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Identifying the binding site(s) for antidepressants on the *Torpedo* nicotinic acetylcholine receptor: [^{3}H]2-azidoimipramine photolabeling and molecular dynamics studies

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ABSTRACT

Radioligand binding, photoaffinity labeling, and docking and molecular dynamics were used to characterize the tricyclic antidepressant (TCA) binding sites in the nicotinic acetylcholine receptor (nAChR). Competition experiments indicate that the noncompetitive antagonist phencyclidine (PCP) inhibits [^{3}H]imipramine binding to resting (closed) and desensitized nAChRs. [^{3}H]2-azidoimipramine photoincorporates into each subunit from the desensitized nAChR with ~25% of the labeling specifically inhibited by TCP (a PCP analog), whereas no TCP-inhibitable labeling was observed in the resting (closed) state. For the desensitized nAChR and within the α subunit, the majority of specific [^{3}H]2-azidoimipramine labeling mapped to a ~20 kDa *Staphylococcus aureus* V8 protease fragment (αV8-20; Ser^{173}-Glu^{338}). To further map the labeling site, the αV8-20 fragment was further digested with endoproteinase Lys-C and resolved by Tricine SDS-PAGE. The principal labeled fragment (11 kDa) was further purified by rpHPLC and subjected to N-terminal sequencing. Based on the amino terminus (αMet^{243}) and apparent molecular weight, the 11 kDa fragment contains the channel lining M2 segment. Finally, docking and molecular dynamics results indicate that imipramine and PCP interact preferably with the M2 transmembrane segments in the middle of the ion channel. Collectively, these results are consistent with a model where PCP and TCA bind to overlapping sites within the lumen of the *Torpedo* nAChR ion channel.

1. Introduction

The main pharmacological action of antidepressants (ADs) is to increase the synaptic concentrations of norepinephrine, serotonin, and/or dopamine by inhibiting the reuptake of these neurotransmitters from the synaptic cleft. Moreover, additional studies have shown that ADs act as noncompetitive antagonists (NCAs) of muscle- and neuronal-type nicotinic acetylcholine receptors (nAChRs) (reviewed in [1]). The muscle-type nAChR, in particular the nAChR isolated from *Torpedo californica* electric organ, has been studied extensively over the past several decades and serves as the archetype of the Cys-loop ligand-gated ion channel superfamily. This genetically linked receptor superfamily includes both muscle- and neuronal-type nAChRs, type A and C γ-aminobutyric acid, type 3 5-hydroxytryptamine (serotonin), and glycine receptors (reviewed in [2–4]).

Previous studies from our laboratory determined the molecular mechanisms underlying the noncompetitive inhibition of AChRs elicited by tricyclic antidepressants (TCAs) [5]. These earlier studies further established that imipramine inhibits the binding of the well-characterized NCA [piperidyl-3, 4-^{3}H(*N*)]-(*N*-(1-(2 thienyl)cyclohexyl)-3,4-piperidine) ([^{3}H]TCP), a structural and functional analog of the dissociative anesthetic phencyclidine (PCP), to both resting and desensitized nAChRs (K_is = 6.7 ± 0.4 and 0.75 ± 0.04 μM, respectively [5]). However, the structural components of the TCA binding sites in conformationally distinct nAChRs have not been characterized in detail. Thus, to further characterize the TCA binding sites, we have employed [^{3}H]2-azidoimipramine (see Fig. 1), a photoreactive analog of imipramine, to covalently tag the TCA binding sites in the *Torpedo* nAChR ion channel. We choose the *Torpedo* nAChR as a receptor model mainly because of its high abundance in the electroplaque tissue of *T. californica* fish compared to cells and neurons expressing neuronal-type nAChRs.

