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A microtechnique for quantification of detergent-solubilized muscarinic and nicotinic acetylcholine receptors using a semi-automated cell harvestor

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A specific method for the rapid assay of muscarinic acetylcholine receptors (mAChR), either detergent-solubilized or in neuroblastoma cells, is described. This method is also applicable to the assay of nicotinic acetylcholine receptors. The procedure employs a cell harvestor and microtiter plates, and has the advantage of requiring small quantities of radioligand, microgram quantities of detergent-solubilized cholinergic receptor or less cells. The binding parameters such as the equilibrium dissociation constants (K_d) of mAChR and nicotinic acetylcholine receptor (nAChR) and inhibition constants (K_i) for antagonists determined by the present method are in excellent agreement with values determined by other methods. This assay procedure for mAChR and nAChR should facilitate the rapid screening of cholinergic agonists/antagonists and also the further purification and characterization of mAChR.

Muscarinic acetylcholine receptor; Nicotinic acetylcholine receptor; Cell harvestor; Detergent solubilization

1. INTRODUCTION

Quantitative assay of radiolabeled ligands bound to solubilized mAChRs requires the availability of an assay to determine the relative concentrations of bound and/or free ligand. Until now, mAChR has been measured by equilibrium dialysis [1], charcoal precipitation [2,7], DEAE cellulose adsorption [3,4,5], ammonium sulfate precipitation [5,6], gel permeation [8], and PEG precipitation [3,7]. Assays employing PEG-6000 or ammonium sulfate to precipitate the receptor-ligand complex, which is collected onto glass-fiber filters (mounted on an Amicon vacuum manifold) are time consuming, and only a limited number of assays can be accomplished each day. The equilibrium dialysis and gel filtration procedures are even more cumbersome if a large number of samples are to be analyzed. The assay of mAChR by DEAE-filter disk is dependent on the retention of the receptor-ligand complex on anion-exchange disks followed by removal of the unbound radioligand by washing the disks, usually by tumbling in a beaker. However, removal of the unbound ligand is not efficient by the latter procedure, and background retention of the radioligand tends to be higher. Moreover, considerable fragmentation of the paper disks is inevitable, making quantitative transfer of the disks difficult.

In this report, we describe the use of a cell harvestor and a 96-well tissue culture plate to

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Abbreviations: mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; PEG, polyethylene glycol-6000; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine; QNB, quinuclidinyl benzilate; PMSF, phenylmethylsulfonyl fluoride

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facilitate rapid separation of PEG precipitated ligand-receptor complex. The assay procedure provides a rapid, efficient, and microtechnique for studying ligand binding to solubilized mAChR and nAChR.

2. MATERIALS AND METHODS

2.1. *Reagents*

Tritium labeled *N*-methylscopolamine ([^{3}H]-NMS; 72 Ci/mmol) was purchased from Dupont-New England Nuclear and α-[^{3}H]bungarotoxin (93 Ci/mmol) was purchased from Amersham. EDTA, bovine serum albumin, PEG 6000, digitonin (lot no.124F-0261), PMSF, sodium azide, and atropine were purchased from Sigma. Unlabeled α-bungarotoxin was purchased from Boehringer Mannheim. The remaining chemicals were the highest quality commercially available.

2.2. *Preparation of bovine brain membranes*

Bovine brain membranes were prepared from frozen brain as described by Haga and Haga [9] with minor modifications. All procedures were carried out at 0–4°C. Briefly, 800 g of frozen bovine brain was thawed and homogenized in 2 l of buffer A (50 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 0.1 mM PMSF and 0.02% sodium azide). The suspended tissue was homogenized in a Waring blender, and the homogenate was centrifuged at 16000 × *g* for 1 h. The pellet was resuspended in 2 l of buffer A and again centrifuged at 16000 × *g* for 1 h. The pellet was resuspended in buffer A containing 2 M sucrose to make the final sucrose concentration 0.8 M and a final volume of 1.35 l. The suspension (50 ml/tube) was layered over 1.14 M sucrose (10 ml/tube) in centrifuge tubes and centrifuged at 235000 × *g* for 1 h. The layer at the interface between 0.8 and 1.14 M sucrose was collected, homogenized with a Waring blender, and stored at −70°C until the next step.

