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Covalent Labeling of Muscarinic Acetylcholine Receptors by Tritiated Aryldiazonium Photoprobes

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ABSTRACT. *p*-dimethylamino **(A)** and *p*-dibutylamino **(B)** benzenediazonium salts, previously characterized as efficient labels of membrane-bound and solubilized muscarinic receptor sites, are endowed with overall interesting photochemical and alkylating properties that allow their use as structural probes of the muscarinic ligand binding domain to be considered. Under reversible binding conditions, these antagonists display no binding selectivity towards the 5 muscarinic acetylcholine receptor (mAChR) subtypes. They were used here, in a tritiated form, as photoaffinity labels of purified muscarinic receptors from porcine striatum, and their irreversible binding was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. When irradiated under energy transfer conditions, [^{3}H]**A** and [^{3}H]**B** were both found to covalently label purified muscarinic receptor sites in a light-dependent and atropine-protectable manner. The electrophoretic migration properties of the alkylated sites were similar to those of [^{3}H]propylbenzilylcholine mustard (PrBCM)-labeled mAChRs. Specific radioactive incorporation showed a clear dependency on probe concentration. Labeling efficiency was rather high, with up to 30% and even 60% of the receptor population being photolabeled by [^{3}H]**A** and [^{3}H]**B**, respectively. These two photoactivatable ligands have proven to be powerful tools for the structural analysis of other cholinergic targets (acetylcholinesterase and the nicotinic acetylcholine receptor) by allowing the characterization of a number of different residues belonging to their acetylcholine-binding domain. Altogether, these results reinforce the interest of our site-directed labeling approach because [^{3}H]**A**- and [^{3}H]**B**-alkylated mAChRs may now be considered as suitable materials to investigate the muscarinic receptor-binding pocket through peptide mapping, sequence analyses, and identification of radiolabeled amino acid residues. BIOCHEM PHARMACOL 53:4:501–510, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. muscarinic acetylcholine receptor; covalent labeling; photoaffinity; aryldiazonium salts; tritiated probes

The mAChRs† (m1–m5) are members of the superfamily of membrane-bound receptors that regulate cellular activity via coupling to heterotrimeric G-proteins [1]. The binding site for acetylcholine and competitive antagonists on muscarinic receptors appears to be located within the seven transmembrane helices characteristic of all G protein-coupled receptors [1, 2].

Site-directed mutagenesis and the study of chimeric receptors have allowed the identification of a number of residues and domains likely to be involved in the binding of muscarinic agonists and antagonists, and in the coupling with G proteins [3–5]. Structural knowledge of the muscarinic ligand binding domain certainly requires additional approaches, such as molecular modeling and protein labeling.

In particular, site-specific labeling, followed by peptide mapping and sequencing studies, may point at amino acids directly involved in ligand binding. Several affinity probes (mostly nitrogen mustard derivatives [6–13]) and photoaffinity ligands (azido derivatives [13–16]) were synthesized (most under nonradioactive form) and shown to behave as irreversible blockers of mAChRs.

Up to now, only two affinity labels, [^{3}H]propylbenzilylcholine mustard [17, 18] and [^{3}H]acetylcholine mustard [19] have led to the identification of covalently modified residues. Their labeling pattern was, however, restricted to the same conserved aspartate-105 residue of the m1 muscarinic receptor.

We introduced the dimethylamino **(A)** and dibutylamino **(B)** benzenediazonium derivatives (Fig. 1) as new photoactivatable ligands for the mAChRs endowed with very interesting and efficient labeling properties [20, 21].

As reviewed by Kotzyba-Hibert et al. [22], these aryldiazonium salts present several advantages over other photoaf-

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† *Abbreviations:* mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; PrBCM, propylbenzilylcholine mustard; AChM, acetylcholine mustard.

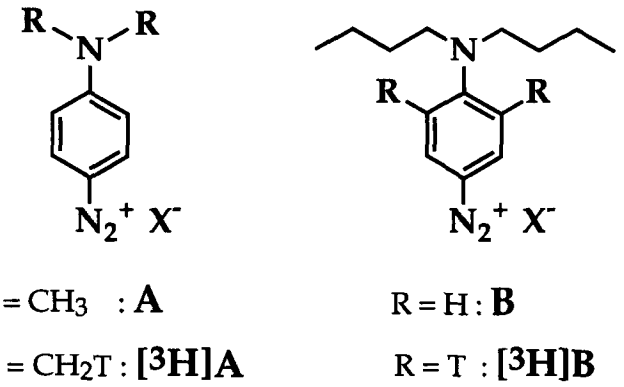


FIG. 1. Chemical structures of unlabelled (A, B) and tritiated ([^{3}H]A and [^{3}H]B) aryldiazonium probes.

finity reagents in regard to efficient site-specific labeling. For example, their spectroscopic properties allow preferential photoactivation of bound probes through energy transfer from photoexcited tryptophan residues of the protein. Such a photosuicide labeling process, using diazonium salts **A** and **B**, proved its efficacy towards several cholinergic targets such as acetylcholinesterase [23], the nicotinic acetylcholine receptor [24] and, more recently, the muscarinic receptors [21].

Moreover, their activation by light generates short-lived aryl cation species with high and nondiscriminative reactivity towards all kinds of residue.

This was demonstrated by the exceptional reactivity of [^{3}H]**A** (DDF), which allowed the identification of different amino acid residues (including hydrophobic ones) belonging to the acetylcholine binding site of acetylcholinesterase (three residues [25, 26]), and of the nicotinic receptor α-subunit (eight residues [27, 28]). As a general consequence, these photoactivatable ligands, when located within a binding site, can react with any vicinal amino acid and behave as very powerful topographical markers.

This essential property, added to the very encouraging results we obtained using unlabeled diazonium salts **A** and **B** on membrane-bound [20, 21] and digitonin-solubilized [29] muscarinic receptors, prompted us to characterize their photolabeling properties at purified mAChRs and to examine the covalent incorporation of [^{3}H]**A** and [^{3}H]**B**.

