Aryldiazonium salts as photoaffinity labels of the nicotinic acetylcholine receptor PCP binding site

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Several aryldiazonium salts are described as irreversible blockers of the phencyclidine binding site of the nicotinic cholinergic receptor. A partial hydrophobic character increases the affinity of these salts for the phencyclidine binding site. Photoaffinity labelling with a tritiated diazonium salt in the presence of either carbamylcholine or α-bungarotoxin leads to incorporation of radioactivity into the 4 subunits of the receptor. Among these diazonium salts, an imidazole derivative is unique in that the photoinduced irreversible blocking in only effective when the receptor is in a desensitised state.

Aryldiazonium salt *Acetylcholine receptor* *Phencyclidine binding site* *Photoaffinity labelling* *Non-competitive blocker*

1. INTRODUCTION

The acetylcholine receptor of *Torpedo* electric organ is a pentameric complex composed of 4 different polypeptides with apparent M_r values of 40000 (α), 50000 (β), 60000 (γ) and 66000 (δ). Two α-subunits are present in each pentamer. At least 3 pharmacological sites can be distinguished in the acetylcholine receptor complex: (i) the acetylcholine binding site which complexes other agonists and competitive antagonists such as α-bungarotoxin and d-tubocurarin; (ii) the non-competitive binding site able to bind histrionicotoxin, phencyclidine and a large variety of molecules which share very little, if any, structural homology; (iii) the ionic channel which allows cation translocation through the membrane [1,2].

Whilst the involvement of the 40-kDa subunit in the acetylcholine binding site on the receptor complex is clearly demonstrated, localization of the non-competitive binding site using photoaffinity labelling experiments is more complex to investigate. In fact, the labelling pattern is dependent on the structure of the photosensitive ligand as well as the state (resting, opened or desensitized) and the source of the receptor [3,6]. Interpretation of experiments, attempting to localise the NCB binding site is difficult due to the long-lived photogenerated species and/or the poorly understood photochemical processes. Thus, the regioselectivity of labelling remains uncertain.

To overcome these difficulties, we synthesized aryldiazonium derivatives which are non-competitive blockers giving rise to highly reactive species upon irradiation (fig.1). These diazonium salts, chemically stable in the dark under physiological conditions [7,8], generate with irradiation one of the most powerful reagents yet known, the aryl cation [9]. The properties of some of these diazonium salts as photosensitive non-competitive blockers of the acetylcholine receptor are described here.

Abbreviations: carb, carbamylcholine; αBgtx, α-bungarotoxin; PCP, phencyclidine; NCB, non-competitive blocker; AcHR, acetylcholine receptor; DDF, *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate

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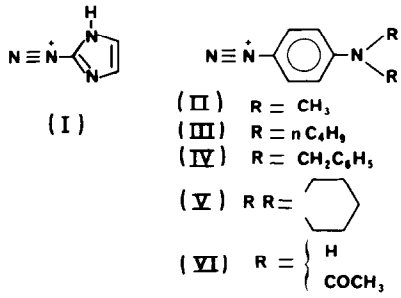
2. EXPERIMENTAL

Acetylcholine receptor rich membrane fragments from *Torpedo marmorata* were prepared as described in [10]. The concentration of acetylcholine binding sites was measured by equilibrium binding [11,12]. pH 11 treated membranes were obtained according to Neubig et al. [13].



All the diazonium salts were synthesized by diazotization of the corresponding arylamine with sodium nitrite (5% molar excess) in 34% (w/v) fluoroboric acid at −10°C and were recrystallized from acetone [14]. Irradiation experiments were done in phosphate buffer (sodium phosphate (pH 7.2), 10 mM; NaCl, 150 mM) at 10°C in 1 cm quartz cell. Reaction mixture contained a given concentration of diazonium salts and 500–800 nmol of ^{3}H-labelled α-toxin binding sites for an assay volume of 0.5 ml.

Monochromatic light was obtained from a 1000 W xenon mercury lamp connected to a grating monochromator. The light beam formed a spot 10 mm high and 2 mm wide. Light intensity was measured with a thermopile (Kipp and Zohnen).

Dissociation constants of the ligands for the agonist binding site were determined by measuring the decrease of the binding rate of the *Naja nigricollis* α-toxin to the receptor rich membrane fragments [15,16].

