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Solubilization and characterization of guanine nucleotide-sensitive muscarinic agonist binding sites from rat myocardium

Christopher P. Berrie, Nigel J.M. Birdsall, Edward C. Hulme[1], Mary Keen & Jane M. Stockton

Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA

1 Muscarinic receptors from rat myocardial membranes may be solubilized by digitonin in good yield at low temperatures in the presence of Mg^{2+}.

2 Under these conditions, up to 60% of the soluble receptors show high affinity binding for the potent agonist [^{3}H]-oxotremorine-M ($K_A = 10^9\,M^{-1}$), which is inhibited by 5'-guanylylimidodiphosphate.

3 The muscarinic binding site labelled with [^{3}H]-oxotremorine-M has a higher sedimentation coefficient (13.4 *s*) than sites labelled with a ^{3}H antagonist in the presence of guanylylimidodiphosphate (11.6 *s*) and probably represents a complex between the ligand binding subunit of the receptor and a guanine nucleotide binding protein.

Introduction

In the accompanying paper, we describe the hydrodynamic properties of muscarinic receptors from the rat forebrain which have been solubilized in several different detergents (Berrie *et al.*, 1984). The receptors are present both as monomers and as higher molecular weight complexes, the nature and specificity of which are not known.

Several modes of receptor-effector coupling are thought to occur in the forebrain (Birdsall & Hulme, 1983). In contrast, myocardial muscarinic receptors present a much simpler picture. In myocardial membranes, a high proportion of the receptor binding sites are modulated by guanine nucleotides such as GTP (see for example Berrie *et al.*, 1979; Wei & Sulakhe, 1979; Ehlert *et al.*, 1980). The effect of the guanine nucleotides is to convert high affinity agonist binding sites to low affinity sites. In receptor systems showing this type of modulation, it is thought that high affinity agonist binding states represent complexes of the receptor with one or more of a class of guanine nucleotide binding proteins (Rodbell, 1980; Sternweis *et al.*, 1981; Bokoch *et al.*, 1983). The effect of guanine nucleotides is to dissociate these complexes. Direct evidence of such a process has been provided for the β-adrenoceptor (Limbird *et al.*, 1980) and the α_2-adrenoceptor (Smith & Limbird, 1981).

It has been shown that the nucleotide-sensitive agonist binding state of the myocardial muscarinic receptor is favoured by low temperature and the presence of divalent cations, notably Mg^{2+} and Mn^{2+} (Hulme *et al.*, 1983b) and that it can be monitored readily using the potent tritiated agonist [^{3}H]-oxotremorine-M. Using this information, we have solubilized and partially characterized this complex, which may represent a stable and functionally intact agonist-receptor-effector complex.

Methods

(−)-[^{3}H]-N-methylscopolamine ([^{3}H]-NMS, 53.5 Ci mmol^{-1}) and [^{3}H]-oxotremorine-M ([^{3}H]-oxoM, 82.5 Ci mmol^{-1}) were obtained from New England Nuclear. Guanylylimidodiphosphate (GppNHp) was obtained from Boehringer. Other reagents were of the highest possible grade.

Digitonin

Digitonin was obtained from Wako (Osaka) or Sigma (London). The Wako preparation had superior prop-

[1] Correspondence.

erties as far as solubility was concerned, but essentially identical properties could be obtained for the Sigma preparation by ether/chloroform precipitation of a dimethylsulphoxide solution of the detergent as described by Janski *et al.* (1980). Stable 4% aqueous solutions of digitonin thus pretreated were obtained readily.

Myocardial membrane preparation

Myocardial membranes were prepared by a modification of the method of Bers (1979). Unless otherwise indicated, all operations were conducted at ice-bath temperatures. Whole rat hearts were homogenized in 9 vol. of 20 mM NaHEPES, pH 7.5, using 3×15 s at Polytron setting 5 with 1 min cooling between bursts. The resultant homogenate was subjected to ten strokes of Potter-Elvehjem homogenization and then filtered through cheese-cloth. The preparation was supplemented with 0.8 vol. of 20 mM NaHEPES, and 0.2 vol. of 3 M potassium chloride and 250 mM disodium pyrophosphate (PPi), mixed rapidly and centrifuged at 140,000 *g* for 50 min. The pellet was then resuspended in 20 mM NaHEPES to a volume equivalent to the original 1:10 tissue dilution, supplemented with EDTA (adjusted to pH 7.5 with sodium hydroxide) to a final concentration of 10 mM, incubated at 30°C for 15 min, cooled to 0°C, and centrifuged at 100,000 *g* for 30 min. The pellet was resuspended to the same volume in 100 μM EDTA and recentrifuged twice, to remove excess EDTA. The resultant membrane fraction was frozen rapidly (dry ice/methanol) and stored at −20°C until used. There was no apparent change in the binding properties of this material over the maximum period of storage, approximately 14 days. Preliminary experiments indicated that the KCl/PPi pretreatment not only improved the specific:non-specific binding ratio of muscarinic ligands ca. 2 fold without significant loss of total binding sites but also yielded preparations in which muscarinic binding was somewhat more stable, both before and after solubilization, than when such pretreatment was omitted.

Labelling of membrane fractions with muscarinic ligands

Membrane fractions to be prelabelled for solubilization were resuspended in 20 mM NaHEPES pH 7.5 to a protein concentration of 5 mg ml^{-1} and supplemented with divalent cations and [^{3}H]-oxoM or [^{3}H]-NMS as indicated. Incubation times were as given in the figure legends and the temperature was usually 0°C. The progress of membrane binding was monitored by dilution into 5–10 volumes of ice-cold NaHEPES, followed by immediate microcentrifugation and scintillation counting of the resultant pellets as previously described (Berrie *et al.*, 1979). Non-specific binding was defined as that fraction of the binding activity which persisted in the presence of 1 μM 3-quinuclidinylbenzilate (QNB).

