Br. J. Pharmac. (1985), **86**, 375–383

Molecular characterization of muscarinic receptor subtypes in bovine cerebral cortex by radiation inactivation and molecular exclusion h.p.l.c.

Osamu Shirakawa & Chikako Tanaka[1]

Kobe University School of Medicine, Department of Pharmacology, Kobe 650, Japan

1 Muscarinic receptor subtypes in bovine cerebral cortex were investigated by means of radiation inactivation and molecular exclusion high performance liquid chromatography (h.p.l.c.).

2 The functional molecular size of the muscarinic receptor *in situ* was determined by the radiation inactivation method. The value for the muscarinic receptor labelled with [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) was 91,000 daltons, while that labelled with [^{3}H]-pirenzepine ([^{3}H]-PZ) was 157,000 daltons.

3 The muscarinic receptor solubilized with digitonin could be labelled with [^{3}H]-PZ as well as with [^{3}H]-QNB. 3-[(3-Cholamidopropyl)-dimethylammonio] - propane sulphonate (CHAPS) solubilized the muscarinic receptor labelled with [^{3}H]-QNB but not that labelled with [^{3}H]-PZ, in agreement with the low affinity of pirenzepine for inhibiting [^{3}H]-QNB binding in CHAPS-solubilized preparations.

4 The size of the muscarinic receptor in solution was estimated by molecular exclusion h.p.l.c. The digitonin-solubilized muscarinic receptor had a molecular weight of 290,000 and the [^{3}H]-QNB and [^{3}H]-PZ binding activities behaved identically. The CHAPS-solubilized muscarinic receptor labelled with [^{3}H]-QNB was apparently of high molecular weight ($>$ 1,000,000 Mr), indicating the formation of aggregates and/or micelles. In the presence of digitonin this form was dissociated into a lower molecular weight species (580,000 Mr).

5 These data indicate that the ligand binding component of the muscarinic receptor species labelled by both [^{3}H]-QNB and [^{3}H]-PZ exists on the same receptor protein, but that the [^{3}H]-PZ binding component *in situ* is probably coupled to other components in the membrane.

Introduction

Muscarinic receptors are present in various tissues and their activation mediates a wide variety of physiological and biochemical responses. In view of the large number of different responses mediated by muscarinic receptors, distinct subclasses of these receptors may exist. Although it has been widely considered that there is only one type of muscarinic receptor, evidence derived from binding studies suggests that the muscarinic receptor population may be heterogeneous (Birdsall *et al.*, 1978; Hammer *et al.*, 1980; Birdsall & Hulme, 1983).

By the use of pirenzepine, a tricyclic compound with selective antimuscarinic activity, different subclasses of muscrinic receptors can be distinguished (Hammer *et al.*, 1980; Watson *et al.*, 1982; Hammer & Giachetti, 1983; Hirschowitz *et al.*, 1983; Watson *et al.*, 1983). However, it is not clear whether these subclasses of muscarinic receptors represent distinct molecular entities. We have determined the functional molecular sizes of muscarinic receptors labelled with [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) and [^{3}H]-pirenzepine ([^{3}H]-PZ) in bovine cerebral cortex by the radiation inactivation method. In addition, we solubilized the muscarinic receptors from the bovine cerebral cortex using two different detergents, digitonin and 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulphonate (CHAPS), and have further characterized the [^{3}H]-QNB binding sites and the [^{3}H]-PZ binding sites in solution, using molecular exclusion h.p.l.c.

[1]Author for correspondence

Methods

Brain membrane preparations

Crude synaptic membrane preparations from bovine cerebral cortex were prepared as described by Kuno *et al.* (1983). The bovine cerebral cortex (obtained from a local slaughterhouse and stored at −75°C) was homogenized in 20 volumes of ice cold 50 mM Tris-HCl buffer, pH 7.4 at 4°C, with a Brinkmann Polytron PT-10 (setting 7, 20 s). The homogenates were centrifuged (50,000 *g*, 10 min) three times, with resuspension of the intermediate pellet in fresh buffer. The final pellet was stored at −75°C until use.

Receptor solubilization

Membrane preparations (700 mg original wet weight) were thawed and resuspended in 6 ml of 50 mM Tris-HCl buffer, pH 7.6 at 4°C containing 1% digitonin or 10 mM CHAPS. The suspension was stirred slowly at 4°C for 30 min, centrifuged (100,000 *g*, 60 min) at 4°C and used immediately.

Receptor binding assay

Muscarinic receptor binding assays were performed as follows unless otherwise specified. Membrane preparations (200 µl, 0.2–0.3 mg protein per 200 µl) were incubated for 90 min at 25°C in a total assay volume of 0.6 ml of 20 mM Tris-HCl buffer, pH 7.4 at 4°C. Specific [^{3}H]-QNB and [^{3}H]-PZ binding was in equilibrium after incubation for 90 min at 25°C. The incubation was terminated by rapid filtration through Whatman GF/B filters which were presoaked in 0.3% polyethyleneimine for at least 60 min before use, in order to reduce nonspecific absorption to glass fibre filters. The filters were rinsed three times with 5 ml of ice cold 20 mM Tris-HCl buffer, pH 7.4 at 4°C. Nonspecific binding for [^{3}H]-QNB and [^{3}H]-PZ was determined in the presence of 1 µM atropine.

