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[^{3}H]*N*-METHYLSCOPOLAMINE BINDING TO MUSCARINIC RECEPTORS IN INTACT ADULT RAT BRAIN CELL AGGREGATES

JONG-HWA LEE and ESAM E. EL-FAKAHANY*

Department of Pharmacology and Toxicology, University of Maryland School of Pharmacy, Baltimore, MD 21201, U.S.A.

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Abstract—Intact brain cell aggregates were dissociated from adult rat brains, by a simple sieving technique, and were used to study the binding characteristics of [^{3}H]*N*-methylscopolamine to muscarinic acetylcholine receptors. The magnitude of binding of this ligand was related linearly to the amount of cell protein in the binding assay, with a high ratio of total to nonspecific binding. In addition, specific binding showed saturability and high affinity. Muscarinic receptor antagonists displaced specific [^{3}H]*N*-methylscopolamine binding according to the law of mass-action, while it was possible to resolve displacement curves using receptor agonists into high- and low-affinity components. The results are discussed in terms of the usefulness of dissociated intact rat brain cells in studying muscarinic acetylcholine receptors in the central nervous system.

Muscarinic acetylcholine receptors are abundant in the central nervous system. Radiolabeled ligand binding studies have demonstrated several important features of brain muscarinic acetylcholine receptors [1]. For example, classical muscarinic receptor antagonists bind with high affinity to a single population of the receptors [2]. On the other hand, agonists interact with a heterogeneous population of muscarinic receptors, consisting of high- and low-affinity binding sites [3]. In some instances, the existence of agonist "super-high-affinity" sites has been demonstrated [4].

Several tissue models have been used to study the binding properties of brain muscarinic acetylcholine receptors *in vitro*. Most of the muscarinic receptor binding studies have been conducted using brain homogenates of various purities [5–8]. The popularity of this tissue preparation stems from the fact that it is easy to obtain in large quantities. Brain synaptosomes have also been used in some studies to investigate the properties of muscarinic acetylcholine receptor binding in a more refined preparation than crude brain homogenates [9]. To study the binding of ligands to muscarinic acetylcholine receptors under more physiological conditions, brain slices have been utilized [10, 11]. In addition, intact cells in culture have been employed as a model for muscarinic acetylcholine receptors in the central nervous system. These include both clonal nerve cell lines [12–20] and primary cultures of fetal rat brains [21, 22]. In the present study, we report on the use of dissociated intact adult rat brain cell aggregates as a model to study the binding properties of muscarinic acetylcholine receptors in the brain. The results are discussed in terms of comparing this model to the other models mentioned above.

* To whom all correspondence should be addressed.

MATERIALS AND METHODS

Preparation of dissociated rat brain cells. Adult male or female Sprague–Dawley rats were decapitated, and brains were immediately dissected on ice to remove the cerebellum. Tissue was dissociated at 4° using a modification of the sieving technique of Honegger and Richelson [23]. Brains were minced into a paste using a razor blade, then placed in a nylon mesh bag (210 μm pore diameter, Nitex 210, Tetko, Elmsford, NY), and submerged in a modified Puck's D_1 solution (medium I) of the following composition (mM): NaCl, 138; KCl, 5.4; Na_2HPO_4, 0.17; KH_2PO_4, 0.22; glucose, 5.5; and sucrose, 58.4 (pH 7.35, 340 mOsm). Tissue was dissociated by gently stroking the bag from the outside with a glass rod. The resulting suspension was filtered by gravity flow through a tighter nylon mesh bag (130 μm pore diameter, Nitex 130), and the resulting tissue was washed twice by centrifugation (400 *g* for 3 min at 4°) in a physiological buffer solution (medium II) of the following composition (mM): NaCl, 110; KCl, 5.3; $CaCl_2$, 1.8; $MgSO_4$, 1; glucose, 25; sucrose, 70; and HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], 20 (pH 7.4, 340 mOsm). Viability tests performed by the trypan blue exclusion method usually yielded viability values greater than 90%. Although we have not measured the size of the prepared aggregates, it is estimated that their size is about 100 μm [24]. In addition, the preparation probably contains different cell populations.