Following their purification by affinity chromatography and the analysis of their pharmacological characteristics, muscarinic receptors were irradiated under energy transfer conditions in the presence of the radioactive probes.

Available at a high radioactive specific activity, [^{3}H]**A** and [^{3}H]**B** displayed light-, concentration-, and atropine-dependent alkylating properties at mAChRs. After electrophoretic analysis of the radiolabeled species, specific [^{3}H]**A** and [^{3}H]**B** covalent labeling was located within a single broad 60–80 kDa molecular mass peak, as were specifically [^{3}H]PrBCM-affinity-labeled mAChRs.

These two photoactivatable probes may now be regarded as promising tools to investigate the receptor binding domain through peptide mapping, sequence analyses, and identification of [^{3}H]**A**- or [^{3}H]**B**-labeled amino acid residues.

MATERIALS AND METHODS

Drugs and Chemicals

Synthesis, tritiation, diazotization, and purification of the *p*-N,N-dimethyl amimo (**A**) and *p*-N,N-dibutylamino (**B**) benzenediazonium photoactivatable compounds (Fig. 1) were as described previously [20, 30]. Note that, in the literature, probe **A** has also been referred to as DDF [22–25, 27, 28]. Their radioactive specific activities were adjusted through isotopic dilution and varied from 14 to 22 and from 14 to 16 Ci/mmol for [^{3}H]**A** and [^{3}H]**B**, respectively.

[^{3}H]Quinuclidinyl benzilate (QNB, 33 Ci/mmol) and [^{3}H]Propylbenzilylcholine mustard (PrBCM, 74.7 Ci/mmol) were from N.E.N. (Du Pont de Nemours, Les Ulis, France). Atropine sulfate, chloride salts of carbamoylcholine and trimethylamine, bromide salts of phenyltrimethylammonium and of tetramethylammonium, dithiothreitol, bovine gamma globulin, and bovine serum albumin were all purchased from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). Pirenzepine, 4-DAMP, methoctramine, AF-DX 116, and *p*-fluorohexahydro-siladifenidol were gifts from Marion-Merrell-Dow (Strasbourg, France). Digitonin, sodium cholate, and activated charcoal (Norit A) were from Serva (Bio-Whittaker, Gagny, France). Hydroxylapatite (Bio-Gel HTP), electrophoresis reagents and Mr protein standards were supplied by Bio-Rad (Ivry s/Seine, France).

Preparation of aminobenzhydryloxytropane-Sepharose (ABT-agarose) followed a well-described procedure [31] and the content of bound ABT (2–4.5 μmol/mL) was determined from the UV absorption spectrum of the gel suspension. All common reagents were of the highest purity available.

Materials

For irradiation experiments, monochromatic light was obtained from a 1000W xenon-mercury lamp (Hanovia, Newark, NJ) connected to a grating monochromator (Jobin-Yvon, Longjumeau, France). The light intensity was monitored with a thermopile (Kipp and Zohnen, Sevran, France) coupled to a microvoltmeter (AOIP, Evry, France) and adjusted through an iris diaphragm. The light beam was focused through a quartz lens on the refrigerated assay quartz cell.

Solubilization and Purification of mAChRs

The striatum from porcine brains was dissected out and microsomal membranes, prepared by differential centrifugation [29], were stored at −80°C. Solubilization of mAChRs was performed in buffer A (20 mM potassium dihydrogen phosphate/50 mM NaCl/1 mM EDTA, pH 7.2) supplemented with 1% digitonin and 0.1% sodium cholate,

at a detergent/protein ratio (w/w) close to 2, as detailed in [29]. The 140,000g (r_{av}) supernatant was immediately used as soluble extract.

Purification of mAChRs followed the overall procedure described by Haga et al. [31, 32], with a few modifications. After a 2-fold dilution in buffer A, the soluble extract (450 mg protein, 300 pmoles specific [^{3}H]QNB binding sites, 250 mL) was applied (0.2 mL/min) on an ABT-agarose column (25 mL) equilibrated in buffer A supplemented with 0.1% water-soluble digitonin. The gel was washed with 20 mM potassium-dihydrogen phosphate/0.15 M NaCl/0.1% digitonin, pH 7.2 (buffer B) until the absorbance of the eluate at 280 nm became negligible. The outflow-end of the ABT-gel column was then connected to a small hydroxylapatite column (HA; 0.7 mL) and elution proceeded with buffer B (250 mL) supplemented with 0.1 mM atropine at the same flow rate as above. Finally, the HA-column was disconnected from the affinity column and submitted to sequential elution (0.3 mL/min) with 0.02, 0.15, 0.5, and 1 M monopotassium-dihydrogen phosphate buffer (pH 7.2) containing 0.1% digitonin.

mAChR-containing fractions, as assessed by specific [^{3}H]QNB binding, were pooled, desalted, and concentrated in buffer C (20 mM sodium-dihydrogen phosphate/0.1% digitonin, pH 7.2), with no receptor loss, by ultrafiltration on CM10 or CM30 (Amicon) microconcentrators before storage at −80°C.

[^{3}H]QNB Binding Assays

Chinese hamster ovary (CHO-K1) cells, transfected with human genes encoding each of the 5 subtypes of mAChRs, were grown, harvested, and homogenized to give crude membrane preparations as described [33].

Membrane-bound muscarinic receptors from porcine striatum or from CHO cells were quantitated as previously reported [20].

Solubilized and purified mAChRs were assayed for [^{3}H]QNB binding in buffer A or in buffer C with 2.5 nM or 4 nM [^{3}H]QNB, respectively. After incubation (1 hr at 30°C) in the presence of various concentrations of unlabeled drugs, ligand-receptor complexes were separated from free ligand by means of a charcoal adsorption procedure [34]. A 50 μL aliquot of BSA-coated charcoal (10% activated charcoal and 2% bovine serum albumin in water) was added to the 0.4 mL precooled incubation medium, rapidly mixed, and centrifuged at 11,000 g for 4 min. A 0.3 mL fraction of the supernatant was counted for radioactivity. Specific binding was defined as the difference between total binding and nonspecific binding measured in the presence of 2 μM atropine.

