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NEW PHOTOAFFINITY LABELS FOR RAT BRAIN MUSCARINIC ACETYLCHOLINE RECEPTORS

BRIGITTE ILIEN,* ANNICK MEJEAN and CHRISTIAN HIRTH
Laboratoire de Chimie Bio-Organique (URA 1386 du C.N.R.S.), Université L. Pasteur, Faculté de Pharmacie, BP 24-67401 Illkirch Cedex, France

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Abstract—Localization of the ligand binding site on muscarinic acetylcholine receptors is one of the new fields of interest opened by the recent determination of their primary structures. Owing to their interesting photochemical properties, aryldiazonium salts may be considered as appropriate tools for "tagging" the agonist/antagonist binding domain and to get precise identification and positioning of covalently labelled residues along the primary sequence of these receptors. A series of aryldiazonium derivatives and some of their azido-analogs were synthesized and their reversible muscarinic binding component was assessed through competition experiments involving either the whole population of receptor sites ([^{3}H]QNB assay) or the super high affinity of their agonist binding sites ([^{3}H]OXO-M assay). Three compounds fulfilled the criteria for efficient photolabels, allowing substantial and irreversible occupation of the receptor sites to be obtained. Interestingly, the two diazonium derivatives which were selected have been previously described as potent photoprobes of the peripheral nicotinic receptor and of acetylcholinesterase, though displaying lower binding affinities for these acetylcholine binding proteins than for the muscarinic receptors. These findings, together with the all-to-none photolabelling efficiency observed for a quinuclidine derivative, substituted either by an azido or a diazonium group, are discussed. Finally, the apparent lack of binding selectivity of these new photoaffinity probes towards muscarinic receptor affinity states or subtypes should allow comparative studies of the acetylcholine binding site on different muscarinic receptor proteins, obtained either through purification procedures or expression of separate gene products.

Muscarinic acetylcholine receptors belong to a class of integral membrane glycoproteins in which signal transduction is mediated by guanine nucleotide binding regulatory proteins [1, 2].

Purification studies [3, 4] followed by sophisticated cloning strategies have pointed to the existence of five distinct and functional muscarinic receptor genes products [5–11]. Their secondary and tertiary structures have now to be elucidated as well as their binding domains for ligands or G-proteins and their mechanisms of signal transduction.

One approach to localize the ligand binding site on muscarinic receptors is to use irreversible site-specific labelling, peptide mapping and sequencing techniques with complementary informations provided by receptor mutagenesis.

Crosslinking reagents, affinity and photoaffinity probes display in common the ability to form covalent bonds within their binding sites, thus offering a mean of "tagging" the receptor macromolecule. Indeed, several affinity (nitrogen mustard derivatives, Refs 12–16) and photoaffinity (azido-derivatives, Refs 17–19) probes have been previously synthesized and used mainly in order to detect muscarinic receptors at various stages of their purification [3, 4].

However, a site-specific labelling, representative of the agonist/antagonist binding domain, requires appropriate probes able at once to react very rapidly and undiscriminatively with their direct amino-acid environment. Under such conditions, more complete identification and precise positioning of covalently labelled residues (belonging to the ligand binding domain) along the primary structure of the receptor protein may be achieved.

Affinity labels are relatively stable species which react preferentially with some nucleophilic residues [20]. Thus, site-specific labelling by using such tools is not expected to be satisfactory unless high affinity probes are assayed on purified target proteins whose ligand binding site contains the appropriately oriented residues [20].

The latter requirements were met with propylbenzilylcholine mustard, whose alkylation site on purified receptor proteins was identified as a buried aspartate residue, presumably located within the second transmembrane segment of the M1-muscarinic receptor [21].

Photoaffinity ligands present several advantages over affinity ones in being reversibly bound, in the dark, and in generating, upon light irradiation, highly reactive species. Irradiation of azides produces nitrenes [20, 22, 23] whose reactivity (life time 10^{-3} to 10^{-8} sec; Refs 20, 23) is however shaded by the diversity in the reactions they may undergo [20, 22].

Photoactivation of aryldiazonium derivatives [24–26] yields extremely unstable arylcations (life time $<10^{-9}$ sec; Ref. 27) which do not undergo any rearrangement reactions and display an undiscriminative chemical reactivity with solvents, C—H bonds and nucleophilic groups [28–31]. In that sense, aryldiazonium salts may be regarded as tools of choice to get a productive site-specific labelling and some topographical insight of the ligand binding site, including its hydrophobic domain.

* To whom all correspondence should be addressed.

