Photoaffinity Labeling of the Acetylcholine Binding Sites on the Nicotinic Receptor by an Aryldiazonium Derivative†

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ABSTRACT: *p*-(Dimethylamino)benzenediazonium fluoroborate (DDF) behaves, in the dark, as a reversible competitive antagonist of the electrical response of *Electrophorus electricus* electroplaque to acetylcholine and of the acetylcholine-gated single-channel currents recorded in the C2 mouse cell line. This chemically stable but highly photoreactive compound binds irreversibly to the acetylcholine receptor when irradiated by visible light. In vivo, it irreversibly blocks the postsynaptic response of *E. electricus* electroplaque to agonists. In vitro, it reduces the α-bungarotoxin-binding capacity of acetylcholine receptor rich membrane fragments prepared from *Torpedo marmorata* electric organ. Once reversibly bound to the *T. marmorata* acetylcholine receptor, this ligand can be selectively photodecomposed by an energy-transfer reaction involving a tryptophan residue(s) of the protein. By use of reagent concentrations that are below the dissociation constant at equilibrium, up to 60% of the agonist-binding sites are covalently labeled. Under these conditions the α subunit of the acetylcholine receptor is preferentially labeled, and this labeling is partially prevented by agonists or competitive antagonists. This protective effect is substantially increased by prior incubation with phencyclidine, a compound known to prevent the binding of DDF at the level of the high-affinity site for noncompetitive blockers [Kotzyba-Hibert, F., Langenbuch-Cachat, J., Jaganathen, J., Goeldner, M. P., & Hirth, C. G. (1985) *FEBS Lett. 182*, 297–301]. The incorporation of about one molecule of label in an agonist/competitive antagonist protectable manner per α-bungarotoxin-binding site suffices to fully block α-bungarotoxin binding to the membrane-bound receptor. Thus, DDF behaves as a monovalent photoaffinity label of the acetylcholine-binding site.

The nicotinic acetylcholine receptor (AcChoR)[1] from fish electric organ and vertebrate neuromuscular junction is a well-characterized membrane-bound allosteric protein [reviews in Anholt et al. (1984), Changeux et al. (1984), Popot and Changeux (1984), and Hucho (1986)] made up of four polypeptide chains associated into a heterologous pentamer with an accepted stoichiometry of $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). The cDNAs coding for all four of the subunits have been cloned and sequenced in several species [reviews in Numa et al., (1983), Changeux et al. (1984), and Stroud and Finer-Moore (1985)] and several models for the transmembrane organization of these subunits inferred from the primary structure data [reviews in Popot and Changeux (1984) and Stroud and Finer-Moore (1985)]. The test of these models by the localization of functional domains within the primary structure of the subunits remains one of the more challenging issues in AcChoR research. One approach consists in the use of affinity and photoaffinity ligands to covalently label defined sites (Jacoby & Wilchek, 1977; Chowdry & Westheimer, 1979) followed by the identification and positioning of the labeled amino acid(s) along the primary structures of the subunits [see Kao et al. (1984), Giraudat et al. (1986, 1987), and Oberthür et al. (1986)].

The AcChoR protein carries several distinct categories of binding sites. The nicotinic agonists and competitive antagonists bind, at equilibrium, to two primary acetylcholine (AcCho) binding sites that are selectively tagged by snake venom α-toxins [reviews in Karlin (1983) and Changeux et al. (1984)]. The noncompetitive blockers (NCB) of the permeability response bind to several classes of sites, among which is a high-affinity site present as a single copy per receptor that specifically binds histrionicotoxin and phencyclidine (PCP) (Heidmann et al., 1983; Oswald et al., 1983).

The first probe used to label the agonist-binding sites was *p*-(trimethylammoniumyl)benzenediazonium fluoroborate (TDF) (Changeux et al., 1967; Mautner & Bartels, 1970; Wieland, et al., 1979). Incorporation of about one molecule of this affinity reagent per α-toxin-binding site was sufficient to completely block AcCho binding to the receptor (Wieland et al., 1979). Several less chemically reactive alkylating analogues of AcCho have also been used with success [reviews in Karlin (1969, 1983)]; however, these sulfhydryl-directed affinity ligands require the prior reduction of at least one

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[1] Abbreviations: DDF, *p*-(dimethylamino)benzenediazonium fluoroborate; TDF, *p*-(trimethylammoniumyl)benzenediazonium fluoroborate; PCP, phencyclidine; AcCho, acetylcholine; AcChoR, acetylcholine receptor; NCB, noncompetitive blocker; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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disulfide bond on the AcChoR and it is known that this modification alters the functional properties of the AcChoR protein (Karlin, 1969; Chang & Bock, 1977; Barrantes, 1980; Walker et al., 1984). In all of these experiments, the affinity label was incorporated exclusively into the α-subunit [review in Karlin (1983)], suggesting that the two AcCho-binding sites are carried, at least in part, by the two α-chains of the AcChoR molecule.

Photoaffinity labels appear more appropriate to study the topology of the AcCho-binding sites since the photogenerated species possess much higher reactivity (Chowdry & Westheimer, 1979; Bayley & Knowles, 1977). The photosensitive derivatives used up to now with the nicotinic AcChoR were analogues to either AcCho (Kiefer et al., 1970; Hucho et al., 1976; Witzeman & Raftery, 1977) or α-bungarotoxin (Witzeman & Raftery, 1978; Hucho, 1979; Witzeman et al., 1979; Nathanson & Hall, 1980), and they all labeled the α-subunit and, to variable extents, the other AcChoR subunits. However, no stoichiometries of irreversible inactivation were presented for these compounds, making difficult the interpretation of these data in terms of authentic photoaffinity labeling.

In this paper we study the photoaffinity labeling of the primary AcCho-binding sites on the native, unreduced AcChoR using *p*-(dimethylamino)benzenediazonium fluoroborate (DDF). This diazonium ion is fairly stable under physiological conditions and gives rise, after irradiation, to the corresponding aryl cation, which is known for its hyperreactivity (Ambroz & Kemp, 1979, 1982; Himeshima et al., 1985; Grieve et al., 1985; Kieffer et al., 1986). We have previously shown that DDF can be used to label the high-affinity-binding site for NCBs (Kotzyba-Hibert et al., 1985). In the present study we demonstrate that, in the presence of PCP, DDF is an efficient photosensitive irreversible probe of the AcCho-binding sites on the native receptor. The efficiency of the labeling experiments was improved by inducing the photoaffinity labeling via an energy-transfer reaction between a tryptophan of the receptor protein and the photosensitive ligand (Goeldner & Hirth, 1980). Such selective labeling, based on the preferential photodecomposition of the ligand when bound to the active sites, enables us to extend to a receptor protein the concept of "photosuicide inactivation" previously described with acetylcholinesterase (Goeldner et al., 1982).

