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Pharmacological and structural integrity of muscarinic M_2 acetylcholine receptors produced in Sf9 insect cells

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Abstract

Muscarinic acetylcholine receptors (human m2 subtype), expressed in Sf9 cells, using the baculovirus system, were purified and found to display the expected ligand binding properties, whether membrane-bound or affinity-purified. The purified recombinant receptors were specifically photolabelled with *p-N,N*-[^{3}H]dimethylamino and *p-N,N*-[^{3}H]dibutylamino benzene diazonium derivatives. Electrophoretic patterns for covalent radioactive incorporation of the probes were essentially similar to those for [^{3}H]propylbenzilylcholine mustard-labelled receptor sites but were dependent on the infection time of Sf9 cells. Pharmacological properties of the recombinant receptors being unaltered did not reflect structural integrity of the protein as substantial proteolytic fragmentation was detected at a prolonged infection time, i.e., at the highest level of expression. Selection of overexpression conditions, as illustrated here for muscarinic receptors, thus requires not only pharmacological controls, but also analysis of the covalently labelled protein under strongly dissociating conditions.

Keywords: Muscarinic receptor; Muscarinic receptor, m2 subtype; Sf9 cells; Covalent labelling; Aryldiazonium salt

1. Introduction

Structural analysis of G-protein-coupled receptors (Baldwin, 1993, 1994; Strader et al., 1994; Fraser et al., 1994), a superfamily of seven-helix integral membrane proteins, has largely been limited to site-directed mutagenesis (Savarese and Fraser, 1992; Schwartz, 1994; Van Rhee and Jacobson, 1996).

This is primarily due to the difficulty in the production and purification of sufficient receptor protein to permit structural characterization using biochemical and biophysical methods. This applies to muscarinic acetylcholine receptors (Hulme et al., 1990; Caulfield, 1993; Wess, 1993) so that efficient methods for overexpression and purification of the native form of each of the five subtypes (m1–m5) are of great interest.

Several G-protein coupled receptors have already been expressed in mammalian cells but large scale expression is often problematic. Other hosts have also been tested for overexpression of integral membrane proteins (Grisshammer and Tate, 1995; Tate and Grisshammer, 1996). For example, *Saccharomyces cerevisiae* and *Escherischia coli* generally yield low expression levels though the latter system has yielded promising results for bacterio-opsin (Chen and Gouaux, 1996), as well as for the neurotensin receptor, expressed as a fusion product with maltose binding protein (Tucker and Grisshammer, 1996).

The baculovirus-Sf9 (*Spodoptera frugiperda*) cell is an attractive alternative for convenient, efficient and large-scale production of recombinant proteins, including muscarinic acetylcholine receptors (Parker et al., 1991; Rinken et al., 1994; Dong et al., 1995). High levels of expression of individual m1–m5 subtypes (0.5–6 nmol/l culture), endowed with characteristic ligand binding specificity, have been obtained (Rinken et al., 1994; Dong et al., 1995). The m2 protein showed the best yields so that this muscarinic receptor subtype was selected for our labelling experiments.

We had shown efficient photolabelling of muscarinic acetylcholine receptors, either on the membrane-bound

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(Ilien et al., 1989; Ilien and Hirth, 1989), solubilized (Ilien and Hirth, 1991) or affinity-purified forms (Autelitano et al., 1997), using two aryldiazonium probes. Specific alkylation of the muscarinic receptor protein by these *p-N,N*-dimethylamino and *p-N,N*-dibutylamino benzene diazonium derivatives was, however, not expected to be subtype-selective. Under reversible binding conditions, $Me_2NArN_2^+$ and $Bu_2NArN_2^+$ did not distinguish between muscarinic acetylcholine receptors subtypes (Autelitano et al., 1997). When their tritiated counterparts were used to photoaffinity label purified muscarinic acetylcholine receptors from porcine striatum, at least two subtypes, probably m1 and m4, were involved in the label (Autelitano et al., 1997). Under these conditions, the results of photoaffinity labelling analysis of the amino-acid residues lining the muscarinic receptor ligand binding domain (located in the transmembrane core of the protein where homology between muscarinic receptor subtypes is highest) would have been very difficult to interpret and to assign to a definite receptor subtype. This problem, added to the low amount of purified receptor protein available from natural sources, prompted us to reevaluate our site-directed labelling strategy.

Muscarinic acetylcholine receptors expressed in Sf9 cells, using a recombinant baculovirus containing cDNA encoding the human m2 subtype, were purified by affinity chromatography and characterized by their reversible drug binding properties. Covalent labelling of the purified receptors by tritiated affinity and photoaffinity probes allowed the electrophoretic patterns of the alkylated receptor proteins to be compared. The results for reversible and irreversible binding and the dependence on receptor expression levels are discussed.

2. Materials and methods

2.1. Materials

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

2.2. Drugs and chemicals

Synthesis, tritiation, diazotization and purification of the photoactivatable compounds *p-N,N*-dimethylamino ($Me_2NArN_2^+$) and *p-N,N*-dibutylamino ($Bu_2NArN_2^+$) benzene diazonium were described previously (Ilien et al., 1989; Autelitano et al., 1996). Specific activities of [^{3}H]$Me_2NArN_2^+$ and [^{3}H]$Bu_2NArN_2^+$ were adjusted by isotopic dilution.

[^{3}H]quinuclidinyl benzilate (52.3 Ci/mmol) and [^{3}H]propylbenzilylcholine mustard (74.7 Ci/mmol) were from N.E.N. (Du Pont de Nemours, France).

