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Purification of the muscarinic acetylcholine receptor from porcine atria

(NaDodSO$_4$/polyacrylamide gel electrophoresis/receptor subunits/ligand binding/receptor stability)

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ABSTRACT **The muscarinic acetylcholine receptor from porcine atria has been purified 100,000-fold to homogeneity by solubilization in digitonin/cholate and sequential chromatography on wheat germ agglutinin-agarose, diethylaminoethyl-agarose, hydroxylapatite, and 3-(2′-aminobenzhydryloxy)tropane-agarose. The yield of purified receptor was 4.3% of that found in the membrane fraction, and the purified receptor bound 11.1–12.8 nmol of L-[^{3}H]quinuclidinyl benzilate per mg of protein, corresponding to a binding component M_r of 78,400–90,000. The purified receptor preparation consisted of two polypeptides in approximately equimolar amounts when examined on silver-stained sodium dodecyl sulfate/polyacrylamide gels. The larger polypeptide (M_r 78,000 on 8% polyacrylamide gels) was specifically alkylated with [^{3}H]propylbenzilylcholine mustard, whereas the smaller polypeptide (M_r 14,800) was not labeled. The possibility that the small polypeptide is a contaminant fortuitously appearing in equimolar amounts with the large polypeptide cannot be ruled out at this time. The purified preparation was highly stable, with no measurable change in the number of ligand binding sites or the gel pattern after 1 month's storage on ice. Scatchard analysis showed a single class of binding sites for the antagonist L-[^{3}H]quinuclidinyl benzilate with a dissociation constant of 61 ± 4 pM. Equilibrium titration experiments demonstrated that the antagonist L-hyoscyamine displaced L-[^{3}H]quinuclidinyl benzilate from a single class of sites (K_d = 475 ± 30 pM), whereas the agonist carbamoylcholine interacted at two populations of sites (53% ± 3% high affinity, K_d = 1.1 ± 0.3 μM; 47% ± 3% low affinity, K_d = 67 ± 14 μM). The ligand binding data were very similar to that for the membrane-bound receptor, suggesting that the receptor has not been altered radically during purification.**

The muscarinic acetylcholine receptor (mAcChoR) modulates central nervous system activity and heart and smooth muscle contraction. Labeling studies with the specific alkylating agent [^{3}H]propylbenzilylcholine mustard ([^{3}H]PrBChoM) have identified a similar 80,000-dalton ligand binding protein in brain, heart, and smooth muscle (1). However, more detailed understanding of the structure of the functional mAcChoR has not been possible due to the lack of a purified preparation. André *et al.* (2) reported an affinity-purified preparation from calf brain in which a major 70,000-dalton protein was eluted from a dexetimide agarose column by either atropine or PrBChoM and subsequently detected on NaDodSO$_4$/polyacrylamide gels by radioiodination. Receptor activity, however, could not be regained in their preparations. Partial purification of porcine brain mAcChoR by chromatography of the digitonin-solubilized receptor on 3-(2′-aminobenzhydryloxy)tropane-agarose has been reported recently by Haga and Haga (3). This latter preparation is estimated to be about 5% pure on the basis of an approximate value reported for the specific activity (protein was reported too low to accurately measure).

In this study we report the purification of the mAcChoR from porcine atrial tissue. The purified preparation is fully active, stable, and in sufficient quantities for further biochemical studies.

MATERIALS AND METHODS

L-[^{3}H]Quinuclidinyl benzilate (L-[^{3}H]QNB; 30–40 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]PrBChoM (33.1 Ci/mmol) were from New England Nuclear. DEAE-agarose (DEAE Bio-Gel A) and hydroxylapatite (Bio-Gel HT) were from Bio-Rad. The 3-(2′-aminobenzhydryloxy)tropane-agarose affinity resin was prepared according to Haga and Haga (3). Buffer A consisted of 10 mM sodium phosphate/1 mM Na$_2$EDTA, 0.02% NaN$_3$/0.1 mM phenylmethylsulfonyl fluoride (added fresh as used)/0.4% (wt/vol) digitonin/0.08% (wt/vol) cholic acid, pH 7.4. Buffer B was the same except for exchange of 25 mM imidazole for 10 mM sodium phosphate. Other materials were as described (4, 5).

