0026-895X/92/061100-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:1100–1106

Photoactivatable Agonist of the Nicotinic Acetylcholine Receptor: Potential Probe to Characterize the Structural Transitions of the Acetylcholine Binding Site in Different States of the Receptor

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Received October 23, 1991; Accepted February 29, 1992

SUMMARY

The nicotinic acetylcholine receptor exhibits at least four different affinity states for agonists such as acetylcholine. In order to identify the structural changes occurring at or near the agonist binding site during the allosteric transitions, three photoactivatable compounds designed to display agonist activity were synthesized. Inhibition constants of these compounds for the cholinergic and the noncompetitive blocker binding sites were determined for the resting and the desensitized states of the receptor. Among these probes, two ligands, AC_5 and AC_7, displayed a high affinity for the agonist binding site and were poorly recognized by the binding site for noncompetitive blockers. Electrophysiological experiments revealed that these ligands behaved as agonists at low concentrations. We used these two compounds in photolabeling experiments and observed that they were able to inactivate the agonist binding site. Up to 50% of these sites were irreversibly inhibited, depending on the ligand, the irradiation conditions, and the selected receptor state. The compound with the most interesting properties (high affinity and selectivity for the acetylcholine binding site, as well as agonist activity and high photolabeling yield) is AC_5, a structural analogue of the fluorescent agonist dansyl-C_6-choline, which has been previously used to characterize the different states of the nicotinic receptor. After radioactive synthesis, [^{3}H]AC_5 was shown to label all four receptor subunits, in a protectable manner. This radioligand, thus, appears suitable for investigation of the dynamics of allosteric transitions occurring at the activated acetylcholine binding site.

The nicotinic AChR from *Torpedo* electric organ and vertebrate neuromuscular junction is an oligomeric membrane-spanning protein (1, 2) that is composed of four different subunits and has a pentameric structure, with a stoichiometry of $\alpha_2\beta\gamma\delta$ (3, 4). The cDNAs coding for the four subunits have been cloned and sequenced in several species (reviewed in Refs. 5–7).

The AChR protein has two distinct categories of binding sites, (i) sites for nicotinic agonists and competitive antagonists that exist in duplicate on the receptor and involve mainly but not exclusively the α subunits (8, 9) (snake venom α-toxins are powerful pseudoirreversible blockers of these two sites) and (ii) high affinity sites for NCBs of the permeability response, such as histrionicotoxin and PCP. These sites are present in a single copy per receptor complex (10, 11). The AChR is an allosteric protein that is able to undergo agonist activation leading to channel opening, as well as desensitization upon prolonged exposure to the neurotransmitter (12). Functional properties of the AChR have been successfully explored (13), using rapid mixing techniques, with a fluorescent agonist (Dns-C_6-Cho). Kinetic studies of the Dns-C_6-Cho response point out several discrete conformational states, which are interconvertible. At least four states, referred to as resting, active, intermediate, and desensitized, show different affinities for agonists and competitive antagonists.

Using irreversible probes, the sulfhydryl-directed affinity reagents 4-(*N*-maleimido)benzyltrimethylammonium iodide and 4-(*N*-maleimido)phenyltrimethylammonium iodide, two cysteinyl residues (Cys-192 and Cys-193) located on the α chain of the receptor were labeled (14, 15). It should be noted that this labeling required prior reduction of at least one disulfide bridge of the AChR, a treatment inducing alteration in the

This work was supported by the CNRS.
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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; NCB, noncompetitive blocker; Dns-C_6-Cho, dansyl-C_6-choline; PCP, phencyclidine; TFA, trifluoroacetic acid; Carb, carbamylcholine; DDF, *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate; AC_5, [*N'*-methyl,*N'*-4-diazonium phenyl][*N*-6-hexanoic acid, 2-(trimethylammonium bromide)ethyl ester]urea; AC_7, [*N'*-methyl,*N'*-4-diazonium phenyl][*N*-8-octanoic acid, 2-(trimethylammonium bromide)ethyl ester]urea; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EtOAc, ethyl acetate.

functional properties of the receptor (16). Recently, a tyrosine residue (Tyr-190) on the α chain of the native AChR was labeled using the reduced form of lophotoxin (17).

Photoaffinity labeling is likely to be a more appropriate technique to study the topographical structure of the ACh binding site, because, under suitable photochemical conditions, no alteration of the native AChR occurs. In addition, photogenerated species have a much higher reactivity than conventional labeling agents and, thus, represent more efficient and specific labeling tools (18, 19). The first photosensitive probes of AChR were either analogues of ACh (20–22) or neurotoxin derivatives (23–25). Their photocoupling to the receptor led to the covalent labeling of the α subunit and, to variable extents, of the other subunits. More recently, regions of the α subunit of the AChR were probed with the photoaffinity ligand DDF (26), which generates an extremely reactive aryl cation upon photolysis (27). After cleavage with CNBr, the major [^{3}H]DDF-radiolabeled peptide was purified and identified by sequence analysis as the α179–207 fragment (15). Finally, the amino acids involved in the binding process were identified as Trp-149, Tyr-190, Cys-192, Cys-193 (28), and Tyr-93 (29).