Photoaffinity labeling of Purified mAChRs With [^{3}H]A and [^{3}H]B

Purified receptor samples (20 to 100 pmoles) were diluted in buffer C and preincubated for 15 min at 30°C in the absence or the presence of 10 μM atropine. The photoactivatable probes [^{3}H]**A** (50–200 μM) or [^{3}H]**B** (5–50 μM) were added to reach the final concentrations, as indicated. After an additional 20-min incubation period at 30°C in the dark, the incubation mixture (200–500 μL) was cooled at 4°C and introduced in a 1-cm path-length quartz cell that had been carefully siliconized.

Irradiation was routinely performed (at 10°C under gentle stirring) at 295 nm with an incident light energy of 30 μV, for 20 to 30 min, depending on sample volume. Unless otherwise stated, the unreacted probe was destroyed after irradiation by the addition of 10 mM dithiothreitol. The sample was then submitted to a twice-repeated dilution-concentration step on CM30 filter units, using 50 mM Tris-HCl buffer (pH 7.5) supplemented with 0.1% SDS as the washing medium, which removed up to 90% of contaminating radioactivity.

Affinity labeling of Purified mAChRs with [^{3}H]PrBCM

The labeling protocol was essentially as described in [31]. Cyclized [^{3}H]PrBCM, equivalent in concentration to 4 times the concentration of receptor sites (20 to 100 nM), was allowed to react in buffer C for 30 min at 30°C with the purified receptor sample. Subsequently, the residual aziridinium reactive species was quenched by the addition of 1 mM sodium thiosulfate.

Parallel experiments were performed by preincubating the receptor with 2 μM atropine to determine the nonspecific binding level of the radioligand. Specific [^{3}H]PrBCM incorporation, as assessed from specific binding determined by the classical charcoal adsorption procedure, represented nearly 60% of the initial [^{3}H]QNB binding capacity.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Slab gel electrophoresis (160 × 200 × 1.5 mm gels) was carried out essentially as described by Laemmli [35]. The stacking and running gels contained 4.5% and 10% acrylamide, respectively. Concentrated receptor samples were mixed for 90 min at 30°C with an equal volume of sample buffer (5% SDS, 20% glycerol, 0.1 M dithiothreitol and 0.01% Bromophenol Blue in 125 mM Tris-HCl buffer, pH 6.8).

Samples (25 to 250 μL per lane) were then electrophoresed at 100V constant voltage for approximately 16 hr. Prestained protein standards were run in parallel lanes.

For quantitative determination of radioactivity, each lane of separating gel was cut into 2.1-mm slices that were digested for 20 hr at 80°C with 250 μL hydrogen peroxide. Then, 250 μL 4 M urea/1% SDS and 4.5 mL of scintillation cocktail (Emulsifier-Safe™; Packard Instruments, Rungis, France) were added in each vial and the mixture was allowed to stand overnight before counting.

Determination of Protein Concentration

The Bio-Rad protein microassay [36] was used for membrane-bound and crude solubilized preparations. Protein

concentration of purified receptor samples was determined using different methods (listed here according to decreasing sensitivity, protein amounts per assay) such as the colloidal gold (20–200 ng, [37]) and fluorescamine (0.1–2 μg, [38]) assays, or a modified micro-Bradford procedure (2–10 μg, [39]).

Phosphate interference was avoided through desalting of purified receptor samples prior to the protein assay. With the exception of the colloidal gold method, digitonin at less than 1% did not interfere with the assays. Bovine gamma globulin was used as a standard in all cases.

RESULTS

Receptor Purification and Pharmacological Characterization

Muscarinic acetylcholine receptors (mAChRs) from porcine striatal membranes (1–2 pmoles/mg prot.) were purified by submitting a digitonin-cholate solubilized extract (0.70 ± 0.05 pmoles/mg prot.; mean ± SEM, n = 23; 45% of the initial membrane-bound receptor sites and 75% of the proteins) to affinity chromatography on ABT-agarose according to Haga's procedure [31, 32].

Subsequent elution of the protein material adsorbed onto small hydroxylapatite columns with 0.5 M and 1 M phosphate buffer permitted the recovery of 32 ± 2% and 11 ± 1% (mean ± SEM, n = 23) of the initially applied receptor sites, respectively. Good overall yields in receptor sites (55%) and proteins (100%) were reproducibly obtained.

Fractions containing significant amounts of receptor sites were pooled, desalted, and concentrated over CM10 filter units, and tested for their protein content. Depending on the type of protein assay, mean-specific [^{3}H]QNB binding activities ranging from 0.5 (fluorescamine assay) to 2 nmoles/mg prot. (colloidal gold and modified micro-Bradford assays) were estimated. A 700- to 3000-fold enrichment in receptor sites, with an approximate purity of 3–10%, was thus obtained.

Pharmacological characterization of the enriched receptor material was undertaken by saturation and competition experiments using [^{3}H]QNB as the radioligand (Table 1). K_d-values for [^{3}H]QNB binding and K_i-values for most muscarinic antagonists and carbachol were slightly higher than those previously reported for solubilized receptors [29], but were in complete agreement with binding affinity constants (QNB, atropine, pirenzepine, AF-DX 116, and carbachol) determined on similarly purified mAChRs from porcine brain [41].

Photoactivatable probes **A** and **B** competed for [^{3}H]QNB binding with similar potencies to digitonin-solubilized [29] and purified receptor sites. Their antagonist nature was assessed by their ability to inhibit oxotremorine-stimulated [^{35}S]GTPγS binding to membranes from Chinese hamster ovary (CHO) cells [42] stably expressing individual m1–m4 mAChR subtypes.† Whatever the receptor subtype that was expressed in these cells, similar affinity constants were found, indicating a lack of selectivity of these diazonium compounds.