Atropine sulfate, carbamoylcholine hydrochloride and dithiothreitol were purchased from Sigma-Aldrich (France). Chlorhydrates of pirenzepine, methoctramine and of *p*-fluorohexahydrosiladifenidol (*p*-F-HHSiD) were from Research Biochemicals International (France). AF-DX 116 was a gift of Marion Merrell Dow Research Institute (Strasbourg, France).

Digitonin, sodium cholate and activated charcoal (Norit A) were from Serva (Bio-Whittaker, France). Hydroxylapatite (Bio-Gel HTP), electrophoresis reagents and Mr protein standards were supplied by Bio-Rad (France). All common reagents were of the highest purity available.

2.3. Expression of the human muscarinic M_2 acetylcholine receptor subtype

Subcloning of human m2 cDNA, isolation of recombinant baculoviruses, Sf9 cell culture and transfection were performed as previously described (Heitz et al., 1995).

Sf9 cells were grown in TNM-FH medium (Tricoplusia Ni Medium modified by Dr. F. Hink) supplemented with 10% fetal calf serum in spinner flasks at 27°C. For receptor expression, 500 ml flasks (2.10^6 cells/ml) were infected with recombinant baculoviruses at a multiplicity of infection of 2.

2.4. Preparation of Sf9 cell membranes

At 3 or 6 days post infection (dpi), recombinant virus-infected Sf9 cells were collected by centrifugation at 1000 $\times g$ for 10 min and the pellet was homogenized in 100 ml buffer solution (20 mM Na-HEPES, 2 mM $MgCl_2$, 1 mM EDTA, 100 μg/ml bacitracin and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0) using a Potter-type homogenizer. The pellet resulting from a centrifugation at 40 000 $\times g$ for 20 min was homogenized in 100 ml of the same buffer and centrifuged again. After resuspension of the final pellet in Na-HEPES buffer, membranes (1–15 mg protein/ml) were stored at −80°C.

2.5. Solubilization and purification of muscarinic M_2 acetylcholine receptors

Thawed Sf9 cell membranes were diluted in buffer A (20 mM KH_2PO_4, 50 mM NaCl, 1 mM EDTA, pH 7.2) supplemented with 1% digitonin and 0.4% sodium cholate, according to the optimized conditions described by Rinken et al. (1994), and gently mixed for 1 h at 4°C. After centrifugation at 140 000 $\times g$ (r_{av}) for 1 h, the supernatant was immediately used as soluble extract.

Purification of muscarinic M_2 receptors followed the procedure described by Haga et al. (1990), with a few modifications (Autelitano et al., 1997).

After 2-fold dilution in buffer A, the soluble extract (140 mg protein and 375 pmol specific [^{3}H]quinuclidinyl benzilate binding sites or 95 mg protein and 870 pmol specific [^{3}H]quinuclidinyl benzilate binding sites for 3 and 6 dpi-solubilized receptors, respectively) was applied (0.2 ml/min) to an affinity gel (aminobenztropine-agarose) column equilibrated in buffer A supplemented with 0.1% water-soluble digitonin. Then, the gel was washed with buffer B (20 mM KH_2PO_4, 0.15 M NaCl, 0.1% digitonin, pH 7.2) until the absorbance of the eluate at 280 nm became negligible. Muscarinic receptors were eluted (0.2 ml/min) from the aminobenztropine gel with buffer B, supplemented with 0.1 mM atropine, onto a hydroxylapatite column (0.7 ml) which was finally eluted sequentially (0.33 ml/min) with 0.02 (20 ml), 0.15 (25 ml), 0.5 (10 ml) and 1 M (10 ml) KH_2PO_4 buffer (pH 7.2) in the presence of 0.1% digitonin.

Fractions enriched in muscarinic M_2 receptors, as assessed by specific [^{3}H]quinunuclidinyl benzilate binding, were pooled, desalted and concentrated in buffer C (20 mM NaH_2PO_4, 0.1% digitonin, pH 7.2) by ultra-filtration on CM30 (Amicon) micro-concentrators before storage at −80°C.

2.6. [^{3}H]quinuclidinyl benzilate binding assays

Sf9 membranes were incubated for 1 h at 37°C in 20 mM NaH_2PO_4 (pH 7.2) buffer containing various concentrations of [^{3}H]quinuclidinyl benzilate and unlabelled drugs (total volume 2 ml). After rapid vacuum filtration on glass fibre filters (Whatman GF/B), membrane-bound radioactivity was quantitated by liquid scintillation counting as described (Ilien et al., 1989).

Solubilized and affinity-purified muscarinic M_2 acetylcholine receptor sites were assayed for [^{3}H]quinuclidinyl benzilate binding in buffer C and incubated (1 h at 30°C) in the presence of various concentrations of unlabelled drugs. Ligand-receptor complexes were separated from free radioligand by charcoal adsorption (Gorissen et al., 1981; Autelitano et al., 1997).

Absolute amounts of membrane-bound and purified receptor sites were quantitated at saturating [^{3}H]quinuclidinyl benzilate concentrations, 0.4 nM and 3 nM, respectively. Specific binding was defined as the difference between total binding and non-specific binding measured in the presence of 2 μM atropine.

2.7. Photoaffinity labelling of purified receptors with [^{3}H]$Me_2NArN_2^+$ and [^{3}H]$Bu_2NArN_2^+$

Purified receptor samples (8–35 pmol) 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]$Me_2NArN_2^+$ or [^{3}H]$Bu_2NArN_2^+$ were then added in order to reach the final concentration as indicated. After an additional 15-min incubation period at 30°C in the dark, the incubation medium (130–150 μl) was cooled to 4°C and put into a 1 cm path-length quartz cell which had been carefully siliconized.

