Gating-enhanced Accessibility of Hydrophobic Sites within the Transmembrane Region of the Nicotinic Acetylcholine Receptor's δ-Subunit

A TIME-RESOLVED PHOTOLABELING STUDY*

Received for publication, December 10, 2004, and in revised form, January 20, 2005 Published, JBC Papers in Press, January 21, 2005, DOI 10.1074/jbc.M413911200

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General anesthetics often interact more strongly with sites on open than on closed states of ligand-gated ion channels. To seek such sites, Torpedo membranes enriched in nicotinic acetylcholine receptors (nAChRs) were preincubated with the hydrophobic probe 3-(trifluoromethyl)-3-(m-iodophenyl) diazirine (TID) and exposed to agonist for either 0 ms (closed state), 1.5 and 10 ms (activated states), or ≥1 h (equilibrium or slow desensitized state) and then rapidly frozen (<1 ms) and photolabeled. Within 1.5 ms, the fractional change in photoincorporation relative to the closed state decreased to 0.7 in the β- and γ-subunits, whereas in the α-subunit, it changed little. The most dramatic change occurred in the δ-subunit, where it increased to 1.6 within 10 ms but fell to 0.7 during fast desensitization. Four residues in the δ-subunit’s transmembrane domain accounted for the enhanced photoincorporation induced by a 10-ms agonist exposure both when TID was added simultaneously with agonist and when and it was preincubated with membranes. In the published closed state structure, two residues (δThr274 and δLeu278) are situated toward the extracellular end of helix M2, both contralateral to the ion channel and adjacent to the third residue (δPhe283) on M1. The fourth labeled residue (δIle288) is toward the end of the M2-M3 loop. Contact with these residues occurs on the time scale of a rapid phase of TID inhibition in Torpedo nAChRs, suggesting the formation of a transient hydrophobic pocket between M1, M2, and M3 in the δ-subunit during gating.

Among the most sensitive of these targets are the ligand-gated ion channel superfamily of receptors that include anion channels gated by γ-aminobutyric acid (GABA1) and glycine and cation channels gated by acetylcholine (ACh) and serotonin (1, 2). In general, the action on anion channels is to shift the agonist concentration-response curve to the left (enhancement) (3), probably as a result of stabilizing the open state (4). In cation channels, only a few of the smallest general anesthetics (e.g. urethane and ethanol) act similarly, whereas most are noncompetitive open channel inhibitors (5). General anesthetics interact with these receptors in a conformationally sensitive manner, often having their highest affinity for those transient conformational states that occur immediately after the agonist binds to the closed state (5). Structural information is key to a complete understanding at the molecular level of anesthetic interactions with the open state, but, given its transitory nature, other techniques will be required for the foreseeable future.

Currently, the structure of the transmembrane domain of the Torpedo nicotinic acetylcholine receptor (nAChR) is known to 4.0 Å in the closed state (6). The nAChR consists of four subunits, α, β, δ, and γ with a stoichiometry 2:1:1:1, each having a bundle of four transmembrane helices (M1–M4, 27–34 residues in length). The five M2 helices are arranged about a central axis orthogonal to the membrane forming the channel lumen. A 9 Å structure of the open state (7) is of too low a resolution to resolve secondary structure.

Electrophysiological studies combined with site-directed mutagenesis provide kinetic evidence that general anesthetics interact with an enhancing site in anion channels that is located within the four-helix bundle of a given subunit (8) and with an inhibitory site in cation channels located in the channel lumen (9). Whereas these models of anesthetic action are self-consistent, much work is necessary to rule out alternative explanations. For example, the mutations might increase the affinity for a distant general anesthetic site by stabilizing a conformation that has high affinity for the anesthetic (5). Thus, a complementary approach that can provide structural information such as ligand-protein contact points is desirable.

Photolabeling is an approach that has provided abundant information about agonist and antagonist sites on the nAChR, particularly at synapses.

* This research was supported by National Institutes of Health Grant GM-58448, by the Department of Anesthesia and Critical Care, Massachusetts General Hospital, and by an award to the Harvard Medical School from the Howard Hughes Biomedical Research Support Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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$ The abbreviations used are: GABA, γ-aminobutyric acid; GABAAR, subtype A γ-aminobutyric acid receptor; ACh, acetylcholine; Carb, carbamylcholine; EndoLys-C, endoprotease Lys-C; HPIC, high performance liquid chromatography; nAChR, nicotinic acetylcholine receptor; OPA, o-phthalaldehyde; TID, 3-(trifluoromethyl)-3-(m-iodophenyl) diazirine; V8 protease, S. aureus endopeptidase Glu-C; Tricine, N-[2-hydroxy1,1-bis(hydroxymethyl)ethyl]glycine.
a member of the superfamily that is abundantly available from the electric tissue of Torpedo (10, 11), and some, more difficult to achieve, information on GABA_α receptors (GABA_αRs) from the brain (12). With some exceptions (13, 14), these studies have been on the closed and desensitized states, conformational models that exist at equilibrium, but an improved method of time-resolved photolabeling has now been introduced that has allowed nanomolar of nAChR to be efficiently photolabeled following exposure to agonists and other ligands for times as short as 1 ms (15).

The interaction of the lipophilic photoactivatable probe 3-(trifluoromethyl)-3-imidophenyl) diazirine (TID) with every subunit of the nAChR in the closed and desensitized states has been thoroughly characterized (16–19), as has the ability of general anesthetics, such as barbiturates, to modulate its photocentrification (20). Preincubation of nAChRs with TID causes closed state inhibition, which develops on the same 100-ms timescale (21) as the increase in photolabeling of the channel lumens, whereas equilibration with the lipid-protein interface is complete within a few milliseconds (22). In addition to the above closed state inhibition, rapid perfusion studies of mouse muscle nAChRs reveals a phase of inhibition that occurs on the 10-ms time scale immediately following agonist-induced activation (23).