One approach to studying the dynamics of changes in receptor conformation at the submolecular level is to develop photosensitive agonists that are able to react covalently and instantaneously with surrounding residues. In this article, we describe the synthesis of photoactivatable agonists, the properties of their binding to the agonist and the NCB binding sites, and their electrophysiological properties. Use of a tritiated photosensitive agonist, [^{3}H]AC_5, enabled us to study the photolabeling of the AChR in the desensitized state and to analyze the distribution of the label in the different receptor subunits.

Experimental Procedures

Materials

Native and tritiated *Bungarus* α-bungarotoxin were purchased from Boehringer/Mannheim and Amersham, respectively; Carb was from Sigma, proadifen from SKF, and [^{3}H]PCP from NEN. PCP was generously provided by Dr. A. Jaganathen, and ^{3}H-labeled *Naja nigricollis* α-toxin was a generous gift from Dr. A. Ménez (CEN Saclay). Live *Torpedo marmorata* were purchased from the Station Biologique de Roscoff (France). All commercial reagents were of the highest purity available. The radioactive precursor [^{3}H]-**9** was synthesized in the laboratory of Dr. B. Rousseau (CEA Saclay, France).

Synthesis

Synthesis of 4-[*N*-methyl,*N*-(2-ethylacetate)]benzene diazonium trifluoroacetate, 1. Reaction of the monoprotected *N*-terbutoxycarbonyl-*para*-phenylenediamine (1.04 g, 5 mmol) (30) and triethylamine (766 μl, 5.5 mmol) with 2-bromoethyl acetate (612 μl, 5 mmol) in 10 ml of dimethylformamide yielded, after 20 hr at 50°, the monoalkylated derivative, which was purified by silica gel chromatography (eluant, EtOAc/hexane, 35:65) (50% yield). This compound (600 mg, 2.04 mmol) was methylated by reductive amination for 12 hr, in 15 ml of ethanol with 1.2 ml of a 37% aqueous solution of formaldehyde (20 mmol), using 60 mg of Pd/C (Engelhard) as catalyst for the hydrogenation. After removal of the catalyst, the crude product was chromatographed on silica gel (eluant, EtOAc/hexane, 25:75) (80% yield). The diazotization step was performed as follows. The amino terbutoxycarbonyl protecting group was removed by stirring for 30 min in TFA, at room temperature. The solution was then cooled at −15°, and 1.1 eq of $NaNO_2$ (in water) was added in small amounts in the dark over a period of 30 min. After lyophilization, the diazonium compound **1** was kept in a pH 2 water solution and stored at −30°.

Synthesis of AC_5 and of AC_7. AC_5 and AC_7 were synthesized, using an identical pathway, from 6-aminohexanoic acid **2a** and 8-aminooctanoic acid **2b**, respectively. The structures of AC_5, AC_7, and their precursors are given in Fig. 1.

The amino acids **2a** (3 g, 22.9 mmol) and **2b** (3 g, 18.75 mmol) were first activated with thionyl chloride (1.8 ml, 40.5 mmol, for **2a** and 1.5 ml, 33.75 mmol, for **2b**) for 1 hr at 0°. Acid chlorides were esterified with 2-bromoethanol (15 ml) for 1 hr at 60°. The products were recrystallized from a mixture of isopropanol/hexane (90% yield). *N*-Methyl,*N*-(*N'*-terbutoxycarbonyl)-4-anilino)carbamoyl chloride, **9**, was synthesized as described previously (31).