In the solubilized preparations, specific [^{3}H]-QNB and [^{3}H]-PZ binding was in equilibrium after incubation for 30 min and 45 min at 25°C, respectively. Assays of the solubilized receptor preparations (200 µl, 0.2–0.3 mg protein per 200 µl), as described by Bruns *et al.* (1983), were the same as for the membrane preparations, except for the incubation time of 45 min at 25°C and the total assay volume of 1.8 ml. Radioactivity in the filters was counted in a toluene-based scintillator by LS-7000 Beckman scintillation spectrometer at 45% efficiency. Protein was assayed by the method of Bensadoum & Weinstein (1976).

Radiation inactivation

Crude synaptic membranes from bovine cerebral cortex prepared as above (2–3 mg protein) in 2 ml of ice cold 20 mM Tris-HCl buffer, pH 7.4 at 4°C, were frozen immediately at −75°C. The samples were stored at −80°C until irradiated. For the calibration experiments, the mixture of standard enzymes was added and the membrane preparations lyophilized and then sealed *in vacuo*. Radiation inactivation was performed with a ^{60}Co gamma ray source at the Radiation Centre of Osaka Prefecture (Osaka, Japan). ^{60}Co sources were of the coin type (10 mm in diameter and 1 mm in thickness) made by Oakridge National Lab. and the pellet type (1 mm × 1 mm) made by Atomic Energy of Canada, Ltd. or by General Electric Co.. These coins and pellets were encapsulated in stainless steel rods (30 cm in length and 14 mm in diameter). The basket type holder contained 32,000 Ci of ^{60}Co in 14 vertical rods disposed in a radial symmetry around the irradiation chamber (inside diameter; 7.0 cm, height; 27.5 cm) to form a uniform field of radiation. The average dose was about 4.5 Mrad h^{-1}, and samples were exposed in the chamber to various doses of radiation. The temperature in this chamber during irradiation was kept within 0–4°C by placing crushed ice just beneath the sealed test tubes.

Irradiated samples were rehydrated and assayed for muscarinic receptor binding and for standard enzyme activity. The size of the target was calculated by the method of Lo *et al.* (1982). When the standard enzymes were subjected to irradiation, enzymatic activity declined as a simple exponential function of the radiation dosage. In enzymes simultaneously irradiated, the inactivation ratio (Sx/Sp) was determined by the ratio of the slopes of the semilogarithmic plots of each enzyme (Sx) to the slope of pyruvate kinase (Sp). The ratio Sx/Sp for each standard (x) was related to its molecular weight (Mx) normalized relative to the molecular weight (Mp) of pyruvate kinase; i.e., a plot was made of Sx/Sp against Mx/Mp. This gave a good linear fit (Figure 2). Therefore, the molecular weight of any other enzyme or receptor (y) could be determined from this plot, represented by the equation: Sy/Sp = My/Mp.

The calibration enzymes were: calf intestine alkaline phosphatase, horse liver alcohol dehyrogenase, yeast alcohol dehydrogenase and rabbit muscle pyruyate kinase. The enzymes (0.5–2.0 mg ml^{-1}) containing 2 mg ml^{-1} of bovine serum albumin were frozen in test tubes in combination with the membranes, lyophilized, sealed *in vacuo*, and irradiated. Alkaline phosphatase was measured by the method of Malamy & Horecker (1966), alcohol dehyrogenase as described by Racker (1955) and pyruvate kinase as described by Bueher & Pfleiderer (1955).

High performance liquid chromatography (h.p.l.c.) of the solubilized muscarinic receptors on a molecular exclusion column

Digitonin- and CHAPS-solubilized samples (500 μl) were chromatographed on a Toyo Soda TSK 4000 SW molecular exclusion column (7.5 × 600 mm) or a Toyo Soda TSK 3000 SW molecular exclusion column at a flow rate of 0.8 ml min^{-1} at 4°C, using a Toyo Soda (HLC-803D) h.p.l.c. pump and u.v. detector (UV-8 model II). In some experiments, the digitonin-solubilized preparations were preincubated for 45 min at 25°C with [^{3}H]-QNB (2nM) or [^{3}H]-PZ (7.5 nM) in the absence or the presence of 1 μM atropine, and then chromatographed as described above.

The mobile phase was 20 mM Tris-HCl (pH 7.4 at 4°C) containing 150 mM NaCl, and 0.1% digitonin or 1.5 mM CHAPS. 0.4 ml or 0.7 ml fractions were collected and assayed. This chromatographic step yielded a modest 3–5 fold increase in the specific activity of [^{3}H]-QNB and [^{3}H]-PZ binding. Continuous protein profiles were determined by monitoring the absorbance of the column eluate at 280 nm. The TSK 4000 or 3000 column (7.5 × 600 mm) was calibrated with proteins of known molecular weight; bovine serum albumin (Mr = 67,000), γ-globulin (Mr = 160,000), glutamate dehydrogenase (Mr = 280,000), apoferritin (Mr = 460,000) and thyroglobulin (Mr = 669,000).