Materials and Methods

Materials

Electrophorus electricus were obtained from Paramount Aquarium (Ardsley, New York), and live *Torpedo marmorata* were provided by the Institut Universitaire de Biologie Marine (Arcachon, France). [^{3}H]-α-Toxin from *Naja nigricollis* was a gift from Drs. Morgat, Menez, and Fromageot from the Commissariat à l'Energie Atomique (Saclay, France). [*methyl*-^{3}H]-*N*,*N*-Dimethyl-*N*'-(butyloxycarbonyl)-*p*-phenylenediamine was a gift of Drs. Van Hove and Rousseau. α-Bungarotoxin from *Bungarus multicinctus* was from Boehringer (Mannheim, FRG). Millipore filters (HAWP 02500, 0.45 μm) were obtained from Millipore (Mutzig, France). DEAE 81 paper circles were from Whatman (England), and *d*-tubocurarine and carbamoylcholine were from Sigma (St. Louis, MO). All other chemicals were of analytical grade from Prolabo (Paris, France), E. Merck (Darmstadt, FRG), or Fluka (Buchs, Switzerland).

Fluorescence spectra were recorded with a Jobin-Yvon (JY 3C) spectrofluorometer using 1 cm × 1 cm quartz cells. Absorbance spectra were obtained with a Jobin-Yvon (Duospac 203) spectrophotometer. For irradiation experiments, monochromatic light was obtained from a 1000-W xenon–mercury lamp (Hanovia) connected to a grating monochromator (Jobin-Yvon, France). The light intensity was measured with a thermopile (Kipp and Zohnen, Netherland) coupled to a microvoltmeter, the response of which is independent of the wavelength of the incident light between 260 and 500 nm. For example, at 350 nm light measurement of 1 mV corresponds to an incident energy of 2.17×10^{-6} einstein s^{-1} cm^{-2}. An iris diaphragm was introduced in the light beam between the source and the monochromator and adjusted in order to vary the number of incident photons in the assay cuvette. *E. electricus* physiological solution was 1.5 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. Phosphate buffer for irradiation experiments and for initial velocity measurements of [^{3}H]-α-toxin binding was 10 mM sodium phosphate (pH 7.0) and 150 mM NaCl. The buffer for the determination of [^{3}H]-α-toxin-binding sites at equilibrium was 10 mM Tris-HCl (pH 7.0), 10 mM NaCl, and 1% Triton X-100.

Methods

(*1*) *Synthesis of* [*methyl*-^{3}*H*]*DDF*. The starting material was [*methyl*-^{3}H]-*N*,*N*-dimethyl-*N*'-(butyloxycarbonyl)-*p*-phenylenediamine (specific activity 60 Ci/mmol) synthesized at the Commissariat à l'Energie Atomique (Saclay, France).

The radioactive precursor (100 mCi in 5 mL of ethanol) was diluted by 29.5 mg (0.125 nmol) of unlabeled material. After cooling to −80 °C, the solvent was removed under vacuum (10^{-2} mmHg). The remaining solid residue was then dissolved in 0.25 mL of 34% (w/v) fluoroboric acid at room temperature under inert atmosphere and stirred for 10 min. After the solution was cooled at −10 °C, sodium nitrite (8.5 mg, about 10% excess) was added in small amounts over a period of 30 min and stirred for an additional 45 min in the dark. The yellow crystals were filtered off (4.2 mg), and the filtrate was taken up in a minimum of acetone and precipitated with anhydrous ether (7.2 mg). The overall yield was 40% and the specific activity 0.28 Ci/mmol. The purity of the diazonium salt was checked by UV absorbance spectrum with reference to a pure sample. DDF can be kept in aqueous solution (10^{-2} M) at −30 °C without noticeable damage for over 1 year.

(*2*) *Electroplaque Experiments*. The pharmacological effect of DDF was examined on an isolated electroplaque preparation from *E. electricus* according to the methods of Schoffeniels and Nachmansohn (1957) and Higman et al. (1963) by recording the membrane potential with a glass microelectrode.

(*3*) *Single-Channel Recordings*. AcCho-activated single-channel currents were recorded in the dark in the "outside-out" mode from myotubes of the mouse cell line C2, which was kindly provided by C. Pinset (Institut Pasteur, Paris). Myotubes were bathed in a solution of the following composition: NaCl, 140 mM; KCl, 4 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1 mM; glucose, 11 mM; and Hepes, 5 mM; pH 7.2. Electrodes were filled with an intracellular solution containing 150 mM KCl and a low concentration of Ca^{2+} ($<10^{-7}$ M). After isolation of an outside-out membrane patch (Hamill et al., 1981), the patch was generally hyperpolarized at a holding potential of −80 mV, and various concentrations of AcCho (100 nM, 500 nM, and 1 μM), alone or in conjunction with DDF (100 nM, 1 μM), were perfused in the bath. A few experiments were performed under the "cell-attached" mode in order to examine the effects of DDF when applied alone in the pipet. Data were stored on tape (Racal FM/4) at a 2.5-kHz bandwidth and were analyzed with an LSI 11/23 DEC computer. Single-

channel recordings were filtered at 1 kHz with an 8-pole Bessel filter and then digitized with a sampling interval of 0.2 ms.

(*4*) [^3H]-α-*Toxin-Binding Experiments.* AcChoR-rich membrane fragments (800–1800 nmol of [^{3}H]-α-toxin-binding sites/g of protein) were purified from fresh *T. marmorata* electric organ following a described procedure (Saitoh et al., 1980). The concentration of α-toxin-binding sites was measured under equilibrium conditions by the DEAE-cellulose filter assay (Fulpius et al., 1972; Schmidt & Raftery, 1973) with [^{3}H]-α-toxin from *N. nigricollis* (27 Ci/mmol) and solubilized AcChoR-rich membrane fragments. The association kinetics of tritiated α-toxin with AcChoR in its membrane-bound state was followed by the rapid Millipore filtration technique (Weber & Changeux, 1974a,b) except that the experiments were performed in phosphate buffer (sodium phosphate 10 mM, pH 7.0; NaCl 150 mM) supplemented with 0.01% NaN_3 and 0.1% bovine serum albumin. Further purification of the AcChoR-rich membrane fragments by alkali treatment was as described previously (Neubig et al., 1979).