This was in complete agreement with competition studies (Table 2) performed on recombinant receptors expressed in CHO cells, which indicated that **A** and **B** competed with similar binding affinities for [^{3}H]QNB binding to all 5 human mAChR subtypes.

TABLE 1. Drug binding affinity properties of purified mAChRs from porcine striatum

Compounds	K_i-values*	N_H*
Muscarinic drugs		
[^{3}H]QNB (nM)†	0.95 ± 0.01 (4)	–
Atropine (nM)	5.6 ± 0.5 (10)	0.97
4-DAMP (nM)	5.4	0.87
*p*FHHSiD (nM)	68	0.84
Pirenzepine (nM)	358	0.99
Methoctramine (nM)	383	1.3
AF-DX 116 (nM)	5800 ± 230 (2)	1.06
Phenyltrimethylammonium (μM)	33	0.63
Carbachol (μM)	297	1.08
Tetramethylammonium (μM)	2900	0.76
Trimethylamine (μM)	7300	0.76
Photoactivatable probes		
A (μM)	75 ± 10 (6)	1.04
B (μM)	7.1 ± 1.6 (5)	0.92

†K_d-values for [^{3}H]QNB binding were from saturation experiments.

*Competition experiments were performed as described in Materials and Methods using [^{3}H]QNB as the radioligand. K_i-values were determined from IC_{50} values using the method of Cheng and Prusoff [40], assuming competitive interactions. The average and standard error mean (SEM) values are indicated with the number of experiments in parentheses. In other cases, a single determination was made. N_H values represent slope factors of binding isotherms.

Preliminary Conditions for Photolabeling of Purified mAChRs

UV stability of the purified receptors was first examined. Under the irradiation conditions (290 nm; 30 μV) previously selected for digitonin-solubilized receptors [29], purified receptor samples showed an enhanced photosensitivity ($t_{1/2}$: 65 min vs 87 min for solubilized receptors).

Photodenaturation of the receptor sites, monitored as a decrease in specific [^{3}H]QNB binding, could not be prevented by argon purging. The stability of the receptors was improved ($t_{1/2}$: 95 min) by shifting the irradiation wavelength to a higher value (295 nm) still compatible with energy-transfer photolabeling [22, 23].

Control experiments performed with unlabeled **A** (10^{-5} M) and **B** (6 10^{-6} M) probes showed that, under the previously described irradiation conditions (290 nm; [20, 21]), similar extents of photolabeling (70–75%) of mAChRs were obtained using either rat brain or porcine striatal membranes. At 295 nm, the labeling efficacy of these diazonium probes was lower (50–55%).

Prior to photolabeling experiments of purified receptors with unlabeled diazonium **A** or **B**, an efficient dissociation procedure had to be designed in order to distinguish be-

† Dr. M. Richards, personal communication.

TABLE 2. Receptor binding profile of diazonium salts A and B

mAChR subtype	[^{3}H]QNB binding K_d (pM)	[^{3}H]QNB binding B_{max} (fmol/mg prot.)	A K_i (μM)	B K_i (μM)
CHO-m1	28 ± 4	1546 ± 129	66	2.9
CHO-m2	35 ± 2	489 ± 73	52 ± 5	2.6 ± 0.8
CHO-m3	66 ± 14	1558 ± 91	53 ± 14	2.6 ± 1.6
CHO-m4	51 ± 15	749 ± 44	64 ± 8	4.9 ± 1.0
CHO-m5	136 ± 8	477 ± 54	67 ± 12	4.6 ± 0.7

Saturation and competition experiments were performed, at equilibrium, on membrane-bound muscarinic receptor subtypes from CHO cells, using [^{3}H]QNB as the radioligand (Materials and Methods). Binding parameters (± SEM values) are representative of at least 2 independent experiments.

tween reversible and irreversible binding components of the probes. A dilution-centrifugation procedure and dialysis were previously selected as the methods of choice for monitoring residual [^{3}H]QNB binding to membrane-bound [20, 21] and solubilized [29] receptors, respectively. Subsequently, they allowed an accurate definition of all parameters for photolabeling of the receptor sites with probes **A** and **B.**

Unfortunately, none of the dissociation procedures (extensive dialysis, adsorption on hydroxylapatite or on BSA-coated charcoal, gel filtration, or repeated dilution-centrifugation steps) we tested on purified mAChRs simultaneously met all criteria required for a labeling study on purified mAChRs: reproducibility, high recovery in receptor sites, and complete dissociation of reversible complexes elicited by the interaction, in the absence of light, of the receptor sites with either atropine or the probes. Examination, under reliable conditions, of the parameters for photolabeling of purified mAChRs by the unlabeled diazonium salts **A** and **B** was, thus, impossible, as was the subsequent estimation of the stoechiometry of irreversible incorporation of the tritiated probes.

Therefore, photolabeling of purified receptors was undertaken with the tritiated probes directly, in the knowledge that the ultimate step of the experiment (i.e. SDS-polyacrylamide gel electrophoresis) was a strongly dissociating procedure.

Experimental Conditions for Photolabelling of Purified mAChRs With Tritiated Diazonium Probes

Just before use, aryldiazonium salts [^{3}H]**A** and [^{3}H]**B** were checked for purity by reversed-phase HPLC [30] and concentrated as needed. To avoid unnecessary receptor dilution and to reduce the absolute amount of radioactivity introduced in the assay, small samples (200–500 μL) were incubated with the probes and irradiated at 295 nm (30 μV) as described in Materials and Methods.

