MOLECULAR SIZE OF MUSCARINIC ACETYLCHOLINE RECEPTORS OF RAT BRAIN

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1. Introduction

The muscarinic acetylcholine receptor of mammalian brain has been identified and its pharmacological properties have been studied in detail by the use of a radiolabeled ligand, 3-[^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) [1], and other ligands (reviewed [2]). Molecular properties of the receptor, however, have not yet been well characterized because trials to solubilize the receptor by common detergents have been generally unsuccessful. Digitonin is the sole detergent known to solubilize a certain portion of the receptor in a form capable of binding its ligands [3–6]. The sedimentation coefficient of the solubilized component was estimated to be 9 S [4,6]. In this case, however, the [^{3}H]QNB binding activity is presumed to reside in the receptor–digitonin complex, and the contribution of digitonin to the weight of the complex has remained to be measured for the estimation of the molecular weight of the receptor. Birdsall et al. [7] adopted a radiolabeled ligand that binds covalently to the muscarinic receptor, and solubilized a labeled component in 1% SDS by heating for 3 min at 100°C. The component was est. mol. wt 83 000 by SDS–polyacrylamide gel electrophoresis.

Here, the receptor in membrane fractions of rat brain was labeled with [^{3}H]QNB and solubilized by a mild detergent, Lubrol PX. The protein portion of the solubilized receptor was est. mol. wt 86 000 by a gel filtration chromatography and the sucrose density gradient centrifugation in H_2O and D_2O. The close similarity between the value obtained here and in [7] suggests that the muscarinic receptor is monomer.

2. Materials and methods

Synaptic and microsomal membrane fractions were prepared from the whole brain of male Wister rats as in [8]. Essentially the same results were obtained for the 2 membrane fractions.

Membranes (1 mg protein/ml) were incubated with 5 nM D,L-[*benzilic*-4,4′-^{3}H]3-quinuclidinyl benzilate ([^{3}H]QNB) (29.4 Ci/mmol, New England Nuclear, Boston) in 4 mM Hepes buffer (pH 7.5), 0.4 mM $MgCl_2$ and 0.2 mM EDTA at 30°C for 30 min. Atropine was added to the above suspension before the incubation for control tubes and after the incubation for experimental tubes in 1 μM final conc. Lubrol PX was then added in 0.32% final conc. to the incubation suspension, and the suspension was stood for 60 min at 0°C and centrifuged for 1 h at 100 000 $\times g$. The clear supernatant was subjected to gel filtration chromatography or sucrose density gradient centrifugation together with calibrating enzymes.

The column for gel filtration (Ultrogel AcA 34, 0.9 × 30 cm) was equilibrated and eluted with a Lubrol solution (0.1% Lubrol PX, 0.15 M NaCl, 20 mM Hepes–KOH buffer (pH 7.5), 2 mM $MgCl_2$ and 1 mM EDTA) at 4°C. The sample was applied in 0.5 ml and fractions of 0.33 ml were collected. Linear sucrose gradients (4 ml) in H_2O and D_2O were prepared from 5% and 20% sucrose in the Lubrol solution in H_2O and D_2O, respectively. The sample (0.25 ml) was applied to the top of the gradient and was centrifuged at 1°C and 43 000 rev./min for 14 h in a Hitachi RPS 50 rotor. After centrifugation the bottom of the tube was punctured and 20–23 fractions of 13 drops each were collected.

The binding of [^{3}H]QNB to membranes was assayed by the use of a glass fiber filter (Whatman GF/C), and the bound form of [^{3}H]QNB in solubilized preparations was assayed by counting [^{3}H]QNB eluted in the void volume of a pencil column of Sephadex G-50. The column (0.5 × 7 cm, 1.3 ml) was equilibrated with 10 mM phosphate buffer (pH 7.0) and

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0.1–0.2 ml of the sample was applied. In the typical experiment, the incubation of the labeled membranes in the Lubrol solution resulted in the loss of 64% of bound [^{3}H]QNB, and 73% of the remaining bound [^{3}H]QNB, that is 26% of original bound [^{3}H]QNB, were recovered as solubilized bound [^{3}H]QNB in the supernatant. The similar low yield of the muscarinic receptor has also been reported when digitonin was used as a solubilizing agent [4–6]. The recovery of bound [^{3}H]QNB in gel filtration and sucrose density gradient centrifugation was 60–70%.

