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SOLUBILIZATION OF MUSCARINIC RECEPTOR SUBTYPES FROM BACULOVIRUS INFECTED *Sf*9 INSECT CELLS

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Abstract—Five different subtypes (human m1, m2, m5 and rat m3, m4) of muscarinic acetylcholine receptors (mAChR) were produced in insect *Sf*9 cells by infection with recombinant baculoviruses. *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) has a similar affinity to each of these mAChR subtypes in cell membranes, while pirenzepine, 11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido-(2,3-b)(1,4)benzo-diazepin-6-on (AF-DX 116) and (±)-*p*-fluoro-hexahydrosiladifenidol (p-F-HHSiD) have a higher affinity for m1, m2 and m3, respectively, than for the other subtypes, indicating the maintenance of subtype specificity of mAChR in this system. Digitonin (1%, w/w) with sodium cholate (0.1%, w/w) solubilized 51% of m1, 36% of m2, 3% of m3, 28% of m4 and 17% of m5 mAChR from these cell membranes with retention of the [^{3}H]NMS binding activity. Optimization of cholate concentrations resulted in solubilization of up to 50–60% for m1, m2 and m4, but up to 25% for m5 and 7% for m3. Optimal concentrations of cholate differed from one subtype to another. Sucrose monolaurate solubilized 21–43% of m1, m2 and m4, but only up to 12% for m5 and 2% for m3. 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS) was practically ineffective in mAChR solubilization from *Sf*9 cell membranes for all subtypes investigated. Solubilization with digitonin and cholate had little influence on [^{3}H]NMS affinity for m2 and m4, but decreased m1 and m5 affinity by 10-fold and that of m3 by more than 50-fold. These results indicate that the solubility and stability of mAChR in detergents differ among the subtypes, in spite of their structural similarities. These differences should be taken into account when comparing the five subtypes, particularly when determining the proportion of each subtype in a given tissue by precipitating the solubilized mAChR with subtype-specific antibodies.

Key words: muscarinic receptors; solubilization; subtype-specific solubilization; receptor subtypes; *Sf*9 insect cells; detergents

Four different subtypes of mAChR§ (M1–M4) have been defined by their different ability to bind specific muscarinic ligands [1, 2]. Thus, pirenzepine and telenzepine have a high affinity for M1, AF-DX 116, himbacine, methoctramine and gallamine for M2, and HHSiD and p-F-HHSiD for M3. Tropicamine has been proposed to be specific for M4 mAChR [3, 4]. Molecular cloning studies have identified five distinct mAChR genes (m1–m5) widely expressed in brain and peripheral tissues. m1–m4 correspond to pharmacologically defined M1–M4 mAChR, whereas the ligand-binding properties of m5 have not been characterized *in vivo* [1, 2, 5]. These subtypes are specifically coupled with specific effector systems: m1, m3 and m5 initiate phosphatidylinositol hydrolysis, while m2 and m4 cause inhibition of adenylate cyclase [6, 7].

For biochemical characterization, mAChR are usually solubilized and purified in digitonin or in a mixture of digitonin and sodium cholate. By using these detergents, more than 50% of mAChR can be solubilized with retention of ligand-binding activity [8, 9]. Sucrose monolaurate, dodecyl-β-D-maltoside and CHAPSO have also produced high yields (up to 80%) in mAChR solubilization, but the stability of ligand-binding activity of these preparations was lower than when digitonin was used [10–12]. The best yields in mAChR solubilization have been achieved with atrial membranes (mostly m2); using brain tissues (mixture of m1–m4) resulted in lower yields. Systematic data about solubilization of mAChR from other tissues do not appear to have been published. Solubilization of membrane proteins with retention of their activity usually depends on protein structure and the protein:detergent ratio, the lipid composition of the membranes and the lipid:detergent ratio during solubilization [13–15]. To determine the role of the structure of receptor proteins in maintaining an active conformation for ligand-binding of mAChR, the solubilization of different subtypes from similar cell cultures were studied. Considerable differences in the solubilities

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§ Abbreviations: mAChR, muscarinic acetylcholine receptor; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine; AF-DX 116, 11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepin-6-on; p-F-HHSiD, (±)-*p*-fluorohexahydrosiladifenidol; CHAPS, 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate; KPB, 20 mM K-phosphate buffer (pH 7.5).

of different subtypes were found, indicating an important role of receptor structure in maintaining its active conformation. The results help to explain several discrepancies between biochemical and pharmacological results in studies of mAChR.

