Desformylflustrabromine (dFBr) and [³H]dFBr-Labeled Binding Sites in a Nicotinic Acetylcholine Receptor[§]

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ABSTRACT

Desformylflustrabromine (dFBr) is a positive allosteric modulator (PAM) of $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs that, at concentrations $>1 \mu\text{M}$, also inhibits these receptors and $\alpha 7$ nAChRs. However, its interactions with muscle-type nAChRs have not been characterized, and the locations of its binding site(s) in any nAChR are not known. We report here that dFBr inhibits human muscle ($\alpha \beta \epsilon \delta$) and *Torpedo* ($\alpha \beta \gamma \delta$) nAChR expressed in *Xenopus* oocytes with IC_{50} values of $\sim 1 \mu\text{M}$. dFBr also inhibited the equilibrium binding of ion channel blockers to *Torpedo* nAChRs with higher affinity in the nAChR desensitized state ([³H]phencyclidine; $\text{IC}_{50} = 4 \mu\text{M}$) than in the resting state ([³H]tetracaine; $\text{IC}_{50} = 60 \mu\text{M}$), whereas it bound with only very low affinity to the ACh binding sites ([³H]ACh, $\text{IC}_{50} = 1 \text{ mM}$). Upon irradiation at 312 nm, [³H]dFBr photoincorporated

into amino acids within the *Torpedo* nAChR ion channel with the efficiency of photoincorporation enhanced in the presence of agonist and the agonist-enhanced photolabeling inhibitable by phencyclidine. In the presence of agonist, [³H]dFBr also photolabeled amino acids in the nAChR extracellular domain within binding pockets identified previously for the nonselective nAChR PAMs galantamine and physostigmine at the canonical α - γ interface containing the transmitter binding sites and at the noncanonical δ - β subunit interface. These results establish that dFBr inhibits muscle-type nAChR by binding in the ion channel and that [³H]dFBr is a photoaffinity probe with broad amino acid side chain reactivity.

Introduction

nAChRs are pentameric ligand-gated ion channels that are expressed at the vertebrate neuromuscular junction (muscle-type nAChR) and at postsynaptic and presynaptic nerve terminals throughout the nervous system (neuronal-type nAChR) (Dani and Bertrand, 2007; Gotti et al., 2009). Drugs that modulate nAChR function have potential therapeutic uses in the treatment of many cognitive and neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (Gotti and Clementi, 2004; Jensen et al., 2005; Taly et al., 2009).

Positive allosteric modulators (PAMs) of nAChRs have been introduced as a novel physiologic approach to enhance nAChR function (Faghih et al., 2008; Arias, 2010; Williams et al., 2011). A nAChR PAM, by definition, binds at site(s) distinct from the ACh binding (i.e., orthosteric) site and enhances the

effects of ACh. Endogenous and exogenous compounds with a variety of structural motifs have been shown to potentiate the action of ACh at nAChRs, which suggests the presence of more than one class of nAChR PAM binding sites (Bertrand and Gopalakrishnan, 2007; Changeux and Taly, 2008). In agreement with this notion, potential PAM binding sites have been identified within the extracellular domain (ECD) and transmembrane domain (TMD) of nAChRs. Within the TMD, an intrasubunit PAM binding site in $\alpha 7$ nAChR was identified by mutational analyses (Young et al., 2008; daCosta et al., 2011), and photoaffinity labeling identified an intersubunit site in muscle-type nAChR (Nirthanan et al., 2008). Within the ECD, PAM binding sites were identified in secondary pockets at the agonist-binding canonical interfaces (Ludwig et al., 2010; Hamouda et al., 2013), at noncanonical interfaces (Seo et al., 2009; Hamouda et al., 2013; Olsen et al., 2014), and near the M2-M3 loop (Bertrand et al., 2008).

Desformylflustrabromine (dFBr) (Fig. 1), isolated originally from the marine bryozoan *Flustra foliacea* (Peters et al., 2002), acts as a neuronal nAChR PAM, enhancing, at concentrations $<1 \mu\text{M}$, peak ACh responses of $\alpha 4\beta 2$ (Sala et al., 2005; Kim et al., 2007; German et al., 2011) and $\alpha 2\beta 2$ (Pandya and Yakel, 2011) nAChRs expressed in *Xenopus* oocytes. At higher concentrations, dFBr inhibited these receptors and $\alpha 7$ nAChRs (Kim et al., 2007). Subcutaneous

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ABBREVIATIONS: α -BgTx, α -bungarotoxin; Carb, carbamylcholine chloride; dFBr, desformylflustrabromine; ECD, extracellular domain; PAM, positive allosteric modulator; PCP, phencyclidine hydrochloride; rpHPLC, reversed-phase high-performance liquid chromatography; TMD, transmembrane domain.

Fig. 1. Structure of dFBr.

administration of dFBr to rats resulted in reduced nicotine self-administration (Liu, 2013). Whereas the effects of dFBr at several neuronal nAChRs have been characterized, its interactions with muscle-type nAChRs have not been characterized, and there has been no localization of its binding site(s) in any nAChR.

Here, we use two-electrode voltage clamp recording to assess the effects of dFBr on human and *Torpedo* muscle-type nAChRs expressed in *Xenopus* oocytes. We also use the intrinsic photo-reactivity of the newly synthesized ligand, [^3H]dFBr, to identify its binding sites within the *Torpedo* nAChR. Adult human and *Torpedo* muscle-type nAChRs have subunit stoichiometries of $\alpha_2\beta\epsilon\delta$ and $\alpha_2\beta\gamma\delta$, respectively. Each nAChR subunit comprises an extracellular N-terminal domain consisting of a 10-strand β sandwich, a TMD consisting of four transmembrane helices (M1–M4), and an unstructured cytoplasmic domain comprised of the M3–M4 intracellular loop (Unwin, 2005). ACh-binding sites are located within the ECD at the two α subunit interfaces (α - ϵ/γ and α - δ) and the ion channel is lined by each subunit's M2 helix. We found that dFBr inhibits muscle-type nAChRs due to high-affinity binding in the ion channel, but it also binds within the *Torpedo* nAChR ECD at binding sites identified previously for galantamine and physostigmine, i.e., nonselective nAChR PAMs (Hamouda et al., 2013).

Materials and Methods

dFBr hydrochloride and des-methyl dFBr hydrochloride (6-bromo-2-(1,1-dimethylallyl)tryptamine) were synthesized as previously described (German et al., 2011). [^3H]dFBr (76 Ci/mmol) was prepared by custom tritiation (Vitrex, Placentia, CA) by reaction of des-methyl dFBr hydrochloride with [^3H]CH $_3$ I.

nAChR Expression in *Xenopus* Oocytes. Plasmids with cDNA encoding human $\alpha 1$ (pSP $\alpha 64\text{T}$), $\beta 1$ (pBSII), δ [pBS(SK)], and ϵ (pBSII) nAChR subunits were generously provided by Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia). Plasmids with cDNA encoding the *Torpedo* $\alpha 1$, γ and δ (pMXT), and *Torpedo* $\beta 1$ (pSP6) were gifts from Dr. Michael M. White (Drexel University College of Medicine, Philadelphia) and Dr. Henry Lester (California Institute of Technology, Pasadena, CA), respectively. Plasmids were linearized with BsaAI ($H\delta$), FspI ($H\alpha 1$ and $T\beta$), ScaI ($H\beta 1$), XbaI ($T\alpha$, $T\gamma$, and $T\delta$), and XhoI ($H\epsilon$). cRNA transcripts were prepared from linearized plasmids using SP6 (*Torpedo* α , β , γ , and δ and Human $\alpha 1$) or T3 (Human $\beta 1$, δ , and ϵ) polymerase using mMESSAGE mMACHINE high-yield capped RNA transcription kits (Ambion/Life Technologies, Grand Island, NY) following the manufacturer's protocol.

