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European Journal of Pharmacology 389 (2000) 155–163

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5-Hydroxytryptamine interaction with the nicotinic acetylcholine receptor

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Received 15 July 1999; received in revised form 19 November 1999; accepted 26 November 1999

Abstract

The present study examines the interaction of the neurotransmitter 5-hydroxytryptamine (5-HT) with muscle-type nicotinic acetylcholine receptors. 5-HT inhibits the initial rate of [^{125}I]α-bungarotoxin binding to *Torpedo* acetylcholine receptor membranes (IC_{50} = 8.5 ± 0.32 mM) and [^{3}H]5-HT can be photoincorporated into acetylcholine receptor subunits, with labeling of the α-subunit inhibitable by both agonists and competitive antagonists. Within the agonist-binding domain, [^{3}H]5-HT photoincorporates into αTyr^{190}, αCys^{192} and αCys^{193}. Functional studies using the human clonal cell line TE671/RD, show that 5-HT is a weak inhibitor (IC_{50} = 1.55 ± 0.25 mM) of acetylcholine receptor activity. In this regard, agonist-response profiles in the absence and presence of 5-HT indicate a noncompetitive mode of inhibition. In addition, 5-HT displaces high affinity [^{3}H]thienylcyclohexylpiperidine binding to the desensitized *Torpedo* acetylcholine receptor channel (IC_{50} = 1.61 ± 0.07 mM). Collectively, these results indicate that 5-HT interacts weakly with the agonist recognition site and inhibits receptor function noncompetitively by binding to the acetylcholine receptor channel.

Keywords: Nicotinic acetylcholine receptor; Photoaffinity labeling; Antagonist, noncompetitive; Antagonist, competitive; 5-HT (5-hydroxytryptamine, serotonin)

1. Introduction

The nicotinic acetylcholine receptor is the best characterized member of a family of ligand-gated ion channels that includes both muscle (e.g., *Torpedo*) and neuronal acetylcholine receptor subtypes, as well as receptors for γ-aminobutyric acid ($GABA_A$), glycine and 5-hydroxytryptamine ($5\text{-}HT_3$; reviews include: Jackson and Yakel, 1995; Arias, 1997). In each ligand-gated ion channel receptor, the subunits are arranged as a pentamer around a central pore, with the *Torpedo* acetylcholine receptor comprised of four homologous subunits (2α,β,γ,δ) while a and b subunits have been identified for the $5\text{-}HT_3$ receptor. In the acetylcholine receptor, affinity labeling and site-directed mutagenesis studies have identified residues in the α-subunit involved in acetylcholine binding, i.e., Cys^{192} and Cys^{193}, the adjacent residues Tyr^{190} and Tyr^{198}, as well as residues which are located more distantly in the primary structure (reviewed in Galzi and Changeux, 1994; Arias, 1997). Similar studies have also implicated these same residues as important in the binding of competitive antagonists such as D-tubocurarine and α-bungarotoxin (αBgTx; Chiara and Cohen, 1997). The two agonist/competitive antagonist binding sites on the acetylcholine receptor are located at the interfaces of the α–γ and α–δ subunits, and amino acid residues involved in ligand binding have also been identified by photoaffinity labeling in the γ and δ-subunits (Chiara and Cohen, 1997; Chiara et al., 1998). While the agonist binding domains are less well characterized for other ligand-gated ion channel family members, homologous residues have been implicated in agonist binding for each receptor, including the $5\text{-}HT_3$ receptor (Boess et al., 1997). Furthermore, in a recent study employing analogs of the acetylcholine receptor competitive antagonist D-tubocurarine, strikingly similar structure/activity relationships were found for interaction

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0014-2999/00/$ - see front matter
PII: S0014-2999(99)00855-9

with the acetylcholine receptor and 5-HT_3 receptor, leading the authors to conclude that a significant degree of structural homology exists in the ligand-binding domains of the two receptors (Yan et al., 1998, 1999).

In the present study, we wished to determine the structural components by which 5-HT interacts with the acetylcholine receptor. Previous electrophysiological studies have yielded conclusions that 5-HT is both a competitive as well as noncompetitive antagonist with half-maximal inhibitory values (IC_{50}) ranging from 40 μM to 1.2 mM (reviewed in Arias, 1998). We found that while 5-HT interacts weakly with the agonist recognition site on acetylcholine receptors, [^{3}H]5-HT is a useful photoaffinity label for residues in that domain. 5-HT inhibits acetylcholine receptor function noncompetitively, displaces binding of the noncompetitive antagonist [^{3}H]thienylcyclohexylpiperidine, reflecting interactions with the acetylcholine receptor channel.

2. Materials and methods

2.1. Materials

Torpedo californica electric organ was obtained from Aquatic Research Consultants (San Pedro, CA). [^{3}H]5-hydroxytryptamine trifluoroacetate (91 Ci/mmol) and [^{125}I]α-bungarotoxin (185 Ci/mmol) were purchased from Amersham Life Sciences (Arlington Heights, IL). [^{3}H]thienylcyclohexylpiperidine ([^{3}H]TCP) (57 Ci/mmol) and ^{86}Rubidium$^+$ came from New England Nuclear (Boston, MA). Liquid scintillation chemicals came from Research Products International (Mount Prospect, IL). Carbamylcholine, α-bungarotoxin, tetracaine, and other chemicals were obtained from Sigma/RBI (St. Louis, MO). Endoproteinase Lys-C was obtained from Boehringer-Mannheim (Indianopolis, IN) and trifluoroacetic acid from Pierce Chemical (Rockford, IL).