For the coupling reaction, 2 eq of triethylamine were added slowly

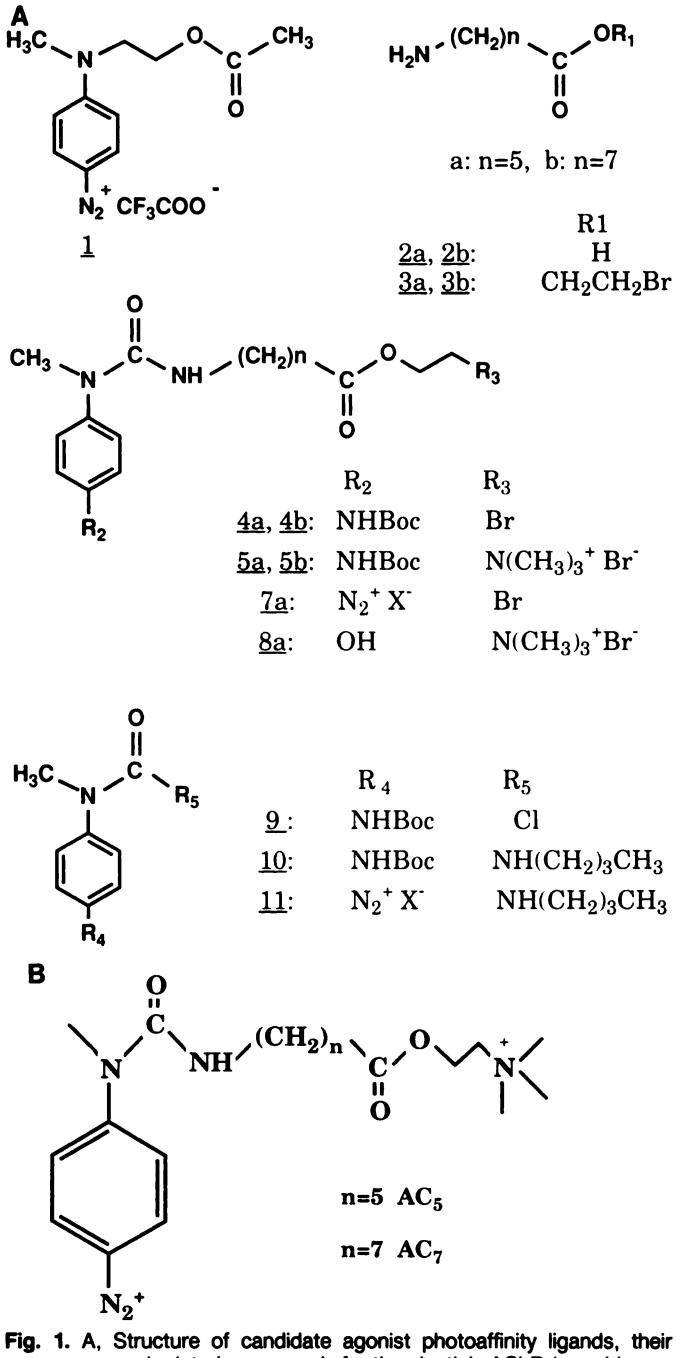

Fig. 1. A, Structure of candidate agonist photoaffinity ligands, their precursors, and related compounds for the nicotinic AChR (*a* and *b*, n = 5 and n = 7, respectively). Substituted groups are indicated by R and are listed beside the various compounds. B, Structure of the photosensitive agonists AC_5 and AC_7.

to a CH_2Cl_2/dimethylsulfoxide (4:1) mixture containing one of the esters (**3a**, 549 mg, 2 mmol; **3b**, 1 g, 3.3 mmol) and equimolar amounts of carbamoyl chloride, **9**. The mixture was stirred at room temperature for 2 hr and purified by silica gel chromatography (eluant, EtOAc/hexane, 6:4), and the products were recrystallized in EtOAc (**4a**: m.p., 113°; 83% yield; **4b**: m.p., 107°; 81% yield). At this step, some of compound **4a** was diazotized as described above, to obtain the corresponding compound **7a**.

A 50-fold molar excess of trimethylamine in toluene was slowly added to a solution of bromoester **4a** (488 mg, 1 mmol) or **4b** (3.2 g, 54 mmol) in acetone. The mixture was stirred at room temperature under nitrogen for 48 hr, and the final product (**5a** or **5b**) was isolated by ether/water continuous extraction (50% yield).

Diazotization of **5a** and **5b** proceeded as stated above, and the compounds AC_5 and AC_7 were purified by high performance liquid chromatography (C_{18} μ-Bondapack analytical column; flow rate, 1.5 ml/min; solvent A, 0.2% TFA in water; solvent B, acetonitrile; linear gradient from 0 to 25% B in A, in 50 min) (R_t = 20 min). The diazonium derivative **11** was synthesized in two steps, by first reacting *n*-butylamine (434 μl, 4.4 mmol) with carbamoyl chloride, **9** (570 mg, 2 mmol), for 1 hr at room temperature. The corresponding urea derivative **10** was isolated in quantitative yield, as crystals (m.p., 110°). The diazonium derivative **11** was obtained as described above, with a 60% overall yield.

Synthesis of tritiated ligands. The synthesis of [^{3}H]AC_5 was similar to the method described for AC_5. The radioactive precursor [^{3}H]-**9** was synthesized as detailed elsewhere (31), with a specific radioactivity of 77 Ci/mmol. Before use, the radioactive reagent was isotopically diluted to a final specific radioactivity of 1.22 Ci/mmol. The three reaction steps leading to [^{3}H]AC_5 were done in a more dilute solution, compared with the compound AC_5 (final volume of 250–500 μl). [^{3}H]AC_5 was chromatographed using the same conditions as for AC_5 (see above). For [^{3}H]-**4a** and [^{3}H]-**5a**, the products were purified by high performance liquid chromatography, using a C_{18} μ-Bondapack column (flow rate, 1.5 ml/min; solvent A, 0.2% TFA in water; solvent B, acetonitrile; linear gradient from 10 to 60% acetonitrile in water, in 30 min), and were eluted with retention times of 21 min and 14 min, respectively.