MATERIALS AND METHODS

Materials. [^{3}H]NMS (79.5 Ci/mmol) was obtained from New England Nuclear (Du Pont de Nemours, Germany), scintillation solution Ecoscint A from National Diagnostics (U.K.), digitonin from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Wako (Japan), and CHAPS from Calbiochem (La Jolla, U.S.A.); sucrose monolaurate (SM-1200) was kindly donated by Mr Hajime Machida (Mitsubishi-Kasei Food Co., Japan).

Recombinant baculoviruses. Baculoviruses containing cDNA for human m1 or m2 subtypes were donated by Dr E.M. Ross (University of Texas, Southwestern Medical Center at Dallas, U.S.A.); rat m3 and m4 cDNAs were obtained from the late Dr S. Numa (Kyoto University, Japan) and human m5 subtype from Dr T. I. Bonner (N.I.H., U.S.A.). Recombinant DNA was manipulated according to standard protocols, as described elsewhere [35]. Baculoviruses which contained cDNAs for m3, m4 and m5 subtypes were constructed as follows. A 1.8 kb m3 or a 1.6 kb m4 cDNA, obtained by digestion with EcoRI of plasmids pKRM3 or pKRM4, respectively, were ligated into the EcoRI site of a transfer vector pVL1392. A plasmid HM5pCDp2 containing the m5 cDNA was treated in two ways: one sample was digested with BstNI, blunted and then digested by BglII, resulting in a 0.2 kb fragment; the other sample was digested by BglII and PstI, resulting in a 1.5 kb fragment. These two fragments were ligated into pVL1392, which had been digested with SmaI and PstI. The transfer vectors containing cDNA of mAChR were co-transfected with wild type AcNPV DNA by a calcium phosphate method [35]. Positive recombinant viral clones were isolated by plaque assay and checked for their ability to direct the expression of mAChR subtype as revealed by [^{3}H]QNB binding activity. Southern blotting analysis of each recombinant viral DNA was also carried out.

Sf9 *cell culture and membranes.* The *Sf*9 cells were grown in a medium using IPL-41-Insect Medium (JRH Biosciences, U.S.A.); 2% fetal calf serum and 0.1% Pluronic F68 (a detergent from BASF Chemicals, Holland) were added immediately before use. *Sf*9 cells were cultured in a 3 L spinner bottle (1 L medium) with aeration at 28°, recombinant viruses were proliferated in a small spinner bottle (250 mL) in 100 mL medium. The latter was added to the cell culture ($\sim 2 \times 10^9$ *Sf*9 cells/bottle) and incubation continued for 60 hr. The suspension was centrifuged at 1500 *g* for 10 min and the pellet homogenized in 100 mL buffer solution (20 mM Na–HEPES, pH 7.0, 2 mM $MgCl_2$, 1 mM EDTA) using a Potter-type homogenizer. The suspension was centrifuged at 40,000 *g* for 20 min and the pellet homogenized in the 10–50 mL buffer solution and stored at −80°.

Solubilization of mAChR. For solubilization, the thawed membranes were diluted in 20 mM KPB, pH 7.5, 5 mM imidazole, 1 mM EDTA to a protein concentration of 0.7–1.2 mg/mL, incubated with different concentrations of detergents for 30 min at 4° and centrifuged at 100,000 *g* for 90 min at 4°. The supernatant fraction was used as a solubilized fraction. The binding activity of mAChR was determined by the specific binding of [^{3}H]NMS (10 nM, incubation for 60 min at 30° or 24 hr at 4°). Non-bound ligands were removed by gel chromatography on Sephadex G-50 (fine) or by filtration through a glass-fibre filter (A/E, Gelman Sciences Inc. (Ann Arbor, MI, U.S.A.)), for solubilized and membrane-bound mAChR, respectively, as described elsewhere [16]. Specific binding was defined as the difference between total and non-specific binding, which were measured in the absence and presence of 1 μM atropine, respectively. Protein concentrations were determined by the modified Lowry method [17], using BSA as standard.