Leucine-to-serine substitutions at position M2-9' ($\alpha\text{Leu}251$, $\beta\text{Leu}257$, $\gamma\text{Leu}260$, and $\delta\text{Leu}265$) were introduced into plasmid expression vectors

coding for the *Torpedo* nAChR subunits using the Quick Change II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and custom-designed oligos containing the desired mutation (Integrated DNA Technologies, Coralville, IA). Mutations were confirmed by sequencing the entire coding region.

Ovarian lobules were removed surgically from adult, female *Xenopus laevis* following animal use protocols approved by the Texas A&M Health Sciences Center Institutional Animal Care and Use Committee. The lobules were treated with 3 mg/ml collagenase type 2 (Worthington Biochemical, Lakewood, NJ) with gentle shaking for 3 hours at room temperature in Ca^{+2} -free OR2 buffer (85 mM NaCl, 2.5 mM KCl, 1 mM MgCl_2 , 5 mM HEPES, pH 7.6). Stage V and VI oocytes were selected, injected with cRNA, and incubated at 18°C in ND96-gentamicin buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, 50 $\mu\text{g}/\text{ml}$ gentamicin, pH 7.6) for 24–72 hours to allow receptor expression. For *Torpedo* and human muscle nAChRs, oocytes were injected with 20–50 ng total mRNA mixed at ratios of 2 α :1 β :1 γ :1 δ and 2 α :1 β :1 δ :1 ϵ , respectively.

Electrophysiological Recording. The effects of dFBr on ACh-induced current responses were examined using standard two-electrode voltage clamp techniques. Oocytes in the recording chamber were perfused with ND96 recording buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl_2 , 0.8 mM MgCl_2 , 1 mM EGTA, 10 mM Hepes, pH 7.5), and voltage-clamped at -50 mV using Oocyte Clamp OC-725B (Warner Instruments, Hamden, CT), and whole cell currents were digitized using Digidata 1440A/Clampex 10.4 (Axon Instruments/Molecular Devices, Sunnyvale, CA). An automated perfusion system (Warner Instruments) was used to control recording chamber perfusion and drug application. Each recording run included 3–6 applications of ACh or dFBr alone or in combination. Drug applications were separated by 1–3-minute wash intervals. Each drug concentration was tested at least three times and repeated on at least two oocytes. Currents were quantified using pCLAMP 10.4 (Axon Instruments), and then normalized to the peak current elicited by ACh alone. Data (average $\pm$ S.E.) were fit to a single-site model of inhibition (see Data Analyses).

Radioligand Binding Assays. Centrifugation assays (Hamouda et al., 2011) were used to determine the effects of dFBr on the equilibrium binding by *Torpedo* nAChR of [^3H]ACh (1.9 Ci/mmol), phencyclidine hydrochloride (PCP; 27 Ci/mmol; PerkinElmer Life Sciences, Billerica, MA), and [^3H]tetracaine (30 Ci/mmol; SibTech, Brookfield, CT), and the effects of dFBr, PCP, and tetracaine on the equilibrium binding of [^3H]dFBr. nAChR-rich membranes, isolated from *Torpedo californica* electric organs as previously described (Middleton and Cohen, 1991), were resuspended in *Torpedo* physiologic saline (250 mM NaCl, 5 mM KCl, 3 mM CaCl_2 , 2 mM MgCl_2 , and 5 mM sodium phosphate, pH 7.0). Binding of [^3H]ACh (15 nM) was determined using 40 nM ACh binding sites to allow detection of either enhancement or inhibition of binding. These membrane suspensions were pretreated with 0.5 mM diisopropylphosphofluoridate to inhibit acetylcholinesterase. Binding of [^3H]tetracaine (10 nM), [^3H]PCP (10 nM), and [^3H]dFBr (4 nM) was determined using 0.7 μM ACh binding sites in the presence of the competitive antagonist α -bungarotoxin (α -BgTx; 5 μM) or the agonist carbamylcholine chloride (Carb; 1 mM) to stabilize the nAChR in the resting or desensitized state, respectively. Bound and free ^3H were separated by centrifugation (18,000g for 1 hour), then quantified by liquid scintillation counting. Specifically bound ^3H (total-nonspecific) was normalized to the specifically bound ^3H in the absence of competitor. Binding (total-nonspecific) were: for [^3H]ACh (± 1 mM Carb, $7200 \pm 60/83 \pm 3$ cpm); for [^3H]PCP (+Carb, ± 300 μM proadifen, $9000 \pm 80/1310 \pm 15$ cpm; + α -BgTx, ± 300 μM tetracaine, $3300 \pm 90/1520 \pm 15$ cpm); and for [^3H]tetracaine (+ α -BgTx, ± 300 μM tetracaine, $23,600 \pm 1800/4360 \pm 35$ cpm). For direct [^3H]dFBr binding, the total bound ^3H was converted to the concentration (in nanomolar) of bound dFBr.

Data Analyses. Current responses and binding data were analyzed using SigmaPlot 11.0 (Systat Software, San Jose, CA). The effects of drugs on [^3H]dFBr binding were fit to one or two-site models

using eqs. 1 and 2, respectively. All other inhibition data were normalized and fit to a single-site model using eq. 3

$$f_x = f_0 / [1 + (x/IC_{50})] + B_{ns} \quad (1)$$

$$f_x = f_{0,H} / [1 + (x/IC_{50,H})] + f_{0,L} / [1 + (x/IC_{50,L})] + B_{ns} \quad (2)$$

$$f_x = 100 / [1 + (x/IC_{50})] \quad (3)$$

where f_x is radioligand binding or ACh current measured in the presence of inhibitor at concentration x ; f_0 is the specific radioligand binding/ACh current in the absence of inhibitor; IC_{50} is the total concentration of inhibitor that inhibits 50% of the total specific radioligand binding/ACh current, with H and L denoting the high- and low-affinity sites; and B_{ns} is the nonspecific binding. For adjustable parameters, best-fit values $\pm$ S.E. are presented.