Muscarinic receptor binding assay. For displacement studies, intact brain cell aggregates (0.07 to 0.1 mg protein/assay tube) were incubated with 0.2 nM [^{3}H]*N*-methylscopolamine ([^{3}H]NMS), (84.8 Ci/mmole, New England Nuclear, Boston, MA). Incubations were carried out in a final volume of 1 ml of medium II in triplicate in the absence and in the presence of increasing concentrations of the

unlabeled muscarinic receptor agonists or antagonists. Nonspecific binding was measured in the presence of 2 μM atropine and was subtracted from all values to yield specific binding. For saturation studies, cell aggregates were incubated in triplicate with increasing concentrations (0.01 to 1.0 nM) of the radioactively labeled ligand in the absence (total binding) or in the presence (nonspecific binding) of 2 μM atropine. In all assays, incubations were carried out for 90 min at 15°, where equilibrium was attained. This temperature was chosen to increase the stability of the preparation and to minimize desensitization in the presence of high agonist concentrations. The binding reaction was terminated by filtration under vacuum through GF/B glass fiber filters (Whatman) using a Cell Harvester (BRANDEL, Gaithersburg, MD). Filters were washed three times with 5 ml of ice-cold isotonic saline (0.9%, w/v). Each filter was placed into a scintillation vial, and then 4.0 ml of a toluene-based scintillation fluid was added. Radioactivity was determined at least 6 hr later in a Beckman LS-6800 liquid scintillation counter with automatic correction for the counting efficiency of each sample, which averaged about 50%. Protein was determined according to the method of Lowry *et al.* [25] using bovine serum albumin as standard.

Data analysis. Displacement curves were analyzed by computerized iterative nonlinear least-squares regression using the LIGAND program [26] adapted for an Apple II computer. The statistical difference between one-site and two-site models was analyzed by comparing the residual variance between the predicted and actual data points, and the F statistic was computed according to the following equation:

$$F = [(SS_1 - SS_2)/(dF_1 - dF_2)]/(SS_2/dF_2)$$

where SS_1 and SS_2 are the sum of squares of residuals for the one- and two-site fits, respectively, and dF_1 and dF_2 are the corresponding degrees of freedom. Saturation isotherms were analyzed by the method of Scatchard [27] using linear least-squares regression analysis.

RESULTS

When different concentrations of rat brain cell aggregates were incubated with 1 nM [^{3}H]NMS in the presence and in the absence of 2 μM atropine, specific [^{3}H]NMS binding to muscarinic acetylcholine receptors was linearly related to the amount of cell protein included in the binding assay, up to 400 μg of protein (Fig. 1). All subsequent binding experiments were conducted using 100 μg protein per assay tube. Increasing the concentration of [^{3}H]NMS in the range of 0.01 to 2 nM resulted in increased total binding, while specific binding of [^{3}H]NMS to muscarinic acetylcholine receptors demonstrated saturability at higher ligand concentrations (Fig. 2A). On the other hand, nonspecific binding measured in the presence of 2 μM atropine increased linearly with the ligand concentration (Fig. 2A). It is noteworthy that the level of nonspecific binding was reasonably low compared to total binding. Analysis of the averaged saturation isotherms of specific [^{3}H]NMS binding shown in Fig. 2A, using Scatchard plots and linear least-squares regression,