Ligand binding to mAChR. The crude membrane homogenates in 20 mM KPB, 1 mM EDTA, or solubilized preparations in the same buffer with detergent, were incubated with [^{3}H]NMS (0.1–15 nM) or, in the case of displacement experiments, with [^{3}H]NMS (2 nM) and other ligands for 1 hr at 30°. Free ligands were removed by fast filtration through glass-fibre filters or by gel-filtration at 4° as described above. All binding data were analysed by non-linear least-squares regression analysis using a standard programme, GraphPAD in Plot (GraphPAD Software, San Diego, CA, U.S.A.).

RESULTS

Expression of mAChR in Sf9 *cells*

Five different mAChR subtypes (human m1, m2, m5 and rat m3, m4) were expressed in *Sf*9 cells using the baculovirus infection system. Up to 0.5 (m1), 5 (m2), 3 (m3), 1 (m4) and 0.5 (m5) nmol receptors, determined by specific binding of [^{3}H]NMS, were produced by 1 L of culture. The corresponding specific activities of [^{3}H]NMS binding were 0.6, 4, 16, 2.5 and 0.8 pmol/mg protein for m1, m2, m3, m4 and m5 subtype, respectively.

Ligand-binding characteristics of mAChR subtypes expressed in Sf9 *cells*

[^{3}H]NMS binding to all subtypes of mAChR in *Sf*9 cell membranes showed high affinity and was saturable with similar K_d values (60–120 pM) (Table 1) and with Hill coefficients close to unity. The displacement curves of [^{3}H]NMS binding by muscarinic antagonists atropine, pirenzepine, AF-DX 116, p-F-HHSiD and an agonist carbachol fitted ($P < 0.05$) the equation derived, assuming that each ligand binds to the receptor with homogeneous affinity in the case of all subtypes of mAChR. The calculated K_i values are listed in Table 1. Atropine bound without preference to any subtype, while pirenzepine had a considerably higher affinity for m1 (Fig. 1a) and AF-DX 116 for m2 (Fig. 1b), but p-F-HHSiD had only moderate specificity for m3 (Fig. 1c). All these data are in good agreement with published data for mAChR subtypes in different tissues [3, 4] or in mammalian cultured cell lines

Table 1. Constants of ligand binding to the muscarinic receptor subtypes expressed in *Sf*9 cells

Ligand	Subtype of mAChR				
	m1	m2	m3	m4	m5
[^{3}H]NMS K_d* (pM)	79 ± 8	81 ± 12	112 ± 10	62 ± 8	93 ± 12
Atropine, K_i† (nM)	0.74 ± 0.02	0.39 ± 0.02	0.99 ± 0.06	0.37 ± 0.02	0.51 ± 0.04
Carbachol, K_i† (μM)	149 ± 6	12 ± 2	123 ± 8	73 ± 4	57 ± 6
Pirenzepine, K_i† (nM)	10 ± 4	236 ± 7	360 ± 7	288 ± 7	194 ± 6
AF-DX 116, K_i† (μM)	2.2 ± 0.3	0.089 ± 0.003	8.0 ± 0.3	1.6 ± 0.1	3.7 ± 0.4
pFHHSiD, K_i† (nM)	19 ± 1	69 ± 1	9.4 ± 0.1	41 ± 1	21 ± 2

* K_d for [^{3}H]NMS was calculated from corresponding binding curves.

† K_i were calculated from displacement curves against 2.2 nM [^{3}H]NMS with correlations by equation of Cheng–Prusoff: $K_i = IC_{50}/(1 + [L]/K_d)$ using corresponding K_d values for every subtype.