Purification of Porcine Atrial mAcChoR. Porcine atrial membranes enriched in the mAcChoR were prepared as described (4) and stored at −80°C. For solubilization of the mAcChoR, membranes accumulated from 5–7 kg of atrial tissue (2.5–3 g of protein) were thawed and extracted by using the dual detergent treatment method and the mixed digitonin/cholate system in buffer B as described (4). This resulted in the preparation of about 400 ml of solubilized receptor (extract), which was loaded directly onto a 1.5 × 29 cm wheat germ agglutinin (WGA)-agarose column prepared as described (5). All column chromatography steps described below were run at 4°C. The lectin column was loaded at 1 ml/min, washed with about 200 ml of buffer B, and eluted at 1 ml/min sequentially with 80 ml of 0.25 M NaCl, 80 ml of 10 mM *N*-acetylglucosamine, and 100 ml of 200 mM *N*-acetylglucosamine, all in buffer B. The receptor eluted with 200 mM *N*-acetylglucosamine (WGA pool; 30–35 ml) was loaded at 0.10–0.15 ml/min directly onto a 1.5 × 13 cm DEAE-agarose column in buffer B. The DEAE column was then washed with 8 ml of buffer B at 0.25 ml/min and eluted at 0.1 ml/min with a 70-ml linear 0–0.25 M NaCl gradient in buffer B. The peak of mAcChoR eluted from the DEAE column at about 0.075 M NaCl, whereas most of the contaminating protein eluted at either lower or higher salt concentrations. The high specific activity DEAE pool (6.5–7.5 ml) was loaded at 0.1 ml/min onto a 1.5 × 2.3 cm hydroxylapatite column that had been equilibrated in 0.4% (wt/vol) digitonin/0.08%

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Abbreviations: mAcChoR, muscarinic acetylcholine receptor; PrBChoM, propylbenzilylcholine mustard; L-QNB, L-quinuclidinyl benzilate; WGA, wheat germ agglutinin.

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(wt/vol) cholate/25 mM *N*,*N*-bis(2-hydroxyethyl)glycine/1 mM Na_2EDTA, pH 8.9, and carefully washed to remove fines. After loading, the hydroxylapatite column was washed with 3–5 ml of the equilibration buffer and then eluted at 0.15 ml/min sequentially with 10 ml of 0.12 M potassium phosphate (pH 9), 10 ml of 0.05 M potassium phosphate (pH 7.4), and 15 ml of 0.5 M potassium phosphate (pH 7.4), all containing 0.4% (wt/vol) digitonin, 0.08% (wt/vol) cholate, 1 mM Na_2EDTA, and fresh 0.1 mM phenylmethylsulfonyl fluoride. The mAcChoR was eluted in the final 0.5 M phosphate solution (hydroxylapatite pool; 4–5 ml) and was used directly in the final affinity purification step.

The final purification step involved specific binding and elution of the mAcChoR from the 3-(2'-aminobenzhydryloxy)tropane-agarose affinity resin. Five volumes of hydroxylapatite pool were added to 1 vol of affinity resin in small plastic disposable-type chromatography columns, the ends were sealed, and the mixture was agitated gently at 4°C overnight. The liquid was removed from the resin by draining under vacuum. The resin was washed with 5 vol of buffer A containing 0.25 M NaCl and drained under vacuum. Three to 5 vol of 200 nM L-[^{3}H]QNB or 100 mM carbamoylcholine in buffer A containing 0.25 M NaCl were added to the resin, the column ends were sealed, and the mixture was agitated gently for 6–8 hr at 4°C. The eluate was removed from the resin by draining under vacuum and, in the case of carbamoylcholine elution, dialyzed for 2 days against four changes of 200–300 vol of buffer A containing 1/5 the usual amount of detergent.

Receptor Activity Measurements. The mAcChoR was quantitated in terms of specific binding of L-[^{3}H]QNB by using the DEAE filter disc assay as described (4). Total protein was determined by the Folin phenol method (6) in the early stages of purification and either by A_{280} or by a sensitive *o*-phthalaldehyde fluorescence method (6) in later steps. The absolute protein concentration of the purified receptor was determined by fluorescence assay of the total amino acids released by acid hydrolysis (6).