[³H]dFBr Photolabeling of *Torpedo* nAChR. Analytical (150 pmol ACh binding sites/condition) and preparative (15 nmol ACh binding sites/condition) [³H]dFBr photolabeling of *Torpedo* nAChR-rich membranes were performed at 2 mg protein/ml in *Torpedo* physiologic saline and 0.5 μ M [³H]dFBr, as previously described (Garcia et al., 2007; Hamouda et al., 2011). In initial analytical photolabeling experiments, [³H]dFBr photoincorporation into the nAChR was compared after photolysis at 254 nm (5 minutes), 312 nm (5 and 10 minutes), and 365 nm (30 minutes) using Spectronics Corporation (Westbury, NY) hand-held lamps. Only photolysis at 312 nm resulted in significant photoincorporation of [³H]dFBr into nAChR subunits, with equal photoincorporation observed at 5 and 10 minutes. For subsequent experiments, *Torpedo* nAChR-rich membranes were incubated with [³H]dFBr for 40 minutes at 4°C in the absence or presence of other drugs and then irradiated on ice at 312 nm for 5 minutes at a distance of less than 2 cm. Photolabeled nAChR-rich membranes were denatured in SDS sample buffer and then resolved on 8% polyacrylamide gels, with the polypeptides visualized by Coomassie Blue R-250 stain (White and Cohen, 1988). In analytical experiments, [³H]dFBr photoincorporation into polypeptides was visualized by fluorography and quantified by liquid scintillation counting of excised gel bands. For photolabeling on a preparative scale, gel bands containing nAChR subunits were excised. The α subunit band was transferred directly to 15% polyacrylamide gels and subjected to in-gel digestion with 100 μ g *Staph aureus* endoprotease Glu-C (V8 protease) (MP Biomedical, Santa Ana, CA) as previously described (Blanton and Cohen, 1994). The β , γ , and δ nAChR subunits and α nAChR subunit fragments (α V8-20, α V8-18, α V8-10, and α V8-4) were recovered from gel bands by passive elution, and then concentrated and resuspended in digestion buffer (15 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 8.1).

Proteolytic Digestion, Peptide Isolation, and N-Terminal Sequencing. Enzymatic digestions of nAChR subunits and subunit fragments, reversed-phase high-performance liquid chromatography (rpHPLC), and automated Edman protein microsequencing were performed as detailed previously (Chiara et al., 2009a). Briefly, rpHPLC was performed on an HP1100 binary system (Agilent Technologies) using a Brownlee Aquapore BU-300 column (PerkinElmer) with material eluted at 0.2 ml/min using a linear (0–100%) or nonlinear gradient (25–100%) of acetonitrile/isopropanol (3:2, 0.05% trifluoroacetic acid) over 100 minutes. Peak ³H rpHPLC fractions were loaded on trifluoroacetic acid-treated glass fiber filters and sequenced on an Applied Biosystems PROCISE 492 protein sequencer (Life Technologies). At each cycle of Edman degradation, 1/6 was used for amino acid identification/quantification and 5/6 for ³H counting. The efficiency of photolabeling (E_x , in cpm/pmol) for an amino acid in cycle x of Edman degradation was calculated using the equation $E_x = (cpm_x - cpm_{(x-1)}) / 5I_0R^2$; where cpm_x is the counts per minute in cycle x , I_0 is the calculated initial mass in picomoles of the peptide sequenced, and R is the calculated repetitive yield of Edman degradation.

To isolate nAChR subunit fragments beginning at β Met²⁴⁹, the N-terminus of β M2, at δ Phe²⁰⁶ before δ M1, and at δ Met²⁵⁷, the N-terminus of δ M2, L-1-tosylamido-2-phenylethylchloromethylketone-treated trypsin (Worthington Biomedical) digests of the β subunit (Chiara et al., 2009a) and *Lysobacter enzymogenes* endoprotease Lys-C (EndoLys-C) (Roche Diagnostics, Basel, Switzerland) digests of the δ subunit (Arevalo et al., 2005) were fractionated on small pore (16.5%T, 6%C) Tricine SDS-PAGE gels (Schägger and von Jagow, 1987). The polypeptides eluted from gel bands with apparent molecular masses of ~8 kDa (β) and 10–14 kDa (δ) were then fractionated by rpHPLC to isolate the peptide beginning at β Met²⁴⁹, which elutes at ~80% organic (Chiara et al., 2009a), and the peptides beginning at δ Phe²⁰⁶ and δ Met²⁵⁷, which elute at ~60 and 75% organic, respectively (Arevalo et al., 2005).

Peptides beginning at the N-terminus of α M2 (α Met²⁴³) and at α His¹⁸⁴ were isolated by rpHPLC fractionation of an EndoLys-C digest of the α V8-20 fragment, which begins at α Ser-173 and contains agonist binding site Segment C and the α M1, α M2, and α M3 helices (Blanton and Cohen, 1994; Pratt et al., 2000). A peptide beginning at α Lys⁷⁷ that contains ACh binding site Segment A was isolated by rpHPLC fractionation of an EndoLys-C digest of the α V8-18 fragment, which begins at α Thr⁵² (Ziebell et al., 2004). A fragment beginning at γ Val¹⁰² that contains ACh binding site Segment E was isolated as previously described (Chiara et al., 2003) by rpHPLC fractionation of a V8 protease digest of a 30 kDa γ subunit fragment isolated by Tricine SDS-PAGE from an EndoLys-C digest of [³H]dFBr-photolabeled γ subunit.

Molecular Modeling and Computational Analyses. For docking in the ECD, the Discovery Studio software package (Accelrys, Inc., San Diego, CA) was used to construct a homology model of the *Torpedo* nAChR ECD from the X-ray structure of the Carb-bound form of the L-AChBP PDB ID 1UV6 (Celie et al., 2004). This model (nAChR_TORCA_ECD_from_1UV6.pdb) is available in the Supplemental Material. AutoDock (Scripps Research Institute, La Jolla, CA) was used to dock dFBr in binding site grids centered at (1) the α - γ extracellular interface at the entrance to the agonist binding site, including γ Tyr¹¹¹ and γ Tyr¹¹⁷; (2) the α - γ extracellular interface in the vestibule of the ion channel, including γ Tyr¹⁰⁵; and (3) the δ - β extracellular interface, including δ Tyr²¹².

For dFBr docking in the ion channel, Discovery Studio was used to construct two *Torpedo* nAChR models, one based on the cryoelectron microscopy-derived structure of *Torpedo marmorata* nAChR (PDB ID 2BG9) (Unwin, 2005) and a second based on the X-ray structure of the mouse serotonin 5-HT3 receptor (5-HT3R) PDB ID 4PIR (Hassaine et al., 2014), which also contains a cation selective channel. The full subunit alignments used for the homology model based on the 5-HT3R structure are presented in the Supplemental Material. This model (nAChR_TORCA_from_4PIR.pdb) is available in the Supplemental Material. In the *Torpedo* 2BG9 nAChR and 4PIR 5-HT3R models, the same surface of the M2 helices line the lumen of the ion channel, but the vertical alignment of the M2 helix relative to the M1 and M3 helices differs, with that of the 5-HT3R being similar to the alignment in the other pentameric ligand-gated ion channels of known structure (Hibbs and Gouaux, 2011; Corringer et al., 2012; Miller and Aricescu, 2014). Recent cysteine cross-linking and photolabeling studies provide experimental evidence favoring nAChR homology models based on these X-ray structures for the structure of the nAChR TMD (Mnatsakanyan and Jansen, 2013; Chiara et al., 2014; Hamouda et al., 2014).

The CDocker module of Discovery Studio was used to dock dFBr in the ion channel. For each docking experiment 20 randomly oriented replicas of dFBr were seeded in the center of a binding site sphere (radius = 15 Å) positioned to contain the five M2 helices including amino acids M2-2 to M2-20 of each M2 helix. For each seed, CDocker was used to generate 10 ligand conformations and the 10 lowest-energy orientations were identified using random rigid-body rotations followed by simulated annealing and a full potential final minimization step. Docking results are shown as Connolly surface

representations (1.4 Å diameter probe) of the ensemble of the 100 lowest-energy docking solutions.