(*5*) *Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was done in 1.4 mm thick slab gels according to the method of Laemmli (1970). The running and stacking gels contained, respectively, 10% and 5% acrylamide. Samples of 50 μL containing 20–50 μg of protein were heated to 80 °C for 3 min or kept 1–2 h at room temperature before electrophoresis. After the gel was run, the proteins were stained for 30 min with 0.15% Coomassie brilliant blue in 50% methanol and 8% acetic acid and destained overnight with 6.5% acetic acid and 5% methanol.

For qualitative revelation of radioactivity on the polyacrylamide gels, the method described by Laskey and Mills (1975) was used. For quantitative determinations, the gels were cut either in 2-mm slices or in specific bands corresponding to AcChoR subunits sliced out of Coomassie blue stained gels. Each slice was placed in a glass scintillation vial containing 300 μL of 30% hydrogen peroxide, and the tightly capped vials were heated to 50 °C for 12–18 h, after which time 200 μL of 4 M urea and 1% $NaDodSO_4$ were added. After shaking, the samples were counted following the addition of 10 mL of scintillation solution.

(*6*) *Irradiation Experiments.* During in vivo irradiation experiments, the isolated electroplaque was illuminated with the 40-W microscope lamp (Nikon) normally used for organ dissection. In order to minimize the heating caused by this irradiation, the microscope lamp was turned on only 5 min every 15 min, and the preparation was frequently washed with large volumes of unexposed solution. In vitro irradiation experiments were done in phosphate buffer at 10 °C in a quartz cell with a 1-cm path length under gentle magnetic stirring. Unless otherwise stated, the reaction mixture contained 0.2 mM DDF and 500–800 pmol of [^{3}H]-α-toxin-binding sites for an assay volume of 0.5 mL. A monochromatic light beam was focused on the cell with a quartz lens to form a spot 10 mm high and 2 mm wide. We used as visible light a monochromatic beam of wavelength 435 nm (I = 40 μV) and as UV light a monochromatic beam of wavelength 290 nm (I = 60 μV). These two conditions correspond to the same number of incident photons. In fact, at these two wavelengths, DDF has the same extinction coefficient and we checked that it is photodecomposed with the same rate ($T_{1/2}$ = 15 min at 2 × 10^{-4} M).

Results

In Vivo Experiments with E. electricus Electroplaque. DDF, which possesses a dimethylamino group in the para position, is stable in water in the absence of light, and its decomposition in buffered medium is quite slow ($T_{1/2}$ = 3.5 h at pH 7.2). Therefore, at variance with TDF (Changeux et al., 1967) DDF does not behave as a covalent affinity label of the AcChoR in the absence of light (Mautner & Bartels, 1970).

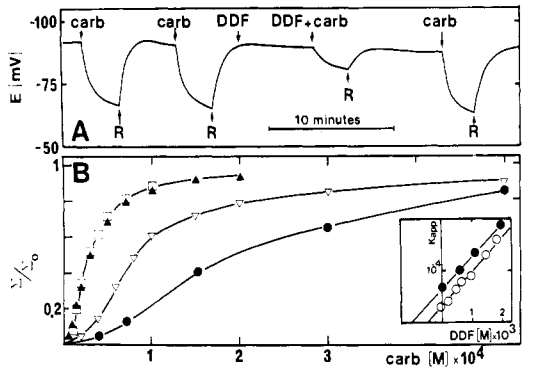

FIGURE 1: Pharmacological effect of DDF on the isolated electroplaque from *Electrophorus electricus* in the dark. (A) Changes in membrane potential caused by bath application of 30 μM carbamoylcholine (carb) are recorded as a function of time in the presence and in the absence of DDF (1 mM). Washings with physiological solution are indicated by R. (B) Concentration effect curves in the presence of 30 μM carbamoylcholine and various concentrations of DDF. The depolarization caused by bath application of various concentrations of carbamoylcholine on the innervated face on the electroplaque were recorded. Σ/Σ_0 is the ratio of the amplitude of the electroplaque response at a given carbamoylcholine concentration to the maximal amplitude of the response: $\Sigma/\Sigma_0 = [(E - E_0)/E_0]/[(E_\infty - E_0)/E_0]$ where E_0 is the membrane potential at rest, E is the membrane potential in the presence of carbamoylcholine, and E_∞ is the membrane potential extrapolated to infinite carbamoylcholine concentration. Response in the presence of (□) carbamoylcholine, (▽) carbamoylcholine and 1 mM DDF, (●) carbamoylcholine and 1.8 mM DDF, and (▲) control after a 15-min exposure of the cell to 0.5 mM DDF followed by a 5-min wash with a physiological solution. (Inset) Competitive blocking action of DDF. Half-effective doses of carbamoylcholine are plotted as a function of DDF concentration: (○) on isolated electroplaque; (●) after exposure of the electroplaque for 10 min to 0.40 mM dithiothreitol and a wash with physiological solution.

DDF has no significant effect on the resting potential of the isolated electroplaque from *E. electricus* when applied on its innervated side at concentrations up to 10 mM. However, in the presence of 1 mM DDF and in the dark, the amplitude of the depolarization caused by 30 μM carbamoylcholine decreases by about 60% (Figure 1). A complete recovery of the response occurs after washing. In the absence of light, DDF thus acts as a reversible inhibitor of the permeability response.

Quantitative analysis of this reversible inhibition shows that, still in the absence of light DDF blocks the response to carbamoylcholine in a competitive manner with an apparent dissociation constant (K_{app}) of 0.4 mM (Figure 1). In the dark, no irreversible inhibition of the response was ever noticed, even after application of DDF at a concentration of 10 mM and for over 2 h. At variance with what occurs with TDF, prior reduction of the electroplaque by dithiothreitol (0.4 mM) does not substantially modify the pharmacological effects of DDF, which remains a reversible antagonist.