Assay of calibrating enzymes and analysis of data of sedimentation experiments were done as in [9] except that ribonuclease A was also employed in this experiment. Ribonuclease was assayed in 0.1 M acetate buffer (pH 5.0) using 0.55 mg/ml yeast RNA as a substrate. The following values were adopted as physical parameters of the enzyme [10]: partial specific volume ($\bar{v}$) 0.703 ml/g, sedimentation coefficient ($s^0_{20,w}$) 1.78 S, diffusion coefficient ($D^0_{25,w}$) 1.25 × 10^{-6} cm^2/s and Stokes radius (a) 1.69 nm.

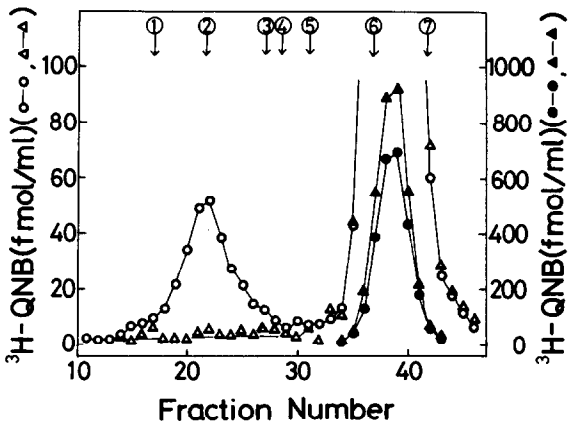

Fig.1. Gel filtration in Ultrogel AcA 34. Membranes were incubated with [^{3}H]QNB in the absence (o——o, ●——●) or presence (Δ——Δ, ▲——▲) of 1 μM atropine, solubilized with Lubrol PX and then applied to the column as described in the text. A portion of each fraction was directly counted with liquid scintillation counter. The first peak corresponds to the receptor–[^{3}H]QNB complex and the second peak to free [^{3}H]QNB. The designated markers are: (1) blue dextran; (2) β-galactosidase; (3) fumarase; (4) lactate dehydrogenase; (5) malate dehydrogenase; (6) cytochrome *c*; (7) cyclic AMP.

3. Results and discussion

The muscarinic receptor binds its ligands with a high degree of specificity, and the binding of [^{3}H]-QNB to membranes is inhibited almost completely by 1 μM atropine. The presumed receptor–[^{3}H]QNB complex in the solubilized preparation should therefore be detected only when membranes were incubated with [^{3}H]QNB in the absence of atropine, but not when atropine is present in the incubation mixture. Fig.1 shows the elution pattern from the Ultrogel of [^{3}H]QNB, which had been incubated with membranes in the absence and presence of 1 μM atropine followed by the treatment with Lubrol PX as in section 2. When membranes were incubated in the presence of atropine, [^{3}H]QNB was observed only at the position where free [^{3}H]QNB was eluted (the second peak in fig.1). A clear peak of [^{3}H]QNB (the first peak in fig.1) besides free [^{3}H]QNB was observed when membranes were incubated in the absence of atropine indicating that the first peak is the receptor–[^{3}H]QNB complex. The receptor–[^{3}H]QNB complex was estimated to have a = 6.9 nm from a standard curve of distribution coefficient (K_d) versus a, constructed from the calibrating enzymes (fig.2).