The samples were irradiated at 295 nm with an incident light energy of 30 μV for 15 min at 10°C. The irradiated sample was then run through two cycles of dilution-concentration on CM30 micro-concentrators, using 50 mM Tris–HCl buffer (pH 7.5) supplemented with 0.1% SDS as washing medium.

2.8. Affinity labelling of purified muscarinic M_2 receptors with [^{3}H]propylbenzilylcholine mustard

The labelling protocol was essentially as described by Haga et al. (1990).

Purified receptors from 3 dpi (2.1 pmol, 10 nM) or 6 dpi (9.5 pmol, 95 nM) Sf9 cells were preincubated for 20 min at 25°C, in buffer C, in the presence or the absence of 2 μM atropine.

Precyclized [^{3}H]propylbenzilylcholine mustard (100 nM or 475 nM, for 3 or 6 dpi-purified receptors, respectively), was then added to the preincubated receptor sample and allowed to react for 45 min at 25°C. Alkylation was terminated by the addition of 1 mM sodium thiosulfate to quench the residual aziridinium reactive species and the washing-concentrating step was as described above for photolabelled samples.

2.9. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Slab gel electrophoresis ($160 \times 200 \times 1.5$ mm gels) was carried out essentially as described by Laemmli (1970). Stacking and running gels contained 4.5% and 10% acrylamide, respectively. Concentrated labelled receptor samples were mixed for 30 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 150 μl per lane) were then electrophoresed at 95 V constant voltage for approximately 15 h. 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, which were digested for 20 h at 80°C with 250 μl hydrogen peroxide. Then, 250 μl of 4 M urea/1% SDS and 4.5 ml of scintillation cocktail (Emulsifier-Safe™, Packard, France) were added to each vial and the mixture was allowed to stand overnight before counting.

3. Results

3.1. Production of human muscarinic M_2 receptors in Sf9 cells, solubilization and purification

Insect cell membranes from Sf9 cells infected with the recombinant m2-baculovirus were obtained as described in Section 2.

Production of muscarinic M_2 receptors, assessed from [^{3}H]N-methylscopolamine saturation binding experiments, ranged from 1.5 ± 0.5 pmol/mg protein (3 days after infection, 3 dpi) to 3.0 ± 0.2 pmol/mg protein (6 days after infection, 6 dpi) and remained fairly stable for two additional days (Heitz et al., 1997).

The time course for M_2 acetylcholine receptors expression in Sf9 cells, as monitored by [^{3}H]quinuclidinyl benzilate binding, was consistent with the data above but the absolute density (B_{max}) in receptor sites was higher (2.6 ± 0.5 and 7.5 ± 0.3 pmol/mg protein at 3 and 6 dpi, respectively). Saturation binding isotherms were indicative of a homogeneous population of binding sites for both radioligands.

Thus, while production was maximal 6 days after infection, its estimation appeared to depend on the physico–chemical properties of the radioligand and, therefore, on its ability to interact with internalized receptor sites. The specific binding activities found here were consistent with that reported for [^{3}H]N-methylscopolamine binding (4 pmol/mg protein) to similarly expressed human muscarinic M_2 receptors (Rinken et al., 1994).

Crude membrane preparations from either 3 dpi- or 6 dpi-harvested Sf9 cells were then digitonin-solubilized (under the optimized conditions described for the m2 receptor subtype by Rinken et al., 1994) before affinity chromatography and adsorption on hydroxylapatite according to the protocol described by Haga et al. (1990).

Solubilization (60%) and purification (50%) yields assessed from [^{3}H]quinuclidinyl benzilate binding were in agreement with earlier reports for this system (Parker et al., 1991; Rinken et al., 1994; Kameyama et al., 1994).

3.2. Pharmacological properties of membrane-bound and affinity-purified M_2 receptors

Competition and saturation experiments were performed under equilibrium binding conditions for [^{3}H]quinuclidinyl benzilate (Table 1). Non-selective ([^{3}H]quinuclidinyl benzilate, atropine), m1 subtype-selective (pirenzepine), m2-selective (methoctramine, carbachol, AF DX-116) and m3-selective (*p*-F-HHSiD) compounds were compared for their binding affinity constants at membrane-bound and affinity-purified muscarinic M_2 receptors from Sf9 cells harvested 3 or 6 days after infection.

Prolonging the infection time (3 to 6 dpi) did not significantly affect the overall ligand binding properties of either membrane-bound or purified muscarinic receptor sites.

The receptor binding profile of membrane-bound [^{3}H]quinuclidinyl benzilate binding sites was in close agreement with that defined earlier for M_2 [^{3}H]N-methylscopolamine binding sites expressed in the same cells (Rinken, 1995; Dong et al., 1995; Heitz et al., 1997). This profile, when compared to those of the four other subtypes individually expressed in the same cells was indeed m2 in nature as shown by the low affinity of the m1-selective antagonist pirenzepine and the high affinity of AF-DX116 and methoctramine (m2-selective compounds) for this material.