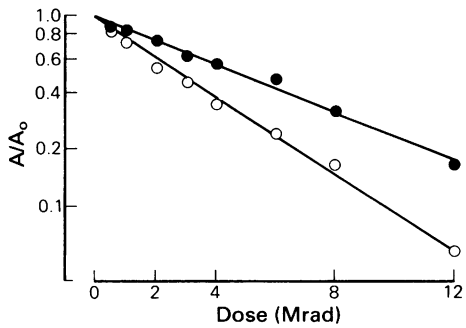

Figure 1 Radiation inactivation-target size analysis of the bovine cortical muscarinic receptor labelled with [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) and [^{3}H]-pirenzepine ([^{3}H]-PZ). Samples of bovine cerebral cortex membranes were irradiated with ^{60}Co gamma ray source, and the muscarinic receptor levels assayed with [^{3}H]-QNB and [^{3}H]-PZ, as described in Methods. The radiation dose-dependent loss of muscarinic receptors was assessed by measuring specific [^{3}H]-QNB (0.1–1 nM) and [^{3}H]-PZ (2–7 nM) binding. A/Ao is the binding activity as a fraction of the initial binding activity. The logarithm of A/Ao was plotted against the radiation dose. (●) Assays with [^{3}H]-QNB binding; (○) assays with [^{3}H]-PZ binding. Each point represents the average of triplicate determinations from five separate experiments. Lines were drawn by least squares linear regression and the correlation coefficient for all of the points was greater than 0.98.

The excluded volume was determined by blue dextran and ascorbate was taken as the included volume. The estimates of molecular size of the receptor-detergent complexes were obtained from a graph of the log of the molecular weight of the standards as a function of their distribution coefficients (K) where K = (Ve-Vo)/(Vt-Vo). Vo: excluded volume, Vt: included volume and Ve: elution volume.

Reagents

[^{3}H]-QNB (30.1 Ci $mmol^{-1}$) and [^{3}H]-PZ (82.3 Ci $mmol^{-1}$) were purchased from New England Nuclear. Digitonin and CHAPS were purchased from Wako (Japan) and Sigma (U.S.A.), respectively. Pirenzepine dihydrochloride was a gift from Boehringer Ingelheim (West Germany). All the calibration enzymes for radiation inactivation and molecular exclusion h.p.l.c. experiments were from Sigma (U.S.A.).

The sources of other drugs and other reagents were as follows: atropine sulphate (Merck, West Germany), scopolamine hydrobromide and polythyleneimine (Nakarai, Japan), carbamylcholine chloride (Yoneyama, Japan), oxotremorine (Aldrich, U.S.A.). All other chemicals were obtained form other commercial sources.

Results

Determination of the molecular size of muscarinic receptors in the membrane

We first determined the effect of lyophilization on [^{3}H]-QNB and [^{3}H]-PZ binding to crude synaptic membrane preparations form bovine cerebral cortex. No loss of binding of [^{3}H]-QNB and [^{3}H]-PZ occurred and the same K_D and B_{max} values were obtained (data not shown). When the lyophilized membranes from bovine cerebral cortex were subjected to gamma irradiation from a ^{60}Co source, muscarinic receptors, as measured by [^{3}H]-QNB- and [^{3}H]-PZ- specific binding, were inactivated as a simple exponential function of the radiation dosage (Figure 1). Specific [^{3}H]-QNB binding was assessed over a range of 0.1–1 nM, that is both above and below the K_D of 0.23 nM for [^{3}H]-QNB binding to muscarinic receptors. Specific [^{3}H]-PZ binding was assessed over a range of 2–7 nM, concentrations both above and below the K_D for [^{3}H]-PZ (4.3 nM). The molecular size of the muscarinic receptor labelled with [^{3}H]-QNB and [^{3}H]-PZ was determined by inclusion of standard enzymes in the membrane preparation (Figure 2). The calculated target size for each ligand was independent of the ligand concentration as there was no change in affinity of specific [^{3}H]-QNB and [^{3}H]-PZ binding during radiation inactiviation (data not shown).

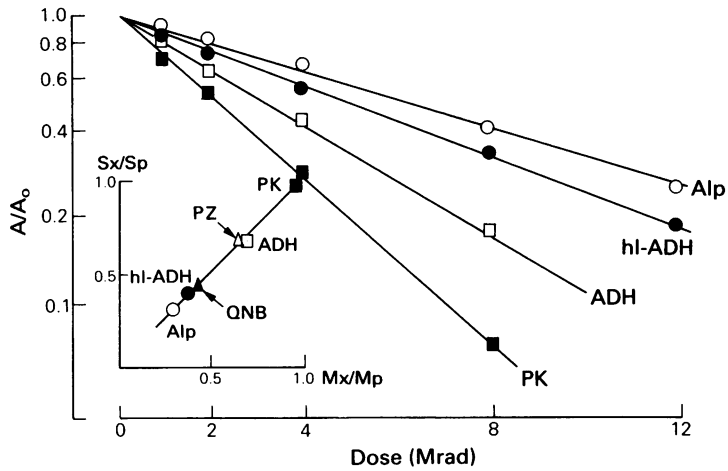

Figure 2 Radiation inactivation molecular weight calibration curve. The radiation dose-dependence of four calibration enzymes: rabbit muscle pyruvate kinase (■ PK, 224,000 daltons), yeast alcohol dehydrogenase (□ ADH, 160,000 daltons), horse liver ADH (● hl-ADH, 84,000 daltons) and calf intestine alkaline phosphatase (○ Alp, 70,000 daltons) was measured. A/Ao is the activity relative to that before irradiation (Ao), plotted on a logarithmic scale. Means of data of triplicate experiments are shown, with lines fitted by least squares. (Inset) Calibration plots for the inactivation ratios of the four standard enzymes. The ratio Sx/Sp and Mx/Mp was determined as described under Methods. The molecular weight of the [^{3}H]-quinuclidinybenzilate (▲) and of the [^{3}H]-pirenzepine (△) binding site was estimated from this plot. The slope of the calibration plot obtained (least-squares) is 0.94.

