Supplemental Material

For the Biochemistry article entitled:

[^{3}H]Chlorpromazine Photolabeling of the *Torpedo* Nicotinic Acetylcholine Receptor Identifies Two State-Dependent Binding Sites in the Ion Channel

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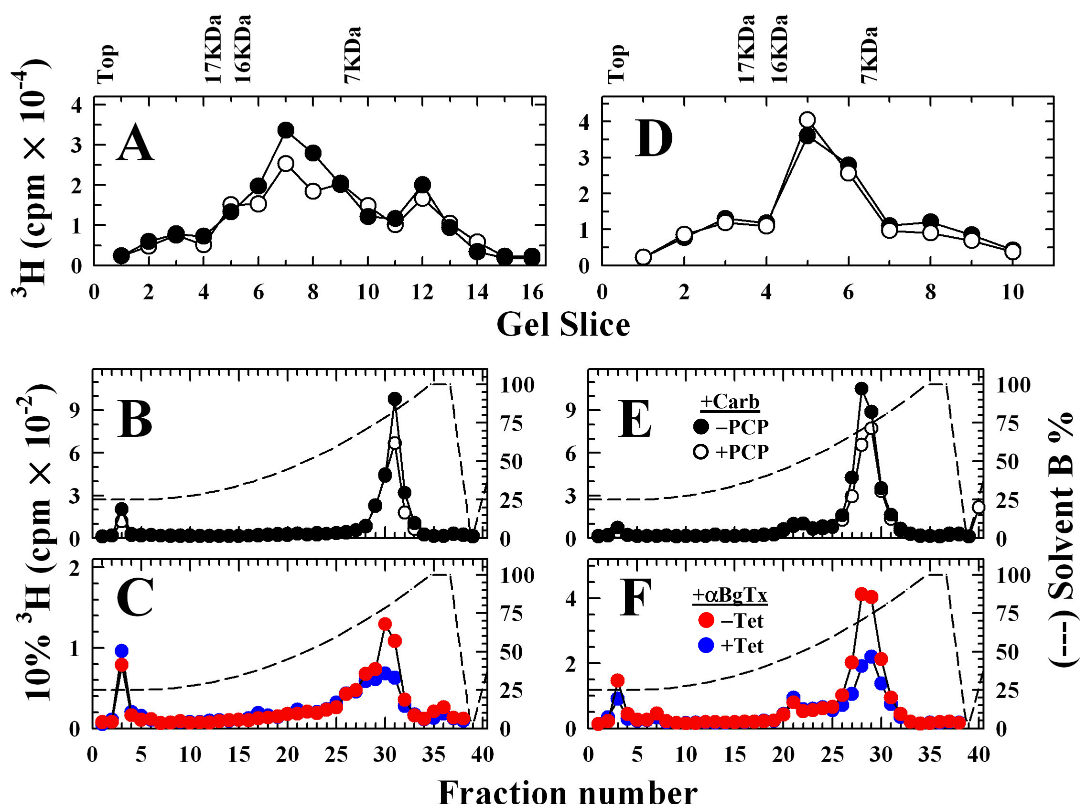
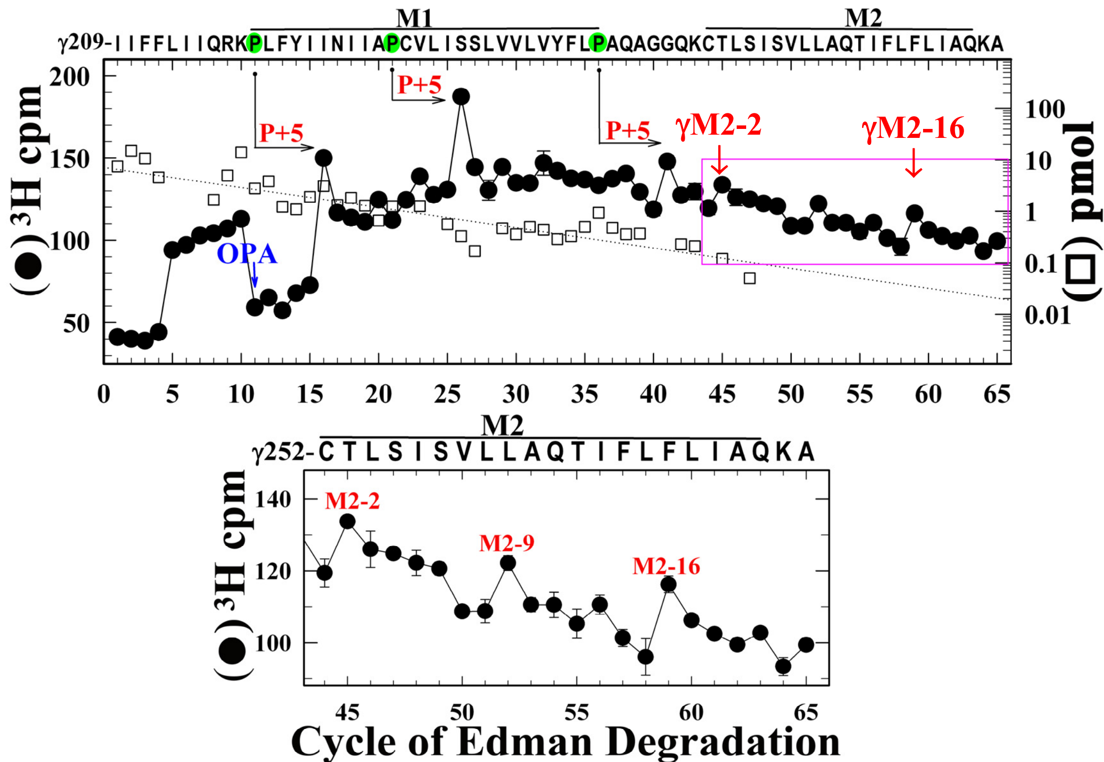
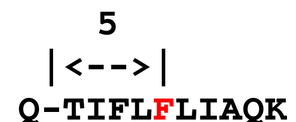
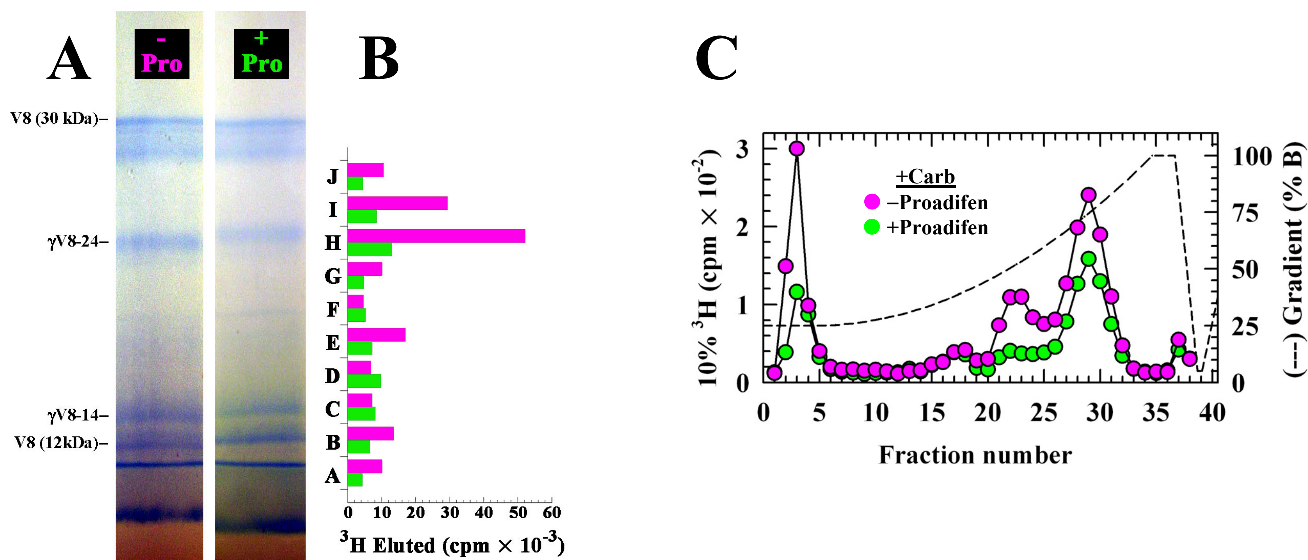