Ligand Binding Studies. The dissociation constant for L-[^{3}H]QNB was determined by Scatchard (7) analysis. Equilibrium titrations were performed for the antagonist L-hyoscyamine and the agonist carbamoylcholine.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The discontinuous buffer system of Laemmli (8) was used for NaDodSO$_4$/polyacrylamide gel electrophoresis with either uniform (8% acrylamide) or gradient pore (8–18% linear acrylamide gradient) gels. The gels were stained with silver according to Wray *et al.* (9).

RESULTS

Purification of Porcine Atrial mAcChoR. The sequential chromatography steps used in purification of the porcine atrial mAcChoR solubilized in digitonin/cholate are shown in Fig. 1. The WGA column bound about 75% of the mAcChoR in the extract and about 25% of the protein. No receptor, but a small amount of protein, was eluted with 0.25 M NaCl, about 40–50% of the bound receptor was eluted with 10 mM *N*-acetylglucosamine at low specific activity (peak = 37 pmol/mg), and the remainder was eluted with 200 mM *N*-acetylglucosamine at high specific activity (peak > 100 pmol/mg), which represented a 15- to 20-fold enrichment over the extract (Fig. 1*A*). Overall receptor and protein recovery was 80%. All of the WGA pool receptor and protein bound to the DEAE-agarose column, and 70% of high specific activity receptor (5-fold enriched over the WGA pool) was eluted in the range of 60–75 mM NaCl. Overall recovery on the DEAE column was 95% for receptor and 82% for protein. All of the DEAE pool receptor and protein bound to the hydroxylapatite column and the bulk of protein was eluted at