The particular features of arylcations, combined to the interesting identity between the positively-charged nitrogen moiety (present in essentially all muscarinic ligands) and the photosensitive group beared by diazonium salts, led us to design new photoactivatable probes and to test their ability to irreversibly label muscarinic receptor sites from rat brain.

MATERIALS AND METHODS

Drugs and chemicals

Atropine sulfate, carbamoylcholine (carbachol) hydrochloride and pilocarpine hydrochloride were purchased from Sigma Chemical Co. (Poole, U.K.). Pirenzepine dihydrochloride was a gift from Boehringer-Ingelheim (Ingelheim, F.R.G.). *p*-Amino-dexetimide was kindly provided by Dr J. E. Leysen (Janssen Pharmaceutica, Belgium).

The ^{3}H-labelled compounds, quinuclidinyl benzilate (QNB, 43.6 Ci/mmole) and Oxotremorine-M acetate (OXO-M, 87 Ci/mmole) were from New England Nuclear (Boston, MA). All common reagents were obtained from different suppliers and were of the highest purity available.

Synthesis and characterization of photoactivatable compounds

N-*phenyl*,N-(4-*nitrophenyl*) *propylamide* ***b***. 26 mmoles of NaH were added to a solution of 4-nitrophenyl, phenylamine **a** (4.19 g, 20 mmoles) in 100 ml of anhydrous tetrahydrofuran. The reaction mixture was refluxed for 30 min at 80°, then cooled to 0°, and propionyl chloride (2.77 g, 30 mmol) was added dropwise and allowed to stand for further 30 min. The solvent was removed *in vacuo*, the residue dissolved in ethylacetate was washed (water, NaCl-saturated water) and dried over Na_2SO_4. After removal of the solvent, the crude product ***b*** was purified by silica gel chromatography and obtained as a yellow oil (4.08 g, 75%).

N-*phenyl*, N-(*oxo*-1-*propyl*), N′-*t*-*butyl*-*oxycarbonyl*, p-*phenylenediamine* ***c***. A solution of *N*-phenyl, *N*-(4-nitrophenyl) propionamide (3.65 g, 13. 5 mmoles) in methanol containing 500 mg of 10% Pd/C was submitted to catalytic hydrogenation (1 atm) for 2 hr. Di-*t*-butyldicarbonate (5.94 g, 27 mmoles) was added and the mixture was stirred under nitrogen atmosphere for 4 hr. After removal of the catalyst by filtration and evaporation of the solvent, the crude product **c** was purified by silica gel chromatography. A white compound (1.1 g, 31%), which was recrystallized from ethylacetate/hexane (m.p. = 191°), was taken as the precursor of compound **5** (Scheme 1).

N-*phenyl*,N-(4-*nitrophenyl*) *carbamoyl chloride* ***d***. This was synthesized according to the above procedure using phosgene (5 equivalents) instead of propionyl chloride (yield: 80%, m.p. = 118°).

N-*phenyl*,N-(4-*nitrophenyl*)-3-*quinuclidinyl carbamate* ***e***. This was prepared as previously described [32], from *N*-phenyl,*N*-(4-nitrophenyl) carbamoyl chloride and 3-quinuclidinol (yield: 20%).

N-*phenyl*,N-(4-*aminophenyl*)-3-*quinuclidinyl carbamate* ***f***. This was obtained through reduction of the former carbamate with sulfurated sodium borohydride [33] and taken as the common precursor (yield: 70%) of compounds **6** and **10** (Scheme 2).

Aryldiazonium derivatives. In a typical experiment, 2 μmoles of protected amine were dissolved in 200 μl of a 1:1 mixture of trifluoroacetic acid (TFA) and concentrated HCl. After a 30 min stirring at room temperature, the solution was cooled to −20° and 2.2 μmoles of $NaNO_2$ in water were added over a period of 30 min. Formation of the diazonium salt was monitored by UV spectroscopy. After evaporation of the acids under vacuum, the residue was resuspended in 1 ml of water and analysed by reverse-phase HPLC (Waters μ-Bondapack C18-column, 3.9 × 300 mm, gradient conditions: 100% H_2O, 0.05% TFA to 100% CH_3CN in 40 min at a flow rate of 2 ml per min).

The diazonium nature of the major peak (85%), monitored at 229 nm, was verified in terms of UV spectroscopic properties and photosensitivity.

We checked, using the crystalline compounds **1** and **2** as models, that our diazotization and chromatographic procedures gave satisfactory results in terms of purity and yields. Molar extinction coefficients were calculated assuming that the conversion of the aromatic amine into the corresponding aryldiazonium salt was complete.

