Supplemental Figures S1-S5

Multiple Transmembrane Binding Sites for \( p \)-Trifluoromethylazirinyl-etomidate, a Photoreactive *Torpedo* Nicotinic Acetylcholine Receptor Allosteric Inhibitor

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Supplementary Figure S1. Isolation of $[^3\text{H}]$TFD-etomidate-labeled $\alpha$M2 (A and B) and $\alpha$M4 by rpHPLC fractionation of EndoLys-C digests of $\alpha$V8-20 (A and B) and trypsin digests $\alpha$V8-10(C). The $\alpha$ subunit bands isolated from preparative scale labelings (10 mg of Torpedo nAChR membranes photolabeled with 0.8 µM $[^3\text{H}]$TFD-etomidate in absence (○) or the presence of Carb (●) or tetracaine (∇)) were digested in-gel with V8 protease as described under Materials and Methods. Alpha subunit fragments $\alpha$V8-20 (A and B) and $\alpha$V8-10 (C) were recovered from the mapping gels and digested with Endo-Lys C (0.5 units, 2 weeks) and trypsin (1:1 protein to enzyme ratio; 2 days), respectively, and the digests were fractionated by rpHPLC. The elution of $^3\text{H}$ was monitored by liquid scintillation counting of a 10% aliquot from each fraction. The $^3\text{H}$ recoveries were for Endo-Lys C digests of $\alpha$V8-20 (A, −/+ tetracaine, 56/48%; B, −/+ Carb, 56/52%) and for the trypsin digests of $\alpha$V8-10 (C, −/+ Carb, >90%). N-terminal sequence analyses of $^3\text{H}$ peak fractions pooled from A (fractions 30-32), B (fractions 30-32) and C (fractions 28-31) are shown in Figures 5A, 7A and 9A, respectively. Additional fractions sequenced from A: f27-29 (−tetracaine), which contained a fragment beginning at $\alpha$His-186 (~15 pmol), with no peaks of $^3\text{H}$ release in 10 cycles, and f35-37 (− and + tetracaine), which contained the fragment beginning at $\alpha$Met-243 as the primary sequence with $^3\text{H}$ release patterns similar to those of Fig. 5A. Additional fractions sequenced from B: f35-37 (− and + Carb), which contained the fragment beginning at $\alpha$Met-243 (1.3 pmol, both conditions) with $^3\text{H}$ release profiles similar to those of Fig. 7A. For the quantification of residue photolabeling within $\alpha$M2 (cpm/pmol, Table 1), the tabulated values are the mean and range of the samples sequenced from rpHPLC fractions 30-32 and fractions 35-37.
Supplementary Figure S2. Fractionation of trypsin digests of $[^3H]$TFD-etomidate labeled β subunit by Tricine-SDS PAGE (A and B) and rpHPLC purification (C and D) of the fragments beginning at βMet-249 (βM2). The β subunits isolated from the $[^3H]$TFD-etomidate nAChR photolabelings described in Supp. Fig. S1 in the absence (open bars, ○) or the presence of tetracaine (gray bars, ▽) or Carb (solid bars, ●)) were digested with trypsin (~16 h), and the digests were fractionated on small pore (16.5%T, 6%C) Tricine SDS-PAGE gels as described under “Materials and Methods”. After electrophoresis, the Tricine gels were cut into equal slices and the polypeptides were recovered from gel slices by passive elution in 12 mls of elution buffer. A and B, the $^3H$ distribution, determined by scintillation counting of 10% of the eluates. Eluates from gel slices 6-9 were each concentrated to less than 500 µL and fractionated by rpHPLC. Representative rpHPLC fractionation profiles for gel slice #7 from A (-/+ tetracaine) and gel slice #6 from B (-/+ Carb), which run with apparent molecular mass of 8-10 kDa, are shown in C and D, respectively. $^3H$ recovery was >95%. HPLC fractions 31-32, containing the fragment beginning at βMet-249, were pooled for amino acid sequence analysis (shown in Figures 5B and 7B, βMet-249, ~40 pmol and 18 pmol, respectively). Additional fractions sequenced from A (-/+ tetracaine): gel slice #6, rpHPLC fractions 29-32, which contained the fragment beginning at βMet-249 (7 pmol) contaminated with trypsin peptides and a $^3H$ release profile similar to that in Figure 5B; gel slice #8, rpHPLC fractions 28-30, which contained the fragment beginning at βAsp-425 (30 pmol) and a $^3H$ release at cycle 15 indicating labeling of βTyr-441 (-/+ tetracaine, 14/12 cpmpmol). Sequence analysis of rpHPLC fractions 28-30 from gel slice #9 from B (-/+ Carb) is described in Figure 8B.