The radioligand binding, photoaffinity labeling, and docking and molecular dynamics data suggest that imipramine interacts with the PCP binding sites in the resting (closed) and desensitized states. More specifically, imipramine binds mainly to the M2 transmembrane segment of the *Torpedo* AChR, which is the principal segment forming

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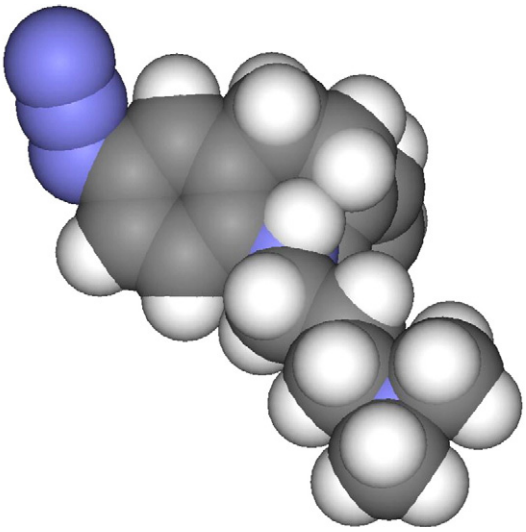

Fig. 1. Molecular structure of [^{3}H]2-azidoimipramine. The molecule was built using the Builder module of Insight 2005L (Accelrys, San Diego, CA), and is rendered as a space-filling surface (CPK). The nitrogen atoms from the azido group are colored in blue.

the ion channel. These studies in the muscle-type nAChR will aid the modeling of the TCA binding site on neuronal nAChRs.

2. Materials and methods

2.1. Materials

[^{3}H]2-azidoimipramine (Fig. 1) (5H-Dibenz(b,f)azepine-5-propanamine, 2-azido-10,11-dihydro-*N*,*N*-dimethyl-) (80 Ci/mmol) was synthesized according to Rotman and Pribluda [6] by American Radiolabeled Chemicals (St. Louis, MO). [Benzene ring-^{3}H(*N*)]-imipramine hydrochloride ([^{3}H]imipramine) (41.3 Ci/mmol) and [piperidyl-3,4-^{3}H(*N*)]-(*N*-(1-(2 thienyl)cyclohexyl)-3,4-piperidine) ([^{3}H]TCP) (45 Ci/mmol) were purchased from PerkinElmer Life Sciences Products, Inc. (Boston, MA). Radiolabeled drugs were stored in ethanol at −20 °C. Carbamylcholine hydrochloride (Carb), suberyldicholine dihydrochloride, l-[l-(2-thienyl)cyclohexyl]pyrrolidine (TCP), imipramine hydrochloride, α-bungarotoxin (α-BgTx), polyethylenimine, and Tricine were purchased from Sigma-Aldrich (St. Louis, MO). Phencyclidine hydrochloride (PCP) was obtained through the National Institute on Drug Abuse (NIDA) (NIH, Bethesda, Maryland). *Staphylococcus aureus* glutamyl endopeptidase (V8 protease) was obtained from MP Biochemicals (Irvine, CA), and Genapol C-100 from Calbiochem (San Diego, CA). Trifluoroacetic acid (TFA) and [1-(dimethylamino) naphtalene-5-sulfonamido]ethyltrimethylammonium perchlorate (dansyltrimethylamine) were purchased from Pierce (Rockford, IL). Prestained low range molecular weight standards were purchased from Life Technologies, Inc. (Gaithersburg, MD).

2.2. Preparation of nAChR-rich membranes and affinity-purified nAChRs reconstituted in lipid vesicles

nAChR-rich membranes were isolated from frozen *T. californica* electric organs obtained from Aquatic Research Consultants (San Pedro, CA) by differential and sucrose density gradient centrifugation, as described previously [7]. The final membrane suspensions in ~38% sucrose were stored at −80 °C. Total AChR membrane protein was determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL). Specific activities of these membrane preparations were determined by the decrease in dansyltrimethylamine (6.6 μM) fluorescence produced by the titration of suberyldicholine into receptor suspensions (0.3 mg/ml) in the presence of 100 μM PCP. The activities ranged from 0.9 to 1.2 nmol of suberyldicholine binding sites/mg total protein (0.45–0.60 nmol AChR/mg protein). Fluorescence titrations were carried out in 5-mm quartz cuvettes using an Olis DM245 spectrofluorimeter (Bogart, GA). Dansyltrimethylamine excitation and emission wavelengths were 295 and 546 nm, respectively. To reduce stray-light effects, a 530 nm cutoff filter was placed in the path of the emission wavelength.