Results

dFBr Is a Potent Noncompetitive Inhibitor of Muscle-Type nAChRs. To characterize the effects of dFBr on muscle-type nAChRs, we recorded ACh-induced currents of *Xenopus* oocytes expressing human muscle ($2\alpha:1\beta:1\delta:1\epsilon$) or *Torpedo* ($2\alpha:1\beta:1\gamma:1\delta$) nAChR. When tested at concentrations up to 10 μM , dFBr neither directly activated these nAChRs nor potentiated ACh-induced responses. Instead, dFBr was equipotent as an inhibitor of both nAChRs (Fig. 2). When co-applied with ACh for 10 seconds, dFBr reversibly reduced peak current responses with IC_{50} values of $1.0 \pm 0.08 \mu\text{M}$. dFBr was 10-fold more potent ($\text{IC}_{50} = 0.1 \pm 0.01 \mu\text{M}$) as a *Torpedo* nAChR inhibitor when it was co-applied with ACh for 10 seconds immediately following a 10-second application of ACh than when co-applied without prior to exposure to ACh (Fig. 3, A and B). In contrast, dFBr was not a potent desensitizing antagonist, because preincubation with 1 μM dFBr for 10 seconds reduced the subsequent ACh-induced response (in the absence of dFBr) by only $\sim 15\%$ (Fig. 3C). These results

Fig. 2. dFBr inhibition of muscle-type nAChRs. *Xenopus* oocytes expressing wild-type (A) human ($2\alpha:1\beta:1\delta:1\epsilon$; ∇) or (B) *Torpedo* ($2\alpha:1\beta:1\gamma:1\delta$; $\blacktriangledown$) nAChR were voltage clamped at -50 mV , and currents elicited by 10 second applications of $10 \mu\text{M}$ ACh were recorded in the absence or presence of increasing concentrations of dFBr. Each drug concentration was tested at least three times and peak currents were normalized to the peak current elicited by $10 \mu\text{M}$ ACh alone. (C) Data (average $\pm$ S.E.) from at least three oocytes were plotted and fit to a single-site model (eq. 3); human nAChR, $\text{IC}_{50} = 1.0 \pm 0.08 \mu\text{M}$; *Torpedo* nAChR, $\text{IC}_{50} = 0.1 \pm 0.06 \mu\text{M}$.

indicated that inhibition by dFBr is state dependent and that dFBr might be most potent as an inhibitor of nAChRs in states stabilized only transiently by agonist. To test this, we examined dFBr inhibition of muscle-type nAChRs containing the leucine-to-serine substitutions at position M2-9' (αLeu^{251} , βLeu^{257} , γLeu^{260} , and δLeu^{265}) that have been shown to decrease ACh EC_{50} values and increase nAChR channel mean open time (Filatov and White, 1995; Labarca et al., 1995). dFBr was 7-fold more potent as an inhibitor of the γ subunit mutant nAChR than of wild-type and ~ 2 -fold more potent for the α and β subunit mutants (Fig. 3D).

We also used equilibrium radioligand binding assays to characterize the effects of dFBr on the binding of ACh and known channel blockers to *Torpedo* nAChR-rich membranes (Fig. 4A). dFBr enhanced the equilibrium binding of [^3H]ACh at concentrations up to $100 \mu\text{M}$ and only inhibited binding at higher concentrations. For *Torpedo* nAChR stabilized in the desensitized state in the presence of the agonist Carb, dFBr was a potent inhibitor of the equilibrium binding of [^3H]phencyclidine ([^3H]PCP) ($\text{IC}_{50} = 3.6 \pm 0.2 \mu\text{M}$), a desensitized state-selective nAChR channel blocker (Oswald et al., 1983). dFBr was ~ 10 -fold less potent as an inhibitor of [^3H]PCP binding to the nAChR stabilized in the resting, closed-channel state in the presence of the competitive antagonist α -BgTx ($\text{IC}_{50} = 49 \pm 6 \mu\text{M}$) or as an inhibitor of binding of [^3H]tetracaine ($\text{IC}_{50} = 63 \pm 11 \mu\text{M}$), a resting state-selective nAChR ion-channel blocker (Middleton et al., 1999).

Equilibrium Binding of [^3H]dFBr. We characterized the equilibrium binding of [^3H]dFBr (4 nM) to *Torpedo* nAChR-rich membranes ($0.7 \mu\text{M}$ ACh binding sites). As shown in Fig. 4B, [^3H]dFBr binding was higher in the desensitized state (+Carb; $\sim 2.2 \text{ nM}$ [^3H]dFBr bound) than in the resting state (+ α -BgTx; $\sim 1.5 \text{ nM}$ [^3H]dFBr bound). In the presence of Carb, PCP at high concentrations reduced [^3H]dFBr binding to the level seen in the presence of α -BgTx. The concentration dependence of inhibition ($\text{IC}_{50} = \sim 1 \mu\text{M}$) was consistent with PCP's affinity for the ion channel binding site (Oswald et al., 1983). Nonradioactive dFBr also inhibited [^3H]dFBr binding in the presence of Carb, but the concentration dependence was not consistent with a simple, single-site model. The inhibition was consistent with a two-site model: (1) a high-affinity component ($\text{IC}_{50} = \sim 6 \mu\text{M}$) that reduced [^3H]dFBr binding to the same extent as PCP, and (2) a low-affinity component that reduced [^3H]dFBr binding with the same concentration dependence as seen for dFBr inhibition in the presence of α -BgTx ($\text{IC}_{50} = \sim 400 \mu\text{M}$). Tetracaine, which in the presence of α -BgTx binds in the nAChR ion channel with high affinity ($K_{\text{eq}} = 0.5 \mu\text{M}$) (Middleton et al., 1999), only inhibited [^3H]dFBr binding at concentrations $> 100 \mu\text{M}$. These results indicate that the high-affinity component of [^3H]dFBr binding in the desensitized state results from [^3H]dFBr binding in the ion channel, and dFBr inhibition of [^3H]PCP binding establishes that dFBr binds in the channel with ~ 10 -fold higher affinity in the desensitized state ($\text{IC}_{50} = \sim 4 \mu\text{M}$) than in the resting state. The low potency of tetracaine as an inhibitor of [^3H]dFBr binding in the presence of α -BgTx indicates that this low-affinity component of binding is unlikely to be in the ion channel.