2.2. Acetylcholine receptor-rich and affinity-purified acetylcholine receptor membranes

Acetylcholine receptor-rich membranes were isolated from the electric organ of *T. californica* according to the procedure of Sobel et al. (1977) with the modifications described previously (Chiara and Cohen, 1997). The final membrane suspensions in ~38% sucrose/0.02% sodium azide were stored at −80°C. The *Torpedo* acetylcholine receptor was isolated from detergent (cholate) extracts of acetylcholine receptor-rich membranes by affinity chromatography in the presence of asolectin lipids. Affinity column purification was performed using an acetylcholine affinity matrix according to the procedure of Ellena et al. (1983) with several modifications according to the methods outlined in Blanton and Wang (1990). Briefly, the affinity column matrix was prepared by coupling cystamine to Affi-gel 10 (Biorad), reduction with dithiothreitol and final modification with bromoacetylcholine bromide. Affinity-purified acetylcholine receptors were reconstituted with asolectin at a lipid–protein ratio of 800:1 on a mole per mole basis and stored at −80°C.

2.3. [^{3}H]5-HT photoaffinity labeling

For labeling experiments, affinity-purified acetylcholine receptor membranes (0.4 mg/ml in vesicle dialysis buffer: 10 mM (3-[*N*-morpholino]propanesulfonic acid (MOPS); 100 mM sodium chloride; 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% sodium azide, pH 7.5) were incubated for 1 h at room temperature with [^{3}H]5-HT (0.28 μM) in the absence or presence of 400 μM carbamylcholine, or 10 μM α-bungarotoxin, or 30 μM tetracaine, and in the absence or presence of the aqueous scavenger oxidized glutathione (1 mM). The samples were then irradiated with a 312-nm ultraviolet lamp (Spectroline EB-280C) for 7 min at a distance of <1 cm (photolytic conditions established in Fig. 1). The membrane suspensions were transferred to 1.5 ml microcentrifuge tubes and centrifuged at $39{,}000 \times g$ for 1 h. Pellets were solubilized in electrophoresis sample buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970). Acetylcholine receptor subunits were resolved on 1.0 mm thick polyacrylamide gels comprised of 8% polyacrylamide/0.33% bis-acrylamide. For analytical gels, polypeptides were visualized by staining with Coomassie Blue R-250 (0.25% w/v in 45% methanol and 10% acetic acid) and destaining in 25% methanol and 10% acetic acid. The gels were then impregnated with fluor (Amplify, Amersham) for 30 min with rapid shaking, dried, and exposed at −80°C to Kodak X-OMAT LS film for various times (2–10 weeks). Incorporation of tritium into individual polypeptides was quantified by liquid scintillation counting of excised gel pieces as described (Middleton and Cohen, 1991). Alternatively, bands corresponding to the acetylcholine receptor α-subunit were excised from the stained 8% polyacrylamide gel (after soaking in distilled water overnight) for proteolytic digestion once the gel piece was transferred to the well of a 15% polyacrylamide mapping gel (Cleveland et al., 1977; Pedersen et al., 1986). Mapping gels were composed of a 4.5% T (total acrylamide), 2.6% C (percent bis-acrylamide relative to the total acrylamide concentration) stacking gel and a 15% T 2.6% C separating gel. The α-subunit gel piece was overlaid with buffer containing *S. aureus* V8 protease (Blanton et al., 1998). Electrophoresis was carried out overnight at 15 mA constant current. Preparative [^{3}H]5-HT photolabeling experiments were done with acetylcholine receptor-rich membranes (7.5 mg protein and 250 μCi [^{3}H]5-HT per condition, ±400 μM carbamylcholine). Acetylcholine receptor subunits were resolved on 1.5 mm thick 8% polyacrylamide gels (one gel per labeling condition) and the α-subunit subjected to proteolytic mapping using *S. aureus*