Supplemental Figure S1. Isolation of [^{3}H]CPZ-labeled fragments beginning at the N-termini of βM2 and δM2. β and δ subunits were isolated from nAChRs photolabeled with [^{3}H]CPZ +Carb with (○) or without 100 μM PCP (●) (**A, B, D,** and **E**) or +α-BgTx with (blue ●) or without 100 μM tetracaine (red ●) (**C** and **F**), digested with trypsin (β) or EndoLys-C (δ), and fractionated by Tricine SDS-PAGE (+Carb**, A** and **D**; +αBgTx, not shown). ***A,*** ^{3}H elution profile of the Tricine gel fractionation of the trypsin digests of the β subunit (+Carb, ±PCP). ***B***, ^{3}H elution profile for the rpHPLC purification of material eluted from the major radioactive gel band, which migrated with an apparent molecular mass of 10 kDa (gel slice 7; -PCP, 28,200 cpm; +PCP, 23,600 cpm). Sequence analyses of the ^{3}H peak (rpHPLC fractions 31-32; -PCP, 12,700 cpm; +PCP, 8,200 cpm) are shown in **Figure 5A.** The corresponding rpHPLC fractions from gel slice 8 contained fragments beginning at βMet-249 (15 pmol), βLys-216 and βAsp-427, each at 2-4 pmol. For gel slice 9, the fragments began at βAsp-427 (~8 pmol), βMet-249 and βLys-216 (each ~4 pmol). ***C***, ^{3}H elution profile for the rpHPLC purification of material eluted from the major radioactive band, which migrated with ~10 kDa (-tetracaine, 7,000 cpm; +tetracaine, 6,830 cpm). Sequence analyses of the ^{3}H peak (rpHPLC fractions 30-31; -tetracaine, 1,925 cpm; +tetracaine, 960 cpm) are shown in **Figure 7B**. ***D,*** ^{3}H elution profile of the Tricine gel fractionation of the EndoLys-C digests of the δ subunit (+Carb, ±PCP). ***E***, ^{3}H elution profile for the rpHPLC purification of material eluted from the major radioactive gel band, which migrated with an apparent molecular mass of 14 kDa (gel slice 5; -PCP, 35,500 cpm; + PCP, 31,300 cpm). Sequence analyses of rpHPLC fractions 27-30 (-PCP, 26,000 cpm; + PCP, 19,900 cpm) are shown in **Figure 5B**. Fractions 24-26 contained the fragment beginning at δPhe-216 (14 pmol). Fractions 28-30 from gel band 6 contained fragments beginning at δMet-257 and δAsn-437 (20 pmol each). ***F***, ^{3}H elution profile for the rpHPLC purification of material eluted from the major radioactive band, which migrated at ~14 kDa (-tetracaine, 14,300 cpm; +tetracaine, 9,840 cpm) Sequence analyses of ^{3}H peak (rpHPLC fractions 27-30; -tetracaine, 10,640 cpm; +tetracaine, 5,460 cpm) are shown in **Figure 7C**.