Efficient solubilization with digitonin and cholate has been shown to affect differentially the binding properties of the five muscarinic acetylcholine receptors subtypes, m2 and m4 subtypes being the least affected (Rinken et al., 1994; Rinken, 1995). We now found that, indeed, even

Table 1
Binding affinities of muscarinic ligands for the membrane-bound, solubilized and purified m2 subtype from Sf9 cells

Ligand	pK_i values [a]					
	Literature data [b]		3 dpi [c]		6 dpi [c]	
	Membrane	Soluble	Membrane	Purified	Membrane	Purified
[^{3}H]QNB [d]	—	—	10.22	9.36	10.09	9.47
Atropine	9.41 (8.95)	9.02	8.28	8.00	8.33	8.27
Pirenzepine	6.63 (5.52)	7.19	6.14	5.73	6.14	6.53
Methoctramine	—	—	7.71	7.27	7.60	7.82
AF-DX116	7.05 (6.80)	6.03	—	—	—	6.06
p-F-HHSiD	7.16 (6.17)	7.75	—	—	—	7.06
Carbachol	4.92 (4.24)	4.18	3.81	3.26	3.50	—
$Me_2NArN_2^+$	—	—	—	4.33	—	4.44
$Bu_2NArN_2^+$	—	—	—	5.41	—	5.41

[a] pK_i values ($-\log K_i$) were calculated from displacement curves against 0.1 nM (membrane-bound state) or 1.5 nM (affinity-purified sites) [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) with corrections by equation of Cheng and Prusoff (1973), using corresponding K_d values. Values are means of two individual experiments carried out in duplicate.

[b] pK_i values were taken from Rinken (1995) and Dong et al. (1995, in parentheses) using [^{3}H]N-methylscopolamine as the radioligand for each preparation of m2 receptor subtype. Sf9 cells were harvested 3 days post-infection.

[c] Infected Sf9 cells were harvested either 3 or 6 days post-infection.

[d] pK_d values for [^{3}H]QNB were calculated from corresponding saturation binding curves.

after purification of the muscarinic M_2 receptor sites, the binding affinities of most of the ligands tested changed only slightly.

The muscarinic receptors expressed in Sf9 cells are thus endowed with ligand recognition and binding properties characteristic of the m2 subtype, whether membrane-bound or purified. The diazonium $Me_2NArN_2^+$ and $Bu_2NArN_2^+$ probes, when tested in the dark under reversible binding conditions, displayed identical binding affinity constants for 3 and for 6 dpi-purified muscarinic M_2 receptors.

These values were close to those previously obtained for similarly affinity-purified muscarinic receptors from