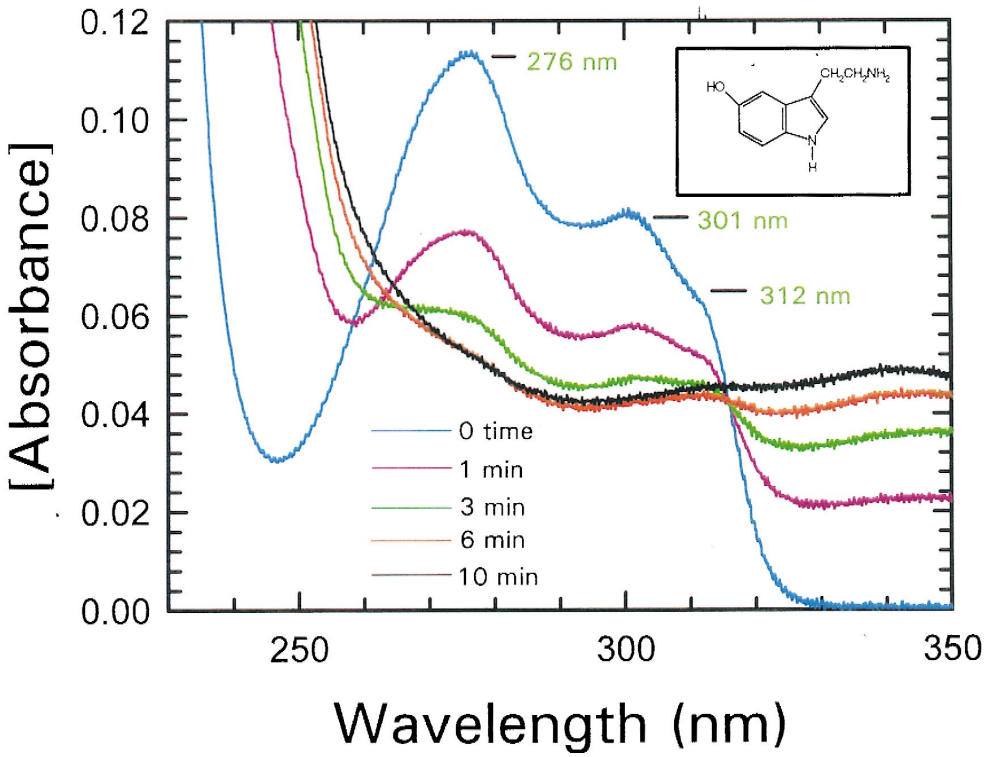

Fig. 1. UV–visible absorbance spectra of reaction products from photolysis of 5-HT. A solution of 5-HT (200 μM) was irradiated with ultraviolet light (312 nm) using a Spectroline EB-280C hand-held lamp (at < 1 cm) for increasing periods of time. The spectra show an ultraviolet light-induced structural change in 5-HT. The inset shows the chemical structure of 5-HT.

V8 protease (1.5 mm thick 15% polyacrylamide mapping gel per labeled α-subunit). Bands corresponding to the [^{3}H]5-HT-labeled αV8-20 proteolytic fragment were excised from the mapping gels and labeled material isolated from the excised gel pieces using a passive elution protocol (Blanton et al., 1998). The eluate was filtered (Whatman No. 1), and the protein concentrated using a Centriprep-10 (Amicon). Excess sodium dodecyl sulfate was removed by acetone precipitation (overnight at −20°C).

2.4. Purification of endoproteinase Lys-C digests of [^{3}H]5-HT-labeled αV8-20

For endoproteinase Lys-C digestion, acetone-precipitated 20 kDa V8 protease fragments of the α-subunit (αV8-20, Ser^{173}-Glu^{338}) isolated from acetylcholine receptors labeled in the absence and presence of 400 μM carbamylcholine were re-suspended in 15 mM Tris–HCl, 0.1% sodium dodecyl sulfate, pH 8.1 at 1–2 mg/ml protein. Approximately 1.5 units of endoproteinase Lys-C (per condition) were added and incubated at room temperature for 7 days. The digests were then separated by reverse-phase high performance liquid chromatography (HPLC) as described by Blanton et al. (1998) using a Brownlee Aquapore C_4 column (100 × 2.1 mm). Solvent A was 0.08% trifluoroacetic acid in water, and solvent B was 0.05% trifluoroacetic acid in 60% acetonitrile/40% 2-propanol. The elution of tritium was monitored by liquid scintillation counting of an aliquot (50 μl) of each 0.5-ml fraction.

2.5. Cell culture

TE671/RD human clonal cells expressing muscle-type acetylcholine receptors were maintained at low passage (less than passage 25) in Dulbecco's modified Eagle's medium supplemented with antibiotics and serum as described previously (Bencherif et al., 1995).

2.6. Assays of acetylcholine receptor function

^{86}Rubidium$^+$ efflux assays using TE671/RD cells were performed according to Bencherif et al. (1995). Specific acetylcholine receptor function was defined as total experimentally determined ion flux in the presence of the agonist carbamylcholine plus or minus 5-HT, minus nonspecific ion flux determined in the presence of 100 μM D-tubocurarine.

2.7. [^{125}I]α-bungarotoxin and [^{3}H]thienylcyclohexylpiperidine binding assays

To assess 5-HT interaction with the acetylcholine receptor agonist recognition site, we tested the ability of 5-HT to inhibit the initial rate of binding of radiolabeled α-bungarotoxin to *Torpedo* acetylcholine receptor rich mem-

branes (Chiara et al., 1999). Binding assays were initiated by adding [^{125}I]α-bungarotoxin (a final concentration of 3 nM) to triplicate samples in a total volume of 450 μl (0.1 mg protein/ml vesicle dialysis buffer). Membranes were pre-equilibrated with increasing concentrations of 5-HT for 2 h prior to addition of [^{125}I]α-bungarotoxin. After a 1-min incubation, [^{125}I]α-bungarotoxin binding was quenched by the addition of 1.3-μM α-bungarotoxin (with a 1-min incubation, [^{125}I]α-bungarotoxin binds ~40% of the available α-bungarotoxin binding sites). The samples were centrifuged (39,000 × *g* for 1 h; Beckman JA-20 rotor), the supernatants removed and the pellets counted in a Packard Cobra II γ-counter. Nonspecific binding of [^{125}I]α-bungarotoxin was determined in the presence of 10-μM α-bungarotoxin (2.9% of total binding).

The equilibrium binding of the acetylcholine receptor noncompetitive antagonist [^{3}H]thienylcyclohexylpiperidine (Katz et al., 1997) with *Torpedo* acetylcholine receptor-rich membranes was assayed by centrifugation. For [^{3}H]thienylcyclohexylpiperidine assays, triplicate 500 μl aliquots of membrane suspensions (0.5 mg/ml in vesicle dialysis buffer, ~0.6 μM acetylcholine receptor) were equilibrated in the presence of 250 μM carbamylcholine with [^{3}H]thienylcyclohexylpiperidine (7 nM) and increasing concentrations of 5-HT for 2 h. Membrane suspensions were equilibrated at room temperature in 10 × 75 mm disposable culture tubes (Corning) and then transferred to 1.5 ml plastic microcentrifuge tubes and pelletted by centrifugation at 39,000 × *g* for 1 h (Beckman JA-20 rotor). After removal of the supernatants, the membrane pellets were solubilized in 100 μl of 10% sodium dodecyl sulfate, and the pellet tritium was determined by liquid scintillation counting. Nonspecific binding of [^{3}H]thienylcyclohexylpiperidine was measured in the presence of the noncompetitive antagonist proadifen (0.2 mM; 3.9% of total binding).