In the present work, we find that during channel gating, TID interacts uniquely with the nAChR δ-subunit, where, within 10 ms after the addition of agonist, photoincorporation has increased about one and a half times but then decreases during fast desensitization. With reference to the nAChR closed state structure (6), three of the additional activation-dependent amino acid residues photolabeled by TID are clustered together on M1 and M2, and the fourth is on the M2-M3 loop close to the start of M3, suggesting the formation of a transient hydrophobic pocket in that region during activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—3-(trifluoromethyl)-3-imidophenyl) diazirine (125I-TID, nominal specific activity of 10 Ci/mmol) was obtained from Amersham Biosciences. Endoprotease Lys-C (EndoLys-C) was obtained from Roche Applied Science, and Staphylococcus aureus (Staphylococcus aureus) from Sigma or Aldrich. Sample buffer for gel electrophoresis was obtained from Invitrogen. All other chemicals were from Sigma or Aldrich. Torpedo physiological saline contained 250 mM NaCl, 5 mM KCl, 2 mM MgCl2, 5 mM sodium phosphate, pH 7.0, and 0.02% NaN₃.

**Preparation of nAChR-rich Membranes—nAChR-rich membranes, prepared from the electric organs of Torpedo californica (Aquatic Research Consultants, San Pedro, CA) as described (24), were stored at 80 °C in 38% sucrose, 0.02% NaN₃. Protein concentrations were determined by the micro-BCA assay method (25), and the ACh binding site concentration was determined by an [3H]ACh (PerkinElmer Life Sciences) binding assay.

**Time-resolved Photolabeling of nAChR-rich Membranes—**The method was detailed previously (15, 22). Briefly, the rapid mixing device was completely filled with buffer from the drive syringes to the two six-sample valves, each of which contained a 0.5-ml sample loop loaded with appropriate reagents. The pneumatic ram delivered sufficient buffer both to force the reagents in the sample loops through the mixer in ~1 ms and to expel only the mixed sample from the aging tube (∼1.3 ms) onto a rotating stainless steel disk, precooled in liquid nitrogen, where it was frozen in a thin film within 1 ms (freeze quenching). Incubation times were varied by changing the velocity of the ram and the length of the aging tube. Typically, one sample loop contained 0.5 ml of membranes (4 mg/ml protein), sometimes premixed with [125I]TID and/or carboxymethylated HABA (5). After mixing, the concentrated concentrations were 3.5 μM [125I]TID and 10 mM Ca++. Stock solutions were irradiated (Blak-Ray UV lamp model UVL-56) on the slowly rotating disk for 30 min at 386 nm at a distance of ∼3 cm. To obtain sufficient material for identification of labeled amino acids (preparative photolabeling), six samples per condition were photolabeled in the frozen state, and the material was subsequently combined for analysis. Twelve samples can be handled on a typical day.

**SDS-PAGE—**Aliquots of the thawed [125I]TID-labeled nAChR-rich membranes were analyzed for protein concentration. The rest of the frozen membranes were thawed in an appropriate amount of 4X sample buffer such that the final mixture contained 10% glycerol, 2% lithium dodecyl sulfate, 140 mM Tris-base, 0.5 mM EDTA, 0.22 mM Serva Blue G250, 0.175 mM phenol red, 0.23 μl N,N-dimethylpropionitrile, pH 8.5. The protein concentrations of the resuspended membranes were used to calculate the appropriate volumes to load for mass-balanced analytical SDS-polyacrylamide gels. The nAChR-rich membrane polypeptides were resolved by SDS-PAGE using the Laemmli buffer system (25) with 8% acrylamide separating gels (1.5 mm thick) containing 0.32% bisacrylamide. Polypeptides were visualized by Coomassie Blue stain, and the bands corresponding to the nAChR subunits as well as bands at 43 and 90 kDa, containing the non-nAChR peptides rapson and the Na⁺K⁺ ATPase 3-subunit, respectively, were excised from the gel for γ-counting (Micromedic 4/800 plus, Tietert Instruments, Huntsville, AL) and/or further processing. EndoLys-C digests were performed on 1.5-mm-thick 16.5% T/6% C Tricine SDS-polyacrylamide gels (19). The labeled bands of interest were detected within the wet gel by phosphorimaging (1–8 h exposure at 25 °C) using a Storm Phosphorimager (Amersham Biosciences), and the resulting image was used as a template for cutting up the gel. Prestained molecular weight standards (M-4038; Sigma) were used to determine the migration distances of the labeled fragments. Subunit or fragment bands of interest were eluted passively (3 days) in 12 ml of elution buffer (100 mM NH₄HCO₃, 0.1% SDS, 2.5 mM dithiothreitol, pH 8.4), filtered, concentrated in Vivaspin 15-mL concentrators (Vivaspin, Hanover, Germany), and either precipitated in 75% acetone (−20 °C, >3 h) or separated directly by reversed-phase HPLC.

**Enzymatic Digestion of [125I]TID-labeled nAChR Subunits—**EndoLys-C digests were performed in 25 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 8.6, for 2 weeks at 25 °C. For V8 protease digestion of material after HPLC, the fragments (~350 μl) were first neutralized by the addition of the adducts of 200 μl of 25 mM Tris, pH 8.6, containing 0.1% SDS and 0.5 mM EDTA. The acid-neutralized pool was then rotary-evaporated to remove most of the organic solvent, and V8 protease (100 μg) was added for 2 days at 25 °C.