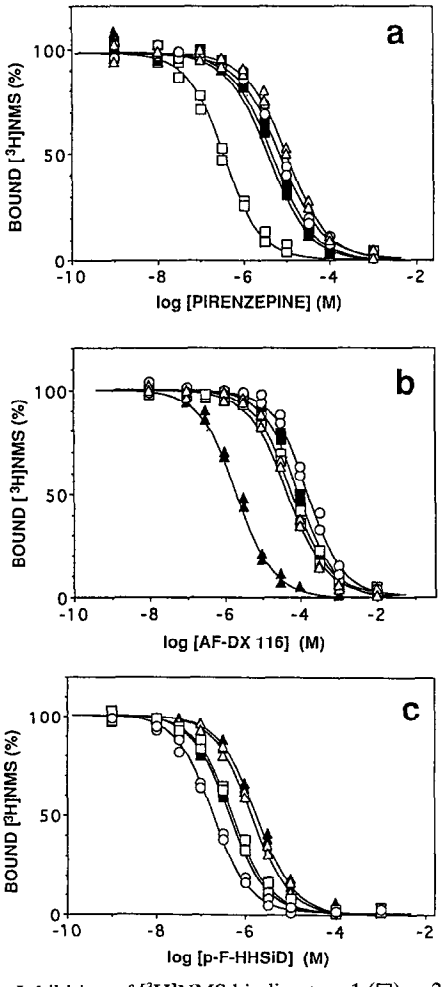

Fig. 1. Inhibition of [^{3}H]NMS binding to m1 (□), m2 (▲), m3 (○), m4 (△) and m5 (■) mAChR by pirenzepine (a); AF-DX 116 (b); p-F-HHSiD (c). Different concentrations of the ligands were incubated with membranes in the presence of 2.2 nM [^{3}H]NMS for 90 min at 30°. Binding of [^{3}H]NMS is presented as a percentage of the specific binding in the absence of the competing ligand. Curves are representative at least two independent experiments carried out in duplicate.

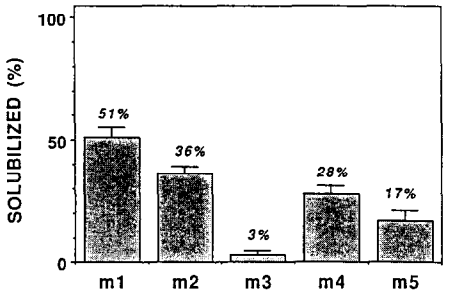

Fig. 2. Solubilization of mAChR subtypes from *Sf*9 cell membranes by 1% digitonin (w/w) and 0.1% sodium cholate (w/w). The amounts of solubilized mAChR in the supernatant fractions were determined by measuring the specific binding of [^{3}H]NMS (90 min, 30°) and expressed as percentage of membrane-bound mAChR, which were assessed under the same incubation conditions. Data are presented as average of three experiments carried out in duplicate.

[18]. Binding of carbachol did not depend on the absence or presence of 0.1 mM GTP (data not shown) and corresponded to the low-affinity binding to membrane mAChR, as described earlier [20].

Solubilization of mAChR subtypes from Sf9 *cell membranes*

A mixture of 1% digitonin with 0.1% sodium cholate has been widely used for solubilization of mAChR. Using this mixture for *Sf*9 cell membranes, up to 51% of m1 mAChR was solubilized, whereas the yields of the other subtypes were considerably lower (Fig. 2). Optimization of solubilization conditions by changing detergent concentrations indicated that yields did not vary with digitonin concentration from 0.4 to 2% (w/w) and that there was no difference between digitonins from Sigma and Wako (data not shown). On the other hand, solubilization yields depended to a great extent on the concentration of sodium cholate. This dependence was different for different subtypes of mAChR (Fig. 3a). Among the subtypes studied, m1 achieved its maximal solubilization (55%) at 0.15–0.2% (w/w) sodium cholate and an increase in bile acid concentration led to a decrease in solubilization.