[^3H]dFBr Photolabeling of *Torpedo* nAChR. Covalent incorporation into *Torpedo* nAChR-rich membranes was determined by SDS-PAGE after membrane suspensions equilibrated

Fig. 3. dFBr inhibition of *Torpedo* nAChR is enhanced in the presence of agonist and by mutations in the ion channel favoring the open state. (A) Representative traces showing the effect of dFBr on ACh-induced current responses when 10 μ M ACh was applied for 10 seconds, followed by co-application of 10 μ M ACh and dFBr for 10 seconds, and then 10 seconds of 10 μ M ACh alone. (B) The concentration dependence of dFBr inhibition when co-applied with ACh as in (A), with the current measured at the end of dFBr co-application normalized to the current just before ($\bullet$, $IC_{50} = 0.12 \pm 0.01 \mu$ M), compared with a parallel experiment quantifying dFBr inhibition of peak ACh responses without prior ACh exposure ($\circ$, dashed line, fit from Fig. 2 for data from 10 oocytes). (C) Representative traces showing the effect of preapplication of 1 μ M dFBr for 10 seconds on nAChR sensitivity to ACh. The ACh responses were inhibited by $\sim 15 \pm 3\%$ ($N = 6$). (D) dFBr inhibition of mutant nAChRs containing leucine to serine substitutions at position M2-9' within the ion channel, quantified from the peak current elicited by 10 μ M ACh with co-application of dFBr normalized to the current in the absence of dFBr. IC_{50} values for wild type, α , β , γ , and δ M2-9' mutants were 1.0 ± 0.07 , 0.2 ± 0.04 , 0.3 ± 0.03 , 0.1 ± 0.02 , and $0.5 \pm 0.06 \mu$ M, respectively. For each dFBr concentration in (B) and (D), average $\pm$ S.E. of several replicas from at least three oocytes were plotted and fit to a single-site model.

with 0.5 μ M [3 H]dFBr were irradiated at 312 nm for 5 minutes (Fig. 5). Based on fluorography (Fig. 5A) and liquid scintillation counting of excised gel bands (Fig. 5B), [3 H]dFBr was incorporated into each nAChR subunit. Carb enhanced 3 H incorporation in each subunit ($\alpha/\beta/\gamma/\delta$, $\sim 25/100/40/60\%$). α -BgTx reduced 3 H incorporation into the α subunit by 40% but by $<15\%$ in the β , γ , and δ subunits. The Carb-enhanced photolabeling within the nAChR subunits was inhibitable by PCP.

State-Dependent [3 H]dFBr Photolabeling in the nAChR Ion Channel. The effects of Carb and PCP on [3 H]dFBr photoincorporation into the nAChR subunits and the results of the competition radioligand binding assays indicated the presence of a state-dependent, high-affinity dFBr binding site within the nAChR ion channel. To identify amino acid residues photolabeled by [3 H]dFBr within the M2 helices that line the lumen of the ion channel, subunit fragments beginning at the N-termini of β M2 (β Met²⁴⁹) and δ M2 (δ Met²⁵⁷) were isolated from trypsin and EndoLys-C digests of [3 H]dFBr-photolabeled β and δ subunits by Tricine SDS-PAGE (data not shown) followed by rpHPLC (Fig. 6, A and B). Upon N-terminal sequencing of the fragment beginning at β Met²⁴⁹ (Fig. 6C), there was a single major peak of Carb-enhanced 3 H release in cycle 9, consistent with [3 H]dFBr photolabeling of β Leu²⁵⁷ (β M2-9'; -Carb/+Carb, 2/50 cpm/pmol). For the fragment beginning at δ Met²⁵⁷, there were peaks of 3 H release in cycles 2, 5, 6, 9, and 16, indicating [3 H]dFBr photolabeling of amino acids that line the ion channel: δ Ser²⁵⁸ (δ M2-2'), δ Ile²⁶¹ (δ M2-5'), δ Ser²⁶² (δ M2-6'), δ Leu²⁶⁵ (δ M2-9'), and δ Leu²⁷² (δ M2-16'). The efficiency of photolabeling of these amino acids was enhanced by ~ 10 -fold in the presence of Carb.

[3 H]dFBr Photolabeling within the nAChR α Subunit: Ion Channel and ACh Binding Site. Similar to the other nAChR subunits, the Carb-enhanced photolabeling within the α subunit was inhibitable by PCP (Fig. 5), suggesting [3 H]dFBr photolabeling within α M2. In contrast to the other nAChR subunits, in the resting state α -BgTx reduced α subunit photolabeling by $\sim 40\%$, suggesting potential [3 H]dFBr photolabeling within the ACh binding site. Photolabeling within α M2 and in ACh binding site segments A (α Tyr⁹³) and C (α Tyr¹⁹⁰/ α Tyr¹⁹⁷) can be readily determined by generating large α subunit fragments by limited in-gel digestion with endoproteinase Glu-C (V8 protease), followed by EndoLys-C digestion of those fragments and rpHPLC fractionation of the digests (see *Materials and Methods*). Limited digestion with V8 protease produces large subunit fragments (Fig. 7A): α V8-18 that begins at α Thr⁵² and contains ACh binding site Segments A and B; α V8-20 that begins at α Ser¹⁷³ and contains binding site Segment C and the M1-M3 helices; and α V8-10 that begins at α Asn³³⁹ and contains the M4 helix (Blanton and Cohen, 1994). Fragments beginning at α His¹⁸⁶, containing ACh binding site Segment C, and at α Met²⁴³, the N-terminus of α M2, can be isolated by rpHPLC from EndoLys-C digests of α V8-20 (Pratt et al., 2000), while the fragment beginning at α Lys⁷⁷, containing binding site Segment A, can be isolated from an EndoLys-C digest of α V8-18 (Ziebell et al., 2004).

We found that the α V8-20 and α V8-18 fragments contained $>75\%$ and $\sim 10\%$ of α subunit 3 H, respectively. When EndoLys-C digests of α V8-20 were fractionated by rpHPLC (Fig. 7B), there was a Carb-inhibitable peak of 3 H at $\sim 60\%$ organic, the expected location of the α His¹⁸⁶ fragment. In the presence of Carb, the major peak of 3 H was at $\sim 80\%$ organic,

Fig. 4. dFBr binds with high affinity to a site in the nAChR ion channel (desensitized state). The effects of dFBr on the equilibrium binding of $[^3H]ACh$, $[^3H]PCP$, and $[^3H]tetracaine$ (A) and the effect of dFBr, PCP, and tetracaine on the equilibrium binding of $[^3H]dFBr$ (B) to the *Torpedo* nAChR in the presence of α -BgTx ($\square$, ∇ , $\triangle$) or Carb ($\bullet$, $\blacktriangle$) were determined using centrifugation assays. (A) For each dFBr concentration, the specific binding of the radioligand was calculated from the total and nonspecific binding, normalized to the specific binding in the absence of dFBr, and fit to a single-site model using eq. 3. dFBr enhanced $[^3H]ACh$ binding (125% at $30 \mu M$), inhibited $[^3H]PCP$ binding (+Carb) with $IC_{50} = 3.6 \pm 0.2 \mu M$, and inhibited $[^3H]PCP$ and $[^3H]tetracaine$ binding in the presence of α -BgTx with IC_{50} values of 49 ± 6 and $63 \pm 11 \mu M$, respectively. (B) For each drug concentration, bound $[^3H]dFBr$ (in cpm/ml) was converted to nM $[^3H]dFBr$. Inhibition curves were fit to a single-site model (eq. 1) or for inhibition by nonradioactive dFBr (+Carb) to a two-site model (eq. 2). For PCP (+Carb), $IC_{50} = 0.9 \pm 0.1 \mu M$ and $B_{ns} = 1.4 \pm 0.1$ nM, respectively. For dFBr (+ α -BgTx), $IC_{50} = 380 \pm 70 \mu M$, $B_{spec} = 1.1 \pm 0.08$ nM, and $B_{ns} = 0.36 \pm 0.09$ nM. For inhibition by dFBr (+Carb), the high-affinity component of binding was fixed as the total binding $[(+Carb) - (+\alpha$ -BgTx)], the low-affinity component was equal to $B_{spec}(+\alpha$ -BgTx), and B_{ns} was the same as in the presence of α -BgTx. With these assumptions, the calculated IC_{50} values for the high- and low-affinity components of binding were 4.1 ± 0.4 and $460 \pm 30 \mu M$, respectively.

the expected location of the α Met²⁴³ fragment. For the EndoLys-C digests of α V8-18 (Fig. 7C), there was a single Carb-inhibitable peak of 3H at ~50% organic, the expected location of the α Lys⁷⁷ fragment.