**Reversed-phase HPLC Purification of [125I]TID-labeled Fragments—**Purification was performed on an Agilent 1100 HPLC with an inline degasser, column heater, and external absorbance detector. Separations were achieved at 40 °C using a Brownlee Aquapore C-4 column (100 × 2.1 mm) with a C-2 guard column. All solvents were HPLC grade. A modified version of one-sixth of each of the amino acid identification and quantification, and five-sixths were collected to measure 125I. HPLC fractions for sequencing were pooled and drop-loaded onto Biobrene-treated glass fiber filters (Applied Biosystems) modified such that one-sixth of each fraction was coated for amino acid identification and quantification, and five-sixths were collected to measure 125I. HPLC fractions for sequencing were pooled and drop-loaded onto Biobrene-treated glass fiber filters (Applied Biosystems) modified such that one-sixth of each fraction was coated for amino acid identification and quantification, and five-sixths were collected to measure 125I. HPLC fractions for sequencing were pooled and drop-loaded onto Biobrene-treated glass fiber filters (Applied Biosystems) modified such that one-sixth of each fraction was coated for amino acid identification and quantification, and five-sixths were collected to measure 125I.
Oocyte Expression of Torpedo nAChRs—Stage V and VI oocytes were harvested from anesthetized Xenopus frogs, in accordance with local and federal guidelines for animal care. Plasmids containing cDNAs for Torpedo γ, γ, and δ nAChR subunits were linearized with restriction endonucleases and used as templates for in vitro mRNA transcription using commercial kits (mMessage Machine, Ambion, Austin, TX). Mixtures of mRNAs at 20:1:8:1:1:10 stoichiometry were injected into oocytes (total = 20–50 ng) and incubated at 17 °C for 48–96 h in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes, pH 7.5). Oocyte membrane patches were excised in the outside-out configuration on borosilicate pipettes (1.2–3 megohms) and voltage-clamped at −50 mV. Patches were positioned in the outflow of a custom-built 2 × 2 quad-barrel superfusion pipette, coupled to two orthogonal piezo electric elements. Application of RC-damped high DC voltages to the piezo elements moved the superfusion pipette and resulted in switching between adjacent superfusion solutions in <1 ms (10–90% rise time of open pipette junction potential). In some experiments, two superfusion barrels were used: one containing K-100 buffer and an adjacent barrel containing ACh or ACh plus TID. If TID preincubation was required, three barrels were used: K-100, TID, and ACh plus TID. Patch currents stimulated by ACh were monitored using a digitizer series interface and pClamp 7.0 software (both from Axon Instruments, Foster City, CA).

Analysis of Electrophysiological Data—Current traces were analyzed offline. Base-line leak currents were subtracted digitally. Current decay rates were determined by fitting exponential decay functions to data points from the peak current to the point at which ACh superfusion ceased. Nonlinear least squares fitting was performed using Clampfit 7.0 (Axon Instruments, Foster City, CA).

RESULTS

Overall Experimental Design Considerations—Our experimental design was based on knowledge of Torpedo nAChR kinetics. Agonist-induced channel activation occurs in ~100 μs (28), within the dead time of our apparatus, and peak Carbinduced cation flux in Torpedo vesicles is linear over at least 10 ms, being terminated by fast desensitization with a time constant of ~200 ms (29, 30). In addition, in the absence of agonist, ~10% of receptors are in the desensitized rather than the closed state (31). Therefore, we chose agonist incubation times of 0 (resting states), 1.5 and 10 ms (activated states), 1 s (fast desensitized state), and ~1 h (slow desensitized state). Initially, the membranes were preincubated with TID before being rapidly mixed with saturating concentrations of agonist so that nAChR-TID interaction kinetics with the closed state (see Introduction) (15, 22) would not be superimposed upon agonist-induced changes. Subsequently, TID was added simultaneously with agonist to obtain a sense of the relative accessibility of the TID interaction sites.

Time Dependence of Agonist Action on TID Photoincorporation into nAChR Subunits—Fig. 1A shows a typical phosphor image of an SDS-polyacrylamide gel of membranes preincubated with TID and exposed for 10 ms either to 10 mM Carb or to buffer before freeze quenching and photolabeling. In this experiment, Carb increased photoincorporation of [125I]TID into the δ-subunit markedly, whereas that into the γ-subunit decreased, and that into all other bands changed little.

The combined results from several separate experiments are shown in Fig. 1B, where the data for each experiment have been normalized to the zero time (no Carb) control (see legend). The overall percentage S.D. (coefficient of variation) for the data set was 12%. The δ-subunit showed the most complex changes with exposure time to agonist. Photoincorporation increased 1.6-fold 10 ms after adding Carb, followed by an equally dramatic decrease first to just below control values at 1 s and then to much lower values similar to the γ- and β-subunits at equilibrium. The α-subunit did not decrease at early times but showed a slight decline at later times. The β- and γ-subunits were the only subunits to decline significantly at 1.5 ms. They declined further by 1 s but did not change thereafter. Modest photoincorporation into the non-nAChR polypeptides was always observed but did not change upon exposure to agonist.

[125I]TID Photoincorporation into the Transmembrane Domain of the δ-Subunit—The δ-subunit was chosen for more detailed study because of its unique kinetics (Fig. 1). nAChR-rich membranes preincubated with [125I]TID were exposed to 10 mM Carb or to buffer (six replicates each) for 10 ms, freeze-quenched, and photolabeled. The membrane polypeptides were separated by SDS-PAGE, and the δ-subunits (visualized by Coomassie stain) were excised, eluted, concentrated, and accordion precipitated. The resuspended δ-subunits were digested in solution with EndoLys-C, which produces subunit fragments of ~21 kDa (δEKC-21, beginning at δHis20/δHis206 and containing most of the extracellular domain), ~10 kDa (beginning at δMet237, the beginning of δM2), and ~12 kDa (beginning at δPhe206 and containing a site of N-linked glycosylation, δAsn208 and δM1) (22, 32).