Prior to SDS-PAGE, it was necessary to remove the excess of tritiated ligand (free or reversibly bound) and to concentrate the irradiated receptor sample. Adsorption on ovalbumin-coated charcoal (a procedure similar to that described in Methods) was designed as a very rapid and non-diluting method, allowing direct analysis of small receptor samples deprived of free aromatic ligands through SDS-PAGE. The classical coating protein BSA was replaced by ovalbumin (45 kDa), whose electrophoretic mobility differed from that of mAChRs. In addition to permitting an excellent recovery (90 to 100%) in specific [^{3}H]QNB binding sites, this charcoal procedure was also found to be very efficient in rapidly adsorbing up to 95% of unlabeled and tritiated probes **A** and **B** at concentrations up to 3 10^{-5} M. Unfortunately, this promising method gave disappointing results when higher concentrations of tritiated probes, concentrated receptor aliquots (with a probable high digitonin concentration), and dithiothreitol were present in the sample. Chloroform/methanol precipitation [43], first retained as a rapid and efficient way to remove more than 95% of the radioactivity and to concentrate the protein material as a pellet gave, in fact, mostly insoluble receptor aggregates, which precluded the characterization of alkylated mAChRs by SDS-PAGE. This was in total agreement with the report of Wheatley et al. [44] on the pronounced hydrophobic character of mAChRs after ethanol precipitation and detergent removal.

Finally, though more time-consuming, gel filtration on PD-10 columns (Pharmacia Biotech, Orsay, France) and repeated dilution-concentration steps on CM30 ultrafilters were retained as methods of choice for giving excellent recovery in receptor sites and allowing the elimination of 90% of contaminating radioactivity. Ultrafiltration, with the additional advantage of concentrating the labeled sample, was preferred to the former method.

Analysis of the Covalent Labeling of mAChRs Using [^{3}H]A and [^{3}H]B

After washing and concentration of the samples, photoincorporation of radioactive aryldiazonium probes into mAChRs was examined by polyacrylamide gel electrophoresis under denaturating conditions.

Typical SDS-PAGE profiles of receptor preparations irradiated with [^{3}H]**A** (Fig. 2a) or [^{3}H]**B** (Fig. 2b), in the absence or the presence of the muscarinic antagonist atropine, are shown. For both probes, the alkylated material was recovered as 2 major broad radiolabeled peaks differing by their molecular weights and their 'sensitivity' to atropine.

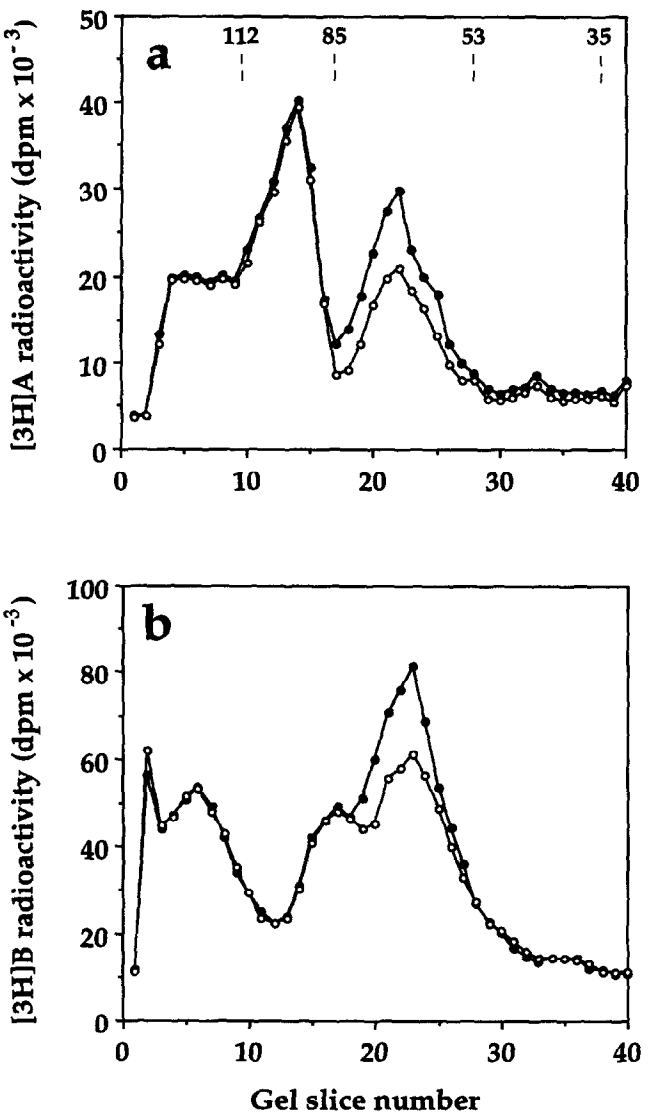


FIG. 2. SDS-PAGE analysis of purified mAChRs labeled with [^{3}H]A and [^{3}H]B. Purified mAChRs were incubated with the tritiated probes (a) 8.5 pmoles mAChRs and 150 μM [^{3}H]A; (b) 5 pmoles mAChRs and 50 μM [^{3}H]B in the absence (●, total binding) or the presence (○, nonspecific binding) of 10 μM atropine, irradiated at 295 nm, and submitted to SDS-PAGE as indicated in Materials and Methods. Prestained molecular weight protein markers were as follows: phosphorylase b (112 kDa), bovine serum albumin (85 kDa), egg albumin (53.2 kDa), and carbonic anhydrase (34.9 kDa).

No labeled species were detected in the lower molecular mass regions (from 35 kDa to the dye front).

The 60–80 kDa peaks (which contained only a small percentage of the radioactivity applied onto the gel) showed a specifically alkylated binding component that was suppressed when labeling was performed in the presence of atropine, which represented 28% ([^{3}H]**A**; Fig. 2a) and 18% ([^{3}H]**B**; Fig. 2b) of the total irreversible binding of the probes. Thus, nonspecific labeling was high.

The pH of the incubation medium was lowered to 6.5 to diminish the chemical reactivity of the diazonium probes. In another experiment, purified receptors were preincubated with phenyltrimethylammonium ($3\ 10^{-6}$ M), tetramethylammonium (10^{-4} M) or trimethylamine ($3\ 10^{-4}$ M) at concentrations that did not interfere with specific [^{3}H]QNB binding to mAChRs (Table 1), to prevent nonspecific interaction of [^{3}H]**A** or [^{3}H]**B** with hypothetical ammonium-recognizing sites present on the receptor protein. None of these conditions led to a significant reduction of the nonspecific incorporation of the probes.