Figure 2A shows that different results are obtained when the innervated face of *E. electricus* electroplaque is illuminated for 5 min with visible light in the presence of DDF. Each illumination of the preparation in the presence of 0.3 mM DDF causes a 20% irreversible decrease in the amplitude of the electrical potential response. Cumulative irradiations under conditions where the physiological solution is changed after

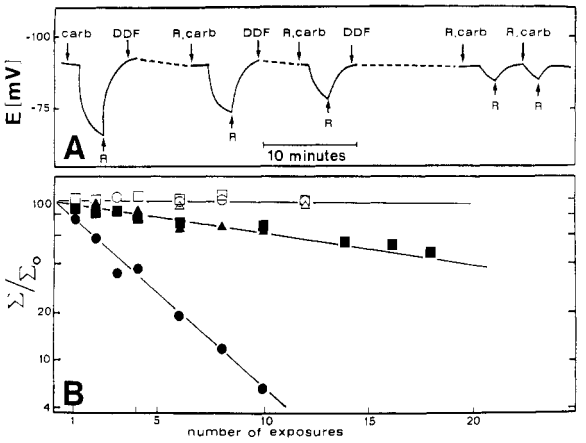

FIGURE 2: In the presence of light, DDF irreversibly blocks the response of *Electrophorus electricus* electroplaque to carbamoylcholine. (A) Changes in membrane potential caused by bath application of 30 μM carbamoylcholine were recorded as a function of time after successive 5-min applications of 0.3 mM of DDF in the presence of light (dotted line represents the illumination period). After each irradiation, the solution containing the unreacted ligand was removed and the innervated face of the cell was washed with physiological solution before the response to 30 μM carbamoylcholine was elicited. (B) Amplitude, expressed as percent of its control value, of the electrical response of isolated electroplaque to 30 μM carbamoylcholine, $(E - E_0)/E_0$), where E and E_0 are the membrane potential in the presence and in the absence of agonists respectively, is represented on a logarithmic scale as a function of the number of 5-min exposes in the dark (open symbols) or in the light (filled symbols) to 0.3 mM DDF (●, ○), 2 μM *d*-tubocurarine (▲, △), or 0.3 mM DDF plus 2 μM *d*-tubocurarine (■, □).

each illumination lead to an exponential decrease of the amplitude of the response. The response is irreversibly reduced to 5% of its initial value after 10 successive illuminations. Finally, *d*-tubocurarine (2 μM) efficiently protects the electroplaque against DDF photoinactivation (Figure 2B).

Effects of DDF on AcCho-Activated Single-Channel Currents from C2 Mouse Myotubes. In order to examine the mechanism of the reversible blocking action of DDF in greater detail, the effect of DDF on AcCho-activated single channel currents was analyzed in patch-clamp experiments, in the absence of light, with myotubes from the mouse C2 cell line (Figure 3). No agonist effect was observed when DDF was applied alone in the pipet at concentrations of 100 nM and 1 μM in the cell-attached mode. DDF, when applied at these concentrations in conjunction with AcCho, did not affect the 35-pS single-channel conductance, which is characteristic of the great majority of AcCho-activated events observed in these myotubes (Changeux et al., 1986).

In the outside-out configuration, the most striking effect of DDF consisted in a decrease of the channel-opening frequency. The ratio r of channel-opening frequency in the presence versus in the absence of the blocking agent decreased with increasing concentrations of DDF (5×10^{-7} M AcCho/10^{-7} M DDF, $r = 0.63$; 5×10^{-7} M AcCho/10^{-6} M DDF, $r = 0.46$) and increased with increasing concentrations of AcCho for a given concentration of DDF (10^{-7} M DDF/10^{-7} M AcCho, $r = 0.44$; 10^{-7} M DDF/5×10^{-7} M AcCho, $r = 0.63$; 10^{-7} M DDF/10^{-6} M AcCho, $r = 0.78$). This is illustrated by the results shown in Figure 3A in which mean channel-opening frequency was measured every 30 s and successive perfusions were performed. The decrease in channel-opening frequency in this experiment cannot be attributed to the irreversible closure of AcCho-activated channels, since the effect of DDF, in the absence of light, was reversed by washing. It also cannot be interpreted in terms of a sequential open-channel block mechanism assuming a very slow unblocking rate. Indeed, in this case the

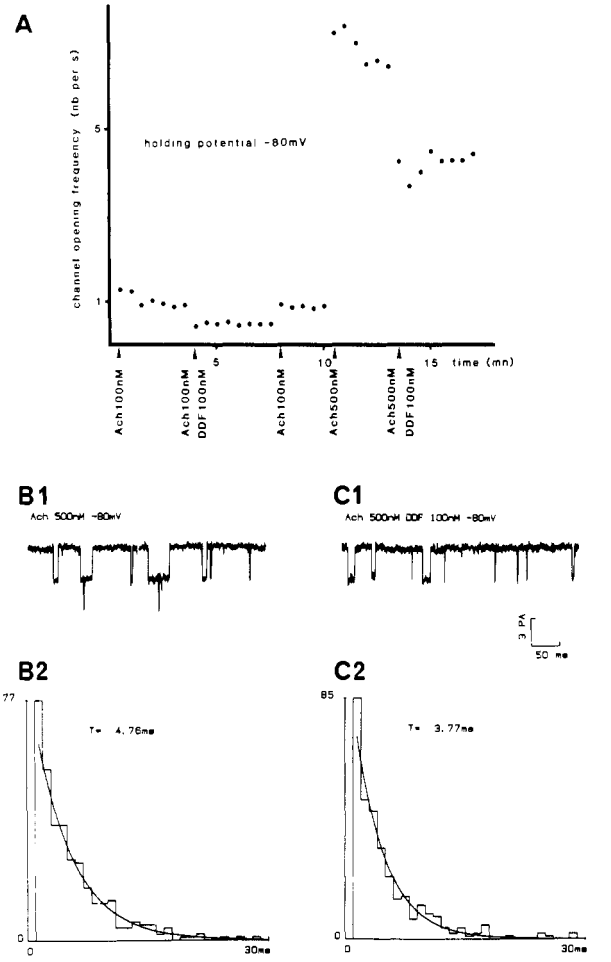

FIGURE 3: (A) Effect of DDF on AcCho-activated single-channel activity recorded from C2 mouse myotubes. Outside-out patch, holding potential −80 mV. The mean channel-opening frequency (number of openings per second) was measured every 30 s in the presence of various concentrations of AcCho and DDF. (B and C) Effect of DDF on mean channel-open time recorded in C2 mouse myotubes. (B1) Single-channel currents recorded from an outside-out C2 patch in the presence of 500 nM AcCho. Holding potential −80 mV. (B2) Open time distribution for the recording of the events presented in (B1). T = time constant. Sampling 5 kHz. Data filtered at 1 kHz. (C1) Single-channel currents recorded from the same patch in the presence of 500 nM AcCho and 100 nM DDF. (C2) Open time distribution.

ratio r should decrease, for a given concentration of DDF, with increasing concentrations of agonist. These results thus support the conclusion that DDF acts as a reversible competitive antagonist, although a contribution of desensitization to the effect of DDF cannot be excluded.