2.8. Data analysis

Prism Software (Graphpad, San Diego, CA) was used to determine IC_{50}, EC_{50}, and Hill coefficients using non-linear least squares regression analysis.

3. Results

3.1. [^{3}H]5-HT photoaffinity labeling of the acetylcholine receptor

In order to initially assess whether [^{3}H]5-HT might be a suitable compound for photoaffinity labeling, we tested the photosensitivity of 5-HT to irradiation with ultraviolet light. As seen in Fig. 1, ultraviolet light irradiation (312 nm) results in a substantial reduction in the 5-HT absorbance spectra with increasing time of photolysis. The ultraviolet light-induced photodecomposition/arrangement of 5-HT may then result in the formation of a reactive intermediate capable of forming a covalent bond with a nearby molecule. Next, acetylcholine receptor membranes were photoaffinity labeled with [^{3}H]5-HT as described in Materials and methods and under photolysis conditions established in Fig. 1 (7 min irradiation, 312 nm, at a distance of < 1 cm). The fluorograph shown in Fig. 2A shows that [^{3}H]5-HT photoincorporates into each of the acetylcholine receptor subunits. Addition of agonist (Fig. 2A, + carbamylcholine lane) results in a significant reduction in the amount of labeling observed in the α-subunit. Based on liquid scintillation counting of excised gel bands, there is a 50% reduction in the extent of [^{3}H]5-HT incorporation into the α-subunit in the presence of agonist, and no significant difference in the labeling of the β, γ, or δ-subunits ($n = 4$). Addition of the competitive antagonist α-bungarotoxin resulted in a similar (60%) reduction in

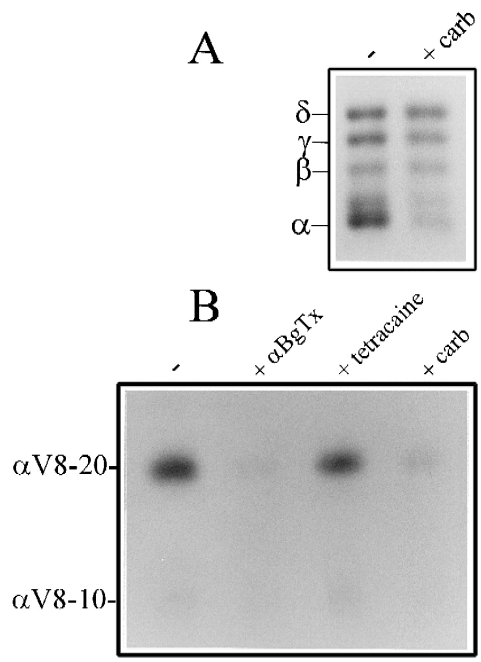

Fig. 2. Photoincorporation of [^{3}H]5-HT into the acetylcholine receptor. Affinity-purified acetylcholine receptor membranes (50 μg) were equilibrated with [^{3}H]5-HT (0.28 μM) for 1 h in the absence and presence of 400 μM carbamylcholine (+carb), or in the presence of 10 μM α-bungarotoxin (+αBgtx), or 30 μM tetracaine (+tetracaine). Acetylcholine receptor membranes were then photolyzed (7 min irradiation at 312 nm, at a distance of < 1 cm), and polypeptides resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and processed for fluorography (A, 4-week exposure). The acetylcholine receptor subunits (α, β, γ, δ) are indicated on the left. (B) For each labeling condition, the α-subunit was further subjected to Cleveland gel analysis (see Materials and methods) which generates four unique fragments. Shown is the corresponding fluorograph of the Cleveland gel (10-week exposure). [^{3}H]5-HT incorporation is restricted to the fragments αV8-20 (Ser^{173}-Glu^{338}) and αV8-10 (Asn^{339}-Gly^{437}), the positions of which are indicated on the left. αV8-20 contains the transmembrane segments M1–M3 as well as Loop C of the agonist-binding site, while αV8-10 contains a portion of the second intracellular loop and the transmembrane segment M4 (Arias, 1997).

[^{3}H]5-HT labeling of the α-subunit (no effect on labeling of β, γ, δ), while addition of the noncompetitive antagonist tetracaine had no detectable effect on labeling of any receptor subunit. Neither tetracaine nor α-bungarotoxin induces receptor desensitization (Moore and McCarthy, 1994), and therefore, the inhibitory effects of carbamylcholine and α-bungarotoxin on [^{3}H]5-HT incorporation result from competitive interaction with the agonist recognition domain. To determine the extent to which photoincorporation occurs after [^{3}H]5-HT has diffused away from its binding site, oxidized glutathione was employed as an aqueous scavenger. Addition of 1 mM oxidized glutathione had no significant effect (> 10%) on the extent of agonist-inhibitable [^{3}H]5-HT incorporation into the α-subunit (subunit data not shown).

[^{3}H]5-HT incorporation into the α-subunit was mapped by proteolytic digestion with *S. aureus* V8 protease under ''Cleveland'' gel conditions (Blanton et al., 1998). The fluorograph of the mapping gel shown in Fig. 2B shows that the majority of [^{3}H]5-HT labeling is in the αV8-20 fragment (Ser^{173}-Glu^{338}) which contains the membrane-spanning segments M1–M3 as well as Loop C (~ Tyr^{190}-Tyr^{198}) of the agonist binding domain (Arias, 1997). In the absence of any other ligands, approximately 60% of the total [^{3}H]5-HT incorporation into the α-subunit maps to αV8-20 and 28% into the fragment αV8-10. Addition of agonist (Fig. 2B, +carb lane) or competitive antagonist (Fig. 2B, +αBgTx lane) results in a 60% or 70% reduction in the total [^{3}H]5-HT incorporation into αV8-20, respectively. In contrast, addition of the noncompetitive antagonist tetracaine had no effect on the labeling of αV8-20 and none of the ligands had any significant effect (> 10%) on the incorporation into αV8-10 ($n = 8$), which contains a portion of the second intracellular loop and the transmembrane segment M4 (Arias, 1997).