Interestingly, the specific alkylation profiles of the tritiated probes (Fig. 3a) revealed the existence of a single radioactive component (60–80 kDa) containing 23% and 64% of the initial receptor sites as [^{3}H]**A**- and [^{3}H]**B**-specifically alkylated material, respectively. It was noteworthy that the covalent incorporation profiles of the probes were superimposable to that of specific [^{3}H]PrBCM labeling (Fig. 3b). Finally, as shown in Fig. 3c, specific alkylation of the mAChRs by [^{3}H]**A** (and [^{3}H]**B**, not shown) was not observed when the irradiation step was omitted, and was thus clearly dependent on a photochemical process.

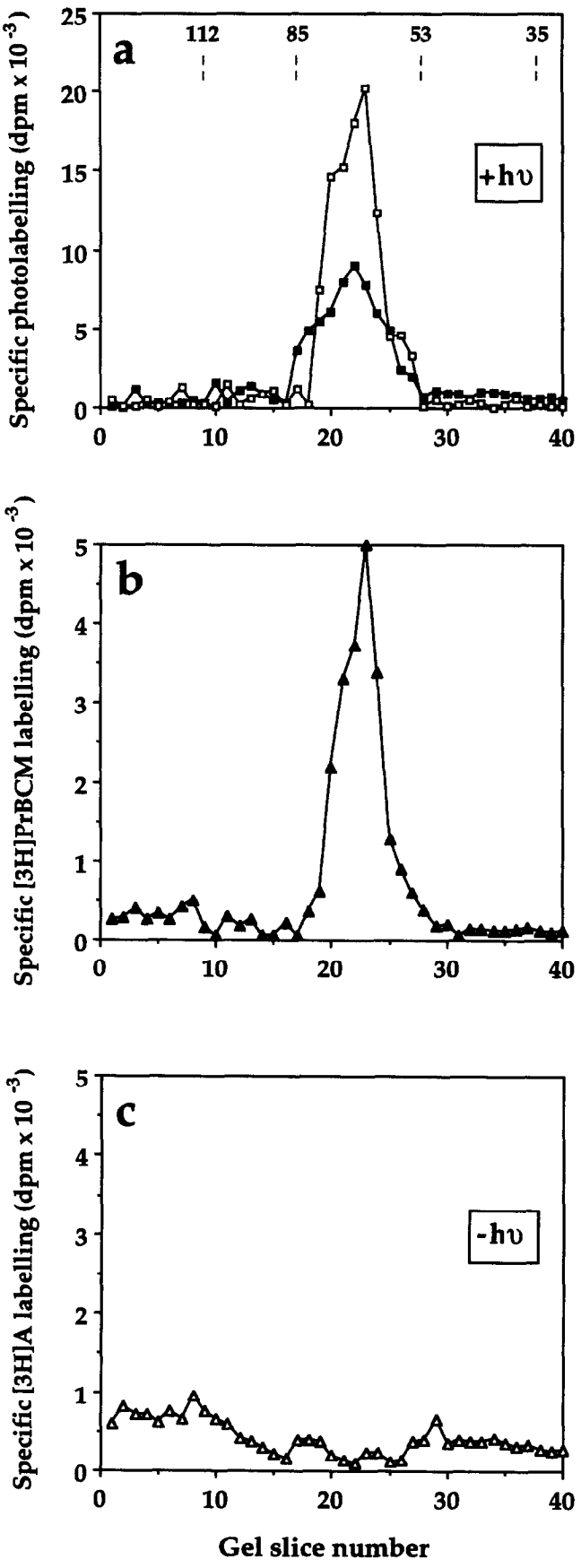
Figure 4 shows that specific covalent incorporation of [^{3}H]**A** and [^{3}H]**B** into mAChRs was clearly concentration-dependent. At concentrations ranging from 0.5 to 3 ([^{3}H]**A**) or from 1 to 7 ([^{3}H]**B**), their respective K_i-values (Table 1), both probes alkylated increasing proportions of the overall receptor population referred to as [^{3}H]QNB binding sites. [^{3}H]**A** was slightly more efficient than [^{3}H]**B** in labeling purified in mAChRs and, despite its lower affinity, gave a better signal-to-noise ratio (specific vs total irreversible binding). This last property might be due to its less hydrophobic character [30].

Finally, as part of this labeling study, it was interesting to see if the 2 main radioactive components (Figs. 2a and 2b) could be fractionated prior to SDS-PAGE.

Further purification of the photolabeled receptor material was, thus, undertaken by size-exclusion chromatography (not shown), a procedure that has proven to be useful in removing the high-molecular-weight material, contaminating the purified receptor protein when using Haga's protocol [31, 32] and nonspecifically labeled, as in our study, by the two affinity probes [^{3}H]PrBCM and [^{3}H]AChM [19]. Figure 5 shows that following size-exclusion chromatography of the irradiated sample, the [^{3}H]**A**-alkylated material was resolved into a single labeled species of molecular mass 60–80 kDa, displaying a marked atropine sensitivity. Such an alkylated receptor preparation, deprived of the high-molecular-mass radioactive contaminant, may now be considered as a starting material directly suitable for enzymatic cleavage in solution and peptide mapping studies.

DISCUSSION

Purification of mAChRs is a prerequisite for their site-directed labeling by tritiated probes. This step was crucial in our study for at least 2 main reasons. First, specific alkylation of membrane-bound receptors by micromolar concentrations of photosensitive probes [^{3}H]**A** and [^{3}H]**B** was indistinguishable from the high radioactive background level.



Second, affinity chromatography was also essential to free the receptor preparation from other biogenic amine receptors (e.g. adrenergic, dopaminergic, serotoninergic, and histaminergic receptors), mechanistically expected to bind through the same aspartate-quaternary nitrogen interaction as do muscarinic receptors [3, 5], which could represent interfering candidates for labeling by our cationic diazonium photoprobes.