2.3. *Muscarinic receptor solubilization procedure*

The homogenate containing mAChR of the previous step was solubilized with digitonin by adding an equal volume of buffer A containing 2% digitonin. The suspension was shaken for 1 h and centrifuged at 47000 × *g* for 1 h. The pellet was again extracted with buffer A containing 2% digitonin and the suspension centrifuged again at 47000 × *g* for 1 h. The supernatant collected after the second extraction contained the mAChR activity. The fraction from the second extraction was considered the solubilized mAChR because further centrifugation at 106000 × *g* did not decrease the mAChR activity in the supernatant.

2.4. *Nicotinic receptor solubilization procedure*

Soluble nAChR was prepared from the frozen electric organ of *Torpedo californica* essentially as described by Lindstrom et al. [18]. Briefly, the electric organ was homogenized in a buffer containing 0.1 M sodium chloride, 0.01 M sodium phosphate buffer (pH 7.5), 0.01 M sodium azide, 0.01 M EDTA, 0.01 M ethylene-bis(oxyethylene-nitrile)tetraacetic acid, 0.01 M iodoacetamide, 0.001 M PMSF at room temperature for 1 min in a Waring blender. The homogenate was centrifuged at 100000 × *g* for 30 min and the pellet was collected and solubilized using sodium cholate as the detergent (final concentration, 1%) at 4°C for 1 h.

2.5. *Binding assays*

2.5.1. mAChR

[^{3}H]NMS binding to purified membranes or N4TG1 neuroblastoma cells and solubilized receptor was carried out in 96-well tissue culture plates at 25°C in a final volume of 0.15 ml. The concentration of [^{3}H]NMS typically was 2 nM and non-specific binding was determined in the presence of 1 μM atropine. To assay for the ligand binding to cells, approx. 5–50 × 10^4 cells were added to each well. To determine the ligand binding to membranes or soluble mAChR, less than 400 μg of protein were added to each well. The plates were then placed on a mechanical rocker for 1 h to mix and incubated at 25°C.

2.5.2. nAChR

α-[^{3}H]Bungarotoxin binding to solubilized nAChR was assayed in tissue culture plates at 4°C as described for the mAChR. For Scatchard analysis [11], the α-[^{3}H]bungarotoxin concentration ranged from 0.1 to 50 nM, and the protein concentration was approx. 0.018 mg in 0.15 ml per 96 well. The incubation period was 20–24 h. The non-specific binding was determined in the

presence of 10 μM cold α-bungarotoxin. The incubation system also included 0.2 mg of bovine serum albumin.

2.6. *Harvesting of samples*

The contents of the wells were harvested with a semi-automated cell harvestor which can harvest 2 rows (24 samples) of the 96-well plate in 30 s (Cambridge Technology, Cambridge, MA) onto glass-fiber filters (grade 934 AH, Cambridge Technology). In the case of cells or membranes, after harvesting, the filters were quickly washed with 24 ml of phosphate buffered saline (PBS, 50 mM potassium phosphate, pH 7.2, and 0.9% sodium chloride (w/v), 1 ml/well). With respect to soluble mAChR or nAChR, prior to harvesting, 0.15 ml of ice-cold 25% (w/v) PEG-6000 (final concentration was 12.5%) in PBS was added to each well to precipitate the receptor-ligand complex and the plate was placed at 4°C for 5–10 min. Then the samples were harvested and the glass-fiber filters were washed quickly with 24 ml of 12.5% (w/v) ice-cold PEG-6000 (1 ml/well). The dried filter disks were placed into minivials and 5 ml of Hydroflour was added prior to scintillation counting.