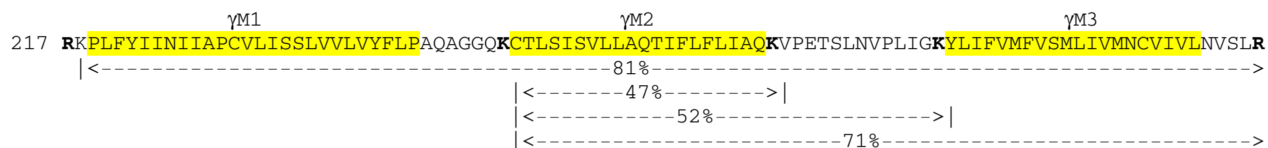
Supplemental Figure S2. Sequence analysis of [^{3}H]CPZ-labeled γM1-M2 illustrates an acid-labile Q-T peptide bond four residues before the labeled γPhe-267 (M2-16) within γM2. γ Subunit was isolated from nAChRs photolabeled with [^{3}H]CPZ (+Carb), digested with V8 protease in solution, and fractionated by rpHPLC. The ^{3}H eluted as a single broad peak centered at ~80% organic (90,700 cpm). Half of that material was sequenced for 65 cycles of Edman degradation, using liquid trifluoroacetic acid in each cleavage step, and the sequencing filter was treated with *o*-phthalaldehyde prior to cycle 11 to chemically isolate the peptide extending through γM1 and γM2 that contained γPro-219 at that cycle. The only sequence remaining after this treatment originally began at γIle-209 (□, I_0 = 7.5 pmol, R = 91 %). At each cycle containing a proline, the sequencer was programmed to increase the duration of exposure to acid for the cleavage step from 5 to 6 min and the temperature was increased from 48 to 53 °C, because the proline bond is relatively refractory to acid cleavage. The ^{3}H release pattern (●) contained an unusual sudden background increase in cycle 5, reduced upon *o*-phthalaldehyde treatment, and peaks of ^{3}H release in cycles 16, 21, and 41, each occurring 5 cycles after cleaving at a proline. While sequencing through γM2, there were minor peaks of ^{3}H release in cycle 59 (20 cpm), consistent with labeling of γPhe-267 (γM2-16), and in cycles 46 and 52, consistent with labeling of γM2-2 and γM2-9. The levels of released PTH-amino acids could be quantified only for the first 45 cycles of Edman degradation, but based upon the observed initial and repetitive yields, the calculated mass level of γPhe-267 indicates labeling at that position at ~100 cpm/pmol, and labeling at γThr-253 at ~20 cpm/pmol. As treatment with liquid trifluoroacetic acid produces preferential cleavage N-terminal of Ser or Thr and C-terminal of Asp or Asn (Hulmes and Pan, Anal. Biochem. 197: 368-376 (1991); Lin et al., Proteomics 1: 1172-1184 (2001)), the increase of background ^{3}H release in cycle 5, 5 cycles after the acid pretreatment of the sample before sequencing, and the peaks of ^{3}H release in cycles 16, 21, and 41, each 5 cycles after the prolonged exposure to acid at the proline cycles, can all be explained by the enhanced acid-lability and selective cleavage of the γGln-262/γThr-263 bond 5 cycles before [^{3}H]CPZ-photolabeled γPhe-267. In separate experiments, we confirmed that for samples containing [^{3}H]CPZ-photolabeled γM2, increased acid exposure at any cycle of Edman degradation resulted in a peak of ^{3}H release 5 cycles later, and that for samples containing γM2 with [^{125}I]TID photoincorporated at γIle-264 (White and Cohen, J. Biol. Chem. 267: 15770-17783 (1982)), increased exposure to acid resulted in ^{125}I release 2 cycles later (data not shown).