Following Haga's procedure [31, 32], highly enriched receptor preparations were reproducibly obtained, with good yields and expected purity. Their pharmacological characterization showed that they retained a typical muscarinic binding profile. More refined interpretation of our data, especially those of the more selective antagonists, was, however, complicated by the heterogeneous receptor subtype composition of the striatum [45, 46], the differential receptor subtype extraction through digitonin-solubilization [47], and the poor selectivity of most antagonists that are known, in addition, to strongly depend on the lipid environment of the receptor sites [47, 48].

Nevertheless, the nanomolar affinity of 4-DAMP, the intermediate affinity of *p*-FHHSiD, and the micromolar binding affinity constants found for AF-DX 116 and methoctramine were indicative of a low m2 and m3 receptor subtype content in our purified receptor preparation, and were in agreement with previous reports [45, 46] describing the coexistence, in the striatum, of predominant m1 and m4 subtypes, present in nearly equivalent proportions.

Purification did not affect the binding affinity parameters of the two diazonium salts **A** and **B** previously reported for digitonin-solubilized receptors [29].

Different experiments were then conducted to set up the optimal conditions for photolabeling of purified mAChRs with unlabeled and tritiated probes.

Examination of the irradiation conditions pointed to the increasing photosensitivity of the receptor sites, as membrane-bound [21], solubilized [29] and, finally, purified mAChRs were considered. This phenomenon was probably related to the drastic environmental changes imposed on the receptor throughout the purification procedure and to the very low protein content of the purified material. The irradiation wavelength was thus shifted from 290 nm to 295

FIG. 3. SDS-PAGE of affinity- and photoaffinity-labeled purified mAChRs. (a) Specific incorporation of [^{3}H]A (■) and of [^{3}H]B (□) was defined as the difference between corresponding total and nonspecific irreversible binding values (Fig. 2), and corresponded to 1.9 and 3.2 pmoles of alkylated receptor sites, respectively. (b) Purified mAChRs (0.4 pmoles) were affinity-labeled with [^{3}H]PrBCM (0.43 μM) as described in Materials and Methods. Specific [^{3}H]PrBCM alkylation (▲) was determined after SDS-PAGE of the labeled receptors as above. (c) In a control experiment, 5.5 pmoles of purified mAChRs were incubated with [^{3}H]A (90 μM), in the absence or the presence of 10 μM atropine, but the irradiation step was omitted. Specific binding of the probe (△) is shown.

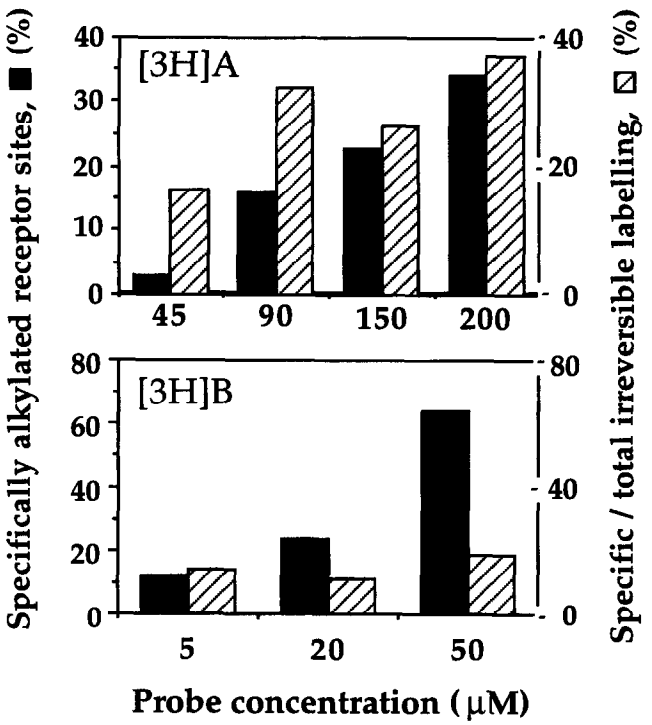


FIG. 4. Concentration-dependence of the covalent incorporation of [^{3}H]A and [^{3}H]B into purified mAChRs. 8.5 pmoles (100%, upper panel) and 5 pmoles (100%, lower panel) of purified mAChRs were incubated and irradiated at 295 nm in the presence of the indicated concentrations of photolabels as described in Materials and Methods. Similar experiments performed in the presence of 10 μM atropine allowed, after SDS-PAGE and radioactivity counting of the 60–80 kDa gel region, the specific alkylation levels of the probes to be determined (black bars, percent of [^{3}H]QNB binding sites recovered as specifically [^{3}H]A or [^{3}H]B-labeled sites; striped bars, percent of specific vs total incorporation of the probes).

nm to restrict the excitation process to the tryptophan residues [22, 23].

Quantitation of irreversible binding of the unlabeled probes **A** and **B** to purified mAChRs was hindered by the lack of a safe and nondenaturating dissociation procedure for the removal of reversibly bound ligands. Nevertheless, the comparison of several dissociation procedures with other methods designed for ligand removal (charcoal adsorption and chloroform/methanol precipitation) allowed us to select repeated dilution-concentration steps on Centricon filters for further experiments, using tritiated probes **A** and **B**. Indeed, such a procedure made it possible to remove, after irradiation of the purified mAChRs with the probes, considerable amounts of unbound radioactivity and to concentrate, without loss, the labeled receptor material.

Photolabeling of purified mAChRs by [^{3}H]A and [^{3}H]B was then undertaken by irradiating the samples at 295 nm and submitting them to gel electrophoresis under denaturating conditions. After SDS-PAGE, the radioactivity associated with the protein material could be attributed exclusively to stable covalent labeling.