When the δ-subunit EndoLys-C digests were separated by Tricine gel SDS-PAGE, phosphorimaging revealed two major photolabeled bands of ~10 kDa (δEKC-10) and ~13 kDa (δEKC-13) (Fig. 2A). For both labeling conditions, the amount of [125I] in bands between 20 and 24 kDa was less than 15% of that in δEKC-10 and δEKC-13, which sets an upper limit on the
amount of $^{125}$I/TID incorporation in the δEKC-21. Using this image as a template, δEKC-10 and -13 were excised, eluted, concentrated, and further purified by reversed-phase HPLC (Fig. 2, B and C, respectively). Below, we first describe the analysis of purified δEKC-10, leading to the identification of photolabeled amino acids in δM2-δM3 (Figs. 3–5). Subsequently, we describe the analysis of fractions purified from δEKC-13 that identify the labeled amino acids in δM1 (Fig. 6).

**Identification of $^{125}$I/TID-labeled Amino Acids in δM2-δM3**—For each condition, HPLC fractionation of the δEKC-10 band produced a single peak of $^{125}$I (Fig. 2B) eluting at 70% organic solvent that contained ~75% of the recovered radioactivity. Sequence analysis (Fig. 3A) of aliquots of the combined fractions 26 and 27 demonstrated the presence of a single fragment beginning at the N terminus of δM2, δMet225 (~Carb, $I_0 = 9$ pmol; +Carb, $I_0 = 8$ pmol), with no other peptide sequences detected (<0.5 pmol). For the ~Carb sample, there was an increase in $^{125}$I release in cycle 9 (cpm $- I_{10} = 4,265$ cpm with lower increase in cycles 13 (670 cpm) and 16 (350 cpm), corresponding to the labeling of δLeu265, δVal269, and δLeu272, residues in the lumen of the closed channel that were labeled by $^{125}$I/TID in previous freeze quench studies (22). After exposure to Carb for 10 ms, peaks of $^{125}$I release were retained in cycles 9 (2,570 cpm), 13 (360 cpm), and 16 (220 cpm), but there was also $^{125}$I release in cycles 15 (290 cpm), 22 (170 cpm), and 32 (340 cpm) that indicated labeling of δThr232, δLeu234, and δLeu238.

Although in the +Carb sample the increased release of 340 cpm in cycle 32 was only ~10% of the 2,570 cpm in cycle 9, δLeu288 was potentially labeled as efficiently as δLeu265 because the repetitive yield of Edman degradation was ~95%/cycle. Two additional experiments were carried out to confirm that the cycle 32 release did result from labeling of δLeu288. First, we used OPA, which prevents subsequent sequencing of all peptides except those with N-terminal prolines, to determine whether the $^{125}$I release in cycle 32 was two cycles after a proline, as predicted by the presence of δPro296. An aliquot of the +Carb sample from fractions 26 and 27 was sequenced, but after 29 cycles of Edman degradation, the sequencing was stopped, the filter was treated with OPA, and sequencing was then continued for another 11 cycles (Fig. 3B). Enhanced release of $^{125}$I in cycle 32 (340 cpm) was preserved after the OPA treatment, consistent with the presence of δPro296 in cycle 30 and δM3 incorporation in δLeu288. Second, we sequenced a sample after digestion with V8 protease, which cleaved the δMet255 fragment after δGlu260, exposing δPro296 after five cycles of Edman degradation. In this sample, the filter was treated with OPA after five cycles, and sequencing was continued for 25 additional cycles. Although the identification of the δ-subunit fragments was prevented by the presence of V8 protease, there was a single peak of $^{125}$I release in cycle 8 (2,670 cpm; Fig. 3C). Thus, the labeling of δLeu288 was confirmed relative to δPro296 in these two separate experiments (Fig. 3, B and C).

In this experiment, no release of $^{125}$I was seen in cycles 11–30, corresponding to the amino acids of δM3. Although TID does photointegrate into δM3 residues at the lipid-protein interface (19), the photolabeling efficiency reported in that study would be below the detection limit for the lower δM3 mass levels analyzed in Fig. 3.
For the +Carb (●) and −Carb (○) samples, the only sequence detected began at δMet236 (+Carb (●) and −Carb, I₀ = 23 pmol, R = 93%). The sequencing filter for the +Carb sample was treated with OPA after cycle 29 (●) (+Carb, 42,770 cpm loaded, 13,680 cpm remaining on the filter; −Carb, 9,610 cpm loaded, 2,955 cpm remaining). The release of δI in cycles 9, 13, and 16 indicated labeling for the −Carb and +Carb samples of δLeu265 (16 and 22 cpm/pmol), δVal266 (4 and 7 cpm/pmol), and δLeu272 (2 and 2 cpm/pmol). Release of δI in cycles 18 (810 cpm) and 32 (380 cpm) was observed only in the +Carb sample, consistent with agonist-dependent labeling at δThr274 (28 cpm/pmol) and δLeu268 (41 cpm/pmol).

For the −Carb and +Carb samples, the efficiencies of photoincorporation at each of the labeled amino acids (cpm/pmol), calculated from the observed increases in δ[125I] and phenylthiohydantoin-derivative releases, are tabulated in Table I. Exposure to Carb for 10 ms reduced by −50% the efficiency of labeling of each of the labeled amino acids within the channel lumen (δM2–9, −13, and −16). For the amino acids labeled only after brief exposure to agonist, δLeu268 (δM2–32) was labeled at 5–9-fold higher efficiency than δThr274 (δM2–18) or δLeu278 (δM2–22) and at similar levels to the most highly labeled amino acid in the channel lumen, δLeu265 (δM2–9).