Compounds **1** and **2** were obtained through standard procedures [34]. Quaternary ammonium derivatives **3** and **4** as well as compound **7** were generous gifts of B. Chatrenet and L. Sabatier. Unprotected *p*-amino dexetimide was taken as the direct precursor of compound **8**. Synthetic steps for compounds **5** and **6** are depicted in Schemes 1 and 2.

Azido derivatives. To a 10^{-2} M basic solution (pH 9) of the corresponding diazonium salt, 10 equivalents of NaN_3 were added at room temperature. After stirring for 30 min, the solution was extracted with ethylacetate. The organic phase was washed with water, dried over Na_2SO_4 and evaporation *in vacuo* with mild external heating. The solid product thus obtained was crystallized from ethylacetate.

All chemical structures were confirmed by ^{1}H NMR, MS and i.r. (azido derivatives) analyses. Special care was taken to avoid light exposure during the diazotization and azidation steps.

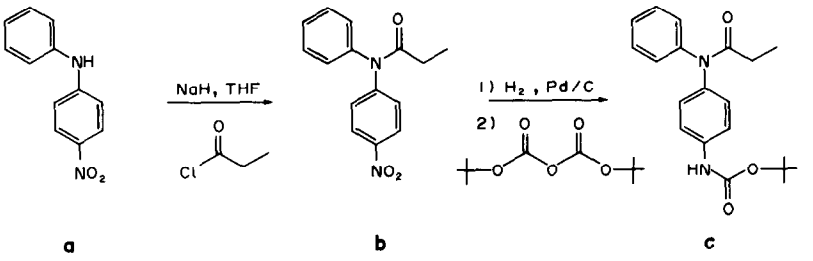
Membrane preparation

Crude rat brain membranes were prepared as described previously in detail [35].

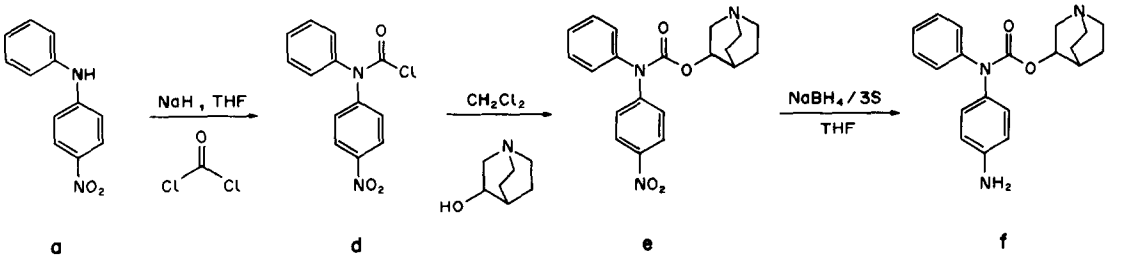
Binding assays

[^{3}H]QNB and [^{3}H]OXO-M binding were carried out in 2 ml (final volume) of 50 mM Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) containing either 0.1 nM [^{3}H]QNB or 0.6 nM [^{3}H]OXO-M, various concentrations of unlabelled drugs and appropriate amounts of rat brain membrane preparation (25 and 145 μg protein for [^{3}H]QNB and [^{3}H]OXO-M binding assays, respectively). Incubation was performed at 37° for 60 min ([^{3}H]QNB) or at 25° for 30 min ([^{3}H]OXO-M) and stopped by rapid vacuum filtration through Whatman glass fibre filters ([^{3}H]QNB: GF/B filters; [^{3}H]OXO-M: GF/C filters presoaked in 0.05% polyethylenimine in water). Filters were rinsed three times with 3 ml-aliquots of ice-cold phosphate buffer and transferred to vials

Scheme 1



Scheme 2



containing 7 ml of Biofluor™ (New England Nuclear). After vigorous shaking, vials were allowed to stand for at least 8 hr and counted for radioactivity with an efficiency of 48%.

Specific binding was defined as the portion of the total radioligand binding which was inhibited by 10^{-6} M atropine. It represented 95% and 85% of the total [^{3}H]QNB and [^{3}H]OXO-M binding, respectively.

Under both assay conditions, the steady state for specific binding was verified and the specifically bound radioligand was found to vary linearly with membrane amounts up to 60 μg ([^{3}H]QNB) or 190 μg ([^{3}H]OXO-M) protein per assay.