In addition, DDF (100 nM) also slightly reduced the mean channel-open time (Figure 3B,C), from 4.5 ± 0.35 ($n = 4$) to 3.4 ± 0.35 ms ($n = 4$), for a holding potential of −80 mV. This effect can be compared to the action of the NCBs chlorpromazine and PCP, which decrease the mean burst open duration of AcCho-activated channels at concentrations ranging from 10 to 200 nM. However, no change in the mean channel-opening frequency was observed with either compound in this concentration range, although higher concentrations of chlorpromazine or PCP (1 μM) caused a large reduction of channel openings and the appearance of extremely long closed times (Aguayo et al., 1986; Changeux et al., 1986; Papke & Oswald 1986). DDF also binds to the high-affinity site for NCBs but with a K_i 100-fold greater than that for PCP. Thus, the concentrations of DDF used in the present study (100 nM, 1 μM) appear very low compared to the concen-

Table I: Irreversible Loss of α-Toxin-Binding Capacity of AcChoR-Rich Membrane Fragments after Direct Illumination (435 nm) or Energy-Transfer Labeling (290 nm) with the Same Number of Incident Photons[a]

DDF (mM)	*d*-tubocurarine (μM)	*hν*	[^{3}H]-α-toxin binding sites available (%)
none	none		100
0.2	none		100
0.2	none	435 nm, 40 μV	79
0.2	2	435 nm, 40 μV	96
0.2	none	290 nm, 60 μV	40
0.2	10	290 nm, 60 μV	97

[a] In this experiment, AcChoR-rich membrane fragments (830–1800 nmol of [^{3}H]-α-toxin-binding sites/g of protein) were diluted in 0.5 mL of phosphate buffer at a final concentration of 0.9–1.4 μM of [^{3}H]-α-toxin-binding sites and incubated with 0.2 mM DDF. After 30 min of irradiation, the membrane fragments were diluted 100-fold with Tris buffer and their [^{3}H]-α-toxin-binding capacity measured on duplicates under equilibrium conditions.

trations of PCP for which a decrease in channel-opening frequency was observed. Thus, the mechanism by which DDF (100 nM, 1 μM) decreases channel-opening frequency is probably different from the mechanism by which high concentrations of PCP and chlorpromazine affect channel-opening frequency.

In conclusion, DDF (100 nM, 1 μM) decreases AcCho-activated single-channel frequency most likely by acting as a reversible competitve antagonist and shortens burst duration by a mechanism comparable to that described for other NCBs.

In Vitro Photoinactivation of the AcChoR from T. marmorata by DDF. The experiments performed in vitro cannot be quantitatively compared with those carried out in vivo for the following reasons: (i) the biological material is different, namely, *T. marmorata* AcChoR-rich membrane fragments instead of *E. electricus* electroplaque or mouse C2 myotubes; (ii) the irradiation conditions differ by the geometry of the system and by the use of monochromatic light instead of visible polychromatic light; and (iii) the binding of [^{3}H]-α-toxin from *N. nigricollis* venom is used to monitor the properties of the AcCho-binding sites instead of the electrical response to AcCho. On the other hand, a qualitative comparison of the effect of DDF on these systems appears legitimate in view of the high degree of conservation of the amino acid sequence of the AcChoR (Stroud & Finer-Moore, 1985).

In the absence of light, DDF causes a reversible decrease of the initial rates of [^{3}H]-α-toxin binding (Figure 4A) without modification of the total number of α-toxin molecules bound at equilibrium (Table I), suggesting a mutual reversible exclusion of DDF and α-toxin for a common site. An apparent dissociation constant of 1.5 mM for DDF binding to the agonist site was evaluated from these experiments (Figure 4A). This value is about 4 times greater than the corresponding K_{app} determined in vivo with *E. electricus* electroplaque.

Irradiation with monochromatic visible light (λ_{irr} = 435 nm) of a mixture of AcChoR-rich membrane fragments from *T. marmorata* and 0.2 mM DDF led to a 20% irreversible decrease in the [^{3}H]-α-toxin-binding capacity of the membrane fragments. Again, as observed with *E. electricus* electroplaque, *d*-tubocurarine protected the membranes against DDF inactivation (Table I). Therefore, with both preparations DDF behaves as a reversible antagonist in the dark and as irreversible antagonist after irradiation by visible light.

The important overlap existing between the fluorescence emission spectrum of the AcChoR and DDF absorption spectrum (R_0 = 27 Å in Forster's approximation if one takes 0.2 for the fluorescence yield of tryptophan residues) makes

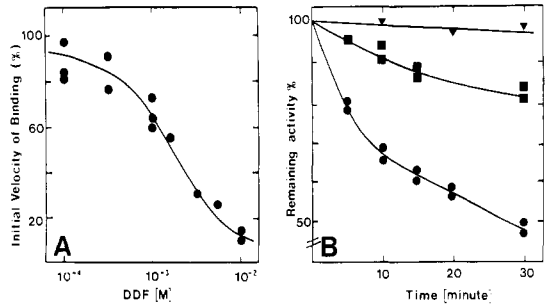

FIGURE 4: DDF decreases the rate of *Naja nigricollis* [^{3}H]-α-toxin binding to *Torpedo marmorata* AcChoR-rich membrane fragments. (A) Receptor-rich membrane fragments (830 nmol of [^{3}H]-α-toxin-binding sites/g of protein: 15.2 mg of protein/mL) were diluted in 7.5 mL of phosphate buffer (supplemented with 0.1 mg/mL bovine serum albumin) to a final concentration of 0.34 nM of [^{3}H]-α-toxin-binding sites and incubated with the indicated concentrations of DDF. The reaction at room temperature (about 20 °C) and in the dark was started by adding 0.5 μL of [^{3}H]-α-toxin (27 Ci/mmol) to a final concentration of 0.79 μM. The amount of toxin bound was determined as a function of incubation time by rapid filtration of 1-mL aliquots. The intial velocity of [^{3}H]-α-toxin binding is expressed as percent of its control value determined in the absence of DDF. (B) Energy transfer between the membrane-bound AcChoR protein and DDF enhances the photoinactivation of the α-toxin-binding sites. AcChoR-rich membrane fragments prepared from *T. marmorata* electric organ (1400 nmol of [^{3}H]-α-toxin-binding sites/g of protein; 7 mg of protein/mL) were diluted in phosphate buffer to a final concentration of 1.2 μM α-toxin-binding sites with 0.2 mM DDF. Irradiation was as described under Methods either at 290 nm with incident energy of 60 μV (●) or at 435 nm with an incident energy of 40 μV (□). A control irradiation was also performed (▼) at 290 nm with an energy of 60 μV in the absence of ligand. The photoinactivation of the AcChoR was followed by its loss of binding capacity for [^{3}H]-α-toxin according to the procedure described in Table I. Results expressed as percent of the initial value are plotted as a function of irradiation time.