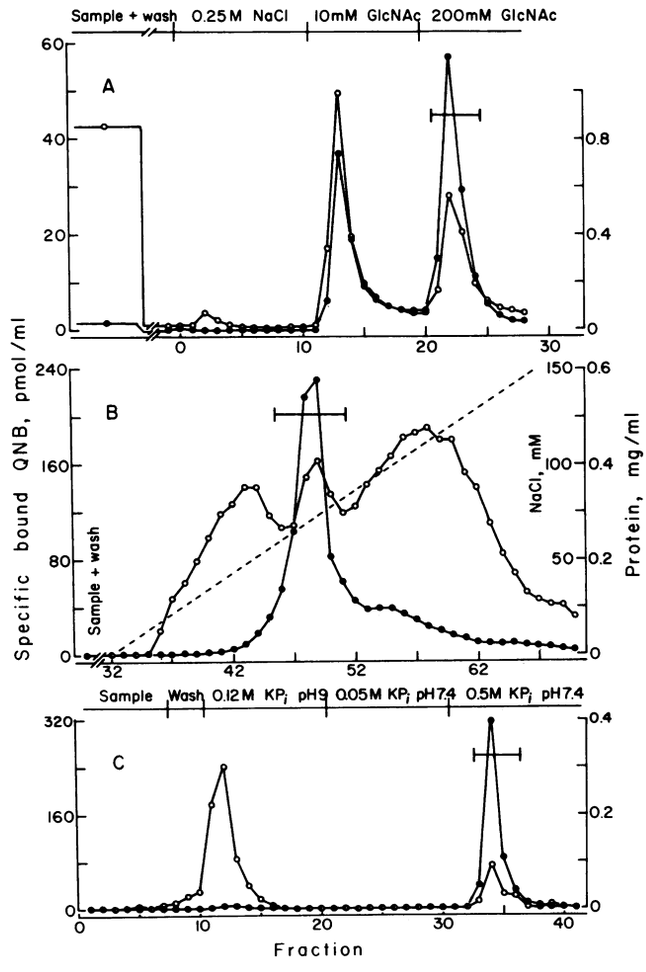

FIG. 1. Sequential chromatography steps during purification of the digitonin/cholate-solubilized mAcChoR from porcine atria. The receptor profile in each column is shown by the ● and the protein profile by the ○. The pooled fractions are indicated by the bar (⟷). (*A*) WGA-agarose column. The detergent-solubilized mAcChoR was loaded onto the column, the column was washed with 230 ml of buffer B, and the unbound material was collected in bulk. The column was then eluted sequentially with the indicated solutions in buffer B and 7.5-ml fractions were collected at 1 ml/min. Protein was determined by A_{280} ($E^{1\ mg/ml}$ = 1.45). (*B*) DEAE-agarose column. The WGA pool from *A* was loaded, then washed with 8 ml of buffer B, and eluted with a linear 0–0.25 M NaCl gradient. Fractions (1.1 ml) were collected at 0.09 ml/min. Protein was determined by the Folin phenol method (6). (*C*) Hydroxylapatite column. The DEAE pool from *B* was loaded onto the column, washed with 3 ml of 0.4% (wt/vol) digitonin/0.08% (wt/vol) cholate in pH 9 *N*,*N*-bis(2-hydroxyethyl)glycine buffer, and eluted with the indicated concentrations and pH of potassium phosphate at the same detergent concentrations. Fractions (1.1 ml) were collected at 0.15 ml/min.

0.12 M potassium phosphate at pH 9, whereas the receptor was eluted at 0.5 M potassium phosphate at pH 7.4 (Fig. 1*C*). This resulted in a further 4-fold purification of the receptor. Overall yield on the hydroxylapatite column was 75% for receptor and near 100% for protein.

Table 1 summarizes the purification of the mAcChoR. The final affinity purification on 3-(2'-aminobenzhydryloxy)tropane-agarose resulted in preparations with specific activities of 11.1–12.8 nmol/mg of protein, which corresponded to a binding component with a minimal M_r of 78,400–90,000. Higher yields (1.8-fold) were obtained when 100 mM carbamoylcholine rather than 200 nM L-[^{3}H]QNB was used to elute the receptor from the affinity resin. The 15% difference in specific activities determined for the two elution preparations was considered to be within experimental error. The

Table 1. Purification of mAcChoR from 5.8 kg of fresh porcine atria

Purification fraction	Total protein, mg	Total receptor, pmol	Receptor yield, %	Specific activity, pmol/mg	Relative purification, fold
Membrane	2820*	8420	100.0	2.99	1.0
Extract	745*	4095	48.6	5.50	1.8
WGA pool	13.4†	1158	13.8	86.4	28.9
DEAE pool	1.94*	892	10.6	460	154
Hydroxylapatite pool	0.30*	509	6.0	1,700	567
Affinity resin eluate					
Carbamoylcholine	0.029‡	324	4.3	11,100	3700
L-[^{3}H]QNB	0.015‡	194	2.4	12,800	4280

*Determined by the Folin phenol method (6).
†Determined by A_{280} ($E^{1\ mg/ml} = 1.45$).
‡Determined by *o*-phthalaldehyde fluorescence assay of total amino acids released by acid hydrolysis (6).

amount of protein in the purified receptor was found to be overestimated by both the Folin phenol method (1.27-fold) and the fluorescence assay on intact protein (1.88 ± 0.12-fold) in comparison to the true concentration measured by quantitation of the total amino acids released by acid hydrolysis.

The total yield of purified receptor was about 30 μg of protein, or 4.3% of the total receptor in the isolated porcine atrial membranes. Overall purification was about 4000-fold from the membranes and about 100,000-fold from atrial homogenates (usually about 0.12 pmol/mg). Attempts were made to increase the yield of purified receptor by processing the lower specific activity fractions from the lectin column or the DEAE column. The preparation from the 10 mM *N*-acetylglucosamine fractions was estimated to be 50% pure, and from the DEAE fractions, 20% pure.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. NaDodSO$_4$/polyacrylamide gel analysis of the purified receptor on uniform 8% acrylamide minigels resulted in the appearance of a single prominent silver-stained band with an apparent M_r of 78,000 at the peak of staining intensity (Fig. 2). This band, however, was broad and covered the M_r range of 56,000–106,000. There was also some silver staining at the position of the tracking dye, indicating protein(s) with $M_r <$ 25,000. Affinity alkylation of the same purified preparation with [^{3}H]PrBChoM and analysis on 8% acrylamide tube gels produced a prominent single band of atropine-sensitive radioactive incorporation that paralleled the staining density of the prominent silver-stained band (Fig. 2). The tube gels were fixed, stained with Coomassie blue R250, and extensively destained to remove any [^{3}H]PrBChoM not covalently bound. No radioactivity was present in the region of the tracking dye. Although Coomassie blue staining of the M_r 78,000 protein was difficult, if not impossible, to detect by eye, high sensitivity scanning revealed a single major staining band discernible above background noise, which corresponded in R_f precisely with that of the radioactive profile and the prominent silver-stained band.