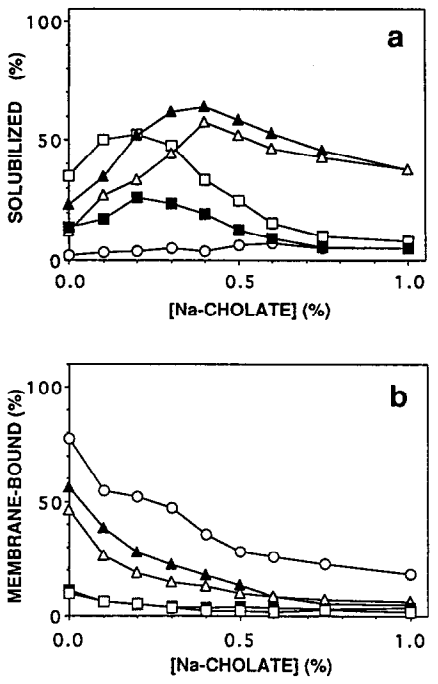

Fig. 3. Effect of concentration of sodium cholate on solubilization of m1 (□), m2 (▲), m3 (○), m4 (△) and m5 (■) mAChR in 1% digitonin. Membranes (0.7–1.2 mg protein/mL) were incubated with different concentrations of detergent and the amount of solubilized mAChR in the supernatant fraction (a) and membrane-bound mAChRs in the whole incubation medium (b) were determined by specific binding of [^{3}H]NMS (90 min at 30°) and expressed as a percentage of the binding with membranes without detergent. Data are the average of two experiments carried out in duplicate.

Similar behaviour was also noted in the case of m5, but the maximal yield was lower (26%). m2 and m4 required higher concentrations of sodium cholate (0.4% w/w) than m1 and m5 to achieve maximal solubilization (63 and 57%) (Fig. 3a). m3 required still higher concentrations of sodium cholate, but only up to 7% of [^{3}H]NMS binding activity was obtained with 1% digitonin and 0.6% sodium cholate. An additional increase of cholate in solution did not increase the solubilization of m3 mAChR (Fig. 3a). At the same time, the specific [^{3}H]NMS binding activity of m3 bound to membrane aggregates, trapped in the glass-fibre filters, was high at a relatively high concentrations of sodium cholate (Fig. 3b), indicating that the very low solubilization of m3 was caused by its complicated integration with membrane structures. m1 and m5 disappeared from the membranes with digitonin only, whereas the disappearance of m2 and m4 corresponded quite well to their appearance in solution (Fig. 3). In all these cases [^{3}H]NMS binding to mAChR was determined at 30°, but incubation at 4° for 24 hr gave similar results (data not shown). Thirty to 50% of bulk protein was solubilized with digitonin/cholate from *Sf*9 cell membranes, without considerable differences between cells expressing different subtypes of mAChR.

In comparison with previous data, the sucrose monolaurate was less effective in solubilizing mAChR from *Sf*9 cell membranes. Up to 21% m1, 32% m2, 2% m3, 43% m4 and 12% m5 mAChR were solubilized with retention of [^{3}H]NMS binding activity at 4° at the narrow concentration interval (0.05–0.2%, w/w) of sucrose monolaurate. Determination of [^{3}H]NMS binding activity at 30° revealed considerably lower yields due to the instability of solubilized mAChR at higher temperatures (data not shown), as was also the case with atrial membranes [10]. The solubilization of bulk protein (35–55%) was similar to that of digitonin/cholate, but considerably lower than in the case of atrial (90%) and cortical (80%) membranes [10].

Up to 15% of the [^{3}H]NMS binding activity of m2 was obtained in the supernatant fraction with 0.4% (w/w) of CHAPS. However, this solubilization could only be termed "apparent", as no loss of [^{3}H]-NMS binding activity from membranes, and all binding sites from supernatant fraction trapped in glass-fibre filter during the filtration assay, could be determined. Further increases in CHAPS concentration led to a loss of [^{3}H]NMS binding activity both in the supernatant and the membrane fraction (data not shown). In the case of other mAChR subtypes CHAPS did not even give a reasonable "apparent" yield of mAChR solubilized from *Sf*9 cell membranes.

Binding of muscarinic ligands to solubilized mAChR subtypes

For the ligand-binding studies the mAChR were solubilized from *Sf*9 cell membranes with 1% digitonin at sodium cholate concentrations which gave maximal solubilization for each subtype (0.2% for m1, 0.4% for m2, 0.6% for m3, 0.4% for m4 and 0.2% for m5). The curves of [^{3}H]NMS binding to solubilized mAChR are shown in Fig. 4a. The straight lines obtained by Scatchard analyses (Fig. 4b) indicate the presence of a single class of [^{3}H]NMS binding sites in the case of all subtypes of mAChR studied. The binding constants are listed in Table 2. [^{3}H]NMS bound with a high affinity to solubilized m2 and m4 mAChR, similar to the values obtained for membrane-bound mAChR. In the case of m1 and m5, solubilization decreased the affinity up to 10 times, and in the case of m3 more than 50 times. In separate experiments it was found that different concentrations of sodium cholate did not cause essential differences in [^{3}H]NMS binding affinities. These losses in binding affinities were taken into account when solubilization yields were recalculated. Similar changes in binding affinities were found for atropine and carbachol in displacement experiments with [^{3}H]NMS (Table 2).