As shown in Figure 2, the molecular weight calibration curve indicated a molecular size of 91,000 daltons for the muscarinic receptor labelled with [^{3}H]-QNB, while that of the muscarinic receptor labelled with [^{3}H]-PZ was estimated to be 157,000 daltons.

Solubilization of muscarinic receptors labelled with [^{3}H]-QNB and [^{3}H]-PZ

The solubilization of the muscarinic receptors was performed by use of two different detergents, i.e., digitonin and CHAPS. The use of these two detergents for muscarinic receptor solubilization has been reported to meet the criteria concerning solubilization and pharmacological characterization (Beld & Ariens, 1974; Gorissen *et al.*, 1981; Gavish & Sokolovsky, 1982; Kuno *et al.*, 1983). Table 1 summarized the equilibrium binding of [^{3}H]-QNB and [^{3}H]-PZ to the membranes and to the solubilized preparations. Scatchard analysis of the binding of [^{3}H]-QNB to digitonin- and CHAPS- solubilized preparations gave similar K_D values (0.24 nM and 0.17 nM, respectively), closely resembling that found in non-treated membranes (0.23 nM). The number of [^{3}H]-QNB binding sites in the digitonin- and CHAPS-solubilized preparations was estimated to be 880 fmol mg^{-1} protein and 340 fmol mg^{-1} protein, respectively. In contrast, the muscarinic receptor labelled with [^{3}H]-PZ was solubilized only by digitonin and with a low yield (180 fmol mg^{-1} protein, 16.5% of that in the membrane) and a lowered affinity (K_D: 8.1 nM). Non-specific binding of [^{3}H]-PZ in the CHAPS-solubilized

Table 1 Equilibrium binding of [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) and [^{3}H]-pirenzepine ([^{3}H]-PZ) to membranes and solubilized preparations from bovine cerebral cortex

	Membrane	*Digitonin extract*	*CHAPS extract*
Specific [^{3}H]-QNB binding			
K_D (nM)	0.23	0.24	0.17
B_{max} (fmol mg^{-1} protein)	1380	880	340
Specific [^{3}H]-PZ binding			
K_D (nM)	4.3	8.1	—
B_{max} (fmol mg^{-1} protein)	1090	180	<15

Solubilization of the muscarinic receptor with digitonin or CHAPS and the receptor binding assays are described under Methods. Specific [^{3}H]-QNB and [^{3}H]-PZ binding was defined as the difference between the binding in the presence and absence of 1 μM atropine. Apparent B_{max} and K_D values were estimated by Scatchard analysis. The same concentration range of [^{3}H]-QNB (0.0625–2 nM) or [^{3}H]-PZ (0.625–15 nM) was used in all experiments. Each value represents the mean of at least three independent experiments, each of which was carried out in duplicate.

Table 2 Comparison of IC_{50} values for specific [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) binding to membrane and solubilized preparations by muscarinic agonists and antagonists

	IC_{50}				
	Membrane (A)	*Digitonin extract (B)*	*CHAPS extract (C)*	*B/A*	*C/A*
Muscarinic agonists (μM)					
Carbamylcholine	320 ± 42	464 ± 35	68.5 ± 5.1	1.45	0.21*
Oxotremorine	3.84 ± 0.62	12.3 ± 1.8	4.38 ± 0.82	3.21*	1.14
Muscarinic antagonists (nM)					
Pirenzepine	321 ± 27	477 ± 59	1365 ± 127	1.49	4.25*
Atropine	2.71 ± 0.18	2.34 ± 0.31	2.00 ± 0.14	0.86	0.74
Scopolamine	2.31 ± 0.43	2.99 ± 0.18	2.88 ± 0.21	1.29	1.25

Solubilization and assay of muscarinic receptors from bovine cerebral cortex was performed as described under Methods. IC_{50} values (drug concentrations which inhibited specific binding of 1 nM [^{3}H]-QNB by 50%) were obtained from displacement experiments. Each value represents the mean ± s.e.mean of three independent experiments, each of which was carried out in duplicate or triplicate. Significance: $*P < 0.05$ (versus membrane preparation).

preparation was over 90% of the total binding and substantial specific binding was not detectable.