State-dependent Labeling in δM2–δM3—The above studies with membranes preincubated with [125I]TID revealed two classes of labeled amino acids: those that preexist in the nAChR closed state and those that appear only after a brief exposure to an activating concentration of agonist. To obtain information about their relative accessibility in the closed and open states, experiments were performed in which [125I]TID, with or without Carb (six replicates each), was added to nAChR-rich membranes for 10 ms before freeze quenching. Subunit labeling was determined by phosphorimaging after SDS-PAGE. As observed in a representative experiment (Fig. 4A), agonist-enhanced photoincorporation occurred only in the δ-subunit. The nAChR subunits as well as non-nAChR polypeptides were excised from such gels, and [125I] incorporation was quantified. In each experiment, the mean of the +Carb samples was normalized to the mean of the no agonist samples, and these values were averaged with propagation of errors. For each nAChR subunit at 10 ms, the ratio of +Carb to −Carb photoincorporation was as follows: α, 1.0 ± 0.3; β, 1.3 ± 0.15; γ, 1.3 ± 0.1; δ, 3.1 ± 0.6 (mean ± S.D. of three different experiments). The addition of Carb resulted in no significant changes in labeling of the non-nAChR polypeptides. Thus, the unusual behavior of the δ-subunit relative to the others was maintained, but the magnitude of this agonist-enhanced subunit

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3 To aid in locating amino acid residues on the structure of the nAChR, two conventions are adopted. First, residues are numbered from the first residue after the charged residue at the N-terminal end of M2; thus, δM2–9 is δLeu265. Second, residues on M1 are located relative to the central conserved Pro; thus, δM1(−F–3) is δPhe398 three residues before the Pro on δM1.
Table I

| Source of sequencing data | This work (see "Results") |
|---------------------------|---------------------------|
| **Equilibrium TID**       | **Photoincorporation in residue** |
| Equilibrium TID            | 10 ms TID                  | Equilibrium TID |
| Equilibrium TID            | 10 ms TID                  | Equilibrium TID |
| Equilibrium TID            | 15 ms TID                  | Equilibrium TID |
| Source of sequencing data | 0 ms Carb | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| 0 ms Carb                  | 10 ms Carb | 10 ms Carb |
| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |
| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |
| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |
| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |
| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |
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| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |
| Photoincorporation in residue<sup>a</sup> | cpm/pmol | cpm/pmol | cpm/pmol |

<sup>a</sup> For each data column, the 125I release was determined from a single sequencing run. To allow comparison of labeling efficiency of amino acids within fragments that were sequenced at times varying from 1 to 2 months after the initial photolabeling, the experimentally determined 125I release (cpm<sub>0</sub> - cpm<sub>n</sub>)/<sub>1</sub>) for each sequence run was corrected for the 125I decay since the date of photolabeling.

<sup>b</sup> An upper limit of labeling efficiency was estimated from the local variability in cpm from cycle to cycle during Edman degradation, which in the absence of specific labeling was characterized by an S.D. of ~10% of the mean cpm over 5–10 cycles. The indicated upper limit was estimated as 20% of the mean cpm.

<sup>c</sup> NS, not sequenced.

<sup>d</sup> Estimated by combining the separate sequencing results (see Fig. 6A and "Results") for HPLC fractions 22 and 23 in Fig. 2C.

**Open State Photolabeling of the Acetylcholine Receptor**

pholabeling relative to the closed state was doubled compared with labeling for nAChRs preincubated with [125I]TID (compare Fig. 1B). This was anticipated, because in the closed state TID equilibrates with its site in the ion channel with a time constant of about 75 ms (15).

For sequencing, two sets of six aliquots of membranes were freeze-quenched after 10-ms exposure to [125I]TID with or without 10 mM Carb and photolabeled. The 6-subunit was then isolated from the membranes by SDS-PAGE and digested with Endo Lys-C. After these digests were separated by Tricine SDS-PAGE, phosphorimaging (Fig. 4B) revealed a major band of 125I at ~13 kDa (M2–13) and a secondary band at ~10 kDa. When the material eluted from M2–13 was further purified by reversed-phase HPLC (Fig. 4C), 125I was distributed in two peaks, fraction 23 (55% organic, 30% of recovered 125I) and fractions 26 and 27 (70% organic, 55% recovered 125I), in each of which 125I in the -Carb sample was ~20% of that in the +Carb sample. The more hydrophobic peak (fractions 26 and 27) contained the M2–13 fragment, whereas the more hydrophilic peak (fraction 23) contained M1 (sequenced in Figs. 5 and 6B, respectively; for details, see below).

Sequence analysis (Fig. 5) of pooled fractions 26 and 27 revealed the presence of a single fragment beginning at Met<sup>325</sup> (−Carb or +Carb, I<sub>0</sub> = 23 pmol). As expected, for the −Carb sample, the major peak of 125I release was in cycle 9 (900 cpm), with lower level release in cycles 13 (170 cpm) and 16 (60 cpm), consistent with labeling within the lumen of the closed ion channel, and there was also a 60-cpm release in cycle 22. For the +Carb sample, in addition to increased release in cycles 9 (1,240 cpm), 13 (305 cpm), and 16 (60 cpm), there was release at cycles 18 (810 cpm), 22 (225 cpm), and 32 (380 cpm, after treatment of the sequencing filter with OPA after the 28th cycle of degradation). Table I shows that the efficiency of [125I]TID incorporation was higher in M2–22 (M2–9) than in M2–26 (M2–13) and M2–16, independent of the presence of Carb. The most efficiently labeled residues, Thr<sup>274</sup> (M2–18) and Leu<sup>278</sup>, were labeled at least 30-fold more efficiently in the presence than in the absence of Carb, whereas for Leu<sup>278</sup> (M2–22), which was labeled at a lower level, Carb enhanced the efficiency 4-fold. For membranes exposed to [125I]TID for only 10 ms (when TID binding within the lumen of the ion channel is at only 10–20% of equilibrium (22)), the agonist dependent labeling at cycles 18 and 32 (28 and 40 cpm/pmol) was actually at higher efficiency than the labeling in cycle 9 (22 cpm/pmol).

We also compared the photolabeling efficiency of amino acids in M2 for nAChRs preincubated with TID and exposed to Carb for as brief a time as possible, 1.5 ms, versus those desensitized by equilibration with 10 mM Carb. As before, the fragments beginning at Met<sup>325</sup> were isolated from Endo Lys-C digests of labeled 6-subunit, but in this case they were sequenced only for 25 cycles of Edman degradation. For samples exposed to Carb for 1.5 ms, M2–9, -13, -16, -18, and -22 were each labeled, with M2–9 labeled at highest efficiency and M2–18 and M2–22 at lowest efficiency (Table I). For the nAChRs preequilibrated with Carb, and therefore in the desensitized state, the efficiency of labeling at M2–9, -16, and -13 was 20–30 times less than that in the sample exposed to agonist for 1.5 ms, and any labeling of M2–18 or -22, if it occurred, was at ~10% the efficiency of that labeling.