Gradient pore NaDodSO$_4$/polyacrylamide gel electrophoresis on 8–18% linear acrylamide gradients revealed the presence of a second polypeptide of low molecular weight (Fig. 3). This polypeptide was apparently enriched along with the high molecular weight polypeptide between the hydroxylapatite pool receptor (Fig. 3*A*, lane 2) and the final purified fraction (Fig. 3*A*, lane 1). A plot of log molecular weight versus log percent total acrylamide produced a straight line (Fig. 3*B*) and linear regression analysis gave apparent M_rs for the two proteins of 95,800 ± 15,600 and 14,800 ± 2,600. Optical density scans taken from the photographic negatives of silver-stained gels from three purified preparations, two carbamoylcholine elutions and one L-[^{3}H]QNB elution, were quantitated to estimate the relative amounts of the two polypeptides. For the three preparations the mass ratio of large to small was 6.9 ± 1.9. The above molecular weight data gave a calculated mass ratio of 6.5 ± 2.3 for equimolar amounts of the two polypeptides. This agreement, however, could be fortuitous in that silver staining may not give an accurate estimate of the mass ratio.

Stability of the Porcine Atrial mAcChoR. The stability of the various purification fractions of the porcine atrial mAcChoR to storage on ice is shown in Table 2. All fractions were at least reasonably stable. The hydroxylapatite pool and purified receptor showed no loss of activity after 1 month's storage. NaDodSO$_4$/polyacrylamide gel electrophoresis of the purified receptor after 1 month's storage on ice showed the identical silver-stained protein profile as the original (data not shown). Glass tubes were suitable for binding assays for the membrane and extract fractions only.

Ligand Binding Studies. Binding of the antagonist L-[^{3}H]QNB to the purified atrial mAcChoR is shown in Fig. 4. At a receptor concentration of about 410 pM, binding was

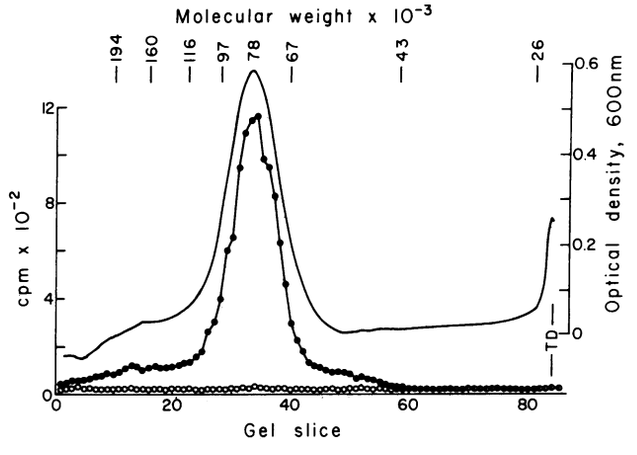

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis pattern of silver-stained and [^{3}H]PrBChoM-labeled mAcChoR purified from porcine atria. Uniform 8% acrylamide gels were run on the same purified preparation by using 0.8 × 80 × 80 mm mini-slab gels for silver staining and 0.6 × 12 cm tube gels for mustard labeling. The silver-stained gel pattern was produced from 120 ng of protein loaded on a 0.8 × 3 mm cross-sectional lane. After silver staining the gel was photographed and the negative was scanned for optical density at 600 nm (solid line). The tube gels each contained 1.12 μg of protein and mustard labeling was performed as described (4) in the presence (○) and absence (●) of 1 μM atropine. The gel slices were about 1 mm thick. TD denotes the position of the tracking dye. The molecular weight markers were myosin (M_r 194,000), debranching enzyme (M_r 160,000), β-galactosidase (M_r 116,000), phosphorylase b (M_r 97,100), bovine serum albumin (M_r 66,300), ovalbumin (M_r 42,800), and chymotrypsinogen A (M_r 25,700).