possible the photoaffinity labeling of the receptor by DDF via an energy-transfer reaction from tryptophan(s). Indeed, under energy-transfer conditions (λ_{irr} = 290 nm; I = 60 μV) and in the presence of 0.2 mM DDF, up to 60% of the α-toxin-binding sites become irreversibly inactivated (Table I). The kinetics of the DDF photoinactivation of the AcChoR by energy transfer (λ_{irr} = 290 nm) differs from the kinetics measured under direct (λ_{irr} = 435 nm) photoaffinity labeling conditions. The comparison was done under conditions such that an equal number of photons were absorbed by DDF at both wavelengths. The results of these experiments are shown in Figure 4B. Clearly, labeling by energy transfer takes place faster than that by direct illumination. For short irradiation times, where the intrinsic photodecomposition of DDF remained negligible, there was about a factor of 5 difference in the inactivation rates. As for direct photoaffinity labeling, *d*-tubocurarine protected the AcChoR against photoinactivation by energy transfer (Table I). Furthermore, the number of AcCho-binding sites inactivated was significantly larger when the inactivation was performed by energy transfer than by direct photoaffinity labeling. For instance, 0.2 mM DDF irreversibly blocked 50–60% of the [^{3}H]-α-toxin-binding sites when activated by energy transfer compared to 15–20% by direct photoaffinity labeling. Thus, an energy transfer induced photodecomposition of DDF bound to the agonist-binding sites takes place.

Incorporation of [^{3}H]DDF into the AcChoR. [*methyl*-^{3}H]DDF (0.28 Ci/mmol) was synthesized to characterize the polypeptide chains that reacted during the photolabeling process and to measure the number of DDF molecules incorporated per α-toxin-binding site. The distribution of radioactivity in the different polypeptide chains was analyzed

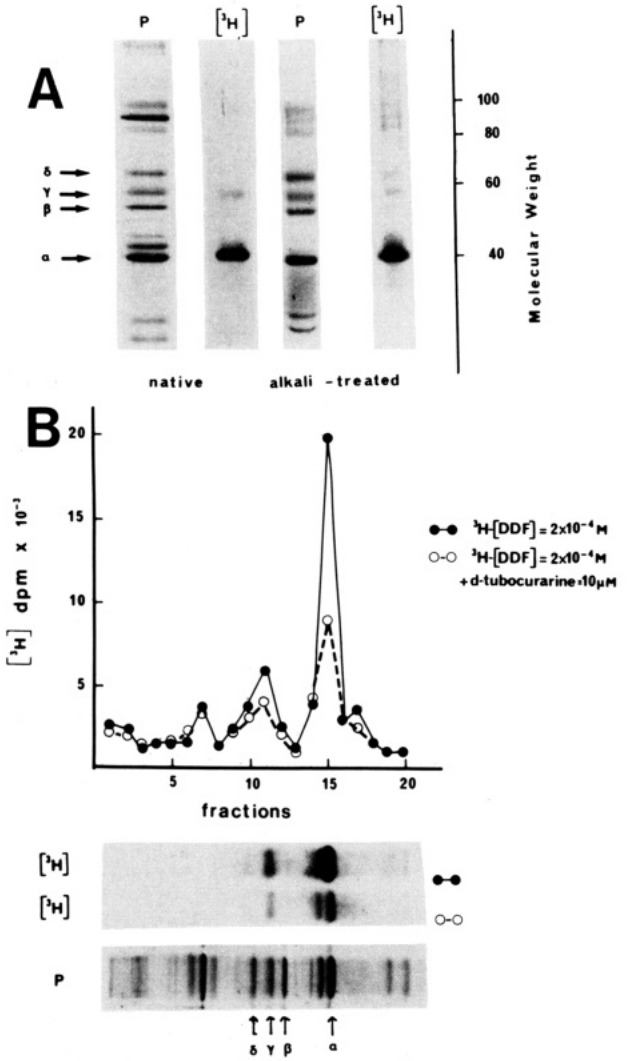

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of AcChoR-rich membrane fragments labeled with [^{3}H]DDF. (A) AcChoR-rich membrane fragments from *T. marmorata* electric organ (1400–1800 nmol of [^{3}H]-α-toxin-binding sites/g of protein for native and alkali-treated membranes, respectively) were diluted to a final concentration of 1.2 μM of α-toxin-binding sites and treated with 0.2 mM [^{3}H]DDF (280 mCi/mmol). After 30 min of irradiation at 290-nm wavelength and 60-μV energy, the samples were dialyzed and submitted to electrophoresis according to the procedure described under Methods. Proteins (P) were stained with Coomassie blue. Radioactivity (^{3}H) was revealed by fluoroautoradiography. (B) Same experiment as in Figure 3A with native AcChoR-rich membranes (1400 nmol of [^{3}H]-α-toxin-binding sites/g of protein). (Upper) Quantification of radioactivity recovered from the $NaDodSO_4$–polyacrylamide gel slices (cf. Methods): (●) membrane preparation labeled by 2×10^{-4} M [^{3}H]DDF and (○) same experiment in the presence of 10μM *d*-tubocurarine. (Lower) Corresponding Coomassie blue staining (P) and fluoroautoradiography (^{3}H): (●) [^{3}H]DDF 2×10^{-4} M and (○) [^{3}H]DDF 2×10^{-4} M *d*-tubocurarine 10 μM. The positions of the AcChoR subunits are indicated by arrows.

after $NaDodSO_4$ polyacrylamide gel electrophoesis both by counting radioactivity in gel slices and by fluoroautoradiography.