Pharmacological profiles of the solubilized muscarinic receptors

In order to elucidate the pharmacological nature of the solubilized muscarinic receptors, the potencies of muscarinic agonists (carbamylcholine and oxotremorine) and muscarinic antagonists (pirenzepine, atropine and scopolamine) to inhibit [^{3}H]-QNB binding were determined. As shown in Table 2, the order of IC_{50} values for specific [^{3}H]-QNB binding was atropine ≒ scopolamine > pirenzepine > oxotremorine > carbamylcholine and was the same in membranes and in solubilized preparations. However, carbamylcholine had a higher potency (IC_{50}: 68 μM) in CHAPS-solubilized preparation compared to membranes, whereas oxotremorine had a lower affinity (IC_{50}: 12 μM) in the digitonin-solubilized preparation. In addition, the potency of pirenzepine in the CHAPS-

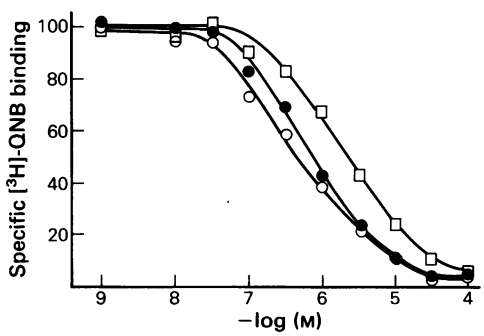

Figure 3 Pirenzepine (PZ) inhibition of specific [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) binding to membranes and solubilized preparations from bovine cerebral cortex. The solubilization and assays were performed as described under Methods. Various concentrations of unlabelled PZ were added to inhibit [^{3}H]-QNB (1 nM) binding to membrane and solubilized preparations. Data shown are the average of the three independent experiments, each performed in duplicate: (○) Membrane preparation, (●) digitonin-solubilized preparation and (□) CHAPS-solubilized preparation.

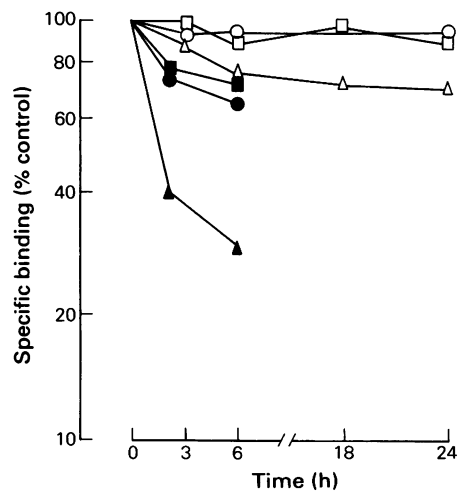

Figure 4 The stability of specific [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) and [^{3}H]-pirenzepine ([^{3}H]-PZ) binding activity of receptor preparations solubilized with digitonin or CHAPS. The solubilized receptor preparations were incubated at 4°C or 25°C in the absence of [^{3}H]-QNB and [^{3}H]-PZ and assayed at intervals for specific [^{3}H]-QNB and [^{3}H]-PZ binding as described under Methods. The concentrations of [^{3}H]-QNB and [^{3}H]-PZ were 1 nM and 3 nM, respectively, in all assays. The results are representative of two similar experiments: 4°C; (○) digitonin (QNB), (□) digitonin (PZ) and (△) CHAPS (QNB); 25°C; (●) digitonin (QNB), (■) digitonin (PZ) and (▲) CHAPS (QNB).

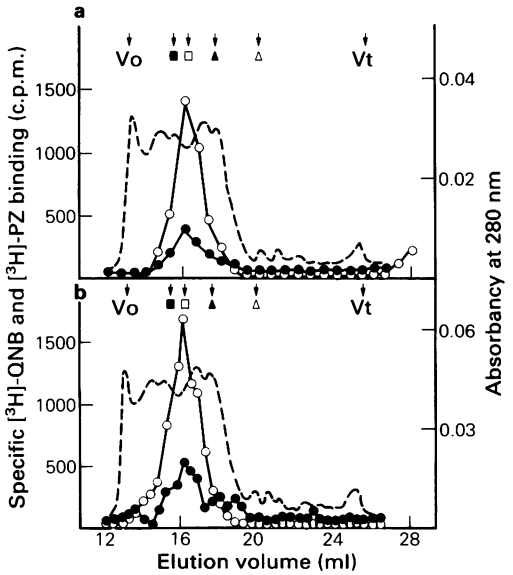

Figure 5 Molecular exclusion h.p.l.c. elution profiles of the digitonin-solubilized muscarinic receptor from bovine cerebral cortex. (a) Elution profiles of muscarinic receptors preincubated with [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) and [^{3}H]-pirenzepine ([^{3}H]-PZ). Digitonin-solubilized preparations were preincubated with 2 nM [^{3}H]-QNB (○) and 7.5 nM [^{3}H]-PZ (●) at 25°C for 45 min. Each solubilized sample (500 μl) was applied to a TSK 3000 column and chromatographed at a flow rate of 0.8 ml at 4°C. The mobile phase was 20 mM Tris-HCl, 150 mM NaCl and 0.1% digitonin (pH 7.4 at 4°C): 0.7 ml fractions were collected. Specific binding was determined by subtracting the counts in the elution profiles of solubilized preparations preincubated in the presence of 1 μM atropine. The overall recovery of the radioactive fractions was approximately 80% and 65% for [^{3}H]-QNB and [^{3}H]-PZ binding, respectively. The results are representative of three separate experiments. (b) Elution profiles of muscarinic receptors not preincubated with [^{3}H]-QNB and [^{3}H]-PZ. Unlabelled digitonin-solubilized preparations were applied to a TSK 3000 column. Each fraction (0.4 ml) was collected and assayed for the specific [^{3}H]-QNB and [^{3}H]-PZ binding, as described under Methods. The results shown are representative of three separate experiments. Calibration enzymes: (△) bovine serum albumin, and (▲) γ-globulin, (□) glutamate dehydrogenase and (■) apoferritin. Continuous protein profiles (---) were determined by monitoring the absorbance of the column elution of a sample at 280 nm. Nonspecific binding for the [^{3}H]-QNB elution profiles in (a) and (b) was less than 10% of the total binding in the peak, while nonspecific binding for [^{3}H]-PZ was 40–45% of total binding in the peak.