N-terminal amino acid sequencing of the hydrophobic 3H peak from the EndoLys-C digest of α V8-20 (Fig. 8A) established the presence of the α Met²⁴³ fragment, with peaks of 3H release at cycles 2, 5, 6, and 9 that indicated Carb-enhanced $[^3H]dFBr$ photolabeling of α Thr²⁴⁴ (α M2-2'), α Ile²⁴⁷ (α M2-5'), α Ser²⁴⁸ (α M2-6'), and α Ile²⁵¹ (α M2-9'), amino acids that line the lumen of the ion channel. Sequence analyses of material in the hydrophilic peak of 3H from the EndoLys-C digest of α V8-20 (Fig. 8B) established the presence of the peptide beginning at α His¹⁸⁶ and peaks of 3H release at cycles 5

Fig. 5. Photoincorporation of $[^3H]dFBr$ into *Torpedo* nAChR-rich membranes. Membranes were photolabeled on an analytical scale with $0.5 \mu M$ $[^3H]dFBr$ in the absence and presence of nAChR ligands. Polypeptides were resolved by SDS-PAGE on duplicate gels that were stained with Coomassie blue (A, lane 1). One gel was prepared for fluorography (A, lanes 2–4), and polypeptide bands were excised from the second gel for 3H determination by liquid scintillation counting (B). Drug additions for the fluorograph in (A): lane 2, no other ligand added; lane 3, +1 mM Carb; lane 4, +1 mM Carb and $100 \mu M$ PCP. The electrophoretic mobilities of the nAChR α , β , γ , and δ subunits, rapsyn (Rn), the Na⁺/K⁺-ATPase α subunit (90K) and calcein (37K) are indicated on the left.

and 13 in the –Carb condition that identified $[^3H]dFBr$ photolabeling of the core aromatic amino acids in ACh binding site Segment C, α Tyr¹⁹⁰ and α Tyr¹⁹⁸. Carb inhibited photolabeling of those residues by >90%. Carb-inhibitable photolabeling of α Tyr⁹³, the core aromatic amino acid in ACh binding site Segment A, was established by sequence analysis of the material in the peak of 3H isolated by rpHPLC from the EndoLys-C digest of α V8-18 (Fig. 8C), with the Carb-inhibitable peak of 3H release in cycle 17 indicating photolabeling of α Tyr⁹³, the core aromatic amino acid in ACh binding site Segment A.

$[^3H]dFBr$ Photolabeling within the nAChR ECD in the Presence of Agonist. In a *Torpedo* nAChR photolabeling study with nonselective nAChR allosteric potentiators (Hamouda et al., 2013), $[^3H]galantamine$ and $[^3H]physostigmine$ were found to photolabel in the presence and absence of agonist γ Tyr¹⁰⁵ and γ Tyr¹¹¹ in ACh binding site Segment E at the canonical α - γ interface and δ Tyr²¹² at the noncanonical δ - β extracellular interface. To determine whether $[^3H]dFBr$ also photolabeled amino acids in binding site Segment E, the fragment beginning at γ Val¹⁰² was isolated for sequence analysis from $[^3H]dFBr$ -photolabeled γ subunits by sequential proteolytic digests, Tricine SDS-PAGE, and rpHPLC (see *Materials and Methods*). During N-terminal sequencing of the γ Val¹⁰² fragment isolated from nAChRs photolabeled in the absence of Carb, there was a peak of 3H release in cycle 10, consistent with $[^3H]dFBr$ photolabeling of γ Tyr¹¹¹ at 18 cpm/pmol (Fig. 9A). This photolabeling was reduced by ~60% in the presence of Carb. There was also a small peak of Carb-enhanced 3H release at cycle 4, indicating that $[^3H]dFBr$ also photolabeled γ Tyr¹⁰⁵ (–Carb/+Carb, 0.2/1.4 cpm/pmol). We characterized $[^3H]dFBr$ photolabeling around δ Tyr²¹² by sequencing the δ subunit fragment beginning δ Phe-206 (Fig. 9B). The Carb-enhanced peak of 3H release at cycle 7 indicated photolabeling of δ Tyr²¹² (–Carb/+Carb, 0.2/1.4 cpm/pmol). $[^3H]dFBr$ photolabeling of γ Tyr¹⁰⁵, γ Tyr¹¹¹, and δ Tyr²¹² and the effect of Carb on that photolabeling mirror those seen for $[^3H]physostigmine$ and $[^3H]galantamine$, which suggests that dFBr binds at the physostigmine/galantamine sites in the nAChR ECD at the canonical α - γ and noncanonical δ - β subunit interfaces.

Fig. 6. Agonist-enhanced $[^3\text{H}]$ dFBr photolabeling in βM2 , and δM2 . *Torpedo* nAChR-rich membranes were photolabeled at preparative scale with $0.5 \mu\text{M}$ $[^3\text{H}]$ dFBr in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of 1 mM Carb. (A and B) Elution of peptides (solid line) and ^3H ($\circ$, $\bullet$) during rpHPLC purifications of an ~ 8 kDa β subunit fragment isolated by from a trypsin digest (A) and an ~ 14 kDa δ subunit fragment isolated from an EndoLys-C digest (B). (C and D) ^3H ($\circ$, $\bullet$) and phenylthiohydantoin amino acids ($\square$, $\blacksquare$) released during sequence analyses of rpHPLC fractions 30 to 31 from (A) and fractions 27–29 from (B), respectively. (C) The only sequence detected began at the N-terminus of βM2 (βMet^{249} ; 5 pmol each condition), and the major peak of ^3H release in cycle 9 indicates photolabeling of βLeu^{257} ($\beta\text{M2-9}$; $-\text{Carb}/+\text{Carb}$, 2/50 cpm/pmol). (D) The only sequence detected began at the N-terminus of δM2 (δMet^{257} ; 10 pmol each condition). The peaks of ^3H release in cycles 2, 5, 6, 9, and 16 indicate photolabeling ($-\text{Carb}/+\text{Carb}$, in cpm/pmol) at δSer^{258} ($\delta\text{M2-2}$; 1/7), δIle^{261} ($\delta\text{M2-5}$; 1/14), δSer^{262} ($\delta\text{M2-6}$; 2/17), δLeu^{265} ($\delta\text{M2-9}$; 0.5/14), and δLeu^{272} ($\delta\text{M2-16}$; 1/12).