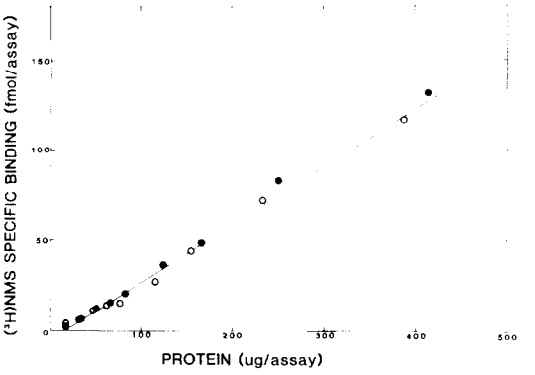

Fig. 1. Tissue linearity of the specific binding of [^{3}H]NMS in adult rat brain cell aggregates. Intact rat brain cells were prepared as described under Materials and Methods. Different tissue concentrations were incubated in quadruplicate with 1 nM [^{3}H]NMS in the absence and in the presence of 2 μM atropine for 90 min at 15°. Data presented are the average specific binding obtained in two independent experiments (○, ●).

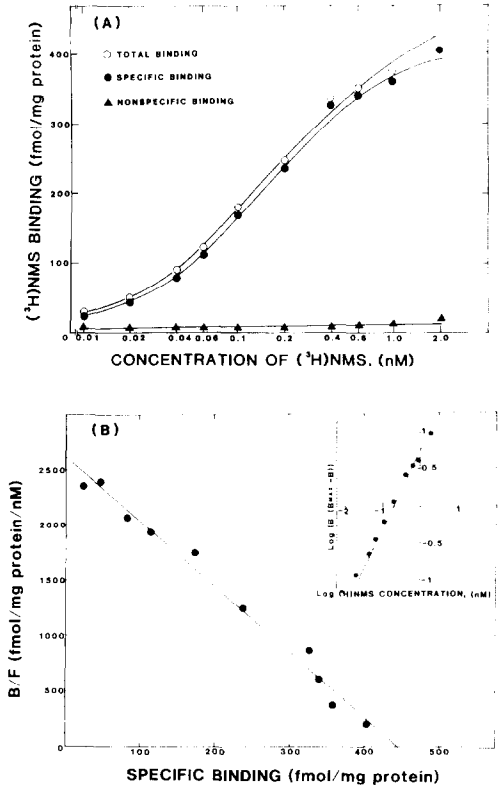

Fig. 2. Saturation isotherm of [^{3}H]NMS binding in adult rat brain cell aggregates. (A) Rat brain cells (100 μg protein) were incubated in triplicate with increasing concentrations of [^{3}H]NMS in the absence (○, total binding) and the presence (▲, nonspecific binding) of 2 μM atropine. Specific binding (●) was obtained by subtracting nonspecific binding from total binding. The data presented are the average of fourteen independent experiments. (B) Scatchard plot of [^{3}H]NMS specific binding presented in (A) ($r = 0.992$). Inset: Hill plot of the saturation isotherm ($n_H = 0.96$).

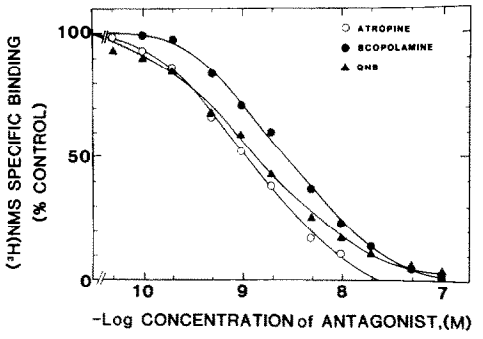

Fig. 3. Displacement of the specific binding of [³H]NMS by muscarinic receptor antagonists in adult intact rat brain cells. Cells were incubated in triplicate with 0.2 nM [³H] NMS in the presence of increasing concentrations of atropine (○), scopolamine (●) or (±)-quinuclidinyl benzilate (▲). Data are presented as a percentage of specific binding obtained in the absence of displacers (with a mean of 18 fmoles/assay tube), by averaging the results of three to five independent experiments.