Dissociation constants of the ligands for the PCP binding site were obtained by competition with [^{3}H]PCP towards the receptor rich membrane fragments as described in [17]. Percentage of inactivation of the PCP binding site in the irreversible experiments was calculated from a reference sample (membranes and carb or/αBgtx) irradiated in

Table 1

Reversible binding characteristics of diazonium salts ((I)–(VI)) to *T. marmorata* receptor rich membranes measured in the dark

Ligand	log P_{exp}[a]	$K_{i_{app}}$ (M) (α-toxin)	K_i (PCP) (M)	
			+ carbamylcholine (10^{-4} M)	+ α-toxin
(I)	N.D.[b]	5×10^{-3}	$1.5 \pm 0.2 \times 10^{-3}$	$1.8 \pm 0.2 \times 10^{-3}$
(II)	−2.3	10^{-3}	$2.5 \pm 0.3 \times 10^{-4}$	$1.7 \pm 0.1 \times 10^{-4}$
(III)	−0.1	10^{-5}	$1.6 \pm 0.2 \times 10^{-6}$	$4 \pm 1 \times 10^{-6}$
(IV)	+0.3	7×10^{-5}	$5 \pm 1 \times 10^{-6}$	–
(V)	−1.4	10^{-4}	$0.7 \pm 0.1 \times 10^{-6}$	$0.8 \pm 0.1 \times 10^{-6}$
(VI)	−1.9	10^{-3}	$2.6 \pm 0.2 \times 10^{-5}$	$8 \pm 1 \times 10^{-5}$
PCP	+0.9	2.5×10^{-4} [5]	$1.5 \pm 0.1 \times 10^{-6}$	$2.6 \pm 0.3 \times 10^{-6}$

[a] Diazonium salts (2×10^{-4} M) are stirred for 30 min in a 1:1 mixture of octanol and buffer (phosphate, 150 mM; NaCl, 10 mM; pH 7.2) at 4°C. After separation of the two phases the partition coefficients P_{exp} are determined by UV measurements of the concentrations of the diazonium salts in the octanol layer

[b] Product (I) is not sufficiently soluble in octanol to get reliable results

The native membranes are preincubated either with αBgtx (2 molar excess for 2 h) or with carb (10^{-4} M for 10 min). After addition of the diazonium derivatives to the incubation medium the samples are irradiated as described in section 2

the same conditions. The number of PCP binding sites is proportional to the number of radiolabelled PCP molecules specifically bound to the membranes when incubated in the presence of a low concentration of PCP (1 nM).

3. RESULTS AND DISCUSSION

3.1. *Aryldiazonium salts as reversible ligands*

Table 1 summarizes the binding characteristics in the dark of two different classes of diazonium used: one derivative of imidazole (I) and several substituted aryldiazonium salts (II–VI) (fig.1). All these compounds are fairly stable in our incubation conditions in the absence of light ($t_{1/2} > 2$ h). They show affinity for the acetylcholine binding site as well as for the PCP high affinity binding site. In all cases, a higher affinity (10 times) is observed for the last site. Such a phenomenon has been noticed with other NCB [5,18].

All experiments were done on the PCP binding site in the presence of either αBgtx (native state of AcHR) or carb (desensitized state) to prevent binding of the diazonium salts to the acetylcholine binding site. There is no significant difference in the affinities of compounds I or II for the two states while compound III, the best ligand of this series, binds slightly more efficiently to the desensitized state. Identical observations have been made with PCP and several non-competitive blockers [5,19,20]. Affinity of the diazonium derivatives increases with their hydrophobicity. Two observations can be made: (i) product (I), which is positively charged and practically insoluble in octanol, interacts with the PCP binding site even though its affinity is low. (ii) Increasing the hydrophobicity of the ligand improves its affinity, but only to a certain extent since compound (IV) which has a higher partition coefficient than compound (III), has a lower affinity. Thus a positive charge and hydrophobicity are two important characteristics of compounds showing affinity for the NCB binding site. Many products such as tricyclic antidepressant [21], perhydrohistrionicotoxin [22,23], PCP [5,24,25], triphenylmethylphosphonium ions [4], local anesthetics [5,20] and our derivatives bind with a good affinity to the NCB binding site (10^{-7} M $< K_D < 10^{-5}$ M) while they have no clear structural homology. We interpret these results proposing the existence in the vicinity of the ionic channel of a hydrophobic NCB binding area much larger and less specific than a binding site.

3.2. *Aryldiazonium salts as irreversible blockers of the PCP binding site*

Table 2 shows the results of irreversible inactivation of the PCP binding site obtained after irradiation of the receptor rich membranes with different diazonium salts in the presence of either carb or

Table 2

Irreversible loss of PCP binding capacity of *T. marmorata* receptor rich membranes

Ligand	Concentration used (M)	Inactivation of PCP binding site in the presence of[a] (%)	
		Carbamylcholine (10^{-4} M)	α-Toxin
(I)	2×10^{-3}	40[b]	0
(II)	2×10^{-4}	40	40
(III)	2×10^{-6}	44[c]	34
(IV)	10^{-5}	20	8
(V)	10^{-4}	40	–