Discussion

In addition to being a potent $\alpha 4\beta 2$ nAChR PAM, the results presented here demonstrate that dFBr is a potent inhibitor of muscle-type nAChRs and that its intrinsic photoreactivity makes it well suited to identify the amino acids contributing to its binding sites in nAChRs by photoaffinity labeling. dayFBr is equipotent as an inhibitor ($\text{IC}_{50} \sim 1 \mu\text{M}$) of adult human and *Torpedo* muscle-type nAChRs expressed in *Xenopus* oocytes. For the *Torpedo* nAChR, equilibrium radioligand binding assays established that dFBr binds with 10-fold higher affinity in the ion channel in the desensitized state ($\text{IC}_{50} \sim 4 \mu\text{M}$) than in the closed-channel state and that it binds only weakly ($\text{IC}_{50} \sim 1 \text{ mM}$) to the ACh binding sites. Photoaffinity labeling of the *Torpedo* nAChR with $[^3\text{H}]$ dFBr allowed the identification of amino acids contributing to the high-affinity binding site in the ion channel and to three classes of binding sites in the nAChR ECD. The locations of the photolabeled amino acids in the ion channel and in the ECD are shown in nAChR homology models in Figs. 10 and 11, along with the orientations of dFBr in these sites predicted by computational docking.

dFBr Is a Broad-Spectrum Photoaffinity Probe. While the photoreactive intermediates formed upon UV irradiation of dFBr are unknown, radical intermediates, based on the diversity of identified photolabeled amino acids (Ser, Leu, and Ile residues in the channel; Tyr in the ECD), are likely. Photolytic cleavage of the C–Br bond to form a reactive radical is one likely mechanism, based on the known susceptibility of haloindoles to photodehalogenation (Yang et al., 1989).

dFBr Binding in the Ion Channel. The photolabeling of the amino acids at M2-2', -5', -6', and -9' in the nAChR α , β , and δ subunits provides an experimental definition of the high-affinity dFBr binding site in the ion channel in the desensitized state. This site, near the cytoplasmic end of the channel, extends over two helical turns ($\sim 10 \text{ \AA}$), a distance similar to the length of dFBr in an extended conformation ($9.1 \text{ \AA}$ from the allyl group to the Br). This site, first identified by photoaffinity labeling with $[^3\text{H}]$ chlorpromazine (Giraudat

et al., 1986), is the binding site in the channel for many other cationic and uncharged channel blockers. The $[^3\text{H}]$ dFBr photolabeling of $\delta\text{M-16'}$ and $20'$ suggests an alternative mode of binding near the extracellular end of the ion channel, as has been found for $[^3\text{H}]$ chlorpromazine (Chiara et al., 2009a). Strikingly, neither $[^3\text{H}]$ dFBr nor $[^3\text{H}]$ chlorpromazine photolabeled the valines at position M2-13', which contribute to the binding site for resting state-selective channel blockers including 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazine, $[^3\text{H}]$ tetracaine, and $[^3\text{H}]$ AziPm, a photoreactive propofol analog (White and Cohen, 1992; Gallagher and

Fig. 7. Isolation of $[^3\text{H}]$ dFBr-photolabeled α subunit peptides. The band for nAChR α subunit from the photolabeling experiment described in Fig. 6 was excised and transferred to a 15% acrylamide mapping gel and subjected to in-gel digestion with V8 protease to produce large nAChR α subunit fragments shown schematically in (A). rpHPLC fractionations of EndoLys-C digests of (B) $\alpha\text{V8-20}$, which begins at αSer^{173} , and (C) $\alpha\text{V8-18}$, which begins at αThr^{52} . Elution of peptides (solid line) and ^3H ($\circ$ - Carb, $\bullet$ + Carb) were monitored. rpHPLC fractions 27–29 and 30–32 from the V8-20 digest and fractions 17–19 from the V8-18 digest were pooled for sequence analysis (Fig. 8).

Fig. 8. [^3H]dFBr photolabeling within α subunit. [^3H] ($\circ$, $\bullet$) and phenylthiohydantoin amino acids ($\square$, $\blacksquare$) released during sequence analyses of rpHPLC fractions 30–32 (A) and 27–29 (B) from the fractionation of Endolys-C digests of $\alpha\text{V8-20}$ (Fig. 7B) and fractions 17–19 (C) from the purification of the Endolys-C digest of $\alpha\text{V8-18}$ (Fig. 7C). (A) The primary sequence began at the N-terminus of αM2 (αMet^{243} , 4 pmol each condition) with a contaminating peptide beginning at βM^{249} (~ 0.5 pmol each condition). The peaks of Carb-enhanced [^3H]dFBr photolabeling (–Carb/+Carb, in cpm/pmol) at αThr^{244} ($\alpha\text{M2-2}$, 4/15), αIle^{247} ($\alpha\text{M2-5}$, 11/36), αSer^{248} ($\alpha\text{M2-6}$, 7/31), and αIle^{251} ($\alpha\text{M2-9}$, 15/101). (B) The only sequence began at αHis^{186} ($\square$ –Carb/ $\blacksquare$ +Carb, 8/10 pmol), with Carb-inhibitable peaks of [^3H]dFBr release at cycles 5 and 13 indicating [^3H]dFBr photolabeling at αTyr^{190} and αTyr^{198} (–Carb/+Carb, 12/1 and 19/2 cpm/pmol, respectively). (C) The pool of material in rpHPLC fractions 17–19 from Fig. 7C was sequenced with the sequencing filter treated before cycle 12 with *o*-phthalaldehyde (OPA), which prevents sequencing of peptides not containing a proline at that cycle (Middleton and Cohen, 1991). After OPA treatment, sequencing continued only for the fragment beginning at αLys^{77} ($\square$ –Carb/ $\blacksquare$ +Carb, 22/31 pmol) that contains αPro^{88} in cycle 12. The peak of [^3H]dFBr release in cycle 17 indicated [^3H]dFBr photolabeling of αTyr^{93} (–Carb/+Carb, 3/0.1 cpm/pmol).

Cohen, 1999; Jayakar et al., 2013). Because the photoreactive intermediates formed upon irradiation of dFBr react readily with aliphatic side chains, the lack of photolabeling of M2-13' indicates that dFBr does not bind at this level in the ion channel.

For the *Torpedo* nAChR homology model based on the 5-HT₃R structure, dFBr docked within the ion channel in two distinct orientations with similar energies: (1) with the allyl group of dFBr at the level of M2-6' and the 6-Br below the level of M2-2', and (2) with the allyl group at the level of M2-4' and the 6-Br at the level of M2-9' (Fig. 10B). Similar docking clusters were seen for dFBr docked within the channel in the homology model based on the *T. marmorata* nAChR structure; however, for both clusters the allyl group was centered near M2-9' with the Br either above M2-2' or near M2-13' (Fig. 10C).

dFBr Binding Sites in the nAChR ECD. To visualize the locations of photolabeled amino acids in the ECD, we used a homology model derived from Carb-bound form of the L-AChBP (PDB ID 1UV6) (Celie et al., 2004). The amino acids photolabeled by [^3H]dFBr in the presence of agonist identified three distinct sites (Fig. 11). Sites I and II are at the canonical α - γ subunit interface, with Site I (Fig. 11, A and D), near the entry to the transmitter binding site (Fig. 11E), and Site II, in the vestibule of the ion channel (Fig. 11F), identified by