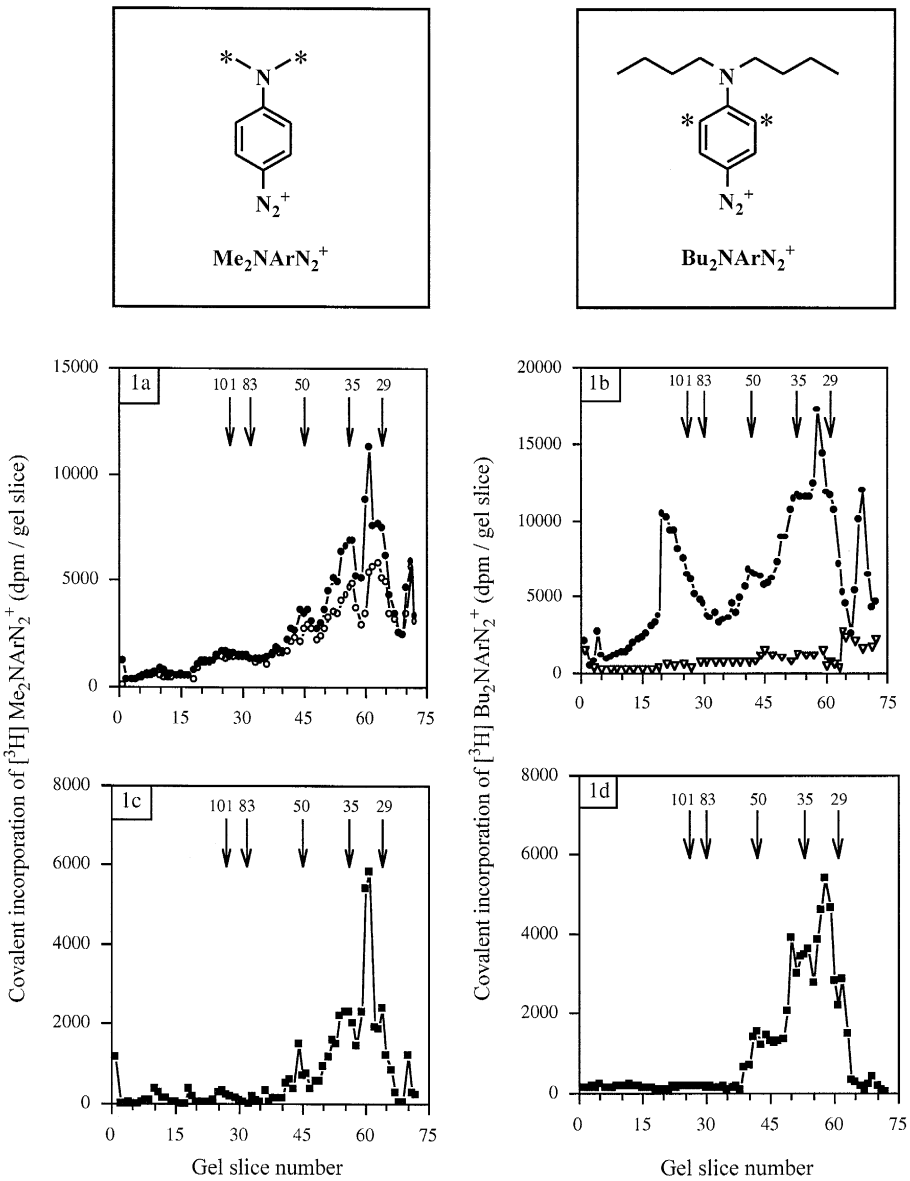

Fig. 1. Electrophoretic radioactive patterns for irreversible labelling by $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ of purified muscarinic M_2 acetylcholine receptors from 6-dpi Sf9 cells. Chemical structures of the $Me_2NArN_2^+$ and $Bu_2NArN_2^+$ aryldiazonium salts are shown. The position of the tritium atoms in the radiolabelled probes is indicated by an asterisk. 8 pmol of purified M_2 receptors from Sf9 cells harvested 6 days after infection was incubated with $[^3H]Me_2NArN_2^+$ (36 μM; 15.1 Ci/mmol) or with $[^3H]Bu_2NArN_2^+$ (15 μM; 13.2 Ci/mmol), used for the photolabelling protocol (unless otherwise stated) and finally analyzed by SDS-PAGE as indicated in Section 2. Prestained molecular weight protein markers were as follows: phosphorylase b (101 kDa), bovine serum albumin (83 kDa), ovalbumin (50 kDa), carbonic anhydrase (35 kDa), soybean trypsin inhibitor (29 kDa). (a) $[^3H]Me_2NArN_2^+$ photoincorporation into receptor samples preincubated in the absence (●, total binding) or the presence (○, non-specific binding) of 10 μM atropine. (b) Purified muscarinic M_2 receptors were incubated in the presence of $[^3H]Bu_2NArN_2^+$ and put through (●) or not (▽) to the irradiation step. Total incorporation of the probe is shown in both conditions. (c) Specific photolabelling profile of $[^3H]Me_2NArN_2^+$, defined as the difference between corresponding total and non-specific binding (as shown in (a) for $[^3H]Me_2NArN_2^+$), is presented. 1.2 pmol of [^{3}H]quinuclidinyl benzilate binding sites was recovered as alkylated sites. (d) Specific photolabelling profile of $[^3H]Bu_2NArN_2^+$ is presented. 2.2 pmol of [^{3}H]quinuclidinyl benzilate binding sites was alkylated by the probe.

porcine striatum (Autelitano et al., 1997), confirming the lack of subtype selectivity of these aryldiazonium derivatives.

3.3. Photoaffinity labelling of purified receptors from 6 dpi-Sf9 cells by $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$

$[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ had been found to label covalently purified muscarinic acetylcholine receptor sites from porcine striatum in an atropine-, probe concentration- and light-dependent manner (Autelitano et al., 1997).

Purified muscarinic M_2 receptors from 6 dpi-Sf9 cells were irradiated under similar conditions in the presence of $[^3H]Me_2NArN_2^+$ (36 μM; 1 K_i) or $[^3H]Bu_2NArN_2^+$ (15 μM; 4 K_i) and then analyzed by SDS-PAGE for covalent incorporation of the tritiated probes (Fig. 1).

Electrophoretic radioactivity profiles for total and non-specific irreversible labelling with $[^3H]Me_2NArN_2^+$ are superimposed on Fig. 1a. Calculation of the difference, defined as the specific or atropine-protectable incorporation profile of the probe, presented in Fig. 1c, shows a specific labelling efficiency (percentage of alkylated receptor sites) close to 15%.

Fig. 1b indicates that covalent incorporation of $[^3H]Bu_2NArN_2^+$ (and of $[^3H]Me_2NArN_2^+$, not shown) into the purified receptor material depended exclusively on UV light exposure (most radioactivity being washed out from the gel when the irradiation step was omitted).

While the two probes have very different total incorporation patterns (Fig. 1a and b), the specific irreversible labelling profile of $[^3H]Bu_2NArN_2^+$ (Fig. 1d) agreed closely with that of $[^3H]Me_2NArN_2^+$ (Fig. 1c), and a labelling efficiency close to 27% was calculated.

In another set of experiments, we also confirmed that the extent of specific alkylation of the purified muscarinic M_2 receptors depends on the concentration of tritiated probes (not shown).

Thus, while most of the above results were consistent with those expected for an efficient site-directed photolabelling process (light-, atropine- and probe concentration-dependence), the low apparent molecular mass of the specifically labelled protein material was unexpected.

The bulk of atropine-protectable $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ labelled material migrated over a large zone (30 to 50 kDa) while intact muscarinic M_2 receptor sites from Sf9 cells are expected to be recovered in a narrow gel band with a 55–59 kDa apparent molecular mass (Parker et al., 1991; Nakata et al., 1994; Kameyama et al., 1994).

It thus seemed possible that the human muscarinic M_2 receptors purified from the enriched Sf9 cells harvested 6 days after their infection by the recombinant baculoviruses, after their alkylation by the diazonium probes, behaved as fragmented muscarinic receptors.

To understand this extensive proteolytic process, we decided to test the structural integrity of the purified receptor preparations by using $[^3H]$propylbenzilylcholine mustard, a well-known affinity label whose reactivity does not require light activation.

3.4. $[^3H]$propylbenzilylcholine mustard affinity labelling of 3 and 6 dpi-purified receptor sites

We compared the electrophoretic patterns for 3 and 6 dpi-purified muscarinic M_2 receptor sites, affinity-labelled with $[^3H]$propylbenzilylcholine mustard (Fig. 2).