Fig.3 shows the result of centrifugation through

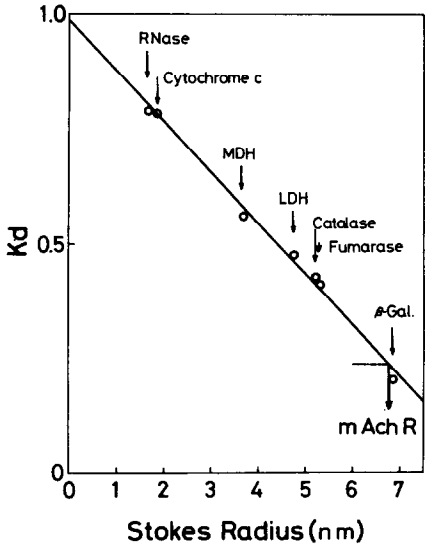

Fig.2. Standard curve of distribution coefficient, K_d, versus Stokes radius (a) for the calibrating enzymes. $K_d = (V_x - V_o)/(V_t - V_o)$ where V_x, V_o and V_t represent the elution volume of calibrating enzymes, blue dextran and cAMP, respectively.

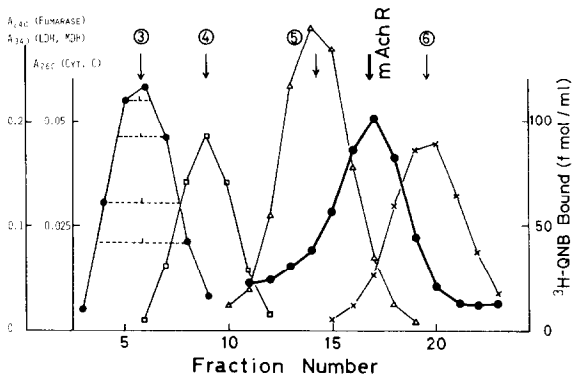

Fig.3. Sucrose density gradient centrifugation in H_2O. Experimental details are described in the text. A portion of each fraction (0.1 ml) was used for assay of bound [^{3}H]QNB by Sephadex G-50 columns. The peak positions of calibrating enzymes and bound [^{3}H]QNB were determined as shown for fumarase, that is the average of midpoints of 4 dotted lines. The designated markers are the same as those in the legend of fig.1.

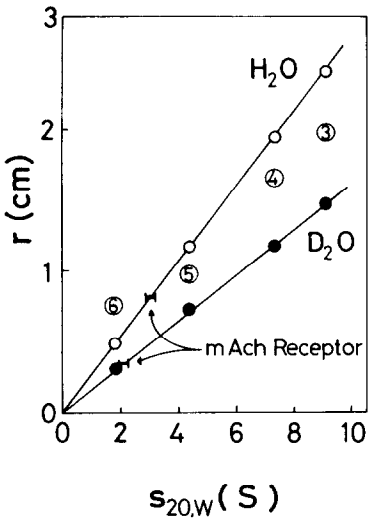

Fig.4. Calibrating curves for sucrose density gradient centrifugation in H_2O and D_2O. The distances traveled by the proteins (r) are plotted versus sedimentation coefficients ($s^0_{20,w}$). The designated markers are the same as those in the legend of fig.1. The apparent values of $s^0_{20,w}$ for the receptor–[^{3}H]-QNB complex are shown (av. ± 1 SD).

the gradient of 5–20% sucrose in H_2O. A clear peak of bound [^{3}H]QNB was detected by the Sephadex G-50 method at near the midpoint between the peaks of malate dehydrogenase and cytochrome *c*. This peak was observed only when membranes were labeled in the absence of atropine. When membranes were incubated with [^{3}H]QNB in the presence of 1 μM atropine, only free [^{3}H]QNB was found around the starting position (not shown). The peak of bound [^{3}H]QNB is therefore considered to represent the receptor–[^{3}H]QNB complex. When centrifugation was carried out in the sucrose gradient in D_2O, a peak of bound [^{3}H]QNB was observed at almost the same position as the peak of cytochrome *c* (not shown).

The distance travelled by calibrating enzymes was determined from the position of the peaks of enzyme activities and was shown to be linearly related to $s^0_{20,w}$ both in H_2O and D_2O (fig.4). Apparent $s^0_{20,w}$ of the receptor–[^{3}H]QNB complex was calculated from the standard curves of calibrating enzymes to be 3.01 ± 0.15 S in H_2O and 2.12 ± 0.13 S in D_2O. The higher value in H_2O than that in D_2O indicates that the receptor–[^{3}H]QNB complex has a higher $\bar{\nu}$ than those of calibrating enzymes. The most likely explanation for the deviation in $\bar{\nu}$ is that the detergent is bound to the complex at hydrophobic regions of the receptor.