[a] General conditions used: $\lambda_{ex} = 290$ nm, $E = 1.32 \times 10^{-7}$ E·s^{-1}·cm^{-2}, $C = K_i$, $\theta = 4°C$, $t = 30$ min

[b] $\lambda_{ex} = 313$ nm

[c] $C = 1/2\ K_i$

αBgtx. In agreement with the high photosensitivity of the ligand and the high reactivity of the generated aryl cation (lifetime $< 10^{-8}$ s [27]), we can reasonably assume that by irradiation we obtain an instantaneous picture of the ligand-receptor complex in a structure similar to the original one. Thus variations in the efficiency of the inactivation of the receptor reflect the diazonium environment in the binding site, since the ligand can react essentially with the target protein or with water molecules. When irradiations are done under comparable conditions (λ_{exc} = 290 nm; ligand concentration = K_i) the desensitized state of the receptor is blocked to the same extent by compounds (II), (III) and (V). Compound (IV), which is more hydrophobic, is less efficient, suggesting that it binds to the PCP binding site in a different way.

Comparison of the amount of inactivation between the desensitized and the native states of the receptor by compounds (II), (III) and (IV) shows no drastic differences. Compound (I) blocks the desensitized state of the receptor very efficiently (λ_{exc} = 313 nm, 50% inactivation at K_i concentration). This result is remarkable since it is obtained in direct photoaffinity labelling conditions which are less efficient than the energy transfer conditions used for the diazonium (II) to (V) [8,26]. No inactivation is observed in the native state; this discrimination might be due to structural differences in the receptor-ligand complex. In the native state (I) reacts more easily with water than with the protein. This could imply either that the PCP binding site is more hydrated in the resting state or that the positioning of (I) in the binding site is different. Whatever the explanation, (I) is an efficient label to discriminate between native and desensitized states of the receptor.

3.3. *Polypeptides involved in the NCB binding area*

We have determined which chains of AcHR are labelled after irreversible binding of a photosensitive ligand. Despite the relatively low affinity of compound (II) (10^{-4} M), it has been chosen to perform preliminary experiments for the two following reasons: (i) it binds with about the same affinity to the resting and desensitized states of the receptor, and (ii) it blocks irreversibly very efficiently the PCP binding site in both states. The labelling experiments with radioactive compound (II) are shown in fig.2. In the presence of carbamylcholine the 4 subunits are labelled. Prior incubation with unlabelled PCP lowers the radioactivity incorporated to about 50% of the initial value. The same pattern of radioactivity is observed when the membranes are incubated in the presence of αBgtx (not shown).

From these results several interpretations are possible: (i) The binding site is located on one subunit and the light generated aryl cation diffuses out of the site and reacts randomly with residues of the surrounding subunits. As discussed previously, the high reactivity of this species makes this possibility unlikely. (ii) The different subunits define a large binding area allowing several orientations for ligand complexation: such a situation was described for cyclodextrin X-complexes [28].

Results obtained with aryl diazonium salts are promising since they are good NCB of AcHR in the dark and are able to block very efficiently the PCP binding site under irradiation. Studies with radioactive ligand (II) suggest that the 4 different subunits are involved in the PCP binding site. The exceptional behaviour of one of them (I) which discriminates between the native and the desen-

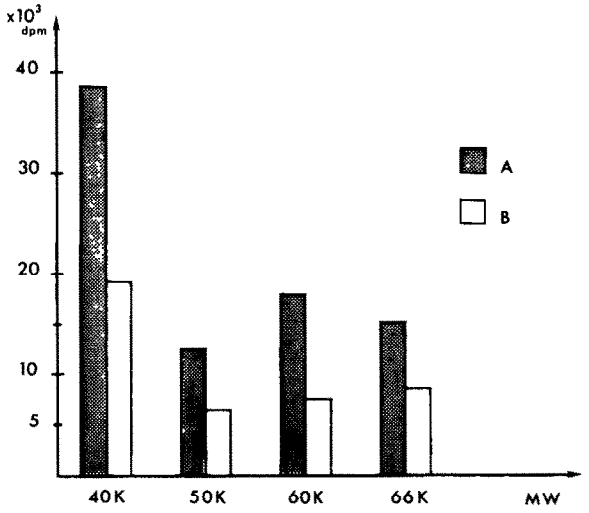


Fig.2. ^{3}H incorporation in the different subunits after irradiation of pH 11 treated *T. marmorata* receptor rich membranes in the presence of 10^{-4} M carb and 2×10^{-4} M [^{3}H]DDF. The radioactivity incorporations are determined without PCP (A) and in the presence of 3×10^{-5} M PCP (B).

sitized state of the receptor might be useful (especially as a radioactive derivative) for conformational prospecting of the NCB binding area. To our knowledge this is the first example of such a discrimination at the reactivity level.

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