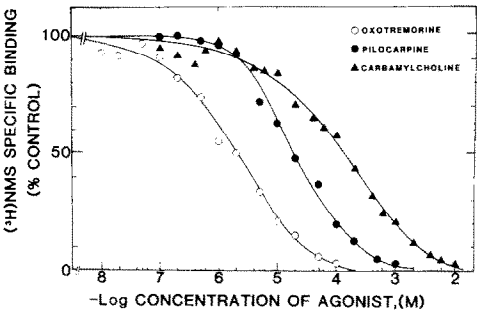

Fig. 4. Displacement of specific [³H]NMS binding by muscarinic receptor agonists in intact adult rat brain cell aggregates. Cells were incubated in triplicate with 0.2 nM [³H]NMS in the presence of increasing concentrations of carbamylcholine (▲), oxotremorine (○), or pilocarpine (●). Data are shown as a percentage of control binding in the absence of displacers, which averaged 14 fmoles/assay tube, and are presented as the mean of two to three independent experiments.

demonstrated a maximal binding capacity (B_{max}) of 446.6 fmoles/mg protein, with an equilibrium dissociation constant of 0.17 nM. Mean B_{max} values obtained from independent experiments were 451 ± 44 fmoles/mg protein (mean ± S.E.M.), while average K_D values were 0.175 ± 0.03 nM. Scatchard plots were linear with an average correlation coefficient of 0.992 ± 0.001, suggesting that [³H]NMS binds to a single homogenous population of muscarinic acetylcholine receptors in rat brain cell aggregates, with no evidence for cooperative interactions, as indicated by a Hill coefficient of unity (Fig. 2B).

Displacement of the specific binding of 0.2 nM [³H]NMS in intact brain cell aggregates by muscarinic receptor antagonists resulted in steep displacement curves, with Hill coefficients close to unity (Fig. 3 and Table 1). Atropine, scopolamine, and (±)-quinuclidinyl benzilate (QNB) displaced the specific binding with average IC_{50} values (concentrations displacing 50% of specific binding) of 0.96 ± 0.16, 2.75 ± 0.25 and 1.09 ± 0.05 nM respectively (mean ± S.E.M.) (Fig. 3). Transformation of these values, as described under Materials and Methods, yielded inhibition constants of 0.42 ± 0.08, 1.27 ± 0.12 and 0.25 ± 0.01 nM for atropine, scopolamine and (−)-quinuclidinyl benzilate respectively (Table 1).

On the other hand, muscarinic receptor agonists displaced specific [³H]NMS binding in intact brain cell aggregates with very shallow inhibition curves extending over three log units (Fig. 4). Hence, the Hill coefficients for carbamylcholine, oxotremorine and pilocarpine were 0.64, 0.65 and 0.78 respectively (Table 1). Nonlinear least-squares regression analysis of displacement of [³H[NMS specific binding in brain cells by agonists resolved the displacement

Table 1. Binding parameters of [³H]NMS in intact adult rat brain cell aggregates

	Displacer		Parameter				
			K_H* (M)	K_L† (M)	$\%R_H$‡	$\%R_L$§	n_H‖
(A)	Antagonists						
	Atropine	(3)¶	$0.42 \pm 0.08 \times 10^{-9}$		100		0.96
	Scopolamine	(4)	$1.27 \pm 0.12 \times 10^{-9}$		100		0.94
	(−)-QNB	(5)	$0.25 \pm 0.01 \times 10^{-9}$		100		0.81
(B)	Agonists**						
	Carbamylcholine	(3)	$4.3 \pm 1.8 \times 10^{-6}$	$8.9 \pm 1.3 \times 10^{-5}$	18.5	81.5	0.64
	Oxotremorine	(2)	8.98×10^{-8}	1.8×10^{-6}	18.9	81.1	0.65
	Pilocarpine	(2)	1.6×10^{-6}	1.81×10^{-5}	34.5	65.5	0.78

Values are expressed as mean ± S.E.M.
* Equilibrium dissociation constant of high-affinity sites.
† Equilibrium dissociation constant of low-affinity sites.
‡ Percentage of high-affinity sites.
§ Percentage of low-affinity sites.
‖ Hill coefficient.
¶ Number of independent experiments.
** A better fit resulted using a two-site model compared to a one-site model, $P < 0.01$.

curves into high- and low-affinity components. The equilibrium dissociation constants of the high-affinity sites for carbamylcholine, oxotremorine and pilocarpine were 4.3 μM, 90 nM and 1.6 μM, respectively, while the respective values for the low-affinity sites were 89 μM, 1.8 μM and 18.1 μM (Table 1). In addition, the proportion of the density of high-affinity sites to total receptor concentration was 18.5, 18.9 and 34.5% for carbamylcholine, oxotremorine and pilocarpine respectively (Table 1).