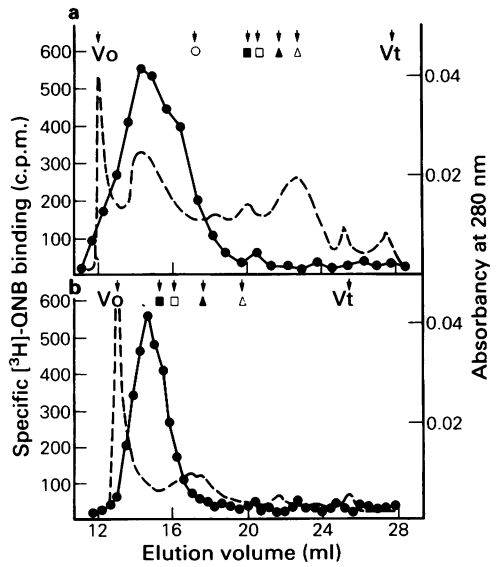

Figure 6 Molecular exclusion h.p.l.c. elution profiles of the CHAPS-solubilized muscarinic receptor from bovine cerebral cortex. (a) Elution profiles of muscarinic receptors not preincubated with [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB). Unlabelled CHAPS-solubilized preparations (500 μl) were applied to a TSK 4000 column and chromatographed at a flow rate of 0.8 ml min^{-1} at 4°C. The mobile phase was 20 mM Tris-HCl, 150 mM NaCl and 1.5 mM CHAPS (pH 7.4 at 4°C): 0.7 ml fractions were collected and assayed for specific [^{3}H]-QNB binding, as described under Methods. The results are representative of three separate experiments. (b) Similar chromatography was performed as described in (a) except that the molecular exclusion column used was TSK 3000 SW, the mobile phase was 20 mM Tris-HCl, 150 mM NaCl and 0.1% digitonin (pH 7.4 at 4°C), and the collected fractions were 0.4 ml. Continuous protein profiles (---) were determined by monitoring the absorbance of the column elutions of a sample at 280 nm. (●) assays with [^{3}H]-QNB binding. Nonspecific binding for the [^{3}H]-QNB elution profiles was less than 10% of the total binding. Calibration enzymes: (△) bovine serum albumin, (▲) γ-globulin, (□) glutamate dehydrogenase, (■) apoferritin and (○) thyroglobulin.

solubilized preparation differed from that in membrane and digitonin-solubilized preparations. The competitive curves (Figure 3) indicate that pirenzepine was clearly of lower affinity in the CHAPS-solubilized preparation; the IC_{50} value in CHAPS-solubilized preparation was 1365 nM, whereas the values in the membrane and in digitonin-solubilized preparations were 321 nM and 477 nM, respectively.

Stability of the solubilized muscarinic receptor preparations

As illustrated in Figure 4, the digitonin-solubilized muscarinic receptors labelled with [^{3}H]-QNB and [^{3}H]-PZ were of a similar stability at 4°C and 25°C. On the

other hand, CHAPS-solubilized muscarinic receptors labelled with [^{3}H]-QNB were less stable than the digitonin-solubilized muscarinic receptors. Therefore further molecular characterizations of the soluble receptors were carried out at 4°C to determine whether the receptors labelled with [^{3}H]-QNB and [^{3}H]-PZ are the same protein.

Molecular exclusion h.p.l.c. elution profiles of the soluble receptor preparations

Figure 5 shows a typical h.p.l.c. profile obtained when the digitonin-solubilized preparation was chromatographed on a TSK 3000 SW column. The elution profile of the digitonin-solubilized muscarinic receptor sites labelled with [^{3}H]-QNB was very similar to that of the receptors labelled with [^{3}H]-PZ, regardless of whether or not this preparation had been labelled with [^{3}H]-QNB and [^{3}H]-PZ before chromatography. The peak of digitonin-muscarinic receptor complex labelled with [^{3}H]-QNB or [^{3}H]-PZ was consistently eluted at 16.0 ml on a TSK 3000 SW column, equivalent to an apparent Mr of 290,000 (Figure 5a,b).

In contrast, CHAPS-muscarinic receptor complex labelled with [^{3}H]-QNB was eluted with the protein void volume on a TSK 3000 SW column (data not shown). On a TSK 4000 SW column (Figure 6a) it was eluted as a broad peak at 14.2 ml, 2.5 ml ahead of thyroglobulin (Mr = 669,000), suggesting a Mr > 1,000,000. However, this macromolecular receptor complex labelled with [^{3}H]-QNB was eluted at 14.8 ml on a TSK 3000 SW column (equivalent to an apparent Mr of 580,000) when chromatographed with a mobile phase buffer containing 0.1% digitonin instead of CHAPS (Figure 6b). This indicates that the CHAPS-muscarinic receptor complex in the absence of digitonin occurred as aggregates and/or as large micelles.