Photolabelling 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) and adjusted through an iris diaphragm placed between the light source and the monochromator. To note that the response of the thermopile varied independently of the wavelength (between 260 and 500 nm) of the incident light which was selected.

Aliquots of membrane preparation (0.45 mg protein/ml) in 50 mM phosphate buffer (pH 7.4) were incubated for 20 min at 25° with the indicated concentrations of photoactivatable compounds, with or without previous addition of 5×10^{-7} M atropine.

Three millilitres of incubation mixture, precooled at 4°, were then introduced in a quartz cell (1 cm-path length) and irradiated for 15 min at 5°, under gentle stirring, at the desired wavelength and intensity of the incident light beam. The latter formed a 10 mm high and 1.5 mm wide spot. After irradiation, the dissociation procedure started with a 9-fold dilution of the incubation mixture with phosphate buffer, followed by a 10-min incubation step at 25°. After centrifugation at 100,000 *g* for 15 min at 20°, the pellet was homogenized in the same buffer, incubated at 25° for 10 min and sedimented again by centrifugation. The final pellet was suspended in phosphate buffer at appropriate dilutions (μg protein/assay) for [^{3}H]QNB (15 μg) and [^{3}H]OXO-M (90 μg) binding assays.

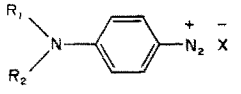
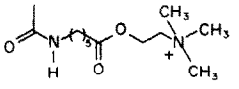
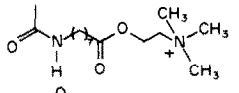
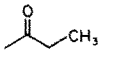
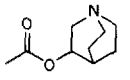
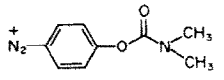
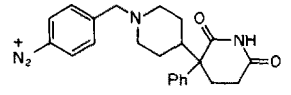
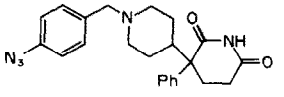
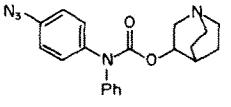
Specific [^{3}H]QNB and [^{3}H]OXO-M binding represented still 90 and 80%, respectively, of the total radioligand binding.

Analysis of binding data

Saturation experiments allowed K_d and B_{max} values to be estimated for specific [^{3}H]QNB (K_d = 16 pM; B_{max} = 1.86 pmoles/mg protein) and [^{3}H]OXO-M binding (K_d = 0.66 nM; B_{max} = 0.19 pmoles/mg protein).

Displacement curves were analysed by non linear least-squares regression. An iterative program originally described by McPherson [36] yielded binding isotherms of the competing ligand for the whole population of muscarinic receptor sites in terms of

Table 1. Structure, spectral properties and chemical stability of aryldiazonium salts and azido derivatives

Compounds			Spectral properties		Stability
			Maximal absorbance wavelength (nm)	Molar extinction coefficient (/M/cm)	Half-life (hr)
Aryldiazonium derivatives					
					
R_1	R_2				
$-CH_3$	$-CH_3$	1	379	37500	16
$-(CH_2)_3-CH_3$	$-(CH_2)_3-CH_3$	2	384	28600	120
$-CH_3$		3	363	23400	-
$-CH_3$		4	363	25700	-
Ph		5	349	16000	5
Ph		6	342	17000	91
		7	286	15000	20
		8	270	8700	1.2
Azido derivatives					
		9	254	10500	>12
		10	259	12200	>12

The chemical stability of these various compounds was estimated in the dark, in Na–K phosphate buffer (pH 7.4) at 10°.

IC_{50} values and slope factors. K_i values of drugs were determined from IC_{50} values using the method of Cheng and Prusoff [37], assuming competitive interactions.

Protein concentrations were determined according to Spector [38], using bovine γ-globulin as standard.

RESULTS

The different aromatic diazonium salts we obtained and some of their related azido-derivatives are presented in Table 1.

Compounds **1** to **6** were synthesized according to the *p-N,N* dialkylbenzene diazonium model, substituted in the *para* position by nitrogen as an electron donating group. These aryldiazonium salts were disubstituted by methyl (**1**) and butyl (**2**) groups or by longer aliphatic side-chains having a quaternary ammonium headgroup (**3** and **4**).

Compounds **5** and **6** displayed close chemical structures, the ethyl group in **5** being replaced by an ester of quinuclidine in **6**. All these compounds showed a major absorption band at wavelengths above 340 nm and were markedly stable in phosphate buffer (pH 7.4).