Biochemical Experiments

AChR-rich membrane fragments from *T. marmorata* were prepared as previously described (32). The concentration of ACh binding sites was measured at equilibrium, by [^{3}H]-α-bungarotoxin binding (33).

Dissociation constants of the ligands from the agonist binding site were determined in the dark, by measuring the decrease of the initial rate of binding of the ^{3}H-labeled *N. nigricollis* neurotoxin (1 nM final) to receptor-rich membranes (1 nM final) (34, 35).

Dissociation constants of the ligands from the NCB binding site were calculated at equilibrium, from competition experiments on receptor-rich membranes, with [^{3}H]PCP (1 nM) as radioligand (36).

Photolabeling Experiments

Monochromatic light was obtained from a 1000-W xenon-mercury lamp (Hanovia) connected to a grating monochromator (Jobin-Yvon). The light intensity was measured (in volts) with a thermopile (Kipp and Zohnen) coupled to a microvoltmeter. The response of the thermopile varied independently from the chosen wavelength (between 260 and 500 nm) of the incident light. Irradiation experiments were performed in a quartz cell (1-cm path length), with magnetic stirring. The monochromatic light beam was focused on the cell to form a 10-mm × 2-mm spot.

Aliquots (1 ml) of membranes (10 nM levels of agonist binding site), in 50 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl, were irradiated for 40 min at 10°, in the presence of 0.56 μM or 0.4 μM levels of the photoactivatable compounds AC_5 and AC_7, respectively. Protection experiments for the alkylation reaction were accomplished by prior incubation of the receptor with Carb (0.5 mM) at room temperature for 30 min. The photocoupling yield was determined by binding of [^{3}H]-α-bungarotoxin to the nonalkylated binding sites. AChR-rich membrane fragments (40 pmol) were photolabeled at 295 nm with [^{3}H]AC_5 (1.22 Ci/mmol, 0.5 μM), in a total volume of 1 ml. After irradiation, dithiothreitol was added to a final concentration of 10 mM, to destroy unreacted [^{3}H]AC_5. After dialysis against buffer (3 × 500 ml of 10 mM sodium phosphate, 2 mM EDTA, pH 7.2), solubilized membranes were analyzed on sodium dodecyl sulfate-polyacrylamide gels, and incorporation of radioactivity was quantified as previously described (15).

Electrophysiology

Single-channel recordings were carried out on Sol8 cells, a mouse muscle cell line. These cells were grown under conditions where they express almost exclusively the embryonic type of nicotinic AChR (37). Before recording, cells were bathed in an extracellular solution of the following composition (in mM): NaCl, 140; KCl, 4; $MgCl_2$, 1; $CaCl_2$, 1; HEPES, 10; D-glucose, 10; pH 7.2. The cell-attached patch pipette contained ACh and ligand AC_5 or ligand AC_7, at various concentrations. Single-channel data were filtered at 2 kHz and digitized at 10 kHz for further analysis using pCLAMP (Axon Instruments, Burlingame, CA).

Results

Aryldiazonium salts as reversible ligands. The binding characteristics, as measured in the dark, of compounds **1**, AC_5, and AC_7 at the agonist and NCB binding sites of the AChR are summarized in Table 1. All three compounds are fairly stable under our experimental conditions ($t_{1/2}$ >1 day). Their affinities for the ACh binding site were determined either without effector (receptor being partially in the resting state) or in the presence of 60 μM proadifen (essentially desensitized state) (13). Affinities for the NCB binding site were measured in the presence of α-bungarotoxin (resting state) or Carb (desensitized state) (Table 1).

Ligand **1** shows poor affinity (K_i about 0.1 mM), with no selectivity, for the two binding sites. In contrast, ligands AC_5 and AC_7 display very high affinity for the agonist binding site, particularly in the desensitized form (K_i values were 9 nM and

TABLE 1

Inhibition constants of ligands 1, AC_5, and AC_7 for the ACh and NCB binding sites