3.2. Identification of the sites of agonist-inhibitable [^{3}H]5-HT photoincorporation

From a preparative scale labeling (Materials and methods), the αV8-20 fragments were isolated from acetylcholine receptors (10 mg acetylcholine receptor-rich membranes) labeled with [^{3}H]5-HT (0.28 μM) in the absence and presence of 400 μM carbamylcholine. αV8-20 fragments isolated from acetylcholine receptors labeled in the absence and presence of agonist contained 434 and 88 cpm/μg, respectively, therefore, approximately 80% of the total [^{3}H]5-HT incorporation into αV8-20 was specific. The αV8-20 fragments were digested with endoproteinase Lys-C for 7 days. The digests were separated by reverse-phase HPLC (Fig. 3B) and the majority of tritium cpm (●, ○) for each labeling condition eluted in a peak centered at 85% solvent B, with a corresponding peak of absorbance. HPLC fractions 34–36 were pooled and subjected to N-terminal sequence analysis (Fig. 3B). For αV8-20 labeled with [^{3}H]5-HT in the absence of agonist (●), sequencing

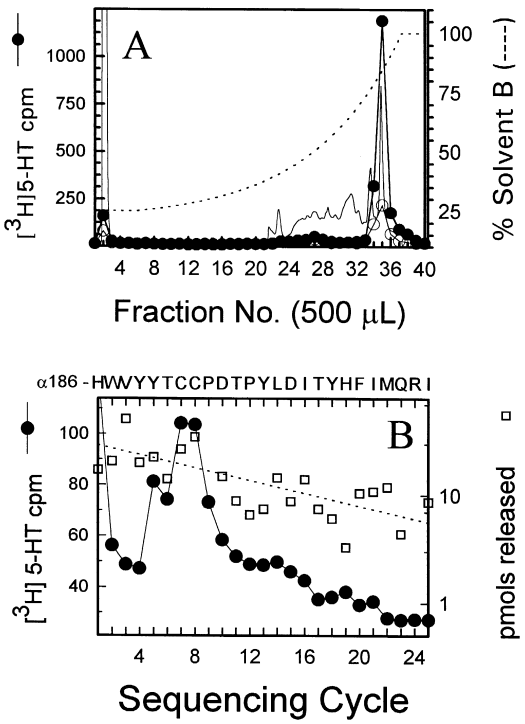

Fig. 3. Reverse-phase HPLC purification and sequential Edman degradation of a [^{3}H]5-HT-labeled endoproteinase Lys-C fragment of αV8-20. The [^{3}H]5-HT-labeled acetylcholine receptor α-subunit proteolytic fragment αV8-20 (Ser^{173}-Glu^{338}) isolated from acetylcholine receptors labeled in the absence (●) and presence (○) of carbamylcholine was further digested in solution with endoproteinase Lys-C for 7 days. The proteolytic digests were separated by reverse-phase HPLC (A) on a Brownlee Aquapore C_4 column (100×2.1 mm) as described under Materials and methods. The elution of peptides was monitored by absorbance at 210 nm (solid line) and elution of tritium (^{3}H) by liquid scintillation counting of 50 μl of each 500-μl fraction (●, ○). For each condition, fractions 34–36 were pooled and subjected to automated sequential Edman degradation (B). Shown is the radio-sequencing profile of fractions 34–36 isolated from acetylcholine receptors labeled in the absence of agonist. Eighty percent of each cycle of Edman degradation was analyzed for released tritium (^{3}H; ●) and 20% for released phenylthiohydantoin (PTH)-amino acids (□) with the dashed line corresponding to the exponential decay fit of the amount of detected PTH-amino acids. A primary peptide was detected beginning at His^{186} of the α-subunit (initial yield, 30 pmol; repetitive yield, 93.3%; 13,308 cpm loaded on sequencing filter; 2511 cpm remaining after 25 cycles). A secondary sequence beginning at Asp^{180} was also present at one-fifth the amount of the primary peptide. The amino acid sequence of the αHis^{186} peptide is shown above panel B.

revealed a primary peptide beginning at αHis^{186} near the N-terminal start of Loop C of the agonist binding domain. Sites of tritium release occur in cycles 5, 7 and 8, a result which indicates [^{3}H]5-HT incorporation into Tyr^{190} (1.53 cpm/pmol), Cys^{192} (1.54 cpm/pmol), and Cys^{193} (1.68 cpm/pmol). The acetylcholine receptor agonist [^{3}H]nicotine photoincorporates into these same residues at very similar levels (Middleton and Cohen 1991). Each of these residues has been shown to contribute to the formation of the agonist/competitive antagonist binding domain of the nicotinic acetylcholine receptor (reviewed in Arias, 1997).