Aryldiazonium salt **7** was designed as an aromatic derivative of carbamoylcholine with a diazonium group as polar head. Compound **8** directly derived from dexetimide, a well-known muscarinic antagonist. The decreased maximal absorbance wavelengths of these two diazonium salts may be due to

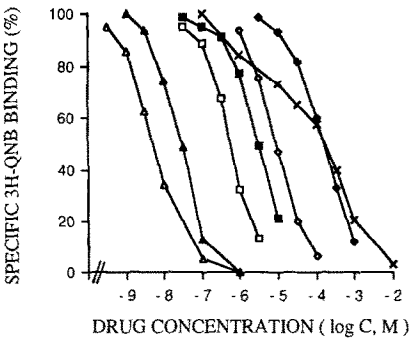


Fig. 1. Competition of photoactivatable compounds and of classical muscarinic drugs for specific [^{3}H]QNB binding in rat brain membranes. Assays were carried out with 0.1 nM [^{3}H]QNB and increasing concentrations of atropine (△), carbachol (×) and of the photoactivatable compounds **1** (◆), **2** (◇), **6** (□), **9** (▲) and **10** (■), using standard binding conditions as described under Materials and Methods.

the lower electron-donating ability (**7**) or to the absence (**8**) of *para*-donating groups, known for their stabilizing properties of the diazonium function [30]. The poor chemical stability of **8** precluded its use in binding experiments.

Two azido derivatives were synthesized for different reasons. Their easy feasibility, when starting from pure aryldiazonium precursors, enabled us to obtain compound **9** which displayed, as expected, a much higher stability in buffered solution than its diazonium congener. The azido homologue **10** of the aryldiazonium quinuclidine derivative **6** was chosen for comparative photolabelling studies and for its analogy with a previously described photoactivatable molecule, *p*-azido-3-QNB [18].

Since all but one of these derivatives displayed a very good stability in Na–K phosphate buffer (pH 7.4), radioligand binding assays using [^{3}H]QNB and [^{3}H]OXO-M have been performed in order to determine their apparent binding affinities for muscarinic receptors from rat brain.

Competition experiments have been performed under equilibrium conditions as shown in Fig. 1. A series of compounds including all photoactivatable derivatives and atropine displayed steep and parallel displacement curves while the agonist carbachol showed a marked biphasic behaviour, when competing with [^{3}H]QNB binding.

Apparent binding affinity constants of the compounds (listed according to decreasing binding affinity for specific [^{3}H]QNB binding sites) are summarized in Table 2.

p-Azido-dexetimide **9** retained the nanomolar binding affinity of dexetimide [39] while the two short diazonium models **1** and **2** were active in the micromolar concentration range. Most photoactivatable derivatives displayed higher binding affinities (QNB/OXO-M ratios less than 1) when tested towards the whole population of brain receptor sites ([^{3}H]QNB binding). An exception was compound **1** and to a lesser extent the derivatives **2** and **10** which were better displacers of [^{3}H]OXO-M binding to super-high agonist affinity state of the receptors. Compounds **5** and **6** displayed similar binding properties though a quinuclidine group was introduced in the latter. To note that replacement of the diazonium group in **6** by an azido function in **10** selectively decreased its affinity for specific [^{3}H]QNB binding sites.

The QNB/OXO-M binding affinity constant ratios for all these derivatives were close to 1 and argued in favour of their antagonistic nature [40]. Moreover, non linear regression analysis of displacement curves provided slope factors close to unity, suggesting the absence of binding selectivity of these photosensitive compounds towards muscarinic receptor subtypes.

The photosensitivity of aryldiazonium and arylazido compounds was examined by sequential irradiation at 290 nm (Fig. 2).

This wavelength was chosen as it represented a mean-value where all our compounds displayed a significant absorption. The diazonium salt **6** showed, in UV spectroscopy, a clean photodecomposition reaction pattern with isobestic points (Fig. 2A), while the one of its azido congener **10** was more complex. The typical absorption band of **10** at 259 nm was less affected upon photolysis but a shoulder peak progressively appeared within 10 min of irradiation (Fig. 2B) and became readily apparent and stable after 90 min of irradiation (Fig. 2C). Such a complicated photodecomposition pattern was not encountered for *p*-azido-dexetimide (**9**) and precluded any reliable determination of the half-life for photolysis of **10** to be done. Photodecomposition rates at 290 nm for all other compounds are listed in Table 3.

Since all our derivatives were inert in solution until they were irradiated, photolabelling experiments, using rat brain membranes, were initiated. Preliminary controls had first to be done in order to test the stability of the biological material throughout the whole procedure (Fig. 3).