Supplementary Figure S3. Fractionation of EndoLys-C digests of [$^3$H]TFD-etomidate labeled δ subunit by Tricine-SDS PAGE (A and B) and purification by rpHPLC of fragments beginning at δMet-257 (δM2) and δPhe-206 (δM1). The δ subunits, isolated from the [$^3$H]TFD-etomidate nAChR photolabelings described in Supp. Fig. S1 in the absence (open bars, ○) or the presence of tetracaine (gray bars, ▽) or Carb (solid bars, ●), were digested with Endo-Lys C (0.5 units, 2 weeks), and the digests were fractionated by Tricine-SDS PAGE. The $^3$H distributions from the gels, which were processed as described in the legend of Supplementary Figure S2, are shown in A and B, and rpHPLC fractionation profiles for gel slice #5 from A and B, which run with apparent molecular mass of ~14 kDa, are shown in C and D, respectively. For nAChR photolabeled in the absence and presence of tetracaine (A, C), sequence analysis of rpHPLC fractions 27-30 is shown in Figure 5C. Sequence analysis of fractions 23-25 revealed the presence of fragment beginning at δPhe-206 (25 pmol), with $^3$H releases at cycles 27 and 31 of Edman degradation indicating labeling (<1 cpm/pmol) at δPhe-232 and δCys-236 within δM1. Sequence analysis of fractions 28-30 from the rpHPLC fractionation of gel slice #6 revealed the presence at equal levels (19 pmol) of peptides beginning at δAsn-200, with the $^3$H release profile similar to that in Figure 5B. For the nAChR labeled in the absence and presence of Carb (B, D), amino sequence analysis of rpHPLC fractions 27-30 is shown in Figure 7C, while sequence analysis of fractions equivalent to fractions 22-25 from a photolabeling at 0.4 µM [$^3$H]TFD-etomidate are shown in Figure 8C. Sequence analysis of fractions 22-25 from D revealed that EndoLys-C had cleaved at δLys-199 rather than δLys-205, with the amount of the fragment beginning at δAsn-200 (10 pmol) too low to allow successful sequencing for the 37 cycles necessary to reach the photolabeled residues in δM1.
Supplementary Figure S4. Fractionation by rpHPLC of V8 protease digests of [3H]TFD-etomidate photolabeled γ (A), δ (B), and β (C) subunits. The subunits (~150 µg), from the nAChR photolabeling of Supplemental Figure S1 in the absence (Ο) and presence (▽) of tetracaine, were digested with V8 protease (200 µg) overnight, and the digests were then fractionated by rpHPLC. N-terminal sequence analyses are shown in Figures 6A-C of the pooled fractions from A (fractions 26-28), B (fractions 27-31), and C (fractions 28-30), respectively.
Supplementary Figure S5. TFD-etomidate docks at the γ-α interface in two orientations.
Views of Torpedo nAChR transmembrane helices γM1-M3 (in green) and αM1-M3 (in gold), from the base of the extracellular domain (upper panels) and from the lipid interface (bottom panels), showing the two orientations of TFD-etomidate docked at the γ-α subunit interface. For each orientation, the volume defined by the ensemble of the 6 docking solutions with the most favorable CDOCKER interaction energies is shown in a Connolly surface representation, with the lowest energy solution in stick format. Also shown in stick format are the amino acids photolabeled by [3H]TFD-etomidate: αSer-252(αM2-10), γMet -295 and γMet-299. One orientation (left panel), with the long axis of the molecule oriented approximately parallel to the transmembrane helices, positions the diazirine carbon 6 Å from αSer-252 and 13 Å from γMet-295 or γMet-299. In the other orientation (middle panel), with the long axis approximately orthogonal to the transmembrane helices, the diazirine carbon is 4 Å from γMet-295, 7 Å from γMet-299 and 15 Å from αSer-252, while the terminal methyl group of the ester is oriented towards γM2 and αM2. The right panel shows the volume defined by the ensemble of the best 12 docking solutions for both orientations combined.