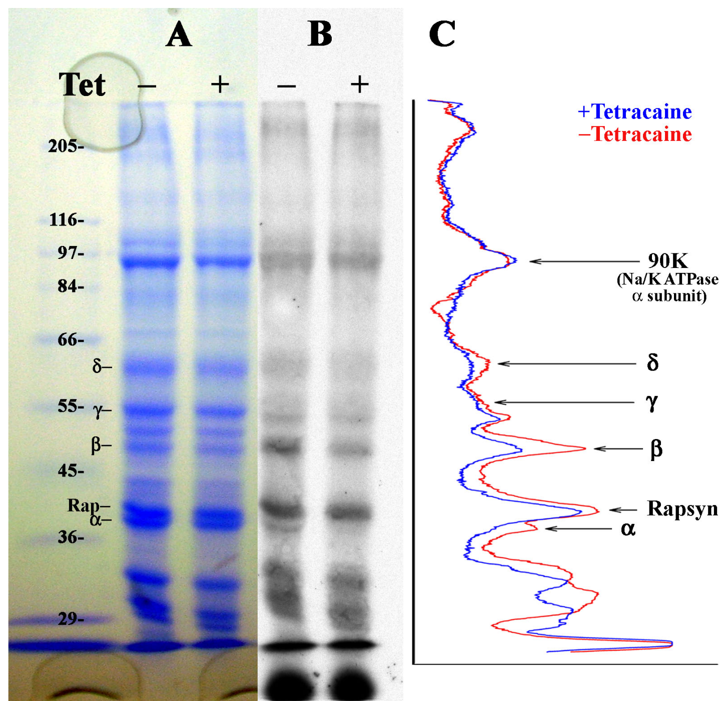


Supplemental Figure S3. Isolation of [^{3}H]CPZ labeled γM2 by "in gel" digestion with V8 protease, trypsin digestion, and reversed-phase HPLC. nAChR-rich membranes were photolabeled with [^{3}H]CPZ in the presence of 1 mM Carb with (●) or without 100 µM proadifen (●) and separated by SDS-PAGE. Gel bands containing γ subunit were placed onto a second gel and digested "in gel" with V8 protease as described in "Experimental Procedures". **A**, Coomassie blue stain of the mapping gel. **B**, Distribution of ^{3}H eluted from 1 cm strips of the gel. Material eluted from gel bands H and I (γV8-24, −proadifen, 95,200 cpm; +proadifen, 20,400 cpm) were pooled, filtered, concentrated, and acetone precipitated. Sequence analysis of an aliquot of γV8-24 confirmed that it began at γAla-167, the N-terminus of γV8-24 (Blanton and Cohen, Biochemistry 33: 2859-2872 (1994)). **C**, rpHPLC fractionation of trypsin digests of γV8-24 (−proadifen, ●, 4,820 cpm injected, 1,400 recovered; +proadifen, ●, 1,080 cpm injected, 1,220 cpm recovered). Fractions 22-23 were pooled for sequencing (**Figure 6D**). Sequence analysis of the pool of fractions 27-29 revealed a primary sequence beginning at the N termini of γM1 (γLys-218, ~0.4 pmol) at 3-5 times the level of the γM2 fragment (γCys-252). Fractions 30-32 contained the same fragments.