Specific [^{3}H]QNB and [^{3}H]OXO-M binding were assayed following a twice-repeated centrifugation step or a 15-min irradiation period at 290 nm with a light intensity of 30 μV. Each separate treatment diminished specific binding by 10% while their combination induced a loss of about 25% of both [^{3}H]QNB and [^{3}H]OXO-M binding sites. Atropine was used as a protector to mimic receptor occupation by a photoactivatable drug. This high affinity muscarinic antagonist was completely washed away from specific [^{3}H]QNB sites, to a lesser extent from [^{3}H]OXO-M binding sites, but its presence did not improve the receptor recovery.

Since our irradiation and dissociation procedures were not destructive for the receptor sites, we decided to investigate the light-dependency of a possible irreversible occupation of muscarinic receptor sites by our photosensitive derivatives (Table 3). Drug concentrations were selected as those allowing at once to occupy, during photolysis, a great fraction of receptor sites and to free them entirely when the irradiation step was omitted. Such was the case (unirradiated samples) for most of our derivatives except for the diazonium salts **4** and **7** which dissociated less readily from [^{3}H]OXO-M binding sites.

In contrast to this, compounds **1**, **2** and **10** pre-

Table 2. Apparent binding affinity constants of drugs for specific [^{3}H]QNB and [^{3}H]OXO-M binding sites

Compounds	Apparent binding affinity constants (K_i, nM) [^{3}H]QNB binding		[^{3}H]OXO-M binding		Ratios
9	3.6	(1.27)	8.6	(1.10)	0.42
5	73	(0.97)	319	(1.35)	0.23
6	78	(1.07)	343	(1.13)	0.23
4	116	(1.00)	341	(1.28)	0.34
7	366	(0.92)	1030	(1.86)	0.35
10	367	(0.99)	258	(0.89)	1.42
3	487	(0.87)	550	(1.07)	0.88
2	1300	(1.17)	1010	(1.10)	1.29
1	29800	(0.93)	8300	(0.94)	3.59
Atropine	0.7	(1.02)	0.8	(0.91)	0.87
Pirenzepine	28	(0.83)	47	(0.55)	0.60
Pilocarpine	2400	(0.84)	—	—	—
Carbachol	14800	(0.50)	9.5	(0.81)	1558.00

K_i values of drugs derived from competition experiments as reported under Materials and Methods. Slope factors of binding isotherms are indicated under parentheses. The QNB/OXO-M ratio is the ratio of the two corresponding apparent binding affinity constants.

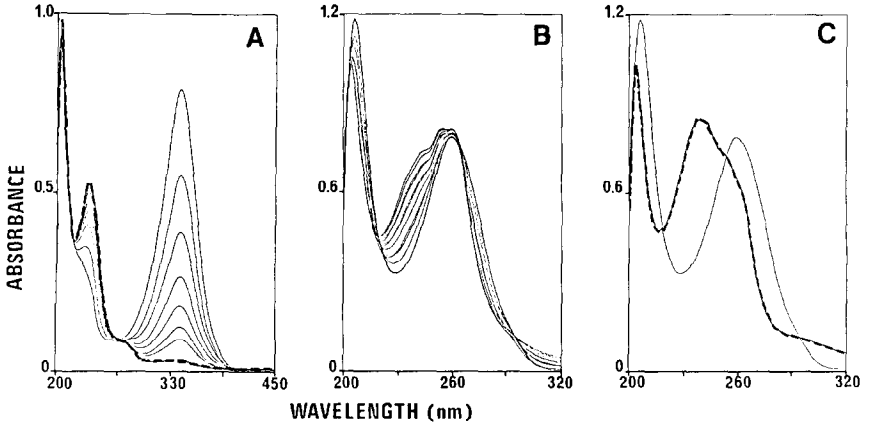


Fig. 2. Effect of UV irradiation on the absorption spectra of compounds **6** (A) and **10** (B, C). Na–K phosphate-buffered (pH 7.4) solutions of the aryldiazonium **6** (5×10^{-5} M) and azido **10** (10^{-4} M) derivatives were sequentially exposed to UV light (wavelength: 290 nm; incident light energy: 30 μV). Samples were scanned for spectral absorption after each irradiation period (A: 10 min; B: 1 min; C: 90 min). Dotted lines followed absorption spectra of the compounds after completion of their photolysis.

sented a marked light-dependency of their receptor site occupation which resisted the dissociation step. The diazonium model **1** was the most effective probe (about 60% of the binding sites were photoinactivated) while the bisbutyl-diazonium **2** and azido **10** derivatives labelled half of the receptor sites population. To note that the diazonium analog **6** of this efficient azido photolabel was completely inactive. The three potent ligands presented a similar photolabelling efficacy at specific [^{3}H]QNB or [^{3}H]OXO-M binding sites which was entirely abolished by 5×10^{-7} M atropine.