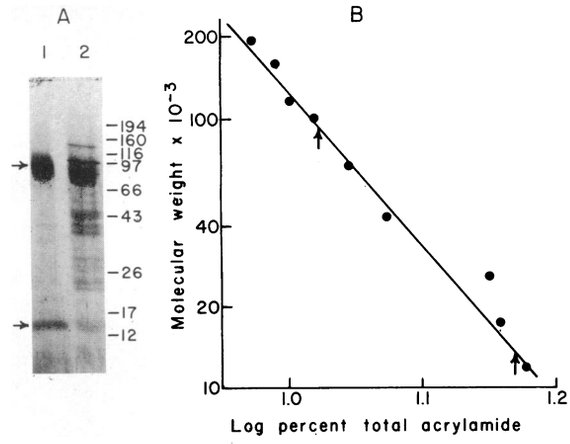

FIG. 3. Gradient pore NaDodSO$_4$/polyacrylamide gel electrophoresis of the purified mAcChoR and the hydroxylapatite pool receptor. (*A*) Silver-stained gel pattern of 120 ng of purified receptor (lane 1) and 200 ng of hydroxylapatite pool receptor (lane 2) on an 8–18% linear acrylamide gradient mini-slab gel. The molecular weight markers were the same as for Fig. 2, but with the addition of myoglobin (M_r 17,200) and cytochrome *c* (M_r 11,800). (*B*) Plot of log molecular weight versus log percent total acrylamide for the gel shown in *A*. The regression line was fitted by least squares; r^2 = 0.980. The positions of the large and small protein bands of the mAcChoR purified preparation are indicated by the arrows.

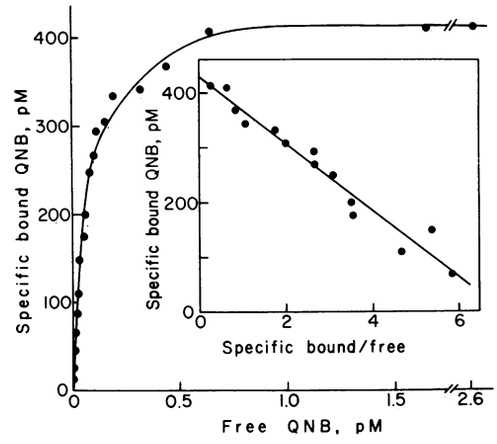

FIG. 4. L-[^{3}H]QNB binding to the purified atrial mAcChoR. The purified receptor was diluted 1:125 in buffer A. L-[^{3}H]QNB at various concentrations was added in the presence and absence of 10 μM atropine and the amount of specific bound L-[^{3}H]QNB was determined after 3.5 hr at 22°C. Scatchard analysis (*Inset*) was fitted by least squares linear regression (r^2 = 0.956) and gave a K_d of 61 ± 4 pM and a maximal binding intercept of 428 ± 12 pM.

maximum at free L-[^{3}H]QNB concentrations >1 nM. Scatchard analysis indicated the presence of a single class of L-QNB binding sites with a dissociation constant of 61 ± 4 pM and a maximal binding capacity of 428 ± 12 pM sites.

Equilibrium titrations of specifically bound L-[^{3}H]QNB with the antagonist L-hyoscyamine and the agonist carbamoylcholine in the purified atrial mAcChoR are shown in Fig. 5. L-Hyoscyamine appeared to interact with L-QNB at a single class of sites and the data were analyzed by a weighted least squares fit to the $\bar{J}$ function as described earlier (4, 10). The calculated dissociation constant for L-hyoscyamine was 475 ± 30 pM. Carbamoylcholine did not obey the law of mass action for a single class of interaction sites and was fitted to a two-site model in which two noninterconvertible subpopulations of agonist sites interact with one homogeneous population of L-QNB sites (4, 10). A weighted nonlinear least squares fit (11) to the two-site model gave the following parameters for the two carbamoylcholine sites: 52.8% ± 2.6% high-affinity sites with K_d = 1.07 ± 0.31 μM; 47.2% ± 2.6% low-affinity sites with K_d = 67.2 ± 14.3 μM.