For the agonist binding site, [^{3}H]α-toxin (1 nM final) and AChR (1 nM final) were incubated with variable concentrations of each ligand (1 nM to 50 μM). After simultaneous addition of α-toxin and ligand, 0.3-ml aliquots were rapidly filtered, at different times, through Millipore HAWP filters and counted for radioactivity. Inhibition constants of the ligands, reflected by their ability to decrease the association rate of the α-toxin to its specific site, were determined as described (34, 35). These experiments were done with the native form of the AChR (column 1) and with desensitized receptor (>90%) by preincubation with proadifen (column 2). For the NCB binding site, inhibition constants were determined at equilibrium (45-min preincubation), using [^{3}H]PCP (1 nM final) and AChR-rich membranes (4 nM final) and variable concentrations of ligand (1 μM to 1 mM). The suspensions were filtered through GF/B filters and counted. These parameters were measured for two different states of the receptor, the resting state (obtained by preincubation of the AChR with 1 μM α-bungarotoxin for 2 hr at room temperature) (column 3) and the desensitized state (obtained by preincubation with 0.1 mM Carb for 30 min at room temperature) (column 4). Inhibition constants were determined according to the method of Eldefrawi *et al.* (36).

Ligand	Inhibition constant, K_i			
	Agonist binding site		NCB binding site	
	Native form	+Proadifen	+α-Bungarotoxin	+CARB
	M			
1	3.2×10^{-4}	1.4×10^{-4}	2×10^{-4}	3×10^{-4}
AC_5	1.4×10^{-7}	9×10^{-9}	5×10^{-4}	1.3×10^{-4}
AC_7	1×10^{-7}	7×10^{-9}	1.5×10^{-4}	3.5×10^{-5}

7 nM for AC_5 and AC_7, respectively, in the presence of proadifen). These values are comparable to the affinity of ACh for the desensitized receptor form (10 nM) (34, 35). It should be noted that ligands AC_5 and AC_7 bind with 15-fold greater affinity to the desensitized than the resting state, as generally expected for agonists. Inhibition constants of AC_5 and AC_7 for the NCB binding site are in the 0.1 mM range, with a 4-fold enhancement in the desensitized state (in the presence of Carb). Thus, AC_5 and AC_7 are remarkably selective for the agonist binding site, when the receptor is in the desensitized form (in the presence of proadifen), with, respectively, 10,000- and 20,000-fold greater affinity for this site than for the NCB site (in the presence of Carb). In contrast, **7** (a precursor of AC_5) and **11** (which lacks the ACh moiety) are poor ligands for the agonist binding site (K_i without effector, 1.2×10^{-4} M for **7** and 1.6×10^{-3} M for **11**).

Electrophysiology of AC_5 and AC_7. As illustrated in Fig. 2, a low concentration (200 nM) of ACh, AC_5, or AC_7 in the patch pipette activates elementary inward currents with comparable single-channel current amplitudes and duration of opening. There are several reasons to believe that these single-channel responses are due to the opening of AChR channels; (i) a similar single-channel conductance, on the order of 35 pS (a value typical of the embryonic type of muscle AChR), is found for ACh, ligand AC_5, and ligand AC_7 and (ii) no single-channel activity is recorded under our experimental conditions when these compounds are not present in the patch pipette or when the muscle cells are preincubated with 500 nM α-bungarotoxin. Ligand AC_5 and ligand AC_7, thus, behave as potent muscle cholinergic agonists.

At concentrations of ACh, AC_5, or AC_7 higher than 3 μM, single-channel openings occur in bursts separated by long periods of silence (Fig. 3). Within a burst, openings are separated by short duration closures. For ACh, this burst-like activity is associated solely with desensitization (38). During long duration closures, the AChR is in a desensitized state. Within a burst, the AChR oscillates between the resting activatable state and the open state. Thus, for ACh, the mean duration of short closures decreases with increasing concentration of agonist, whereas the mean open time can depend on the nature of the agonist but is independent of agonist concentration. For AC_5 and AC_7, this burst-like activity can be attributed partly to desensitization, but we also show that the mean channel open time decreases with increasing concentrations of agonist [ligand AC_5, $t_o = 5.7 \pm 0.7$ msec at 200 nM (three experiments) and 3.4 ± 0.3 msec at 10 μM (three experiments); ligand AC_7, $t_o = 10.1 \pm 0.7$ msec at 200 nM (three experiments) and 2.7 ± 0.5 msec at 10 μM]. This suggests that these compounds can act as channel blockers at higher concentration. In favor of this interpretation, we clearly observe, for concentrations of ligand AC_5 greater than 10 μM, rapid flickering of the single-channel openings that is reminiscent of the effects of fast open channel blockers (39).