Finally, three other photoactivatable diazonium derivatives **4**, **5**, and **7** gave low inactivation yields which seemed to be restricted to specific [^{3}H]QNB binding sites.

DISCUSSION

The recent determination of the primary sequences of different muscarinic acetylcholine receptor proteins [5–11] opens a wide field of investigation where the location of the ligand binding site, its topography and conformation take an important place.

Appropriate radiolabelled probes allowing to alkylate amino-acid residues located exclusively within the receptor binding domain are thus required. In fact, the site-specificity of such an irreversible receptor probing depends mainly upon two factors: the purified state of the receptor protein allowing to minimize the alkylation of non-receptor proteins and the high reactivity of the probe hinder-

Table 3. Irreversible interaction of various photoactivatable derivatives with membrane-bound muscarinic receptors

Compounds	Photolysis half-life (min)	Drug concentration M	Receptor photolabelling [^{3}H]QNB binding (Recovery in specific binding, % of control) Unirradiated	Irradiated	[^{3}H]OXO-M binding (Recovery in specific binding, % of control) Unirradiated	Irradiated
1	52	3×10^{-5}	104	37 (105)	107	39 (78)
2	55	8×10^{-6}	104	53 (105)	104	46 (90)
4*	15	2×10^{-6}	98	86	64	67
5	25	1×10^{-6}	93	77	97	94
6	20	1×10^{-6}	102	101	103	105
7	12	4×10^{-6}	100	91	82	110
9	44	1×10^{-7}	98	100	100	93
10	—	3×10^{-6}	107	52 (96)	99	46 (83)
Atropine	—	5×10^{-7}	97	— (99)	87	— (82)

All irradiation steps were performed at 5° using shortwave UV light (wavelength: 290 nm; light intensity: 30 μV). Half-lives for photodecomposition of the compounds were estimated in the absence of membranes as shown in Fig. 2.

For receptor photolabelling experiments, membranes were first incubated in the presence of the indicated concentration of drugs, then submitted or not (unirradiated) to a 15 min-irradiation period, and finally washed twice by centrifugation. Muscarinic specificity for the photolabelling by compounds **1**, **2** and **10** was controlled by carrying out similar experiments in the presence of 5×10^{-7} M atropine (values under parentheses). Experimental conditions are detailed under Materials and Methods. Results are expressed as percentages of similarly-treated samples where no drugs were added.

* Irradiation using compound **4** was performed at 330 nm (100 μV) and the receptor sites markedly resisted to such irradiation conditions.

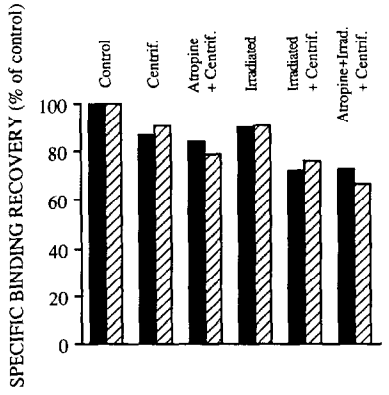


Fig. 3. Influence of membrane treatments on specific [^{3}H]QNB (■) or [^{3}H]OXO-M (▨) binding recovery. Membrane aliquots (0.45 mg prot./ml), preincubated or not with 5×10^{-7} M atropine, were successively submitted, when indicated, to UV irradiation (wavelength: 290 nm light intensity: 30 μV) for 15 min at 5° and to a twice-repeated dilution–centrifugation step. Thereafter, samples were assayed for specific [^{3}H]QNB or [^{3}H]OXO-M binding. All experimental procedures followed those described in detail under Materials and Methods. Results are expressed as percentage values of the untreated membrane control.

ing diffusion-mediated labelling of non specific residues [20, 22]. Though always preferable, the high binding affinity criterion is certainly required for poorly reactive ligands such as affinity labels, this being much less critical when using highly reactive species like those generated upon irradiation by photoactivatable probes [20]. This is particularly true when a photosuicide process [41, 42], involving an energy-transfer mediated decomposition of the probe by prealably photoexcited residues of the binding site, occurs.

