–α interface and at three levels in the ion channel using binding site spheres centered at M2-2’, M2-6’/9’, and M2-13’/17’. For individual binding sites, docking results are displayed as Connolly surface representations defined by a 1.4-Å diameter probe of the 10 solutions with the lowest CDOCKER interaction energies.

**Author contributions**—Z. Y. and J. B. C. designed and analyzed the experiments that were performed by Z. Y. Both authors contributed to the writing of the manuscript and approved the final version of the manuscript.

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