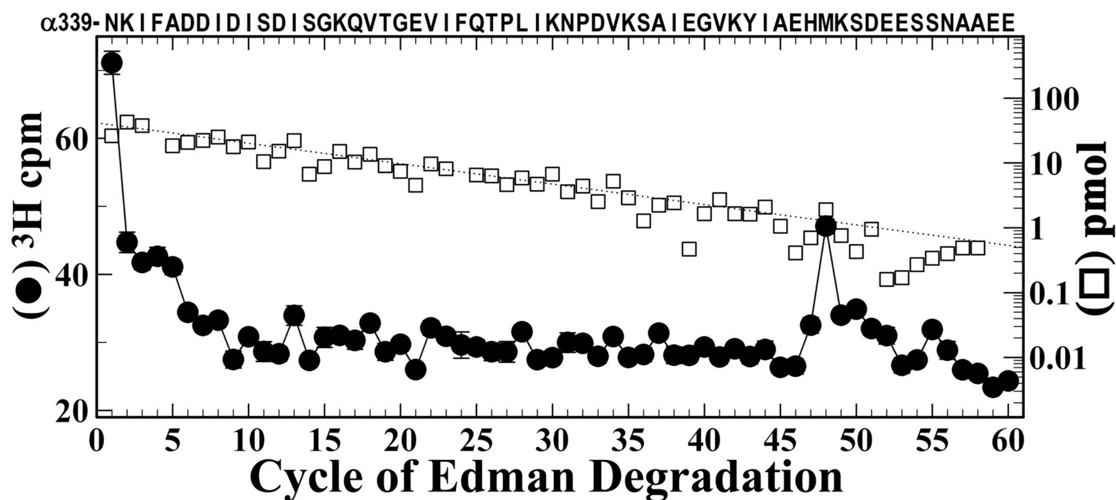
The presence of γM2 in fractions 22-23 is consistent with the predicted hydrophobicity of the fragment beginning at γCys-252 and ending at either γLys-272 or γLys-285 before γM3, as illustrated below:



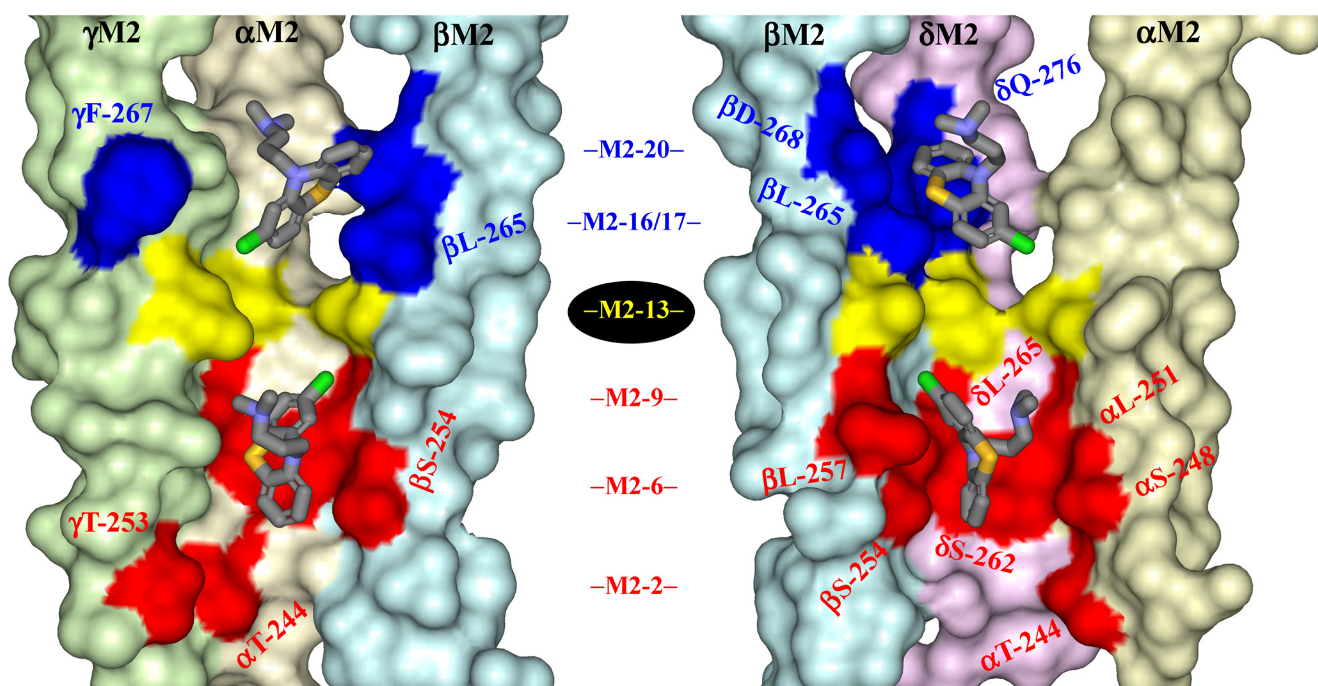
Percentages listed denote predicted organic concentration to elute the denoted peptide, calculated using peptides of known retention times on our rpHPLC system and the peptide retention prediction programs of Kangas (Petritis, K. *et. al.* Analytical Chemistry, 78:5026-5039 (2006)) and Krokhin (Krokhin, O.V. *et al* Molecular Cell Proteomics, 3: 909-919 (2004)) as employed in the Normalized Elution Time (NET) Prediction Utility from the Pacific Northwest National Laboratory. Possible trypsin cleavage sites in bold.