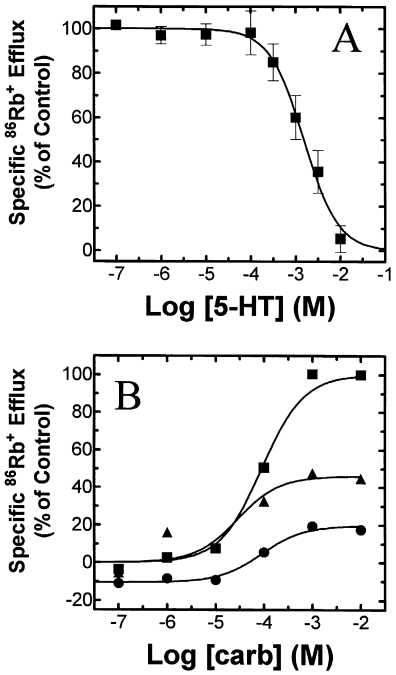

Fig. 4. Effects of 5-HT on ^{86}Rubidium flux in muscle-type acetylcholine receptor expressing TE671/RD cells. Panel A: Inhibition of carbamylcholine-induced ^{86}Rubidium$^+$ flux by 5-HT in muscle-type acetylcholine receptor expressing TE671/RD cells. Acetylcholine receptor functional responses (^{86}Rubidium$^+$ efflux stimulated by 1 mM carbamylcholine; ordinate; percentage of control response where a 100% response is taken as the difference between ^{86}Rubidium$^+$ efflux at 0 or at 100 μM D-tubocurarine) were assessed alone and in the presence of increasing concentrations of 5-HT (see Materials and methods). Data points are the means from at least three experiments, and curve-fitting yielded an IC_{50} value of 1.55 ± 0.25 mM and a Hill coefficient of 1.14 ± 0.21. Panel B: Carbamylcholine concentration–response curves were generated in the absence (■) or presence of 2 mM (▲) and 5 mM (▼) 5-HT. Measurements of specific ^{86}Rubidium$^+$ efflux (ordinate: percentage of 1 mM carbamylcholine control) were made using TE671/RD cells. Data points are the mean of two experiments. The fractional maximum response and the goodness-of-fit, r^2 values (in parentheses) are 100% (0.99) for untreated control, 45.93% (0.87) for 2 mM 5-HT, and 19.68% (0.97) for 5 mM 5-HT. None of the carbamylcholine concentration–response profiles in the presence of 5-HT produced any significant shift in EC_{50} value.

3.3. Effect of 5-HT on acetylcholine receptor function

^{86}Rubidium$^+$ efflux assays using the human clonal cell line TE671/RD, which express muscle-type acetylcholine receptors, were used to evaluate the effect of 5-HT on acetylcholine receptor function. In these studies, cells were exposed simultaneously to test concentrations of 5-HT and a fixed (1 mM) concentration of carbamylcholine. 5-HT produced a concentration-dependent inhibition of ^{86}Rubidium$^+$ efflux (Fig. 4A) with half-maximal block at 1.55 ± 0.25 mM 5-HT. To evaluate the mechanism of blockade by 5-HT, agonist dose–response profiles were obtained either alone or in the presence of 2 or 5 mM 5-HT (Fig. 4B). Functional blockade by 5-HT was insurmountable by increasing concentrations of carbamylcholine suggesting that 5-HT acts noncompetitively to inhibit acetylcholine receptor function.

3.4. Effect of 5-HT on the initial rate of [^{125}I]α-bungarotoxin binding to Torpedo acetylcholine receptor membranes

Given the indication from functional assays of a noncompetitive mode of inhibition by 5-HT, we next tested the ability of 5-HT to inhibit the initial rate of [^{125}I]α-bungarotoxin binding to acetylcholine receptors. As Fig. 5A shows, preincubation with 5-HT results in a concentration-dependent reduction in the amount of [^{125}I]α-

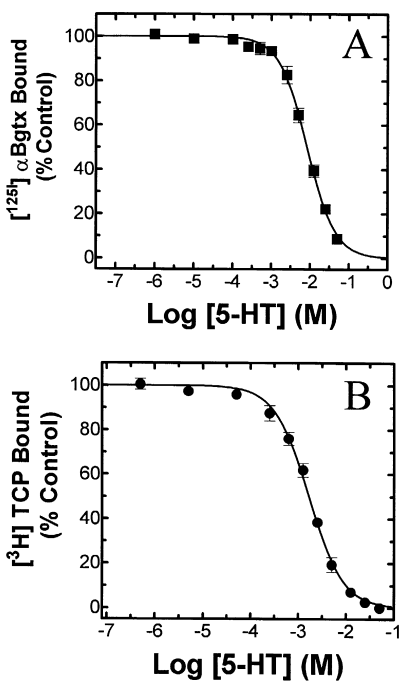

Fig. 5. Effect of 5-HT on the initial rate of [^{125}I]α-bungarotoxin binding and on the equilibrium binding of [^{3}H]thienylcyclohexylpiperidine to acetylcholine receptor-rich membranes. (A) The effect of 5-HT on the initial rate of [^{125}I]α-bungarotoxin binding to *Torpedo* acetylcholine receptor membranes was measured. Incubation with 3 nM [^{125}I]α-bungarotoxin for 1 min followed a 2-h incubation with increasing concentrations of 5-HT (Materials and methods). The data are presented as percentage of control-specific binding and represent mean values obtained from three samples. Nonspecific binding of [^{125}I]α-bungarotoxin was determined in the presence of 10-μM α-bungarotoxin (2.9% of total binding). The solid line represents the non-linear least-squares fit of the binding data ([^{125}I]α-bungarotoxin: $IC_{50} = 8.50 \pm 0.32$ mM, $n_H = 1.19 \pm 0.052$). (B) Acetylcholine receptor-rich membranes (0.5 mg/ml, 0.6 μM acetylcholine binding sites) containing 7 nM [^{3}H]thienylcyclohexylpiperidine and 250 μM carbamylcholine were equilibrated for 2 h with increasing concentrations of 5-HT. Bound tritiated ligand (■) was determined by centrifugation (Materials and methods). Nonspecific [^{3}H]thienylcyclohexylpiperidine binding was determined in the presence of 200 μM proadifen (3.9% of total binding). The data are presented as percentage of control specific binding and represent mean values obtained from three samples for each concentration of competitor. The solid lines represent the non-linear least-squares fit of the binding data ([^{3}H]thienylcyclohexylpiperidine: $IC_{50} = 1.610 \pm 0.0658$ mM, $n_H = 1.211 \pm 0.045$).

bungarotoxin bound to the *Torpedo* acetylcholine receptor. At a 5-HT concentration of 50 mM, [^{125}I]α-bungarotoxin binding is reduced by ~92% of the control value with a calculated IC_{50} value of 8.5 ± 0.32 mM ($n_H = 1.19 \pm 0.05$).