Discussion

Radiation inactivation-target size analysis is the only available method for the determination of the molecular weight of a protein in intact membranes (Kempner & Schlegel, 1979). We have determined the molecular weight of the membrane-bound muscarinic receptor subtypes in bovine cerebral cortex by this procedure. In our study, the membrane-bound muscarinic receptor labelled with [^{3}H]-QNB had a molecular weight of 91,000 daltons. This value was similar to the calculated target size found in other studies (Uchida *et al.*, 1982; Venter, 1983). Furthermore, the target sizes of the muscarinic receptor labelled with [^{3}H]-QNB are the same as those estimated by SDS-polyacrylamide gel electrophoresis (Birdsall *et al.*, 1979; Ruess & Lieflander, 1979; Amitai *et al.*, 1982; Venter, 1983). These studies indicate that the ligand binding component of the muscarinic receptor is a monomeric protein and that there are no clear structural differences in various tissues. In contrast, we have demonstrated, for the first time, that the membrane-bound muscarinic receptor labelled with [^{3}H]-PZ has a molecular size of 157,000 daltons. As shown in Table 1, the B_{max} for specific [^{3}H]-PZ binding to the membranes is about 80% of that for specific [^{3}H]-QNB binding. The difference between the target sizes for [^{3}H]-QNB and [^{3}H]-PZ binding sites is therefore too large, if the slopes of the inactivation curves for [^{3}H]-QNB binding is to be taken as weighed mean of the inactivation curves for high and low affinity PZ binding sites. One possible explanation is that during irradiation, conversion of high to low affinity for PZ may occur in addition to loss of high affinity PZ binding sites. Furthermore, in the characterization of QNB and PZ binding sites, there appears to be an anomaly in the PZ binding properties of the receptors assayed directly with [^{3}H]-PZ and indirectly in competition experiments with [^{3}H]-QNB. The affinity of PZ for muscarinic receptors expected from the K_D values of [^{3}H]-PZ binding in the membranes (4 nM) and in digitonin-solubilized preparation (8 nM) was about ten fold higher than that expected from the IC_{50} values of PZ inhibition of [^{3}H]-QNB binding in the membranes (321 nM) and in the digitonin-solubilized preparation (477 nM) (Tables 1 and 2). This discrepancy may be partly but not totally accounted for by the fact that [^{3}H]-PZ binding labels only high affinity binding sites for PZ, and the PZ–[^{3}H]-QNB competition curves involve high and low affinity sites for PZ.

To elucidate differences between the molecular size of [^{3}H]-QNB binding sites and that of the [^{3}H]-PZ binding site (determined using radiation inactivation, target size analysis), molecular exclusion h.p.l.c. was used. Techniques such as sucrose density gradient centrifugation and conventional gel filtration chromatography have been used for the characterization of the solubilized receptor, but these approaches are tedious and not so reproducible in our hands. Molecular exclusion h.p.l.c. was used as a precise, reproducible and rapid method of assessing size differences in solubilized receptor preparations. The molecular exclusion h.p.l.c. profile of the muscarinic receptor labelled with [^{3}H]-QNB and [^{3}H]-PZ was of a similar pattern and the major peak (Mr = 290,000) was identical. This result indicates that both the [^{3}H]-QNB binding component and the [^{3}H]-PZ binding component probably exist on the same protein of the muscarinic receptor. Using molecular exclusion h.p.l.c., we were not able to determine the molecular weight of the muscarinic receptor protein itself, since the amount of bound digitonin could not be cal-

culated. Berrie *et al.* (1984) reported the molecular weight of about 290,000 for the digitonin-muscarinic receptor complex, in agreement with our result.

The discrepancy betwen the molecular size in the radiation inactivation and that in molecular exclusion h.p.l.c. concerning the [^{3}H]-QNB and the [^{3}H]-PZ binding sites indicates that the muscarinic receptor protein *in situ* labelled with [^{3}H]-PZ can involve both the same receptor protein labelled with [^{3}H]-QNB and other components in the membrane, in agreement with the previous report by Birdsall *et al.* (1984). That is, in the digitonin-solubilized preparation, the components functionally coupled to muscarinic receptor protein involving both [^{3}H]-QNB and [^{3}H]-PZ binding sites probably dissociate during solubilization. This may explain the lower affinity (8.1 nM) and low yield (180 fmol mg^{-1} protein, 16.5% of that in the membrane) of the [^{3}H]-PZ labelled muscarinic receptor in this preparation. In addition, the [^{3}H]-PZ labelled muscarinic receptor could not be solublized with CHAPS, in agreement with the observed low affinity of PZ for [^{3}H]-QNB binding in CHAPS-solubilized preparation.

Thus, we have found that muscarinic receptor subtypes can result from differences in the interaction of the ligand binding protein with other components in the membrane. This study provides the first information concerning the molecular basis of muscarinic receptor subtypes, using radiation inactivation and molecular exclusion h.p.l.c. The techniques we used make feasible the analysis of the heterogeneity of the muscarinic receptor and of the molecular structure and regulation of this receptor system.