DISCUSSION

The baculovirus expression system has been proposed as an excellent method for large-scale production and purification of G-protein-coupled receptors, including mAChR subtypes [20]. In the case of mAChR, this system revealed a relatively

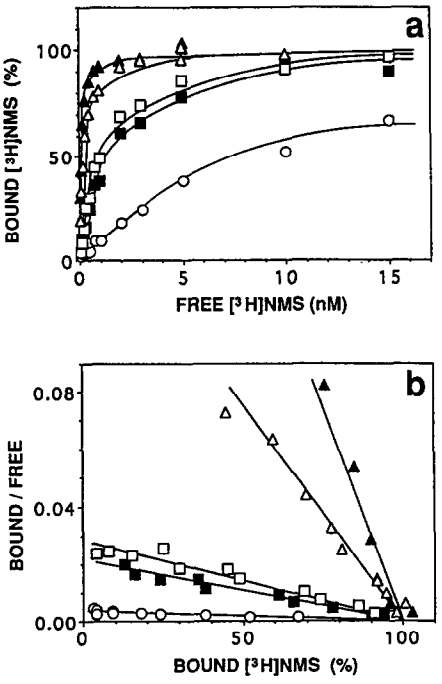

Fig. 4. Equilibrium binding of [³H]NMS to m1 (□), m2 (▲), m3 (○), m4 (△) and m5 (■) mAChR solubilized from *Sf*9 cell membranes (a) linear least-squares fit of these data in the Scatchard plot (b) (data from one typical of three independent experiments). Preparations of solubilized receptors were incubated with different concentrations of ³H]NMS in the absence (total binding) and presence (non-specific binding) of 1 μM atropine for 90 min at 30°. Specific binding was defined as the difference of total and non-specific bindings. Data are normalized against B_{max} values, calculated by non-linear least-squares analysis for each subtype; the values were 0.62, 3.53, 1.12, 1.51 and 0.34 pmol [³H]NMS binding sites/mg protein for m1, m2, m3, m4 and m5 subtypes of mAChR, respectively.

high level of expression of m1–m5 subtypes (0.5–5 nmol/L culture) which retained ligand-binding specificity (Table 1). This system was selected for investigation of the solubilization of different subtypes of mAChR to elucidate the role of receptor protein structure on its solubility. Using the same cell line the same membrane composition may be assumed, eliminating the influence of other membrane components (e.g. lipids, proteins) on the solubility of different subtypes of mAChR. Of the detergents that have been reported to solubilize mAChR (for review see [10]), digitonin/sodium cholate, sucrose monolaurate and CHAPS were used as representatives of three different groups of detergents [14, 15]. The digitonin/sodium cholate system best solubilized mAChR from *Sf*9 cell membranes for all subtypes. Similar conditions resulted in very different solubilization yields for different subtypes of mAChR (Figs 2 and 3).

It may be assumed that the yield of solubilized receptors with ligand-binding activity is determined by two factors: stability in a detergent solution and integration with other membrane components. In this context, mAChR subtypes can be divided into three groups: m1 and m5, m2 and m4, and m3. The m1 and m5 subtypes seem to be most labile in detergent solutions. Their ligand-binding activity disappeared from membrane fractions by treatment with digitonin alone and the maximal yield of solubilized receptors was attained at relatively low cholate concentrations (0.1–0.2%). The 10-fold reduction of affinities of solubilized m1 and m5 subtypes for muscarinic ligands indicates that the solubilization of these receptors is accompanied by conformational changes. On the other hand, the m2 and m4 subtypes of mAChR seemed to be more stable in detergent solutions than the m1 and m5 subtypes. The ligand-binding activity of the former was largely retained in digitonin solution, and the maximal yields of solubilized receptors were attained with higher concentrations of cholate (~0.4%). In addition, the affinities of solubilized m2 and m4 subtypes for muscarinic ligands were not significantly different from those of membrane receptors. The m3 subtype apparently differs from the other subtypes in that the yield of solubilized receptors with ligand-binding activity was less than 7% in digitonin/sodium cholate and 2% in sucrose monolaurate. The m3 subtype may either be more tightly integrated with other membrane components than the other subtypes or be more labile. The first hypothesis is suggested by the finding that the ligand-binding activity of m3 remains bound in membrane fractions even in the presence of 1% digitonin and 1% sodium cholate, whereas no ligand-binding activity was observed in membrane fractions for the other subtypes under the same conditions. On the