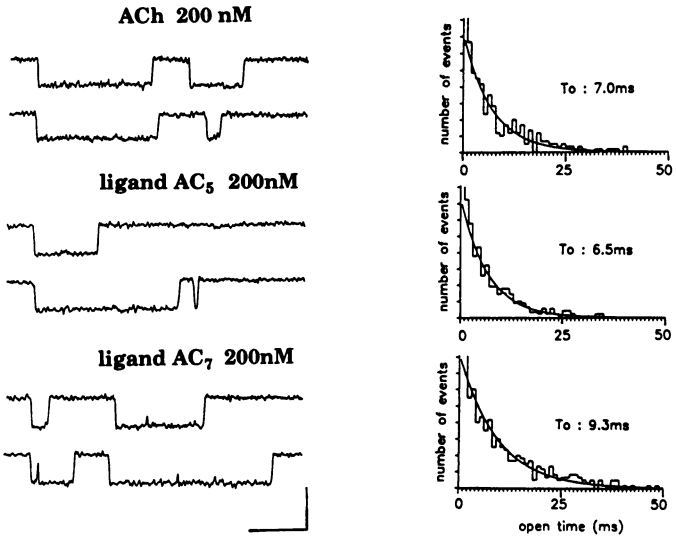

Fig. 2. *Left*, single-channel currents activated by ACh, ligand AC_5, and ligand AC_7, recorded in the cell-attached mode on myotubes from the mouse muscle cell line Sol8. Pipette potential was held at +50 mV, thus giving a transmembrane potential of roughly −110 mV. *Calibration bars*, 5 msec × 5 pA. *Right*, corresponding open time distributions. The distributions (in the range of 1–50 msec) can be well fitted by single exponentials with time constants T_o.

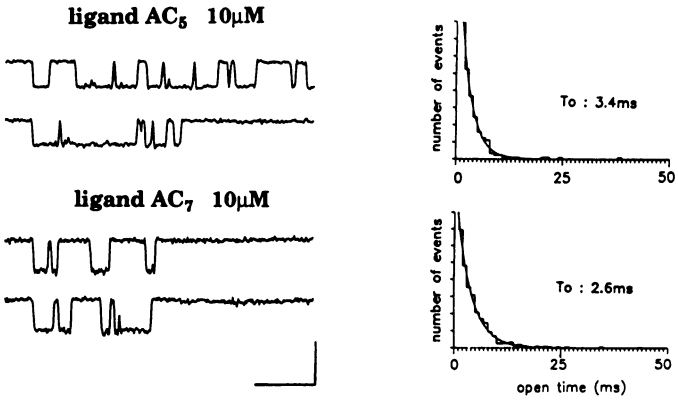

Fig. 3. Single-channel currents activated by 10 μM ligand AC_5 and ligand AC_7 and corresponding open time distribution histograms. Note that the openings occur in bursts. *Calibration bars*, 5 msec × 5 pA.

Aryldiazonium salts as irreversible blockers of the ACh binding site. Only compounds AC_5 and AC_7 were studied further in photolabeling experiments, because of their high affinity and selectivity for the agonist binding site. Two irradiation wavelengths were used for the irreversible experiments, 295 nm for energy-transfer labeling and 405 nm for direct labeling. The latter was chosen because the two compounds had about the same extinction coefficient at both wavelengths, and conditions were determined in which they photodecomposed at the same rate during the irradiation experiment.

Fig. 4 shows the yield of photoinactivation under both irradiation conditions. At 405 nm, ligands are directly photoactivated and the photolabeling is more efficient with AC_5, compared with AC_7. For both compounds, we observe that the photolabeling yields are higher when energy-transfer photoactivation conditions (295 nm) are used (40). As previously reported with other diazonium compounds, this method of photolabeling is more efficient and selective, provided that there is at least one tryptophan residue in the direct neighborhood of the bound ligand (26, 41).

The highest yield is obtained with AC_5 (0.56 μM) when it is irradiated at 295 nm in the absence of proadifen (54 ± 8%). The photolabeling yield may be limited by the high affinity of the photolyzed products of AC_5 and AC_7, which may act as protectors of the binding site. In fact, the affinity of **8a** (9×10^{-7} M), the photolyzed product of AC_5, is similar to that of AC_5 (1.4×10^{-7} M), indicating that the diazonium positive

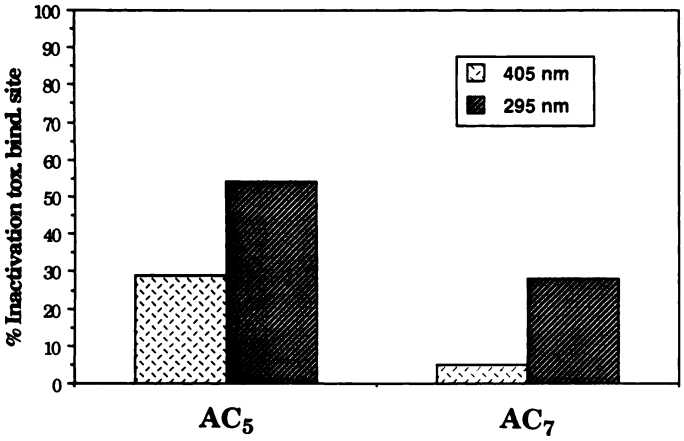

Fig. 4. Photoinactivation of the agonist binding site of the nicotinic AChR at 405 nm (direct irradiation) and at 295 nm (energy-transfer irradiation). AChR-rich membranes (10 nM α-toxin binding sites, final concentration) were irradiated for 40 min at 10°, in the presence of AC_5 (0.56 μM) or AC_7 (0.4 μM). Aliquots were diluted 10-fold in a Tris-Triton X100 buffer (10 mM Tris·HCl, pH 7.2, 100 mM NaCl, 1% Triton X-100), to determine the concentration of nonalkylated sites.