A priori, [^{3}H]DDF is expected to covalently react with several categories of sites: (i) the agonist/antagonist binding sites; (ii) the binding site(s) for NCBs; and (iii) other "nonspecific" sites. Parts A and B of Figure 5 show the distribution of radioactivity after photodecomposition of [^{3}H]DDF by energy transfer in the presence of crude AcChoR-rich membrane fragments or of membranes alkali-treated to remove non-AcChoR peptides (Neubig et al., 1979). With crude membranes a heavy labeling of the α-chain and

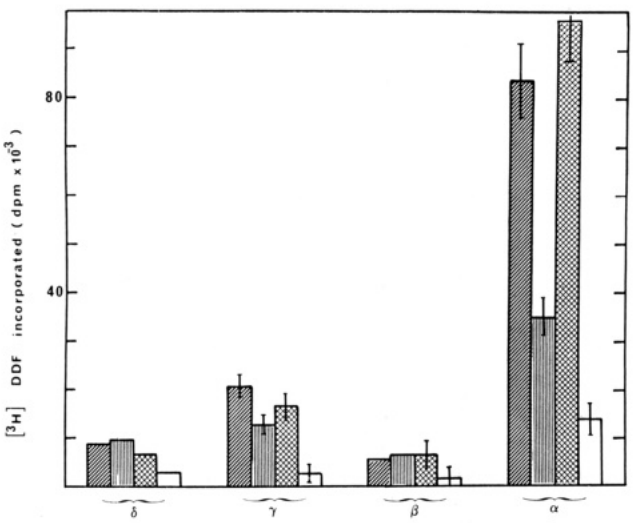

FIGURE 6: Effect of PCP and/or carbamoylcholine on [^{3}H]DDF incorporation into the different AcChoR subunits under energy-transfer conditions. Alkali-treated AcChoR-rich membranes from *T. marmorata* (7.4 μM α-toxin-binding sites) were incubated in phosphate buffer supplemented with 2×10^{-4} M [^{3}H]DDF in the absence or in the presence of PCP (3×10^{-5} M) and/or carbamoylcholine (10^{-4} M) and were submitted to energy-transfer photoaffinity labeling (λ = 290 nm; I = 60 μV; T = 10 °C). In the absence of agonist, 35 and 47%, respectively, of the α-toxin-binding sites were blocked in the absence and presence of PCP. 115 pmol of receptor were layered on the top of a $NaDodSO_4$–polyacrylamide gel. After electrophoresis, the bands corresponding to α-, β-, γ-, and δ-subunits were cut out of the gel and counted (cf. Methods). The four bars in each set represent, respectively, the following conditions: no further additions; 10^{-4} M carbamoylcholine; 3×10^{-5} M PCP; 10^{-4} M carbamoylcholine plus 3×10^{-5} M PCP.

a slight incorporation of radioactivity into the 43 000-Da band and at the level of the γ-chain were observed; preincubation with 10 μM *d*-tubocurarine significantly protected labeling at the level of the α- and γ-chains. With alkali-treated membranes, depleted of the 43 000- and 90 000-Da proteins, the radioactivity present at the level of the α- and γ-chains remained.

The specificity of the labeling of the AcChoR subunits by [^{3}H]DDF was further examined under equilibrium conditions in the presence of the agonist carbamoylcholine (10^{-4} M) and/or the noncompetitive blocker PCP (3×10^{-5} M). The results are shown in Figure 6. Following $NaDodSO_4$–polyacrylamide gel electrophoresis, at least 80% of the total radioactivity was associated with the four subunits of the AcChoR under all experimental conditions. Of the four chains, the α-subunit incorporated the largest amount of [^{3}H]DDF with the highest sensitivity to carbamoylcholine (and *d*-tubocurarine; not shown). The best yields of carbamoylcholine-sensitive incorporation of [^{3}H]DDF into the α-subunit occurred in the presence of PCP (80% versus 60% in its absence). The highest yield of inactivation of α-toxin-binding sites on the AcChoR also occurred in the presence of PCP. A minor, though significant, incorporation of [^{3}H]DDF took place at the level of the other AcChoR subunits. This labeling was particularly marked for the γ-chain, which showed significant carbamoylcholine sensitivity (approximately 80%) in the presence of PCP.

Finally, in the absence of light, a weak incorporation of radioactivity was still observed in the α-subunit. The amount of radioactivity incorporated was less than 15% of that observed in the presence of light. In addition, under direct photoaffinity labeling conditions (λ = 435 nm), [^{3}H]DDF became incorporated in the same polypeptide chains as under energy-transfer conditions: the α-subunit was predominantly

Table II: Relationship between [^{3}H]DDF Incorporation in the α-Chain and α-Toxin Binding Inactivation

	wavelength of irradiation	
	435 nm	290 nm
fraction of inactivated α-toxin sites (mean value)	0.21 ± 0.03	0.56 ± 0.08
total DDF incorporated per α-toxin-binding site (mean value)	0.78 ± 0.1	1.2 ± 0.1
total DDF incorporated per inactivated α-toxin-binding site (ratio 2:1)	3.7 ± 0.4	2.1 ± 0.5

[a] AcChoR-rich membranes from *T. marmorata* (1000–1500 nmol of [^{3}H]-α-toxin-binding sites/g of protein) were incubated at a final concentration of 1.2 μM of α-toxin binding sites with 0.2 mM [^{3}H]DDF (280 mCi/mmol) and irradiated either at 435 nm (40-μV incident energy) or at 290 nm (60-μV incident energy). After 30 min, 5 mM dithiothreitol was added to destroy the unreacted diazonium salt. For each sample, the remaining [^{3}H]-α-toxin-binding sites were measured on 100-fold-diluted aliquots, and the [^{3}H]DDF covalently incorporated into the α-chain was determined by counting the radioactivity found in the 40 000-Da polypeptide after $NaDodSO_4$–polyacrylamide gel electrophoresis.

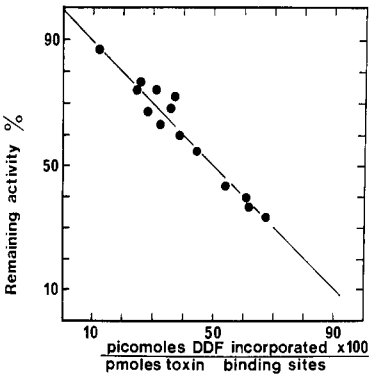

FIGURE 7: Relationship between [^{3}H]DDF specifically incorporated into the α-subunit and the loss of α-toxin-binding capacity under energy-transfer conditions. Energy-transfer photolysis experiments were carried out as described in Table II but for various times of irradiation. The [^{3}H]DDF specifically incorporated into the α-chain was determined by subtracting the amount of radioactivity recovered from the gel band corresponding to the α-chain (cf. Methods) in the presence of *d*-tubocurarine (2 μM) to that obtained in its absence.

labeled, and *d*-tubocurarine or α-bungarotoxin protected against this labeling (not shown).