**State-dependent Labeling in 6M1—Based upon our previous characterization of the sites of photolabeling of [125I]TID and [14C]halothane in the 6-subunit transmembrane domain (22, 39), we expected that the hydrophilic peak of 125I in the HPLC purifications of 6EC-13 (Figs. 2C and 4C) would contain a glycosylated fragment beginning at Phe<sup>206</sup> and extending through 6M1. Fraction 23 from Fig. 2C had been isolated from nAChRs preincubated with [125I]TID and then exposed to buffer or to Carb for 10 ms. When the −Carb sample was sequenced (Fig. 6A), no release of 125I above background was detected during 40 cycles of Edman degradation. In contrast, in the +Carb sample, there was a sharp increase of 125I release in cycle 27 (490 cpm) with possible additional release in cycles 28 and 31. In these samples, the expected 6-subunit fragment was present, but at very low levels (+Carb, I<sub>0</sub> = 0.2 pmol; 4 The release detected in cycle 28 was not observed in a subsequent experiment (Fig. 6B) and probably reflected a lag from the release in cycle 27. Such a lag is expected after ~20 cycles of Edman degradation following treatment with OPA, as was done here before cycle 2.
−Carb, $I_0 = 0.4$ pmol). Although the mass levels were low, the $^{125}\text{I}$ release seen in cycle 27 was associated with labeling of δPro202, since this release was seen after the sequencing filter had been treated with OPA before the second cycle of Edman degradation (δPro207). The low mass level detected during the sequencing of fraction 23 suggested that the labeled and unlabeled fragments may have been separated during HPLC, and, consistent with this, HPLC fraction 22 was sequenced and found to contain the fragment beginning at δPro206 at a much higher level (+Carb, $I_0 = 14$ pmol).

Consequently, for the material isolated from nAChRs exposed to $^{125}\text{I}$TID for 10 ms in the absence or presence of Carb (Fig. 4C), fractions 22 and 23 were pooled before being sequenced (Fig. 6B), with OPA treatment after 19 cycles of Edman degradation corresponding to the expected location of δPro202. The only detected sequence began at δPro200 (+Carb, $I_0 = 13$ pmol; −Carb, $I_0 = 15$ pmol). For the +Carb sample, once again there was a clear peak of $^{125}\text{I}$ release in cycle 27 (360 cpm), corresponding to δPro215, with a shoulder of release in cycle 31 (δCys236). In the absence of Carb, $^{125}\text{I}$ release in cycle 27 was minor (30 cpm). When compared on a cpm/pmol basis (Table I), it was only −2% of that in the +Carb sample.

Electrophysiological Studies of Inhibition by TID—Electrophysiological recordings from outside-out oocyte membrane patches expressing Torpedo nAChRs revealed evidence for multiple steps leading to inhibition by TID, as previously reported for mouse muscle nAChRs (23). Simultaneous exposure to 1 mM ACh and a high concentration of TID (5 μM) resulted in an −10% reduction of peak patch currents (Fig. 7, top and middle), and there was an increase in the rate of the current decay following the peak from 16 to 42 s$^{-1}$. Preexposure of patches to 5 μM TID resulted in complete inhibition of ACh-induced current (not shown). TID preincubation at concentrations of 0.16 μM (not shown) and 0.5 μM (Fig. 7, bottom) prior to activation with ACh and TID resulted in a diminished peak current response and a biphasic decay of the current. We observed both a TID-dependent rapid decay phase and a slow phase comparable with desensitization in the control. Systematic studies (not shown) revealed that the peak response decreased with TID preincubation time at an average rate of 5 ± 1.8 s$^{-1}$ ($n = 6$, mean ± S.D.). This rate was independent of TID concentration. The fast current decay accelerated with TID preincubation time, plateauing after 300 ms at 50 ± 13 s$^{-1}$ at 0.16 μM TID and 150 ± 78 s$^{-1}$ at 0.5 μM TID. Finally, the steady-state current after the fast phase declined more rapidly than peak current (not shown), just as it did in earlier observations with mouse nAChRs (23), indicating that the mechanism of TID inhibition is similar in both species.

**DISCUSSION**

The salient new finding in this study is that within 10 ms of the addition of agonist, when the fraction of nAChRs in the open state is maximum, $^{125}\text{I}$TID photoincorporates into a novel set of amino acids (activation-dependent residues) that are either not or are only inefficiently photolabeled in the closed or desensitized states and that are located only on the δ-subunit within the M1 and M2 transmembrane helices and on the M2-M3 loop. During brief exposure to agonist (1.5–10 ms), only the δ-subunit experienced an increase in photoincorporation. In contrast, the other subunits showed no such increase; instead, they experienced a steady decline with increasing time.

Another set of amino acids (activation-independent residues) on the channel face of the δM2 helix was photolabeled both in the closed state and following exposure to agonist for 10 ms. In earlier studies at room temperature, 80% of TID that photoincorporated into the closed state of the nAChR did so on the channel face of M2, and that labeling was −5-fold less in the equilibrium desensitized state (16, 34). The current-re- solved frozen state data show that, with the exception of the δ-subunit, this decrease is apparent within milliseconds and is maximal within 1 s, suggesting that the reduction is associated with fast desensitization. However, the δ-subunit again differed, experiencing a further decrease in photoincorporation when the conformation changed from the fast to the slow desensitized state. Although no agonist-induced increase was observed in the α-, β-, or γ-subunits, we cannot rule out the possibility that small increases in some locations were masked by larger decreases in other locations.