DISCUSSION

Solubilization of porcine atrial mAcChoR with the mixed detergent digitonin/cholate followed by sequential chromatography on WGA-agarose, DEAE-agarose, and hydroxylapatite led to a preparation that was in the range of 10% pure and permitted visualization of the receptor ligand-binding polypeptide on NaDoDSO$_4$/polyacrylamide gels (Fig. 3*A*, lane 2). Final purification was achieved by using the 3-(2′-aminobenzhydryloxy)tropane-agarose affinity resin developed by Haga and Haga (3). In our experience, the presence of salt (either NaCl or potassium phosphate) was important for specific binding to the affinity resin, in agreement with Haga and Haga (3). Specific binding and elution of the mAcChoR from the affinity resin were slow and at least 6 hr were allowed for each step to insure maximal recoveries.

In our preparations purification was achieved only from the highest specific activity fractions obtained during WGA, DEAE, and hydroxylapatite chromatography. The 10 mM *N*-acetylglucosamine-eluted material was about 1/3 the specific activity of the 200 mM *N*-acetylglucosamine-eluted material from the WGA-agarose column and, when processed through the remainder of the purification scheme, gave a preparation about 50% pure. It is possible that this material represents receptor that is not fully dispersed in the detergent system and thus binds more weakly to the WGA-agarose column and cannot be completely separated from some contaminating proteins.

Table 2. Stability of the various fractions obtained during mAcChoR purification and effect of the use of plastic versus glass tubes for receptor binding assays

Purification fraction	Binding activity, ratio	
	1 month/original*	Plastic/glass†
Membrane	0.93	1.05
Extract	0.71	1.07
WGA pool	0.90	1.46
DEAE pool	0.71	2.05
Hydroxylapatite pool	1.08	2.15
Purified	0.97	2.22

*Activity remaining after 1 month's storage on ice/activity measured at the time of preparation.

†Activity determined by using polypropylene plastic tubes/activity determined by using glass tubes for incubation (2 hr at 22°C) of the receptor and L-[^{3}H]QNB.

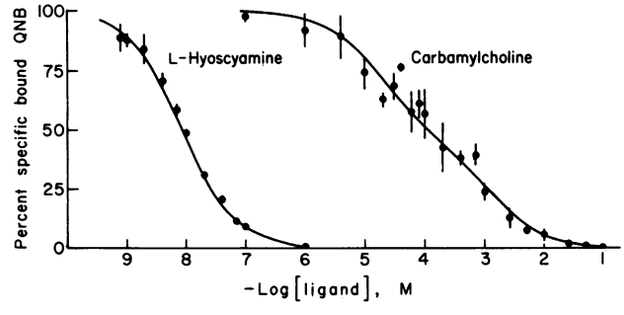

FIG. 5. L-Hyoscyamine and carbamoylcholine titrations of specific bound L-[^{3}H]QNB in the purified atrial mAcChoR. The titrations were performed in buffer A at constant concentrations of receptor sites (388 pM) and L-QNB (1045 pM), and incubations were carried out for 6 hr at 22°C. The titration curve for L-hyoscyamine was fitted to a model predicting a single class of interaction sites, whereas that for carbamoylcholine was fitted to a two-site model (see text and refs. 4 and 10 for further details).

Nevertheless, the porcine atrial mAcChoR is purified in sufficient yield (about 30 μg/5.8 kg of starting material) to permit further biochemical and pharmacological studies. Moreover, the preparation is highly stable both in terms of ligand binding sites and structural integrity (no proteolytic fragmentation is observed after 1 month's storage on ice). The specific activity of the purified receptor (11.1–12.8 nmol/mg) is within range of the theoretical activity (12.5 nmol/mg) for a ligand binding protein of M_r 80,000 (observed M_r 78,400–90,000), which suggests not only that the preparation is pure but also that it is fully active.