Since the α-subunit is the main target of [^{3}H]DDF labeling, the incorporation of radioactivity into this subunit was quantitatively investigated under several experimental conditions. The effect of the irradiation wavelength on the labeling specificity is shown in Table II. In the absence of PCP, direct photoaffinity conditions yielded about 4 mol of DDF/mol of blocked α-toxin-binding sites, but this value dropped to 2 under energy-transfer conditions. This shows that under energy-transfer conditions [^{3}H]DDF attaches more selectively to the agonist-binding sites than under direct photoaffinity conditions. The amount of [^{3}H]DDF incorporated at the level of the agonist/antagonist-binding sites was evaluated by measuring the fraction of [^{3}H]DDF incorporated into the α-subunit in a *d*-tubocurarine-sensitive manner as a function of the number of inactivated α-toxin-binding sites (Figure 7). This comparison reveals a linear relationship that extrapolates to a stoichiometry close to unity. Approximately one molecule of [^{3}H]DDF incorporated per α-subunit in an agonist/competitive antagonist-sensitive manner is thus sufficient to completely block the binding of α-toxin to the AcChoR. The same stoichiometry was obtained when these experiments were performed in the presence of 3×10^{-4} M PCP, under conditions of both direct illumination and energy transfer.

Discussion

Progress in the localization of the primary binding sites for nicotinic agonists and competitive antagonists on *Torpedo* AcChoR has been achieved recently by several techniques. Kao et al. (1984) have identified Cys-192 and -193 as the amino acids of the α-subunit labeled by a maleimido derivative of phenyltrimethylammonium. Analysis of α-toxin binding to proteolytic fragments, synthetic peptides, or deletion mutants of the α-chain localized an α-toxin-binding segment in the region surrounding αCys-192/193 (Neumann et al., 1985, 1986a,b; Wilson et al., 1985; Barkas et al., 1987). On the other hand, site-directed mutagenesis experiments show that modification of Cys-128 or -142 abolishes α-bungarotoxin binding, while that of αCys-192 or -193 does not modify α-bungarotoxin binding but decreases the affinity for agonists (Mishina et al., 1985). In addition, a direct binding of snake venom α-toxin to the synthetic peptide α124–145 has been reported (McCormick & Atassi, 1984). To resolve these discrepancies, a probe that is able to alkylate the agonist/competitive antagonist-binding sites with a high yield had to be developed. Photoaffinity labeling of the AcChoR induced by energy transfer using DDF looked particularly attractive since (i) energy-transfer reaction occurs between AcChoR and some fluorescent ligands like quinacrine (Grünhagen & Changeux, 1976a,b) or C_6 dansylcholine (Heidmann & Changeux, 1979) and (ii) DDF is a competitive inhibitor of acetylcholinesterase and efficiently alkylates it under energy-transfer conditions (Goeldner & Hirth, 1980). With this last system, isolation and sequence determination of a small alkylated peptide showed that DDF can serve as an efficient label of tetraalkylammonium binding sites (Kieffer et al., 1986).

The results obtained from electrophysiological experiments are consistent with the view that, in the dark, DDF acts as a competitive antagonist of the AcChoR. On the isolated electroplaque of *E. electricus*, DDF competitively inhibits the response to carbamoylcholine with a K_{app} of 0.4 mM. Furthermore, the opening frequency for AcCho-activated channels of myotubes from the mouse cell line C2 is decreased by DDF in a manner that is consistent with a competitive inhibitory action.

In the dark, DDF binds reversibly to the α-toxin-binding sites of *T. marmorata* electric organ membranes (K_{app} 1.5 mM). Under irradiation ($\lambda > 320$ nm), we observe an irreversible interaction of DDF with *E. electricus* electroplaque and *T. marmorata* AcChoR-rich membranes that is prevented by *d*-tubocurarine. In vitro, the use of energy-transfer irradiation conditions, as opposed to direct photoaffinity labeling, leads to improved efficiency of irreversible binding (50–60% of the α-toxin-binding sites are irreversibly blocked by using a DDF concentration 8 times lower than its K_{app}) and to a higher signal/noise ratio (Table I). Under these conditions, total radioactivity covalently incorporated into AcChoR was predominantly associated with the α-chain and was markedly decreased by agonists and competitive antagonists.

DDF also reversibly interacts with the high-affinity NCB-binding site ($K_{app} = 2 \times 10^{-4}$ M) (Kotzyba-Hibert et al., 1985). This interaction could account for the decrease in mean open time of AcCho-activated channels in the presence of DDF in patch-clamp experiments with C2 myotubes, an effect similar to that described for several NCBs in other preparations (Changeux et al., 1986; Aguayo et al., 1986; Papke & Oswald, 1986). Interaction of DDF with the high-affinity NCB site becomes irreversible in the presence of light (Kotzyba-Hibert et al., 1985).

In the present study, addition of PCP to the incubation medium in order to prevent incorporation of DDF at the level of the high-affinity NCB-binding site led to an improved specificity of the agonist-binding sites labeling (Figure 6). Under these conditions, more than 90% of the carbamoylcholine-sensitive DDF incorporation was recovered at the level of the α- (and γ-) subunits and over 80% of the labeling of these subunits was abolished by carbamoylcholine. About one molecule of DDF was specifically incorporated (protected by agonists or competitive antagonists) into the α-chain per inactivated α-toxin-binding site whether PCP was present or not. In addition, the fact that up to 60% of the available α-toxin-binding sites could be specifically blocked (Figure 7) indicates that the two agonist-binding sites present on the receptor molecule are susceptible to [^{3}H]DDF inactivation. From these results it is concluded that the α-subunit is the major target of [^{3}H]DDF labeling. This is in agreement with several experiments that favored the localization of the AcCho-binding sites essentially at the level of the α-chains [reviews in Karlin (1983) and Changeux et al. (1984)]. In fact, most of the affinity labels of the agonist-binding site studied react with the α-subunit. Moreover, the α-subunit isolated after NaDodSO$_4$ gel electrophoresis can bind α-toxin with a relatively high affinity and good binding capacity (Tzartos & Changeux, 1984). In addition, the significant incorporation of DDF at the level of the γ-chain opens the possibility that this subunit is involved in AcCho binding.

In this paper we show that DDF behaves as a competitive antagonist of the AcChoR in the dark and as an efficient irreversible probe under energy-transfer conditions. Its charged moiety, which is also the photosensitive function, is probably involved in the interaction with the agonist-binding site (Mautner & Bartels, 1970). In addition, the aryl cation, photogenerated within the active site, is so reactive that it is expected to irreversibly bind to any accessible residue. The identification of the amino acids of the α-subunit photolabeled by DDF should thus provide structural information on the agonist/competitive antagonist binding site. Identification of these residues by microsequencing of purified α-chain fragments is described in the following publication (Dennis et al., 1988).

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Registry No. DDF, 24564-52-1; [*methyl*-^{3}H]DDF, 113010-20-1; AcCho, 51-84-3; HBF_4, 16872-11-0; [*methyl*-^{3}H]-*N*,*N*-dimethyl-*N*'-(butyloxycarbonyl)-*p*-phenylenediamine, 113010-18-7.

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