A special role for the δ-subunit was noted previously during linear free energy analysis of electrophysiological data. It is the slowest subunit to respond during the conformational wave following agonist binding, and it makes a distinct contribution, influencing both agonist binding and channel gating (35, 36).

Although there are several intriguing questions arising from the subunit level data, this study focused on the δ-subunit during gating. At 10 ms, the enhanced photoincorporation into the activation-dependent residues (δThr274, δLeu278, δPhe232, δlle288) is only partially offset by the decrease in the activation-independent residues (δLeu265, δVal269, and δLeu272). The net
increase in Table I accounts for the observed increase in the δ-subunit in Fig. 1, suggesting that there are no other undetected contributions to agonist-induced photolabeling.

When TID was rapidly mixed with nAChRs to reveal relative access rates of open and closed states, the labeling of the activation-dependent residues was greatly enhanced, whereas the labeling of the activation-independent residues was similar in the absence or presence of agonist (Table I). The former result also suggests that preequilibration with TID does not profoundly influence subsequent conformation changes, consistent with previous stopped flow spectroscopic observations (21).

**Inhibition by TID**—Little functional inhibition is observed when nAChRs from either Torpedo or mouse muscle are exposed simultaneously to agonist plus TID, whereas variable length preequilibrium with TID results in inhibition of initial currents that develops at a rate of {\( \sim 10^{-4} \) s{\(^{-1}\)}} (this work) (21, 23). In the closed state, access of TID to the activation-independent site also occurs at a rate of {\( \sim 10^{-4} \) s{\(^{-1}\)}}, which is much slower than access to the lipid bilayer or to the lipid-protein interface (15, 22), and, therefore, prior occupation of this site on the closed state is required if TID is to act as an inhibitor during rapid agonist-induced activation.

In addition to the above pathway of inhibition, continuous current monitoring in rapidly perfused, excised outside-out oocyte membrane patches revealed an additional rapid phase of inhibition that only occurred after preequilibrium with TID. The rate of this phase was faster at {\( \sim 10^{-2} \) s{\(^{-1}\)}} and depended on TID concentration, suggesting a bimolecular action (Fig. 7, lower panel) (23). In the freeze quench experiments, the aqueous concentration of TID has been estimated to fall to a few micromolar to {\( \sim 10^{2} \) nm} (in {\(< 1 \) ms in the case of rapid mixing) upon the addition to membranes (15). Such free aqueous concentrations are comparable with those experienced by oocyte membranes during continuous perfusion experiments. Because this rapid phase of inhibition and the activation-dependent photolabeling of the δ-subunit both occur at similar TID concentrations and within 10 ms of addition of agonist, it seems likely that they are two manifestations of the same underlying process.

**The Location of the TID Sites**—The activation-independent residues in the δ-subunit are all located in the ion channel's lumen in the closed state structure of the nAChR (6) (Fig. 8, dark blue residues). Of the four activation-dependent residues (Fig. 8, cyan residues), two, δThr{\(^{274}\)} and δLeu{\(^{278}\)} (δM2–18 and δM2–22), are situated on the opposite face of the M2 helix from the activation-independent residues. Another, δPhe{\(^{232}\)}, is on the M1 helix, three residues above the conserved proline (δM1–P-3), and adjacent to the δM2–18 and δM2–22 residues noted above. Examination of the Connolly surface in this region (Fig. 8C) reveals that δThr{\(^{274}\)}, δLeu{\(^{278}\)}, and δPhe{\(^{232}\)} are on the surface of an accessible pocket within the M1-M3 helix bundle that measures{\( \sim 9 \times 5 \times 5 \) Å}, which, consistent with the lack of prominent labeling in this region in the closed state, is too small to easily accommodate TID (11 \times 7 \times 7 \) Å). Because TID fits in this pocket in the activated state, it follows that conformation changes during activation must be associated with an expansion of this pocket sufficient to accommodate TID. However, the observed labeling pattern raises two questions about such a simple proposal. First, why are other residues that line this pocket, particularly those on δM3, not photolabeled? Second, why is the other activation-dependent residue (δIle{\(^{288}\)}, M2-M3L) {\( \sim 15 \) Å from the center of this pocket? A simple explanation for these discrepancies is that in the open state δIle{\(^{288}\)} is closer to the other labeled residues than in the closed state model and itself forms part of the TID-binding pocket.

**FIG. 8. Localization on the nAChR closed state transmembrane structure of the residues labeled by \(^{125}\)I-TID after 10-ms agonist exposure.** A, a structural model of transmembrane helices of the nAChR (Protein Data Bank code 1OED (6)) looking down the channel from the synaptic side. The cylinders approximate the individual transmembrane helices from the five subunits. B, the same model rotated 90° (viewed along the red arrow in A) and limited to the δ- and β-subunits as well as one αM2 helix. Colored blue are side chains in the channel that are labeled by TID in the closed state, including the activation-independent residues on the δ-subunit (this work) and previously reported residues on other subunits (16, 34). Colored cyan are the side chains in the δ-subunit labeled by TID in an activation-dependent manner (after 10-ms agonist exposure). Also included are the side chains labeled in the desensitized state either by the anesthetic analogs aizocotanal and/or aizometidate (green) or by halothane (yellow) (32, 33, 43). A Connolly surface representation of TID is included to scale. C, a stereo view of the top (extracellular) part of the δM1, δM2, and δM3 helices as viewed along the black arrow in B. The δM4 helix was removed to allow a better view of the interior Connolly surface of the helical bundle and of a solvent-accessible pocket, which extends down to δCys{\(^{256}\)} and is lined by all of the activation-dependent residues except δIle{\(^{288}\)}. Also included to the same scale is a Connolly surface representation of TID.