DISCUSSION

Muscarinic acetylcholine receptor binding studies presented in this work demonstrate the usefulness of dissociated adult rat brain cell aggregates in characterizing the binding properties of these receptors in an intact cell system. Binding of the potent and specific muscarinic receptor ligand, [^{3}H]NMS, in these cells was proportional to the protein concentration used in the binding assay (Fig. 1). In addition, specific binding of this ligand was saturable, with a very favorable ratio of total and nonspecific binding values (Fig. 2A). Scatchard plots of saturation isotherms of specific [^{3}H]NMS binding are linear (Fig. 2B), suggesting the involvement of a single homogenous population of muscarinic acetylcholine receptors. In addition, [^{3}H]NMS binding in rat brain cells demonstrated high affinity. The muscarinic receptor antagonists atropine, scopolamine and quinuclidinyl benzilate displaced specific [^{3}H]NMS binding with high potency, and with Hill coefficients close to unity (Fig. 3 and Table 1), indicating their interaction with a homogenous population of receptors, and the absence of cooperative interactions. These findings are similar to those obtained in rat brain homogenates [1, 2, 5–8]. On the other hand, the muscarinic receptor agonists carbamylcholine, oxotremorine and pilocarpine displaced specific [^{3}H]NMS binding in these cells with Hill coefficients that were significantly less than unity (Table 1). This binding profile of muscarinic receptor agonists is a common finding in different preparations used to study muscarinic receptor binding, and is probably due to their interaction with multiple receptor sites [1, 3, 12, 19, 20].

Brain homogenates have offered a simple model to study the binding characteristics of muscarinic acetylcholine receptors in the central nervous system. Although this preparation has provided a vast amount of information [1–8], it suffers from several potential drawbacks, resulting mainly from disrupting the cell membrane integrity. This may lead to uncoupling of muscarinic receptors and their effectors or other membrane components that might modulate the receptor conformation. Disrupting the cell membrane also exposes its cytoplasmic side to a nonphysiological environment, while depriving it of the intracellular milieu. Added to this is the common use of nonphysiological buffers in most muscarinic receptor binding assays in brain homogenates. Although purified brain synaptosomes suffer less from these pitfalls compared to brain homogenates, they are quite tedious and time-consuming to prepare. While brain slices have been used to study the binding properties of muscarinic acetylcholine receptors [10, 11], they introduce diffusion barriers which might complicate the interpretation of binding data. Intact cultured clonal nerve cells have been very useful in investigating ligand binding to muscarinic acetylcholine receptors [1, 12–20], in addition to the biochemical responses mediated by these receptors [1, 12–14, 19]. However, it is always questionable whether they actually represent normal differentiated nerve cells. Moreover, the density of muscarinic acetylcholine receptors in the cell line clones studied so far is quite low [12–20]. Although fetal rat brain cell aggregates in primary culture have been used for muscarinic receptor binding experiments, this technique requires a large number of animals and a sterile environment, in addition to the expense of growth media. Moreover, these cells lack a full complement of muscarinic receptors [21, 22].

In conclusion, dissociated adult rat brain cell aggregates may provide a very useful and physiologically-relevant model to study muscarinic acetylcholine receptor binding *in vitro*. This technique offers an easy method to obtain intact differentiated brain cells with minimal diffusion barriers. In addition to the usefulness of these cells in receptor binding experiments, they also provide a tool to study muscarinic receptor-mediated biochemical responses that require intact cells, e.g. cyclic GMP formation and phosphatidylinositol turnover.

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