nAChRs were affinity-purified using a bromoacetylcholine bromide-derivatized Affi-Gel 10 column (Bio-Rad) as described previously [8]. Briefly, the affinity column was prepared by coupling 50 mL of Affi-gel 10 to cystamine, reduction of the cystamine disulphide, and then sulfhydryl coupling to bromoacetylcholine bromide (1.5 g). The column was then equilibrated with ~15 column volumes of lipid (asolectin, a crude soybean lipid extract) in 1% cholate in vesicle dialysis buffer (VDB) (100 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, 10 mM MOPS, pH 7.4) (0.2 mL/min; >15 h). The solubilized material was slowly applied to the affinity column (0.3 mL/min, ~24 h, at 4 °C). The column was then washed extensively with asolectin–lipid solution (0.2–0.9 mg/mL lipid) in 1% cholate in VDB (15 column volumes; >15 h). This extensive wash ensures complete exchange of endogenous lipids for the asolectin–lipid mixture [8]. nAChRs were eluted from the column using the asolectin–lipid solution (0.2 mg/mL) containing 10 mM carbamylcholine (Carb). Peak protein fractions ($A_{280}\times 0.6$; see Ref. [8]) were pooled and the lipid–protein molar ratio adjusted to 400 to 1 except were specified. To remove Carb and reconstitute nAChRs into membranes containing asolectin–lipid, pooled fractions were dialyzed against 2 L of VDB (4 d with buffer change once every day). The reconstituted nAChRs were aliquoted (0.25 mg per tube) and stored at −80 °C.

2.3. Imipramine- and PCP-induced inhibition of [^{3}H]imipramine binding to nAChR in different conformational states

We studied the effect of imipramine and PCP on maximal [^{3}H]imipramine binding to the *Torpedo* nAChR. In this regard, nAChR native membranes (0.3 μM nAChR) were suspended in binding saline buffer (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4), with 10 nM [^{3}H]Imipramine in the presence of 1 mM Carb (desensitized state), or alternatively with 75 nM [^{3}H]Imipramine in the presence of 1 μM α-bungarotoxin [α-BgTx; resting (closed) state], and preincubated for 30 min at room temperature (RT). α-Bungarotoxin is a competitive antagonist that maintains the nAChR in the resting (closed) state [9]. Since imipramine inhibits [^{3}H]TCP binding to nAChRs in the resting (closed) (~6 μM) and desensitized (~0.7 μM) states [5], the nonspecific binding for the imipramine-induced inhibition of [^{3}H]imipramine binding experiments was determined in the presence of 100 μM (desensitized) or 500 μM imipramine (resting/closed), respectively. For the PCP-induced inhibition of [^{3}H]imipramine binding experiments, the nonspecific binding was determined in the presence of 50 μM (desensitized) or 100 μM PCP (resting/closed) according to Arias et al. [10]. The total volume was divided into aliquots, and increasing concentrations of imipramine or PCP (i.e., 0.1 nM–500 μM) were added to each tube and incubated for 2 h at RT. nAChR-bound radioligand was then separated from free ligand by a filtration assay using a 48-sample harvester system with GF/B Whatman filters (Brandel Inc., Gaithersburg, MD), previously soaked with 0.5% polyethylenimine for 30 min. The membrane-containing filters were transferred to scintillation vials with 3 mL of Bio-Safe II (Research Product International Corp, Mount Prospect, IL), and the radioactivity was determined using a Beckman SL6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

The concentration–response data were curve-fitted by nonlinear least-squares analysis using the Prism software (GraphPad Software,