Although uncertainty surrounds the structural changes occurring during it (37), one current concept of gating supposes that upon activation all five M2 helices rotate in a clockwise direction some 15°, which would move the activation-dependent δM2–18 and δM2–22 residues away from M3 and toward the α-δ subunit interface (6, 38). Such a rotation is supported by time-resolved fluorescence studies of rhodamine-labeled βM2–19 (39). The rotation of M2 is driven by agonist-promoted motions in the extracellular agonist-binding domain that are closely coupled to the M2-M3 loop (6, 40). In rotating M2 clockwise, the M2-M3 loop probably moves toward the α-δ interface. If, consequently, during gating M3 tilts toward the space between M2 and M3, it would both force M1 and M2 apart, admitting TID, and decrease the distance between δIle{\(^{288}\}} and the other three activation-dependent residues, perhaps allowing δIle{\(^{288}\}} to form the roof of a cleft whose floor is the cluster of three δM1-M2 residues. While speculative, such a model has the advantage of efficiently rationalizing our time-resolved photolabeling observations. Additional evidence for agonist-induced changes in M1 exposure in nAChRs comes...
from cysteine accessibility studies that found that positions on αM1 and βM1 equivalent to δPhe232 were modified only in the presence of agonist (41, 42).

It is also worth noting that at the current resolution of the closed state structure (6), it is difficult to unambiguously identify individual amino acids, so that some uncertainty exists in the assignments of the M2-M3 loop and the M3 α-helix. As published, three hydrophobic amino acids at the N terminus of M3 (in δ-subunit, Tyr291, Leu292, and Met293) have been positioned one helical turn above the plane of the lipid bilayer. An ~3-amino acid register shift would move δILE288 into the first turn of the δM3 helix at the level of the other activation-dependent residues in M1 and M2, thus completing the pocket. Activation would still be required to enlarge the pocket enough to accommodate TID.

Relevance to General Anesthetic Action on nAChRs—We have used the well characterized hydrophobic photolabel, TID, to probe for hydrophobic cavities that are unique to conformations associated with channel gating. Such cavities, apart from what they reveal about gating, are of pharmacological interest, because general anesthetics trend to selectively target activated states. How does the cavity we have detected relate to what is known about anesthetic action on this superfamily? A number of equilibrium studies with general anesthetic photolabels on the nAChR have been reported. The clinical general anesthetic halothane, a small photolabel with an exclusive preference for the nAChR have been reported. The clinical general anesthetic of equilibrium studies with general anesthetic photolabels on known about anesthetic action on this superfamily? A number of experiments have been done on the TID photolabeled analog of a clinical intravenous agent, and 3-azaotanol both photolabeled δGlh282 (δM2-20; Fig. 8, green) (32, 43). In the closed state structure, the α-carbon of this residue is separated by only 10–12 Å from the α-carbons of our three activation-dependent residues on δM1 and δM2, distances that probably decrease during the hypothesized rotation of M2 during gating discussed above. It will be interesting to see whether analogous of these photolabels with the diazire group in different positions, such as 7-azaotanol (44), provide evidence in favor of a general anesthetic site that spans the interface between the subunits. Such an interfacial site would be analogous to that of the classical allosteric effector of hemoglobin, 2,3-diphosphoglycerate (45).

Most site-directed mutagenesis studies of general anesthetic action in the nAChR have focused on the channel lumen (9, 46), generally in the regions where TID photolabels the activation-independent residues. However, for neuronal nAChRs containing β4 and either α2 or α3 subunits, substitutions at M2–15 cause changes in alcohol sensitivity, and, based on the reactivity of Cys-substituted residues, αM2–15 is in an aqueous environment (47). In addition, at a position in the M2-M3 loop homologous with Torpedo δILE288, an isoleucine to methionine substitution in homomeric chimeras of α2-3nAChRs decreased the sensitivity to halothane inhibition and shifted the ACh concentration-response curve to the left (48). These authors suggested that the mutations acted indirectly by changing gating rather than by ablating anesthetic binding, but our results increase the possibility that binding is part of the explanation.

Relevance to General Anesthetic Action on GABAARs—In the anionic channels of the ligand-gated ion channel superfamily, the hypothesis that there is a site for general anesthetics, central to the channel and bounded by M1, M2, and M3, that is responsible for shifting the agonist concentration-response curve to the left has received detailed attention. Different classes of anesthetic often interact with different subunits of the GABAAR, but in general a strong circumstantial case has been built up using site-directed mutagenesis and cysteine accessibility to support the hypothesis that there are residues at M1-(P-1), M2–15, and at the extracellular end of M3 that modulate the action of general anesthetics (reviewed in Refs. 1 and 49). However, the general anesthetic propofol has been found to compete with cysteine reagents at the extracellular end of M3 but not with the M2–15 residue, suggesting that the later residue is not in the main part of the propofol binding pocket but that its role may be either to indirectly enlarge the site and/or to alter gating so as to enhance anesthetic sensitivity (50). Cysteine accessibility studies have established that amino acids in the outer part of M3 contribute to a solvent-exposed cleft whose accessibility is modulated by agonist (51) and by alcohol (52).

Our finding that on a homologous member of the ligand-gated ion channel superfamily, there is TID photoincorporation in the same general region only during gating adds substantial support to the hypothesis that mutagenesis in this region of the GABAAR and glycine receptors is modulating anesthetic binding directly, although such experiments are complicated by concomitant effects on gating. Two other aspects of our work have bearing on the anion channel studies. First, anesthetics stabilize the GABAAR open state (4, 5), suggesting that the pocket becomes enlarged during gating just as it appears to do on the nAChR δ-subunit. Second, the subunit dependence of TID interaction with the nAChR echoes that of general anesthetic action in the GABAAR mentioned above.

Perspective—Our identification of a novel transient TID site on the nAChR establishes the value of using the rapid mixing, freeze quench technique. In the future, it will be important to use the method with recently developed photoreactive general anesthetics (32, 43) to map transient anesthetic-binding sites on nAChRs and GABAARs.

Acknowledgment—We thank Eileen S. Krenzel for excellent technical assistance.

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