This work was supported by grants form the Ministry of Education, Science and Culture and Ministry of Health and Welfare, Japan. We thank S. Okamoto and T. Ohnishi of the Radiation Centre of Osaka Prefecture and Y. Inoue of Kobe University for helpful suggestions and assistance with irradiation, M. Ohara of Kyushu University for critical reading of the manuscript, and R. Koizumi for secretarial assistance.

References

AMITAI, G., AVISSAR, S., BALDERMAN, D. & SOKOLOVSKY, M. (1982). Affinity labelling of muscarinic receptors in rat cerebral cortex with a photolabile antagonist. *Proc. natn. Acad. Sci. U.S.A.*, **79**, 243–247.

BELD, A.J. & ARIENS, E.J. (1974). Stereospecific binding as a tool in attempts to localize and isolate muscarinic receptors. *Eur. J. Pharmac.*, **25**, 203–209.

BENSADOUM, A. & WEINSTEIN, D. (1976). Assay of proteins in the presence of interfering materials. *Anal. Biochem.*, **70**, 241–250.

BERRIE, C.P., BIRDSALL, N.J.M., HAGA, T. & HULME, E.C. (1984). Hydrodynamic properties of muscarinic acetylcholine receptors solubilized from rat forebrain. *Br. J. Pharmac.*, **82**, 839–851.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1978). The binding of agonists to brain muscarinic receptors. *Mol. Pharmac.*, **14**, 723–736.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1979). A study of the muscarinic receptor by gel electrophoresis. *Br. J. Pharmac.*, **66**, 337–342.

BIRDSALL, N.J.M. & HULME, E.C. (1983). Muscarinic receptor subclasses. *Trends Pharmac. Sci.*, **4**, 459–463.

BIRDSALL, N.J.M., HULME, E.C. & STOCKTON, J.M. (1984). Muscarinic receptor heterogeneity. *Trends Pharmac. Sci.*, Supple. 4–8.

BRUNS, R.F., LAWSON-WENDLING, K. & PUGSLEY, T.A. (1983). A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.*, **132**, 74–81.

BUEHER, T. & PFLEIDERER, G. (1955). Pyruvate kinase from muscle. *Methods Enzymol.*, **1**, 435–440.

GAVISH, H. & SOKOLOVSKY, M. (1982). Solubilization of muscarinic acetylcholine receptor by zwitterionic detergent form rat brain cortex. *Biochem. biophys. Res. Commun.*, **109**, 819–824.

GORISSEN, H., AERTS, G., ILLIEN, B. & LADURON, P. (1981). Solubilization of muscarinic acetylcholine receptors from mammalian brain: an analytical approach. *Anal. Biochem.*, **111**, 33–41.

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J.M., BURTGEN, A.S.V. & HULME, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature (Lond.)*, **283**, 90–92.

HAMMER, R. & GIACHETTI, A. (1983). Muscarinic receptor subtypes; M1 and M2 biochemical and functional characterization: *Life Sci.*, **31**, 2991–2998.

HIRSCHOWITZ, B.I., FONG, J. & MOLINA, E. (1983). Effects of pirenzepine and atropine on vagal and cholinergic gastric secretion and gastrin release and on heart rate in dog. *J. Pharmac. exp. Ther.*, **225**, 263–268.

JEMPNER, E.S. & SCHLEGEL, W. (1979). Size determination of enzymes by radiation inactivation. *Anal. Biochem.*, **92**, 2–10.

KUNO, T., SHIRAKAWA, O. & TANAKA, C. (1983). Regulation of the solubilized bovine cerebral cortex muscarinic receptor by GTP and Na^+. *Biochem. biophys. Res. Commun.*, **112**, 948–953.

LO, M.M.S., BARNARD, E.A. & DOLLY, J.O. (1982). Size of acetylcholine receptors in the membrane. An improved version of the radiation inactivation method. *Biochemistry*, **21**, 2210–2217.

MALAMY, M. & HORECKER, B.L. (1966). Alkaline phosphatase. *Methods Enzymol.*, **9**, 639–645.

RACKER, E. (1955). Alcohol dehydrogenase from Baker's Yeast. *Methods Enzymol.*, **1**, 500–503.

RUESS, K.-P. & LIEFLANDER, M. (1979). Action of detergents on covalently labelled membrane bound muscarinic acetylcholine receptor of bovine nucleus caudate. *Biochem. biophys. Res. Commun.*, **88**, 627–633.

UCHIDA, S., MATSUMOTO, K., TAKEYASU, K., HIGUCHI, H.

& YOSHIDA, H. (1982). Molecular mechanism of the effects of guanine nucleotide and sulfhydryl reagent on muscarinic receptors in smooth muscles studied by radiation inactivation. *Life Sci.*, **31**, 201–209.

VENTER, J.C. (1983). Muscarinic receptor structure. *J. biol. Chem.*, **258**, 4842–4848.

WATSON, M., ROESKE, W.R. & YAMAMURA, H.I. (1982). [^{3}H]-pirenzepine selectivity identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sci.*, **21**, 2019–2023.

WATSON, M., YAMAMURA, H.I. & ROESKE, W.R. (1983). A unique regultory profile and regional distribution of [^{3}H]-pirenzepine binding in the rat provide evidence for distinct M1 and M2 muscarinic receptor subtypes. *Life Sci.*, **32**, 3011–3022.

(*Received January 28, 1985.*
Revised June 7, 1985.
Accepted June 11, 1985.)