Ideally, the photolabelling procedure allows to "freeze", through irradiation, the reversible association between the photoactivatable probe and its binding site into a covalent complex. Among the available photosensitive functions, diazonium salts were selected for a number of favourable properties. Indeed, they are expected to provide a reliable site-specific labelling (unmatched high reactivity and rapid inactivation by surrounding water molecules, Refs 24–27) as well as topographical informations about the ligand binding site (undiscriminative high reactivity with all kind of residues, Refs 28–31). Moreover, it should be noted too that they are synthetic intermediates for azido-derivatives and present a positively-charged and photoactivatable nitrogen moiety.

A series of diazonium derivatives and two azido analogs were tested for their ability to irreversibly occupy membrane-bound specific [^{3}H]QNB and [^{3}H]OXO-M binding sites from rat brain. Three compounds (**1**, **2** and **10**) displayed the typical properties for efficient photoprobes: stability in buffered solution in the dark; high photosensitivity; no irreversible occupation of receptor sites unless irradiation proceeds; and highly productive photolabelling, under non destructive conditions for the biological material, reaching 50 to 60% of inactivation of both antagonist and agonist muscarinic binding sites. Moreover, their labelling was completely prevented by atropine (5×10^{-7} M), a way of assessing its muscarinic receptor specificity.

The azido derivative **10** was a potent photoaffinity probe while its diazonium analog **6** was inactive. Such a great difference in photoaffinity labelling productivity presumably reflects an unfavourable spatial arrangement of the diazonium probe within

the binding site, leading to inactivation of arylcations by surrounding water molecules.

Conversely, the higher water stability of nitrenes should allow the photoproduct of **10** to diffuse to and react with nearby amino-acids. Both compounds showed close binding affinity constants for muscarinic binding sites which were however about two orders of magnitude lower than that afforded for *p*-azido-3-QNB [18]. It should be noted here that the high affinity of azido-QNB, reflected in its slow dissociation kinetics, was a major drawback in photolabelling experiments [18]. Such a problem was not encountered with compound **10** which readily dissociated from the receptor sites in the dark.

Finally, introduction of an azido group into dexetimide led to compound **9** with no significant loss in binding affinity [39]. This apparent advantage may, however, contribute to its non productive labelling, the photo-induced derivatization not occurring directly in the muscarinic receptor binding site.

A surprising finding was the observation of the very high photolabelling efficiency of diazonium derivatives **1** and **2**. Indeed, both compounds have been previously described, in our laboratory, as potent irreversible labels of the high affinity site for non competitive blockers on the peripheral nicotinic acetylcholine receptor [42]. Furthermore, compound **1** which was referred to as DDF, was found to label, also with a high efficiency, the acetylcholine binding sites of both the native, unreduced membrane-bound nicotinic receptor [42, 44] and acetylcholinesterase [41, 45], though it displayed a 100-fold lower apparent affinity for nicotinic binding sites [42, 43] than for muscarinic ones (this study).

Amino-acid and/or sequence analyses of the [^{3}H]DDF-photolabelled α-subunit of nicotinic receptors allowed the identification of five labelled residues, located within three distinct regions of the α-chain primary structure, and provided information on the tertiary folding of the α-subunit at the level of the acetylcholine binding site [44].

The high labelling efficacy of the diazonium salt **1** (DDF) at both the nicotinic receptor and acetylcholinesterase has been attributed to an energy-transfer reaction involving a tryptophan residue(s) of the ligand binding site [41–45]. Our irradiation conditions are compatible with such a so-defined photosuicide inactivation and further investigations should clarify the photolabelling process involved in the labelling of muscarinic receptors by compounds **1** and **2**.

Our results point also to the high sensitivity of the photolabelling reaction upon the chemical structure of the probe and the nature of its photoactivatable function. Moreover, they are in general agreement with the apparent overall photolabelling efficiency of DDF at different acetylcholine binding proteins and make of compounds **1** and **2** very encouraging probes for studying the location and the topography of the acetylcholine binding site on muscarinic receptors.

Their use on purified receptor material, obtained through well-described purification procedures [3, 4] or by high yield expression of separate gene products, should help in overcoming problems linked to their apparent low binding affinity. Finally, the apparent lack of binding selectivity towards the different muscarinic receptor subtypes (slope factors for [^{3}H]QNB displacement curves close to 1) of diazonium salts **1** and **2** and of the azido derivative **10**, may be regarded as an advantage for comparative studies of the acetylcholine binding site on different muscarinic receptor proteins.

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