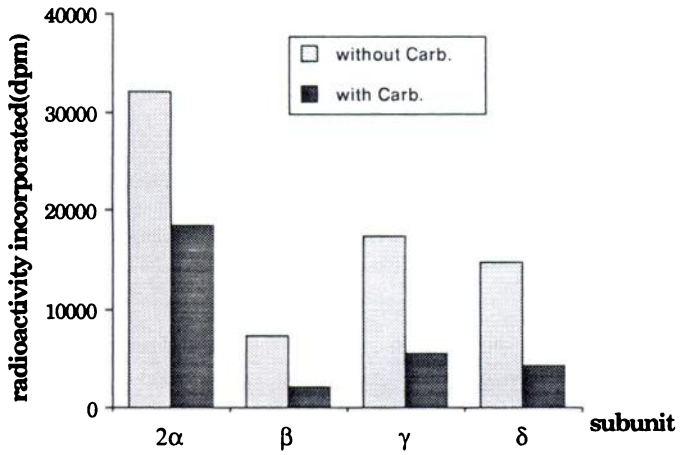

Fig. 5. Carb-specific $h\nu$ incorporation and subunit photolabeling pattern of the desensitized nicotinic AChR. Receptor-rich membranes (40 pmol) were irradiated at 295 nm for 40 min, in the presence of 0.5 μM [^{3}H]AC_5 (1.22 Ci/mmol) and 60 μM proadifen (desensitized form). Distribution of the radioactivity in the four subunits was measured after gel slicing, digestion, and counting. Photoincorporation was determined without and with protecting ligand (0.1 mM Carb).

charge is not primarily involved in the ligand/receptor interaccion.

In all cases, preincubation of the receptor with agonist (Carb) or antagonist (*d*-tubocurarine) largely prevents the inactivation of the agonist binding site (70%), indicating the high specificity of the photolabeling.

We checked, in control experiments, that (i) the AChR is stable under these two irradiation conditions (<10% loss of α-toxin binding site) and (ii) neither proadifen nor Carb and *d*-tubocurarine inhibit the binding of the [^{3}H]α-toxin to the agonist binding site in the dark.

Polypeptides involved in the ACh binding area. We have identified the subunits of the AChR that are labeled after irreversible binding of radioactive derivatives of [^{3}H]AC_5 (Fig. 5). In the presence of proadifen (desensitized receptor form), the four subunits were labeled, predominantly γ and δ (29 and 26%, respectively, of the total specific radioactivity) and, to a lesser extent, α and β (33% for two α and 12% for β). Prior incubation with Carb lowered the radioactivity being incorporated into the different subunits (42% protection for the two α subunits and around 70% for the other subunits). The low protection yield observed for α subunits might be due to nonspecific labeling and/or to labeling of the 43-kDa protein. The 43-kDa protein is always associated with the native receptor and, in our experiments, radioactivity associated with this protein was indistinguishable from that associated with the α subunits.

Discussion

A new family of photoactivatable ligands displaying AChR agonist activity was developed; compounds combining the ACh moiety with the fluorescent dansyl group were used to probe the changes occurring at the cholinergic binding site during AChR conformational transitions (13). As indicated by huge fluctuations in the energy-transfer fluorescence spectra, the region involved in the dansyl binding undergoes large structural changes as AChR switches to the different states. It was, thus, of great interest to use covalent labeling techniques to identify the residues defining the agonist binding sites in different activation states of the AChR. [^{3}H]Nicotine, when used as a photosensitive probe of the agonist binding site, labeled the Tyr-198 residue of the α subunit (42). However, the labeling efficiency was low (around 1%) and the process required extended photolytic conditions that are not compatible with rapid mixing techniques. Because transconformation from the resting to the active state takes place in a millisecond time range (13), it is necessary to have a photogenerated reactive species with a short lifetime that is, thus, able to quench specifically a single state of the AChR. The aryl cation photogenerated from aryldiazonium derivatives fulfills this criterion, because it is highly reactive ($t_{1/2} < 5 \times 10^{-10}$ sec) (27) and able to react with a variety of amino acid side chains defining a given binding site. This has been illustrated by photolabeling of residues Tyr-93, Trp-149, Tyr-190, Cys-192, and Cys-193, which belong to the agonist/competitive antagonist binding site of the α subunit, using DDF as a photoactivatable competitive antagonist (26, 28, 29). Recently, the detailed molecular structural changes occuring during transitions in the cholinergic binding sites have been explored with DDF (43). However, because DDF is a competitive antagonist, its binding domain may not be strictly identical to that of agonists. In an attempt to analyze with efficiency and specificity the conformational transitions of the AChR agonist binding site, a novel class of photoactivatable AChR agonists was developed. These ligands consist of a combination of the ACh moiety itself and a photosensitive aryldiazonium group (Fig. 1B).