Solubilized mAChR was also assayed by the Sephadex G-25 gel filtration assay. Samples (0.15 ml) were applied to a Sephadex G-25 column (4 ml bed volume, Bio-rad Econo-Column). The receptor-ligand complex was eluted by the addition of 2.0 ml of PBS and collected directly into a scintillation vial and counted by adding 20 ml of Hydroflour. Protein was determined according to the procedure described by Lowry et al. [10] and bovine serum albumin was used as the standard.

3. RESULTS

3.1. *Effect of PEG concentration on the precipitation of receptor-ligand complex (PEG cell harvestor assay)*

To optimize [^{3}H]NMS-receptor complex precipitation by PEG, the polyethylene glycol concentration was varied. Quantitative precipitation of the [^{3}H]NMS-receptor complex occurred at PEG concentrations equal to or greater than 12.5% (fig.1). The minimum time required was 5 min at 4°C for the complete precipitation of the receptor-ligand complex.

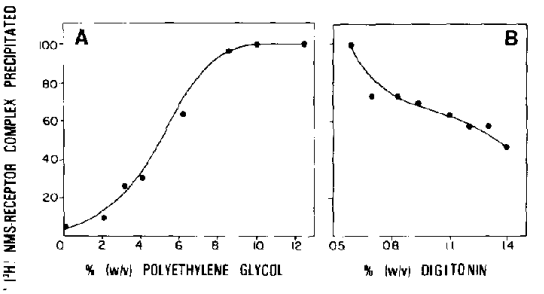

Fig.1. Effect of PEG (panel A) and digitonin (panel B) on the precipitation of [^{3}H]NMS-receptor complex. Percentage precipitation values (ordinate) were calculated by dividing the dpm value for each treatment point by the dpm value obtained at 12.5% PEG with 0.6% digitonin. Each data point is the mean ± SD of triplicate samples. The concentration of [^{3}H]NMS was 2 nM and assay conditions were as described in section 2.

3.2. *Effect of digitonin concentration on binding of [^{3}H]NMS to solubilized bovine brain mAChR*

When digitonin-solubilized bovine brain mAChR was incubated with 2 nM [^{3}H]NMS and varying concentrations of digitonin (0.6–1.4%, w/v), the binding activity of [^{3}H]NMS to the mAChR decreased with increasing concentration of digitonin (fig.1). At 1.4% digitonin, a 50% decrease in ligand binding occurred. This result indicates that [^{3}H]NMS binding to the mAChR receptor could be detected at a high concentration of digitonin by the PEG cell harvestor assay, although digitonin is inhibitory to the binding process.

3.3. *Effect of receptor protein concentration*

[^{3}H]NMS binding to digitonin-solubilized mAChR was linear between 77 and 385 μg of protein (not shown). Thus, the PEG cell harvestor assay described here was quantitative for the receptor-ligand complex precipitated from digitonin buffers.

3.4. *Interaction of solubilized mAChR with [^{3}H]NMS as studied by the PEG cell harvestor assay and comparison with other methods*

Scatchard analysis [11] of [^{3}H]NMS binding to receptors at 25°C produced a dissociation constant

Table 1

Comparison of equilibrium dissociation constants (K_d) obtained by different assay methods[a]

	Assay method		
	Cell harvestor	Sephadex G-25 gel filtration	Literature values
K_d (nM)	0.20 ± 0.02[b]	0.13 ± 0.02[c]	0.2 [7], 0.4 [13], 0.2 [12], 0.52 [9]
B_{max} (fmol/mg protein)	0.21 ± 0.02	0.24 ± 0.03	–

[a] Saturation binding assays were performed as described in section 2, using the cell harvestor or Sephadex G-25 gel filtration. Varying concentrations of [^{3}H]NMS were incubated with solubilized receptor for 1 h at 25°C. K_d values were obtained according to the method of Munson and Rodbard [14]

[b] Mean ± SD of quadruplicate assays

[c] Mean ± SD of duplicate assays

(K_d) of 0.20 nM for the PEG cell harvestor assay and 0.13 nM for the Sephadex G-25 filtration assay. The results for the two methods are in excellent agreement (table 1). These values are in agreement with values obtained from the charcoal adsorption [7], manifold filtration [12,13], and equilibrium dialysis [7] methods. In addition, the non-specific binding of radioligand was 5–10% of the total counts by the PEG cell harvestor and gel filtration procedure.