Fig. 9. [^3H]dFBr photolabels γTyr^{105} , γTyr^{111} , and δTyr^{212} in the presence of agonist. [^3H] ($\circ$, $\bullet$) and phenylthiohydantoin amino acids ($\square$, $\blacksquare$) released during sequence analyses of a γ subunit fragment beginning at γVal^{102} (~ 8 pmol each condition) (A) and a δ subunit fragment beginning at δPhe^{206} (~ 4 pmol each condition) (B), each isolated as described in *Materials and Methods* from the nAChR photolabeling of Fig. 6 (–Carb ($\circ$, $\square$), +Carb ($\bullet$, $\blacksquare$)). (A) The major peak of [^3H]dFBr release at cycle 10 indicates [^3H]dFBr photolabeling of γTyr^{111} (–Carb/+Carb, 18/6 cpm/pmol). The small peak of Carb-enhanced [^3H]dFBr release at cycle 4 indicates photolabeling of γTyr^{105} (–Carb/+Carb, 0.2/1.4 cpm/pmol). (B) During sequencing of the fragment beginning at δPhe^{206} , recovered from rpHPLC fractions 23–25 in Fig. 6B, the peak of Carb-enhanced [^3H]dFBr release at cycle 7 indicates photolabeling of δTyr^{212} (–Carb/+Carb, 0.2/1.4 cpm/pmol). OPA, *o*-phthalaldehyde.

photolabeling of γTyr^{111} and γTyr^{105} , respectively. Site III, identified by photolabeling of δTyr^{212} , is at the noncanonical δ - β subunit interface (Fig. 11, B and C) and is equivalent to the benzodiazepine site in the GABA_A receptor (Sigel and Buhr, 1997; Richter et al., 2012). dFBr affinity for these sites must be much lower than for the site in the ion channel, which was identified as the high-affinity component of [^3H]dFBr binding. These three binding sites in the ECD are the same as those photolabeled by [^3H]physostigmine and [^3H]galantamine, low-efficacy *Torpedo* nAChR PAMs that did not bind with high-affinity, or photolabel amino acids in the ion channel (Hamouda et al., 2013).

Figure 11, C, E, and F depicts dFBr in ball-and-stick format in the lowest-energy orientations predicted by computational docking to these sites in a model containing Carb at the transmitter binding sites. In Site I dFBr is predicted to bind above the level of α subunit ACh binding site Loop C with the bromine carbon (C6) within 5 Å of γTyr^{111} . In Site II dFBr is predicted to bind in the vestibule of the ion channel with C6 within 5 Å of the photolabeled γTyr^{105} and αTyr^{151} , whose photolabeling was not characterized. In Site III at the δ - β subunit interface, dFBr is predicted to bind between δ subunit extracellular loop 10, homologous to ACh binding site Loop C in α subunit, and β strands 5 and 6 of the β subunit (Fig. 11, B and C). dFBr C6 is closest to δAsn^{152} and βAsn^{107} and within 7 Å of photolabeled δTyr^{212} , which is homologous to αTyr^{198} in ACh binding site Loop C.

Differential Effects of dFBr at Muscle-Type and $\alpha 4\beta 2$ nAChR: Relevance to PAM Binding Sites. Our results establish that dFBr binds with high affinity to a site in the ion channel and with much lower affinity to multiple sites in the ECD. Equilibrium radioligand binding assays established that dFBr binds with higher affinity to the ion channel in the desensitized state than in the closed-channel state, and our electrophysiological assays using wild-type and mutant receptors indicate that dFBr interacts preferentially with

Fig. 10. dFBr binding site in the *Torpedo* muscle-type nAChR ion channel. (A and B) Side view (A) and view from within the ion channel toward the α - β - δ subunits (B) of a nAChR homology model based on the X-ray structure of the mouse serotonin 5-HT₃R (PDB ID 4PIR) (Hassaine et al., 2014) and (C) a homology model based on the cryo-electron microscopy structure of *Torpedo marmorata* nAChR (PDB ID 2BG9) (Unwin, 2005). The nAChR α , β , γ , and δ subunits are colored in gold, blue, green, and violet, respectively. Amino acids photolabeled by [³H]dFBr within the α / β M2 helices are colored in green and identified by their position numbers from the N-terminus of the M2 helices. In each model, the ensemble of the 100 lowest energy dFBr docking solutions within the channel is shown as a Connolly surface representation colored by atom (C, black; H, white; Br, red), defining volumes of 905 Å³ (B) and 821 Å³ (C) compared with the dFBr molecular volume of 241 Å³.

the open-channel state compared with the closed-channel or equilibrium-desensitized state. Further studies using rapid perfusion techniques to measure responses at millisecond times will be necessary to determine whether dFBr inhibits nAChRs in the open-channel state without enhancing desensitization, as is seen for classic open-channel blockers. Presumably dFBr inhibits nAChR in the open state by binding

in the ion channel; however, photoaffinity labeling studies using rapid mixing and freeze quench techniques will be necessary to determine whether the binding site in the open-channel state is the same as that seen in the desensitized state, as seen for a photoreactive etomidate analog (Chiara et al., 2009b), or whether there is preferential, transient binding to a nonchannel site (Arevalo et al., 2005; Yamodo et al., 2010).

While inhibition of muscle-type nAChR due to high-affinity binding in the ion channel precludes the ability to study the functional contribution of dFBr binding at the extracellular sites, dFBr binding to one or more of those sites in α 4 β 2 nAChRs is likely to result in potentiation of ACh responses. Our results establish that dFBr can photoincorporate into a wide range of amino acid side chains; however, we did not find photolabeling of amino acids in the TMD that contribute to the known intersubunit or intrasubunit binding sites for *Torpedo* nAChR positive and negative allosteric modulators (Nirathanan et al., 2008; Hamouda et al., 2011, 2014; Jayakar et al., 2013). Mutational analyses provide evidence for PAM binding sites in the ECD at noncanonical interfaces analogous to dFBr Site III in α 3 β 2 nAChRs at the β 2/ α 3 interface (Seo et al., 2009) and in α 4 β 2 nAChRs at the α 4/ α 4 interface, which is also an ACh binding site (Mazzaferro et al., 2014; Olsen et al., 2014). Mutational analyses and computational docking also provide evidence for negative allosteric modulator binding sites equivalent to dFBr Site I at the canonical interfaces in α 4 β 2 and α 3 β 4 nAChRs (Grishin et al., 2010;

Fig. 11. dFBr binding sites in the *Torpedo* nAChR ECD. Views of the homology model, the *Torpedo* nAChR ECD based on the x-ray structure of the L-AChBP in the presence of Carb (PDB ID 1UV6) (Celie et al., 2004) with the α , β , γ , and δ subunits colored in gold, blue, green, and violet, respectively. A view from the top (A) and side views from the exterior (B and D) are shown with dFBr, in space-filling representation, docked at three distinct sites in the presence of the agonist Carb (yellow). Sites I and II are at the agonist-binding canonical interface near entry to the ACh binding site (Site I; A, D, and E) and in the vestibule of the ion channel (Site II; A, D, and F). Site III (A, B, and C) is at the δ - β subunit interface. Views from the exterior (C and E) and a view from the ion channel vestibule (F) showing dFBr docked in its predicted lowest energy orientations in Site I (E), Site II (F), and Site III (C).

Henderson et al., 2012). Based on our results, [³H]dFBr should serve as an appropriate photoreactive PAM to identify its binding sites in $\alpha 4\beta 2$ nAChRs and to identify other allosteric modulators that competitively inhibit photolabeling.

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Authorship Contributions

Participated in research design: Hamouda, Wang, Stewart, Cohen.
Conducted experiments: Hamouda, Wang.
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