Specific incorporation of $[^3H]$propylbenzilylcholine mustard into purified muscarinic M_2 receptors originating from 6 dpi-Sf9 cells (Fig. 2a) was mostly found in a low molecular mass zone (27 to 35 kDa), as were $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ photolabelled receptor sites. Receptor damage was thus not linked to the irradiation step.

Interestingly, 3 dpi-affinity-labelled-muscarinic acetylcholine receptors (Fig. 2b) displayed the electrophoretic radioactivity profile expected for an intact muscarinic M_2

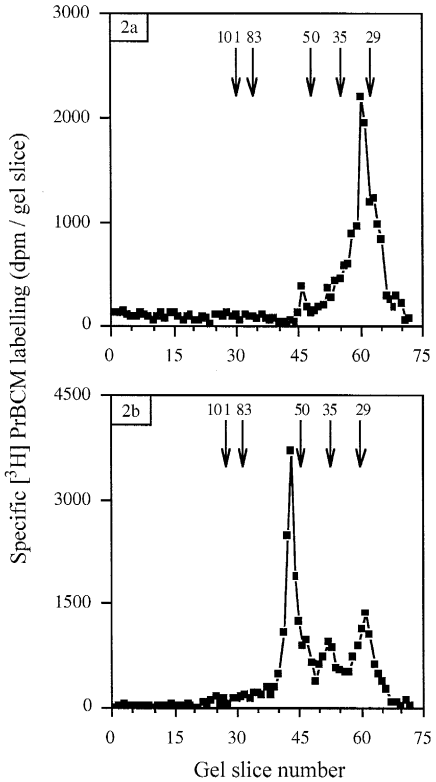

Fig. 2. Comparison of specific $[^3H]$propylbenzilylcholine mustard-labelling at 3 and 6-dpi purified M_2 receptors. Affinity-purified muscarinic M_2 receptors from Sf9 cells harvested 6 days (0.25 pmol, (a)) or 3 days (0.8 pmol, (b)) after infection were labelled with $[^3H]$propylbenzilylcholine mustard (475 nM or 100 nM, respectively) and analyzed by SDS-PAGE as described in Section 2. Specific irreversible incorporation of $[^3H]$propylbenzilylcholine mustard (defined as the difference between total and residual incorporation measured in the presence of 2 μM atropine) is shown in both cases and represented 0.08 and 0.15 pmol of alkylated receptor sites, respectively.

receptor protein (55–59 kDa) though some fragmented material was detectable. As seen by others (Nakata et al., 1994; Kameyama et al., 1994), the proportion of these minor proteolysed components varied from batch to batch (Fig. 2b and Fig. 3).

All these results indicated that prolonged infection time of Sf9 cells leads (i) to an increased expression of muscarinic M_2 receptor sites, (ii) to extensive proteolysis of the receptor protein (a fragment of up to 200 amino-acid residues might have been generated if one assumes a mean value of 23 kDa for a decrease in mass) and (iii) to a truncated receptor architecture retaining appropriate ligand properties.

Sf9 insect cells appear to have limited glycosylation capabilities (Jenkins et al., 1996). When compared to atrial muscarinic M_2 receptor sites (78 kDa; Peterson et al., 1984), the intact muscarinic receptor protein produced in the baculovirus-Sf9 expression system has an apparent molecular mass (55 kDa; Nakata et al., 1994; Kameyama et al., 1994) close to that calculated from the amino-acid composition of the protein (52 kDa; Kubo et al., 1986).

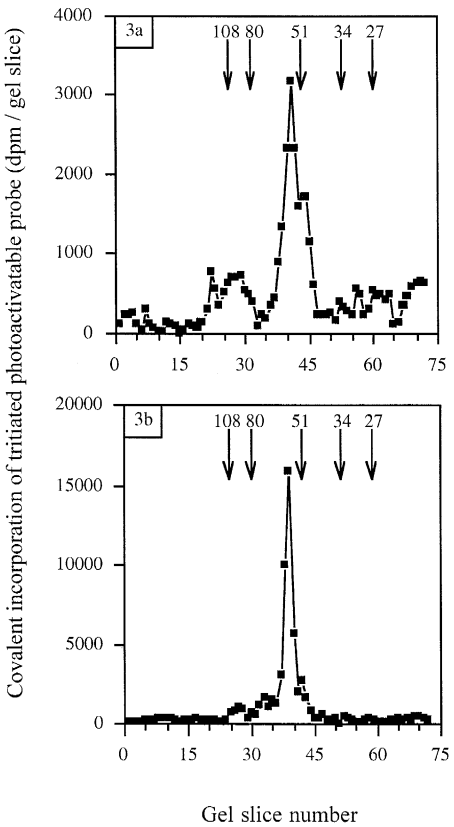

Fig. 3. Photolabelling of purified muscarinic M_2 receptors (from 3 dpi-Sf9 cells) by $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ and analysis on SDS-PAGE. (a) 24.8 pmol of purified muscarinic M_2 acetylcholine receptors was incubated with $[^3H]Me_2NArN_2^+$ (42 μM; 1.9 Ci/mmol) in the absence or the presence of 10 μM atropine, irradiated at 295 nm and put through to SDS-PAGE. Specific irreversible binding is shown and corresponds to 2.5 pmol of labelled receptors. (b) 34.3 pmol of purified muscarinic M_2 receptors was incubated with $[^3H]Bu_2NArN_2^+$ (15 μM; 2.7 Ci/mmol) and processed as described before. Specific irreversible labelling is shown and corresponds to 7.5 pmol of labelled receptors. Prestained molecular weight protein markers were as follows: phosphorylase b (108 kDa), bovine serum albumin (80 kDa), ovalbumin (51 kDa), carbonic anhydrase (34 kDa), soybean trypsin inhibitor (27 kDa).