3.5. Effect of 5-HT on [^{3}H]thienylcyclohexylpiperidine binding to the desensitized acetylcholine receptor

In the presence of agonist, [^{3}H]phencyclidine binds with high affinity ($K_{eq} = 1$ μM) to a single site per acetylcholine receptor (Heidmann et al., 1983) and additional studies indicate a binding site for phencyclidine in the desensitized channel (e.g., Eaton et al., 1997). Thienylcyclohexylpiperidine, which is a structural analog of phencyclidine and [^{3}H]thienylcyclohexylpiperidine, has been shown to bind to the desensitized acetylcholine receptor channel in a manner that is indistinguishable from that of [^{3}H]phencyclidine (Katz et al., 1997; unpublished data from our laboratory). As Fig. 5B shows, in the presence of agonist, addition of 5-HT results in a concentration-dependent reduction in the amount of [^{3}H]thienylcyclohexylpiperidine bound to the acetylcholine receptor. 5-HT completely eliminates all the specifically bound [^{3}H]thienylcyclohexylpiperidine (98.8%) with a calculated IC_{50} value of 1.61 ± 0.07 mM ($n_H = 1.21 \pm 0.05$).

4. Discussion

The acetylcholine receptor and 5-HT_3 receptor exhibit a large degree of sequence similarity and there is a growing body of evidence indicating substantial structural homology as well (Eisele' et al., 1993; Kriegler et al., 1999). In addition, analogs of the acetylcholine receptor competitive antagonist D-tubocurarine interact with both receptors in similar fashion, suggesting common structural features in the agonist/competitive antagonist binding domains (Yan et al., 1998, 1999). The goal of the present work was to characterize the interaction of 5-HT with the muscle-type nicotinic acetylcholine receptor as a means of further examining the structure and ligand-specificities of the agonist recognition domains of these two receptors. We conclude that, perhaps not surprisingly, there is little evidence of any significant cross-talk in neurotransmitter interaction with the agonist binding domains between these two receptors. However, we also conclude that 5-HT is a useful photoaffinity probe of the acetylcholine receptor agonist recognition site, and likely will be an even more useful probe for 5-HT_3 receptors. We also conclude that 5-HT gains access to sites in the acetylcholine receptor channel, where it may exert its effects as a noncompetitive functional antagonist.

Functional studies measuring ^{86}Rubidium$^+$ efflux in TE671/RD cells, which express muscle-type acetylcholine receptors, show that 5-HT is a weak inhibitor of receptor function (Fig. 4A; $IC_{50} = 1.55$ mM). Furthermore, there is no indication of any 5-HT-induced potentiation of ^{86}Rubidium$^+$ efflux (Fig. 4A) as the results of Schrattenholz et al. (1996) suggest. In whole-cell patch–clamp recordings of TE671/RD cells, 3 mM 5-HT reduced the peak current by 59 ± 6% (Grassi et al., 1993), while for recordings in *Xenopus laevis* oocytes expressing mouse muscle-type acetylcholine receptors, 5-HT was slightly more potent, inhibiting acetylcholine-induced currents with an IC_{50} value of 1.2 mM (at 0 mV; Garcia-Colunga and Miledi, 1996). In agonist dose–response profiles obtained in the absence and presence of 5-HT (Fig. 4B), the functional block produced by 5-HT was insurmountable by increasing concentrations of agonist. An identical result was recently reported for 5-HT inhibition of neuronal nicotinic acetylcholine receptor function (Nakazawa and Ohno, 1999). These results suggest that 5-HT acts noncompetitively to inhibit acetylcholine receptor function. This conclusion is consistent with (i) the ability of 5-HT to inhibit binding of [^{3}H]thienylcyclohexylpiperidine (Fig. 5B) to sites presumed to be located in the acetylcholine receptor pore ($IC_{50} = 1.6$ mM), (ii) the concentration at which 5-HT inhibits 50% of the initial rate of [^{125}I]α-bungarotoxin binding to the acetylcholine receptor agonist binding site ($IC_{50} = 8.5$ mM) is ~5-fold greater than that needed to displace 50% of [^{3}H]thienylcyclohexylpiperidine binding to the acetylcholine receptor channel. In electrophysiological studies, functional blockade by 5-HT is significantly affected by the membrane voltage, again consistent with block at sites within the span of the bilayer leaflet. In oocytes expressing muscle-type acetylcholine receptors, half-maximal inhibition of acetylcholine-induced currents by 5-HT occurred at a concentration of 1.2 mM at 0 V and at 40 μM at −50 mV (Garcia-Colunga and Miledi, 1996). The voltage-dependence of 5-HT inhibition, the channel bursting activity in the presence of 5-HT, as well as 5-HT acceleration of agonist-induced desensitization (reviewed in Arias, 1998) lead to a general conclusion that 5-HT is a noncompetitive antagonist of the acetylcholine receptor. Based on the voltage-dependence of 5-HT block [electrical distance ($d = 0.72$); Garcia-Colunga and Miledi, 1996], it was suggested that the binding site for 5-HT is located within the acetylcholine receptor channel at about α-Ser^{248} of the M2 segment (position 6; the sixth amino acid on the carboxy-terminal side of the conserved lysine residue at the N-terminal start of each M2 segment; Unwin, 1995). The noncompetitive antagonists chlorpromazine, TPMP, as well as phencyclidine, each bind to this region of the channel (Giraudat et al., 1986; Hucho et al., 1986; Eaton et al., 1997, 1998). We provide additional support for both channel blocking mechanism of 5-HT inhibition of receptor function and a binding locus at or near α-Ser^{248}. As shown in Fig. 5B, 5-HT competitively displaces binding of the phencyclidine analog [^{3}H]thienylcyclohexylpiperidine (Katz et al., 1997) to the desensitized acetylcholine receptor ($IC_{50} = 1.61$ mM; $n_H = 1.211$). As phencyclidine and