Supplemental Figure S4. For nAChRs equilibrated with αBgTx, there is tetracaine inhibitable [³H]CPZ photoincorporation in the nAChR α, β, and δ subunits, with little if any photincorporation in the γ subunit. nAChR-rich membranes were photolabeled on a preparative scale with [^{3}H]CPZ in the presence of 10 μM αBgTx with or without 100 μM tetracaine, and aliquots (100 μg) were separated by SDS-PAGE. **A**, Coomassie blue stain of the gel. **B**, Fluorogram, after exposure to film for 2 weeks at -80 °C. **C**, Densitometry scans of the fluorogram lanes, quantified using ImageQuant software (Molecular Dynamics). [^{3}H]CPZ was photoincorporated at greater efficiency into rapsyn than in the nAChR α subunit, but only the photolabeling in the α, β, and δ subunits was inhibitable by tetracaine. The mobilities of the molecular weight standards are indicated on the left (A), as are the positions of the nAChR subunits and rapsyn (Rap). The sequencing data characterizing [^{3}H]CPZ photoincorporation in αM2, βM2, and δM2 are shown in **Figure 7**.



Supplemental Figure S5. [^{3}H]CPZ photolabeling in the α subunit cytoplasmic (MA) helix. An additional preparative photolabeling of nAChRs (+Carb) was carried out to further characterize the amino acids photolabeled within the αMA helix. α Subunit was subjected to in-gel digestion with V8 protease. A broad gel band of ~10 kDa was excised, and the polypeptides were eluted and then directly fractionated by rpHPLC. The ^{3}H elution profile was similar to that in Figure 8A (closed circles), and fractions 28-32 were pooled and sequenced, with ^{3}H (●) and PTH-amino acids (□) release determined for 60 cycles of Edman degradation. The primary sequence detected began at αAsn-339 (I_0 = 42 ± 3 pmol, R = 93 %; ~10,000 cpm loaded; 1,700 cpm remained on the filter after 60 cycles). The peaks of ^{3}H release in cycles 48 and 55 indicate [^{3}H]CPZ photolabeling of αMet-386 (2.3 cpm/pmol) and αSer-393 (1.2 cpm/pmol), respectively.



Supplemental Figure S6. Two binding sites for CPZ within the nAChR ion channel in the desensitized state. Shown are Connolly surface representations of three of the five M2 helices (left panel: γ light green; α_γ, light yellow; and β, light blue. right panel: β, light blue; δ, light magenta; and α_δ, light yellow) with the residues labeled by [^{3}H]CPZ at the bottom or top of the channel colored red or blue, respectively. Yellow denotes the M2-13 residues, which are not labeled by CPZ illustrating the separation between the two sites. Included in stick format are the lowest energy solutions for CPZ (carbon, gray; nitrogen, blue; sulfur, yellow; and chlorine, green) docked to each of the sites (see Experimental Procedures).