Ligand binding studies indicated that the purified mAcChoR preparation behaved more like the membrane-bound receptor than the detergent extract. The dissociation constants for L-QNB, L-hyoscyamine, and carbamoylcholine in the purified receptor agreed within a factor of 2 with those reported for the porcine atrial membrane receptor but differed by factors of 3.8 (antagonists) and 16–40 (carbamoylcholine) for the extract (4). Also, the fraction of high-affinity agonist sites observed for the purified receptor (53%) more closely resembles the membrane-bound state (36%) than that of the extract (17%). Detergent solubilization of the membrane receptor may also solubilize effector proteins or other compounds, which then gain free access to the receptor and alter the apparent ligand binding properties. This laboratory has recently reported the identification of one such effector, which is a protease-sensitive and nondialyzable component separated during chromatography on WGA-agarose that affects the rate at which L-[^{3}H]QNB binds to the receptor (5). Purification would isolate the receptor from such effectors with the observation that the native (unmodified) ligand binding characteristics of the receptor would then be measured. More extensive ligand binding studies on the purified receptor will be reported elsewhere.

$NaDodSO_4$/polyacrylamide gel electrophoresis showed that the purified mAcChoR from porcine atria consisted of two polypeptide bands. The larger polypeptide was identified as the ligand binding polypeptide and migrated as a diffuse band (M_r range of 56,000–106,000) with peak M_rs of 78,000 on uniform 8% acrylamide gels and 95,800 on acrylamide gradient gels. The M_r estimate of 78,000 agrees well with the molecular weight of the affinity-labeled mAcChoR from membrane preparations from several species and tissues examined on uniform acrylamide gels (1). The discrepancy with the molecular weight estimate from acrylamide gradient gels and the diffuse banding pattern of this polypeptide, however, suggest that the M_r 78,000 figure must be viewed with caution. Radiation inactivation studies of rat heart and rat and human brain mAcChoR (1) gave an apparent M_r of 78,000–82,000 for the ligand binding polypeptide. This may be an underestimate since target size analysis would give the minimal size of the protein required for L-[^{3}H]QNB binding, with a potential error of 14% or more (12), and could be insensitive to the presence of additional subunits if there were no interactive energy transfer between them (13).

The smaller polypeptide (M_r 14,800 on acrylamide gradient gels) observed in the purified mAcChoR preparation is not alkylated with [^{3}H]PrBChoM, which makes identification of its relationship to the mAcChoR more difficult. Because of its small size and uncertainties in the size measurements of the mAcChoR, it could be accommodated as a receptor subunit without conflicting with existing data. It is not a proteolytic fragment of the M_r 78,000 polypeptide since no other protein bands are observed. The following evidence suggests, but does not prove, that the small polypeptide may be a mAcChoR subunit. It is observed to co-enrich with the M_r 78,000 polypeptide during affinity chromatography (Fig. 3*A*) and appears at the same constant ratio with the larger polypeptide whether affinity eluted with a low concentration of the agonist L-[^{3}H]QNB or a high concentration of the antagonist carbamoylcholine. The estimated mass ratio of the two polypeptides is consistent with an equimolar ratio for the two, which is consistent with the M_r value of 78,400–90,000 obtained from the L-QNB binding capacity of the purified receptor.

Thus, the minimal structure for the ligand binding function of the mAcChoR is either a monomer of the M_r 78,000 polypeptide (the small polypeptide being a contaminant) or a dimer of the large (α) and small (β) polypeptides. Although less likely, uncertainty in the molecular weight assignments and mass ratio analysis would not preclude a possible $\alpha\beta_2$ structure. Venter (1) proposed that the mAcChoR is a monomer based on the evidence that radiation inactivation and [^{3}H]PrBChoM affinity labeling gave the same apparent molecular weight. However, as pointed out above, this evidence does not necessarily prove that the receptor is monomeric. Avissar *et al.* (14) proposed an oligomeric structure for the mAcChoR in which two 40,000-dalton polypeptide chains were covalently linked through alkali-labile bonds to form dimers that bind agonists with low affinity and that two such dimers when covalently linked in a tetramer bind agonists at high affinity. This model is not supported by our findings for the purified mAcChoR from porcine atria since no evidence was found for the existence of 40,000- or 160,000-dalton proteins, despite the fact that fully half of the agonist interaction sites were of the high-affinity type. Further studies are necessary to clarify the relationship of the small polypeptide to the mAcChoR and to more thoroughly examine the molecular weight and possible stoichiometry of the two polypeptides in our purified preparations.

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