3.5. Photolabelling of intact muscarinic M_2 receptors by $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$

In a last set of experiments, we photolabelled intact (3 dpi) purified muscarinic M_2 receptors with $[^3H]Me_2NArN_2^+$ (Fig. 3a) and $[^3H]Bu_2NArN_2^+$ (Fig. 3b) under the classical conditions described in Section 2. However, there were two differences: the specific radioactivity of the tritiated probes (which were isotopically-diluted for stability reasons; Autelitano et al., 1996) and the receptor concentration in the irradiated sample (which was increased by a factor of 3 to compensate for the decrease in radioactive signal).

Both probes specifically labelled a single protein species, migrating as a sharp and symmetrical peak of 55 kDa apparent molecular mass. Of the irradiated receptor sites (quantitated as specific $[^3H]$quinuclidinyl benzilate binding sites), 10% and 22% were specifically alkylated by $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$, respectively.

Thus, the two tritiated aryldiazonium photoactivatable ligands were shown to label intact and active human muscarinic M_2 acetylcholine receptors.

4. Discussion

Expression of G-protein-coupled receptors in insect cells using recombinant baculovirus is widely used and sometimes yields high expression levels of functional protein (Grisshammer and Tate, 1995; Tate and Grisshammer, 1996). The five subtypes of muscarinic receptors (m1–m5) were successfully expressed in Sf9 cells (Parker et al., 1991; Vasudevan et al., 1991; Berstein et al., 1992; Dong et al., 1995). Expression levels differ from one subtype to another (the m2 subtype showing the highest expression yield, up to 6 nmol/l) and ligand binding properties are very similar to those described for mammalian cells (Dong et al., 1995; Rinken, 1995).

Thus, muscarinic acetylcholine receptors overproduced in this system are invaluable tools for obtaining a clear pharmacological picture of each subtype (Dong et al., 1995; Rinken, 1995), for studying the molecular basis of their selective coupling with G-proteins (Parker et al., 1991; Berstein et al., 1992) as well as that of their phosphorylation and desensitization processes (Richardson et al., 1993; Nakata et al., 1994; Kameyama et al., 1994) and ultimately for the structural analysis of ligand and G-protein binding domains of these subtypes.

As much similar work had been done on the human muscarinic M_2 receptor expressed in Sf9 cells, and information was thus available, we chose this receptor subtype for our photoaffinity labelling study.

The time course of expression of human muscarinic M_2 receptors in Sf9 cells was first examined, as a high production level and a high purification yield are both required for structural analysis. Maximal expression in Sf9 cells occurred 6 days after infection, with a level about 3 times higher than that found 3 days post-infection.

These findings were consistent with published data (Heitz et al., 1997), but contrasted with results reported for the expression of β-adrenoceptors in the same system (Parker et al., 1991) where maximal expression was observed 2–3 days after infection. Differences in the transfer plasmid, in the titration of recombinant baculoviruses and/or in the multiplicity factor of infection are likely explanations for such variations in the expression time course.

However, the ligand binding properties of membrane-bound or affinity-purified m2 subtype receptor sites, whatever their expression time in Sf9 cells, were quite similar to those reported for the human muscarinic M_2 receptor (expressed in Sf9 cells harvested 3 days post-infection), either in its native membrane environment (Rinken, 1995; Dong et al., 1995) or after solubilization (Rinken et al., 1994; Rinken, 1995). It is noteworthy that solubilization and purification yields of active receptor protein were not significantly modified when infection of the cells was prolonged.

Thus, since both receptor preparations were apparently undistinguishable on the basis of their ligand binding properties, the 6 dpi-cells appeared to be preferable for their higher expression level for muscarinic M_2 receptors.

Surprisingly, muscarinic M_2 receptors specifically alkylated either by the two photoaffinity probes $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ or by the affinity label [^{3}H]propylbenzilylcholine mustard, when put through a strongly dissociating procedure, i.e., SDS-PAGE, showed an electrophoretic behavior which was very different for the two receptor preparations. Purified muscarinic M_2 receptors (and membrane-bound receptors alkylated by [^{3}H]propylbenzilylcholine mustard, not shown) from 6 dpi-cells displayed a severe fragmentation as most labelled peptides were in the 30–40 kDa molecular mass range. Proteolysis, which could not be prevented by the addition of protease inhibitors during membrane preparation or solubilization, probably occurred before cell harvesting.

It had been reported earlier, regarding β-adrenoceptors expression in the same baculovirus-Sf9 system (Parker et al., 1991), that after 2–3 days infection time, significant receptor proteolysis occurred without loss in binding activity.

Muscarinic M_2 receptors (even from 3 dpi-Sf9 cells) have also been shown to be susceptible to proteolysis as a minor 39 kDa-[^{3}H]propylbenzilylcholine mustard-labelled component (32 kDa after glycanase treatment) appeared after SDS-PAGE analysis of the receptor protein (Nakata et al., 1994; Kameyama et al., 1994).

This latter fragment, which probably corresponds to the radioactive peak at 32 kDa we found after affinity or photoaffinity labelling, was identified as the N-terminal part of the muscarinic M_2 receptor protein, including part of its third intracellular loop (Nakata et al., 1994; Kameyama et al., 1994). As we also found lower molecular mass alkylated peptides, one may suppose that additional fragmentation probably occurred within this receptor region.