The value of 3 S is markedly different from the value of 9 S which had been estimated from sucrose density gradient centrifugation in digitonin for the muscarinic receptor solubilized by digitonin [4,6]. The discrepancy may be explained by assuming that digitonin as well as Lubrol PX binds to hydrophobic regions of the receptor. The receptor–digitonin complex should have a higher $s^0_{20,w}$ than the receptor–Lubrol complex because digitonin has $\bar{\nu}$ = 0.738 ml/g [11] and much lower than that of Lubrol PX (0.958 ml/g) [12]. In addition, it is possible that the receptor binds the micelle of digitonin, the $s^0_{20,w}$ of which has been estimated to be 6.35 S [11].

The calculation of $\bar{\nu}$ and $s^0_{20,w}$ of the presumed receptor–[^{3}H]QNB–Lubrol complex was carried out by combining the data for the centrifugation in H_2O and D_2O [9,13]. Molecular weight of the complex was calculated from $s^0_{20,w}$, $\bar{\nu}$ and a (table 1). The hypothetical amount of bound detergent and the molecular weight of the protein portion of the complex were then estimated on the assumption that the $\bar{\nu}$ represents the average of that of protein (0.71 to 0.76 ml/g) and Lubrol PX (0.958 ml/g). Results are summarized in table 1.

The mol. wt 86 000 estimated for the protein portion of the receptor–detergent complex is very similar

Table 1
Molecular size of the muscarinic receptor

Stokes radius, a (nm)	6.82 ± 0.09 (3)[a]
Partial specific volume, $\bar{\nu}$ (ml/g)	0.814 ± 0.011 (7 × 6)[a]
Sedimentation coefficient, $s^0_{20,w}$ (S)	3.20 ± 0.23 (7 × 6)[a]
Molecular weight, M_r[b]	133 × 10^3
Frictional ratio, f/f_o[b]	1.95
Lubrol PX bound[c]	
g/g protein	0.55 (0.72–0.38)
mol/mol protein	78 (93–60)
M_r of protein portion[c]	86 × 10^3 (77–97)

[a] The values given are the mean ± 1 SD for the no. determinations shown in parentheses. 7 sucrose gradient centrifugations were done in H_2O and 6 were done in D_2O

[b] Molecular weight and frictional ratio were calculated according to the following equations:

$$M_r = 6\pi\eta_{20,w} \cdot N \cdot a \cdot s^0_{20,w} / (1 - \bar{\nu}\rho_{20,w})$$

$$f/f_o = a \cdot [4\pi N/3M_r \cdot \bar{\nu}]^{1/3}$$

where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20°C, and $\rho_{20,w}$ is the density of water at 20°C

[c] These values were calculated from the assumption that the observed represents the average of protein (0.735 ml/g) and for Lubrol PX (0.958 ml/g). The values in parentheses are those obtained when the $\bar{\nu}$ of protein was assumed to be 0.71 and 0.76 ml/g

to the est. mol. wt 83 000 by SDS–polyacrylamide gel electrophoresis [7]. Oligomers of membrane proteins as well as soluble proteins have been known to dissociate into subunits by the treatment with SDS, but usually not by the treatment with mild detergent like Lubrol PX [14]. It is therefore most likely that the muscarinic receptor exists as monomer in membranes. The interaction of the receptor with other membrane components, if it occurs during the function, may be transient and reversible. There still remains another possibility, however, that the population of receptor–ligand complex on intact membranes is heterogeneous (cf. [15]) and that only the monomeric form of the receptor–ligand complexes was selectively solubilized.

It is worthwhile to note that the molecular weight of the muscarinic acetylcholine receptor is much lower than that of nicotinic acetylcholine receptor composed of several subunits [16,17] and comparable to that of α and β adrenergic receptors [9,18]. The difference in size of receptors may reflect the difference in the mode of function of receptors.

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