Consistent alkylation of a receptor material displaying molecular mass characteristics compatible with those of muscarinic receptors (60–80 kDa), an electrophoretic mobility identical to that of [^{3}H]PrBCM affinity-labeled mAChRs, and a selective protective effect of atropine demonstrated the muscarinic nature of the specific irreversible binding of probes [^{3}H]A and [^{3}H]B. Indeed, an apparent molecular weight of 70,000 has been reported for similarly purified and [^{3}H]PrBCM-labeled mAChRs from porcine brain [32]. Mean molecular-mass values ranging from 66 to 80 kDa were consistently found for brain mAChRs [32, 44, 49–51] and are tentatively explained by differences in purification procedures, analytical methods, species, and tissues. The broadness of the [^{3}H]A- and [^{3}H]B-labeled muscarinic receptor band was also in agreement with the 'particular' migrating properties described for this glycoprotein, probably caused by microheterogeneity in the carbohydrate residues and anomalous binding of SDS due to the presence of tightly bound digitonin [32, 52].

Specific covalent incorporation of [^{3}H]A and [^{3}H]B into mAChRs was not observable when the irradiation step was

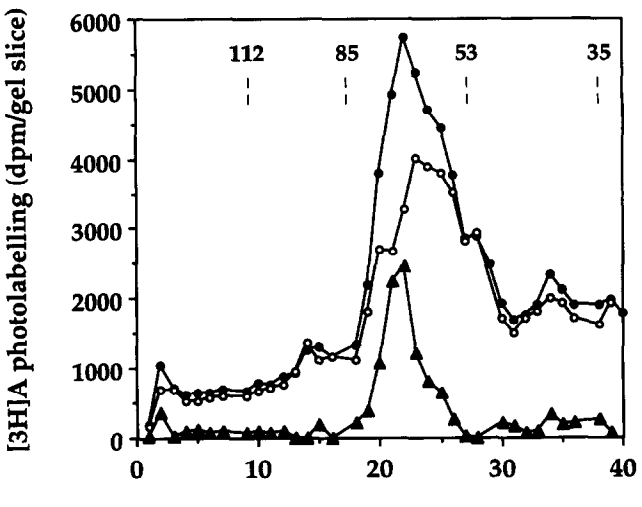


FIG. 5. [^{3}H]A labeling of purified mAChRs after fractionation by size-exclusion chromatography. mAChRs (1.25 pmoles) were photolabeled with [^{3}H]A (120 μM) in the absence or presence of atropine (10 μM) as described in Materials and Methods. Of the mAChRs, 20% were found to be specifically [^{3}H]A-alkylated as assessed from SDS-PAGE analysis. After concentration, the labeled material was reduced and carboxymethylated as reported in [17] and submitted to gel filtration on a TSK G3000SW$_{xl}$ column (TosoHaas, 30 × 7.8 mm) in 0.1 M $Na_1Na_2PO_4$ buffer, 0.1% SDS, pH 7 (0.5 mL/min flow rate). Control experiments were similarly performed using [^{3}H]PrBCM-labeled mAChRs. The high-molecular-weight radioactive component, which was poorly atropine-sensitive, eluted first and free radioactivity was considerably retarded. Fractions containing atropine-sensitive [^{3}H]A binding sites (which corresponded well to those enriched in specific [^{3}H]PrBCM binding) were pooled, concentrated, and finally submitted to SDS-PAGE (●, total binding; ○, nonspecific binding). Specifically, [^{3}H]A-alkylated receptor sites (▲) were recovered (80%, 0.2 pmoles) under the 60–80 kDa peak.

omitted, indicating the occurrence of a typical photochemical process for the labeling. Photolabeling experiments were conducted under energy-transfer conditions and allowed us also to define a clear dependency of the specific alkylation level on the probe concentration. However, the efficacy of such a photosuicide labeling process [22, 23] seemed here to be weaker when compared to earlier results obtained on membrane-bound [20, 21] and solubilized [29] muscarinic receptors.

For example, when irradiated at 290 nm, half of the population of solubilized receptor sites was inactivated at probe **A** and **B** concentrations equal to 0.5- and 1-fold their K_i-value, respectively. In contrast, only 25% of the purified mAChRs were specifically alkylated (irradiation at 295 nm) at concentrations of [^{3}H]**A** and [^{3}H]**B** 2- to 3-fold their respective K_i-values.

Several additional reasons may explain such a decreased labeling efficacy: 1. a weaker photoexcitation of tryptophan residues (λ_{max} = 279 nm) when the irradiation wavelength was shifted from 290 to 295 nm as suggested by control experiments performed on membrane-bound receptors; 2. an enhanced photochemical lability of tryptophan [53] in diluted purified proteins; 3. disruption of specific cystine residues and of hydrogen bonds, responsible for the spatial integrity of the receptor binding domain [3, 4], through energy transfer from photoexcited tryptophan residues [53]; and 4. an underestimation of the extent of specific alkylation by the probes by quantitation restricted to 60–80 kDa molecular mass species (the possible contribution of labeled receptor proteins trapped into aggregates is, thus, neglected).

In conclusion, the 2 tritiated aryldiazonium salts presented here are endowed with interesting properties for a site-directed labeling of mAChRs. When used on purified receptor preparations, under energy-transfer irradiation conditions, [^{3}H]**A** and [^{3}H]**B** led to a significant level of photolabeling of the receptor sites now detectable as irreversible radiolabeled complexes.

A and **B** were also found to behave as nonselective antagonists, a property that might yield, using the same probe, complementary topographical information on the binding 'pocket' of the different muscarinic receptor subtypes, as well as a better understanding of the mechanisms underlying ligand binding selectivity. Because aryldiazonium salts generate, upon irradiation, arylcation species with high and nondiscriminative reactivity leading to the alkylation of all types of residues [22], one should expect, from peptide mapping and sequencing studies of [^{3}H]**A**- and [^{3}H]**B**-covalently bound mAChRs, the identification of several amino acid residues and, thus, additional structural and topographical information on their receptor ligand-binding domain.

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