It is also important to note that the presence of specifically [^{3}H]propylbenzilylcholine mustard-labelled peptides indicates that the third transmembrane segment of the receptor protein is present. This segment is known to contain an aspartate residue alkylated by this affinity label on the m1 subtype (Curtis et al., 1989; Kurtenbach et al., 1990).

The low molecular mass fragments of the 6 dpi-muscarinic M_2 receptor are indeed labelled by [^{3}H]propylbenzilylcholine mustard, $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$, but this does not imply that they are intrinsically able to bind muscarinic ligands. Extensive trypsinolysis of the membrane-bound β-adrenoceptors (Rubenstein et al., 1987) and coexpression in mammalian cells of polypeptide pairs generated by splitting the beta2-adrenergic (Kobilka et al., 1988) or the muscarinic M_2 and M_3 receptors (Maggio et al., 1993; Schöneberg et al., 1995), may lead to functional receptors that can interact with both ligands and G-proteins.

These findings, as do ours, suggest that covalent connection between the helices of these bioamine receptors is not essential for proper arrangement of the transmembrane receptor core, where the binding of small ligands like acetylcholine is thought to occur (Trumpp-Kallmeyer et al., 1992; Wess, 1993; Ballesteros and Weinstein, 1995).

Moreover, the fact that reversible and irreversible binding properties of muscarinic ligands at truncated muscarinic M_2 receptors persist, even after the drastic change in conditions as a result of solubilization and purification procedures, indicates that transmembrane helices, once inserted in the lipid bilayer, interact with each other to form a fairly stable functional protein complex.

One approach for structural analysis of the m2 receptor ligand binding domain, i.e., obtaining constitutive and topological information on the amino-acid residue(s) involved in agonist and antagonist binding, is through site-directed photolabelling of the receptor site followed by identification of the radiolabelled residues and their localization on the receptor primary sequence. This strategy requires, however, high amounts of intact and functional receptor protein.

The two aryldiazonium photoactivatable derivatives we used may be regarded as efficient topographical markers, endowed with high and non-discriminative reactivity towards all kind of amino-acids (Kotzyba-Hibert et al., 1995). As a direct consequence, $[^3H]Me_2NArN_2^+$ has been shown to alkylate several different residues belonging to the acetylcholine binding site of acetylcholinesterase (Harel

et al., 1993; Schalk et al., 1994) and of the nicotinic receptor (Dennis et al., 1988; Galzi et al., 1990).

Our previous work indicating that $Me_2NArN_2^+$ and $Bu_2NArN_2^+$ behaved as efficient photolabels of membrane-bound (Ilien et al., 1989; Ilien and Hirth, 1989), solubilized (Ilien and Hirth, 1991) and purified (Autelitano et al., 1997) muscarinic acetylcholine receptors from brain tissues, was now extended to show that both probes may be used as site-directed alkylating reagents of the recombinant m2 receptor subtype. Since they are known to be non-subtype-selective (Autelitano et al., 1997), one may suppose that these probes can be used for each of the five muscarinic acetylcholine receptor subtypes.

The $[^3H]Me_2NArN_2^+$- and $[^3H]Bu_2NArN_2^+$-specifically labelled protein, purified from 3 dpi-Sf9 cells, migrated in SDS-PAGE as a unique, narrow and almost symmetrical radioactive peak corresponding to a molecular mass of 55 kDa. Such characteristics are typical of an intact muscarinic M_2 receptor protein (Parker et al., 1991; Nakata et al., 1994; Kameyama et al., 1994), probably poorly glycosylated, a feature generally encountered for most G-protein coupled receptors expressed in this baculovirus-Sf9 system (Jenkins et al., 1996; Tate and Grisshammer, 1996).

Finally, we found it interesting to compare photolabelling data for purified muscarinic M_2 receptors, from either 3 dpi (intact receptors) or 6 dpi (fragmented protein) Sf9 cells and to note that labelling efficiencies, at similar probe concentrations, were comparable.

Moreover, while electrophoretic radioactivity profiles for 3 dpi-purified receptors specifically alkylated with [^{3}H]propylbenzilylcholine mustard, $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$ were superimposable, as expected for a single entire protein, the profiles obtained for irreversible labelling of proteolysed receptors (6 dpi) differed according to the probes used. The radioactive pattern, in the 30–50 kDa mass range, was more complex when the tritiated aryldiazonium salts (Fig. 1c, d) were used than for the affinity label (Fig. 2a), so that peak components with variable width and height could be distinguished.

We speculate that this observation could reflect a greater diversity and/or frequency of labelled amino-acid residues when $[^3H]Me_2NArN_2^+$ and $[^3H]Bu_2NArN_2^+$, two probes already known as topographical markers for cholinergic binding sites (Kotzyba-Hibert et al., 1995) are used; in contrast, the [^{3}H]propylbenzilylcholine mustard labelling pattern is restricted to a unique Asp residue (Curtis et al., 1989; Kurtenbach et al., 1990).

More definitive answers should be expected from peptide mapping studies, performed on photoaffinity- and affinity-labelled intact muscarinic M_2 receptors after specific enzymatic and/or chemical cleavage.

Together, our results produced evidence that, when the Sf9-baculovirus system is used for the overexpression of membrane receptors, as done here for the muscarinic receptors, pharmacological controls (density of receptor sites and drug binding affinity measurements) are not the only requisite. Molecular characterization of the receptor (through site-directed labelling) has to be performed in order to assess the quality of the expressed protein.

Once these conditions are fulfilled, structural and topological analysis of a receptor protein can be undertaken under suitable conditions.

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