Ligands AC_5 and AC_7 are potent agonists at low concentrations (200 nM), as indicated by electrophysiological experiments. Their affinities are of the same order of magnitude as those of flexible organic cationic agonists such as Carb and suberyldicholine (K_i of 10^{-8} M, in the desensitized form). The other photoactivatable derivatives in which the ACh moiety is partially or completely missing, **1**, **7**, and **11**, are poor ligands of the ACh binding site (K_i in the 1–0.1 mM range), and ligand **1** displays no selectivity for this site, compared with the NCB site. The low affinity observed for **1**, compared with AC_5 and AC_7, can be accounted for by the lack of a quaternary ammonium moiety, a pharmacophore present in all agonists except

the rigid ones such as anatoxin (44). Although resonance forms of **1** confer a partial positive charge to the aniline nitrogen, this structural analogy with ACh appears to be insufficient to satisfy the structural requirements for an agonist. Substitution of the trimethylammonium moiety (AC_5) for a bromine atom (**7**) results in a 1000-fold decrease in affinity, showing that the quaternary ammonium of ACh is necessary for recognition. In contrast, the acetyl moiety plays a relatively minor role in the recognition process, as indicated by comparison of ligands **11** and **7**.

Compounds AC_5 and AC_7 were, thus, selected for irreversible labeling experiments. Upon irradiation, they efficiently block the ACh binding site. We showed that compound AC_5 is much more efficient than AC_7 as an affinity label of the agonist binding sites; >30% of these sites are blocked using AC_5 in the submicromolar range (0.56 μM), and agonists (Carb) or competitive antagonists (data not shown) protect efficiently against this inactivation. The efficiency of labeling is improved by inducing covalent coupling under energy-transfer conditions (up to 54% inhibition at 295 nm), as previously observed with other aryldiazonium salts (26, 41).

The use of the tritiated agonist [^{3}H]AC_5 allowed analysis of the labeling pattern of the receptor subunits. As for other aryldiazonium derivatives (26), it should be noted that the four subunits of the receptor are labeled. Labeling of the four subunits is in agreement with recent work done on the AChR with the antagonist DDF (26, 29), photoactivatable toxin derivatives (8), and photoactivated *d*-tubocurarine (9), showing that, in addition to the α subunits, the cholinergic binding area may involve the β, γ, and δ subunits. Taken together, these results indicate that the cholinergic ligand recognition site probably requires the concomitant participation of several subunits.

One main difference between [^{3}H]DDF and [^{3}H]AC_5 photocoupling to the ACh binding site is the extent of the α subunit labeling, compared with the other subunits. [^{3}H]DDF labels mainly the α subunits, whereas for [^{3}H]AC_5 the radioactivity is distributed to about the same extent in the four subunits. One possible explanation might be the different size of the two radioligands. Clearly, in the DDF molecule the photosensitive diazonium moiety probes the ammonium binding site, which is not the case for the AC_5 molecule, as shown by the small change in affinity observed for its photolyzed derivative, **8a**. The photoactivatable moiety N_2^+ in AC_5 might be up to 14.5 Å from the ammonium site in an extended conformation, thus likely explaining the broader labeling spectrum observed.

Nevertheless, this diazonium is probably located in a region of AChR where significant topological changes occur during state transitions, as shown by fluorescence experiments with the Dns-C6-Cho agonist. This property might be used to probe the environment of the ACh binding site and to identify the structural changes occurring at the molecular level in this region during AChR transitions.

In conclusion, AC_5 is a potent photoactivatable agonist of the nicotinic AChR that is able to label the ACh binding site irreversibly. Photolabeling of the receptor using [^{3}H]AC_5 results in protectable photoincorporation, mainly in the β, γ, and δ subunits. With rapid mixing techniques, the probes that are reported here should allow a definitive characterization of residues responsible for agonist binding in the active state of the AChR.

Acknowledgments

We thank Dr. Rousseau for tritiating the [^{3}H]AC_5 precursor, Dr. A. Jaganathen for PCP, Dr. A. Ménez for ^{3}H-labeled *N. nigricollis* α-toxin, Dr. J. L. Galzi for fruitful discussions, and Dr. M. Hibert and Dr. K. Takeda for critical reading of the manuscript.

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