Table 2. Constants of ligand binding to the muscarinic receptor subtypes solubilized from *Sf*9 cell membranes

Ligand	Subtype of mAChR				
	m1	m2	m3	m4	m5
[³H]NMS K_d* (pM)	930 ± 80	95 ± 9	8500 ± 400	200 ± 30	1400 ± 110
Atropine, K_i† (nM)	3.45 ± 0.08	0.95 ± 0.07	61 ± 5	0.23 ± 0.05	5.25 ± 0.25
Carbachol, K_i† (μM)	553 ± 26	66 ± 4	1880 ± 120	36 ± 3	450 ± 30

* K_d for [³H]NMS was calculated from corresponding binding curves.

† K_i were calculated from displacement curves against 2.2 nM [³H]NMS with correlations by equation of Cheng–Prusoff: $K_i = IC_{50}/(1 + [L]/K_d)$ using corresponding K_d values for every subtype.

other hand, the fact that the affinities of solubilized m3 subtypes for muscarinic ligands were 100-fold lower than those of membrane receptors suggests that m3 undergoes a large conformational change resulting in the loss of ligand-binding activity for a substantial fraction of solubilized receptors. The third intracellular loop of m3 is the longest among the five subtypes, and that of m5 the second longest [1, 5]. It would be interesting to determine if the large size of the third intracellular loop is responsible for the low yields of solubilized m3 and m5 subtypes.

The solubilization yield of the m2 subtype with sucrose monolaurate from *Sf*9 cell membranes was lower than that for mAChR from atrial membranes (mostly m2) [10]. Thus, components other than receptor proteins may affect the solubilization of mAChR with retention of [^{3}H]NMS binding activity. On the other hand, the large differences in solubility of different mAChR subtypes in sucrose monolaurate were similar to those in digitonin.

Using CHAPS, only an "apparent" solubilization of the m2 subtype of mAChR was found; it was "apparent" in that receptors in the supernatant fraction were trapped in the glass-fibre filter (pore size 1 μm). In this case the first criterion of receptor solubilization (lack of sedimentation in low density media (100,000 g, 60 min) was fulfilled, but the second (no retention on small pore size filters) [14, 22] was not. Further increases in CHAPS concentration lead to a loss of [^{3}H]NMS binding activity in both supernatant and membrane fractions (data not shown). However, solubilization by CHAPS must also be termed "apparent" in these cases, as the binding activity of receptors was determined only by a direct filtration assay of the supernatant fraction [23–25].

The large differences in the solubilities of different subtypes of mAChR should be taken into account when the five subtypes are compared. This consideration is particularly important when the proportion of receptor subtypes in a given tissue is assessed after solubilization. In an increasing number of studies antibodies for different mAChR subtypes are used to determine the regional distribution of the corresponding subtypes [26–30]. In many cases, conclusions have been drawn based on a 20–50% solubilized fraction of mAChR without differences in solubilization of the various subtypes being taken into account. Thus, the very low solubility of m3 can be a reason why such a low level of this subtype was found in brain (2–10%), ileum (2–7%) and urinary bladder (11%) [26, 27, 29], while pharmacological and molecular biological data indicate considerably higher levels [31–34]. Of course, hybridization studies may also give a misleading picture of receptor localization when the mRNA production site is remote from that of receptor protein expression, but this is difficult to explain in the case of peripheral tissues [36]. It should also be noted that the solubility of each subtype may differ from one tissue to another because of possible effects of components other than receptor proteins on the solubility and stability of receptors in detergents.

In summary, it is concluded that mAChR subtypes differ greatly in their solubility and stability in detergents, despite their structural similarities. These differences should be considered when the proportion of each subtype in a given tissue is determined by estimation of solubilized receptors.

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