thienylcyclohexylpiperidine binding to the desensitized acetylcholine receptor is indistinguishable (Katz et al., 1997), these results indicate that 5-HT binds to the desensitized channel at the phencyclidine binding site, which is believed to be at or near the level of α-Ser^{248} (position 6) in the M2 segments. However, while a channel-binding locus for 5-HT is the simplest interpretation of the data, a binding site for 5-HT outside of the channel (i.e., nonluminal) cannot be discarded.

The goal of photoaffinity labeling experiments with [^{3}H]5-HT was to first determine if [^{3}H]5-HT was a suitable photoreactive ligand and then to use it to determine the site(s) of 5-HT interaction with the acetylcholine receptor. Because 5-HT is a very weak inhibitor of receptor function, its affinity for site(s) on the acetylcholine receptor are apt to also be very low, and as a consequence of this, it was likely that nonspecific [^{3}H]5-HT photoincorporation into the acetylcholine receptor would represent a large percentage of the total labeling. Inhibition of [^{3}H]5-HT photoincorporation into the acetylcholine receptor by ligands which bind to pharmacologically important domains was used to screen for labeling that was potentially of importance. The incorporation of tritium into acetylcholine receptor subunits (Fig. 2A) establishes that [^{3}H]5-HT can indeed be used as a photoaffinity ligand, a result that is also of importance for future studies aimed at directly identifying amino acid residues in the agonist recognition site of the 5-HT$_3$ receptor. As predicted, the bulk of [^{3}H]5-HT incorporation into acetylcholine receptor non-α subunits is inhibitable neither by agonists and competitive antagonists nor by noncompetitive antagonists (Fig. 2). However, in the acetylcholine receptor α-subunit, a significant percentage of the total [^{3}H]5-HT incorporation (~60%) is inhibited by addition of either agonist or competitive antagonist. This component of labeling was further mapped to a fragment of the α-subunit (αV8-20), which contains a portion of the agonist-binding domain. The sites of [^{3}H]5-HT labeling were subsequently identified by N-terminal sequence analysis of an HPLC-purified proteolytic digest of αV8-20. The amino acid residues Tyr^{190}, Cys^{192} and Cys^{193} reacted with [^{3}H]5-HT. Each of these residues has previously been shown to contribute to the formation of the agonist/competitive antagonist-binding domain of the acetylcholine receptor (reviewed in Arias, 1997). Agonist-response profiles in the presence of 5-HT (Fig. 4B) and the ability of 5-HT to competitively displace [^{3}H]thienylcyclohexylpiperidine binding (Fig. 5B), strongly suggest a channel binding site for 5-HT. Yet addition of the channel binding noncompetitive antagonist tetracaine (Gallagher and Cohen, 1994) did not result in any detectable inhibition of [^{3}H]5-HT incorporation into acetylcholine receptor subunits (Fig. 2). It is possible that given the predominantly aliphatic nature of the amino acid residues in each of the channel-lining M2 segments, [^{3}H]5-HT photoincorporation into these residues occurs at very low efficiency and therefore, this component of labeling is rather small and not detectable at the level of intact subunit labeling. Further work will be needed to establish whether [^{3}H]5-HT incorporation is detectable in any acetylcholine receptor subunit M2 segment.

Finally, despite the clear indication that 5-HT inhibits acetylcholine receptor function noncompetitively and that 5-HT interaction with the agonist-binding site is extremely weak as judged by an IC$_{50}$ value of 8.5 mM for inhibition of [^{125}I]α-bungarotoxin binding, our photolabeling results are important for several reasons. First, identification of Tyr^{190}, Cys^{192} and Cys^{193} as the sites of agonist/competitive antagonist-inhibitable [^{3}H]5-HT photoincorporation in the acetylcholine receptor, provides additional evidence that these residues contribute to the formation of the acetylcholine receptor agonist recognition domain. 5-HT provides a potentially important ligand for molecular modeling studies of the agonist-binding site. For example, 5-HT is structurally similar to the acetylcholine receptor agonist nicotine and [^{3}H]nicotine photolabels Tyr^{190}, Cys^{192}, Cys^{193} and Tyr^{198} in the α-subunit (Middleton and Cohen, 1991). Finally, the ability to determine specific amino acid residues labeled by [^{3}H]5-HT in the acetylcholine receptor indicates that the photoreacted product is both stable and detectable by proteolytic digestion and HPLC purification and to the harsh conditions of automated N-terminal sequencing. This further validates [^{3}H]5-HT as a photoaffinity ligand useful for determining amino acid residues in the agonist binding domain of the 5-HT$_3$ receptor, such as in future studies using receptors purified from cell lines which express the 5-HT$_3$ receptor at high levels.

Acknowledgements

This research was supported in part by National Institutes of Health NINDS Grant R29 NS35786. Studies done in Phoenix were supported by the Barrow Neurological Foundation and the Arizona Disease Control Research Commission (grants 82-1-098 and 9730 to RJL). The contents of this report are solely the responsibility of the authors and do not necessarily represent the views of the aforementioned awarding agencies.

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