Table 2

Potencies of muscarinic receptor antagonists inhibiting [^{3}H]NMS binding to cell surface or detergent solubilized mAChR

mAChR antagonists	K_i (nM)[a]			
	Cell harvestor assay[b,c]		Literature values	
	Bovine brain solubilized mAChR	N4TG1[d] neuroblastoma cell mAChR	Membrane bound mAChR	Solubilized mAChR
Atropine	1.6 ± 0.2	2.4 ± 0.7	2.5 [12] 2.0 [5]	1.98 [8] 1.20 [9]
QNB	1.3 ± 0.2	0.5 ± 0.2	–	1.43 [5]

[a] The IC_{50} values were first determined using ALLFIT, a computer program for the analysis of inhibition curves [14]. K_i values were calculated from the IC_{50} values (IC_{50} is the dose of drug which inhibits 50% of the binding of the radioactive ligand) according to the calculations of Cheng and Prusoff [15], where $K_i = IC_{50} \times (1 + ([^3H]ligand/K_d)$

[b] Mean ± SD of triplicate assays

[c] The concentration of [^{3}H]NMS was 2 nM and assay duration was 1 h at 25°C

[d] N4TG1 neuroblastoma cells (5×10^5 cells/well) were cultured and prepared in Hanks media containing 25 mM Hepes (pH 7.3) as described [16,17]

3.5. *Inhibition of [^{3}H]NMS binding to mAChR by atropine and QNB*

The ability of two muscarinic antagonists to compete for N4TG1 neuroblastoma cell bound or solubilized [^{3}H]NMS binding sites was determined. The muscarinic antagonists atropine and QNB inhibited [^{3}H]NMS binding to bovine brain solubilized mAChR with K_i values determined by the PEG cell harvestor assay of 1.6 and 1.3 nM, respectively, and agreed well with the findings of other investigators (table 2). Furthermore, atropine inhibited the binding of [^{3}H]NMS to cell surface mAChR of N4TG1 neuroblastoma cells with a K_i value of 2.4 nM, which compares favorably to values reported for membrane bound mAChR (table 2).

3.6. *nAChR assay by the PEG cell harvestor method*

The PEG cell harvestor assay procedure should have general applicability to soluble receptors. By Scatchard analysis of the soluble nAChR, a K_d value of 0.57 ± 0.06 nM was determined using the PEG cell harvestor assay. This K_d value is in excellent agreement with literature values, which range from 0.14 to 0.96 [19,20] for the nAChR from electric organs and determined by other methods than the PEG cell harvestor assay.

4. DISCUSSION

The present assay method, using a cell harvestor and 96-well tissue culture plate and PEG for measuring detergent-solubilized mAChR has several advantages over more conventional assay techniques. First, the receptor protein is pipetted only once directly into each well, thereby minimizing the manual handling errors associated with other methods. Second, only microgram quantities of receptor protein are required for each well. Third, consumption of expensive radioligand materials is minimized. Fourth, a large number of receptor assays can be performed and managed quickly and efficiently. Fifth, this assay is very useful when large numbers of drugs are screened for their potential as anticholinergic agents or as agonists. Sixth, the system can be set up rapidly, conveniently, and inexpensively. Additionally, this procedure should be applicable to the quantitative determinations of other soluble receptors. With respect to another soluble receptor, the nAChR can be assayed by this procedure and the K_d value agrees well with literature values.

However, there is a caution associated with the use of the cell harvestor. The small amounts of cells ($5-50 \times 10^4$ cells/well) or solubilized receptor that can be used without clogging the glass-fiber filters may pose a problem if few receptor sites are present in the tissue or solubilized preparation. In conclusion, the development of this simple and rapid assay procedure for the soluble receptors should facilitate the examination of the effects of many cholinergic receptor agonists or antagonists, and the further purification and characterization of the mAChR.

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