Studies aimed specifically at characterizing membrane binding properties were usually performed at a somewhat lower membrane concentration (1–2 mg ml^{-1} protein) and terminated by microcentrifugation without dilution to avoid loss of rapidly-dissociating components of ligand binding. Membrane binding capacities for assessment of yield in solubilization experiments were conducted in this manner.

Solubilization of membranes and assay of solubilized muscarinic binding activity

Preliminary experiments showed that muscarinic binding sites labelled with [^{3}H]-oxoM could be solubilized with digitonin, and that the optimal detergent:protein ratio was in the range 1:1–2:1. Solubilization of both pretreated and non-treated membranes was typically accomplished by stirring the suspension (5 mg ml^{-1} protein) in 20 mM NaHEPES, pH 7.5 containing 1 mM Mg^{2+} with 0.8% digitonin at 0°C for 15 min, followed by centrifugation at 140,000 *g* for 30 min. The supernatant was used as the solubilized preparation. Binding to supernatants which had not been prelabelled was measured by incubation with ^{3}H-ligands at 0°C. Bound and free ligands were separated by gel filtration of 0.1 ml aliquots on 2 ml columns of Sephadex G50M equilibrated with 20 mM NaHEPES (Hulme *et al.*, 1983a). Non-specific binding was again defined as that fraction of apparent binding which persisted in the presence of 1 μM QNB.

Hydrodynamic characterization of solubilized receptors

Gel filtration of solubilized receptors was conducted on a column of Sepharose 6B (7.6 ml) equilibrated in 20 mM NaHEPES, 1 mM Mg^{2+}, 0.2% digitonin, pH 7.5. The digitonin concentration was reduced to 0.2% to avoid unnecessarily high digitonin: protein ratios with consequent loss of [^{3}H]-oxoM binding. For the same reason, the column was cooled in ice; 0.2 ml samples of solubilized receptor preparations were applied, and eluted at a rate of 0.08 ml min^{-1}. The excluded volume was determined with blue dextran. The free radioligand peak was taken as the included volume, and catalase (bovine liver, Pharmacia) used as a molecular weight reference (M_r = 232,000). Sucrose density gradient centrifugation was carried out using linear 5–20% sucrose gradients in 20 mM HEPES, 1 mM Mg^{2+}, 0.2% digitonin. Samples of 0.5 ml were applied to 4.5 ml gradients, and centrifuged for 1.7×10^6 g.h in a Beckman

SW 55 Ti rotor at 0°C. Fractions (0.25 ml) were collected using a peristaltic pump. The bound ligand was detected by gel filtration of 100 μl aliquots on G50 columns as described. Catalase, β-galactosidase (*E. coli*, Worthington) and lactate dehydrogenase (pig heart, Boehringer) were used as internal standards. Activities were measured as described by Haga *et al.* (1977), and ammonium sulphate was removed by dialysis before addition to the sample to be centrifuged.

Data analysis

Analysis of binding data using non-linear-least squares fitting techniques was performed as previously described (Birdsall *et al.*, 1978).

Results

Binding of [^{3}H]-oxotremorine-M to myocardial membranes

Our previous studies have shown that up to 60% of the total muscarinic binding sites present in rat myocardial membranes bind [^{3}H]-oxoM with an affinity of ca. $5 \times 10^8\,M^{-1}$ under low ionic strength conditions (20 mM NaHEPES, pH 7.5) in the presence of divalent cations (100 μM Mn^{2+} or 1 mM Mg^{2+}) at 30°C. Pretreatment of the membranes with EDTA slightly but significantly enhances the affinity of [^{3}H]-oxoM measured after the addition of divalent cations (Hulme *et al.*, 1983b). Using these findings as a starting point, preliminary experiments showed that (1) the ratio of specific to non-specific binding of [^{3}H]-oxoM, and the stability of the muscarinic cholinoceptor (mAChR)-[^{3}H]-oxoM complex is improved by subjecting the membranes to KCl/pyrophosphate extraction (Bers, 1979), reflecting the partial removal of myofibrils and, possibly, endogenous proteases; (2) the [^{3}H]-oxoM-labelled mAChR is more stable in the presence of Mg^{2+} than Mn^{2+}; and (3) the dissociation rate of [^{3}H]-oxoM is decreased by more than 100 fold by reducing the temperature from 30°C to 0°C; interestingly, lowering the temperature to 0°C also enhanced the level of [^{3}H]-oxoM binding, while reducing the stimulatory effect of Mg^{2+} and Mn^{2+} seen at 30°C (Hulme *et al.*, 1983b).

Figure 1 summarizes an experiment showing several relevant features of [^{3}H]-oxoM binding to the KCl/pyrophosphate-extracted EDTA-washed membrane preparation (see Methods) in the presence of 1 mM Mg^{2+} at 0°C. (1) The association time-course of [^{3}H]-oxoM was markedly biphasic, a rapid initial phase being succeeded by a much slower approach to a final steady state which was maintained for long periods of time (at least 24 h). At 30°C, the [^{3}H]-oxoM binding was much less stable with a half-life of ca. 2 h (data not shown), probably reflecting significant action of endogenous proteases. (2) Binding of [^{3}H]-oxoM was almost entirely prevented by the

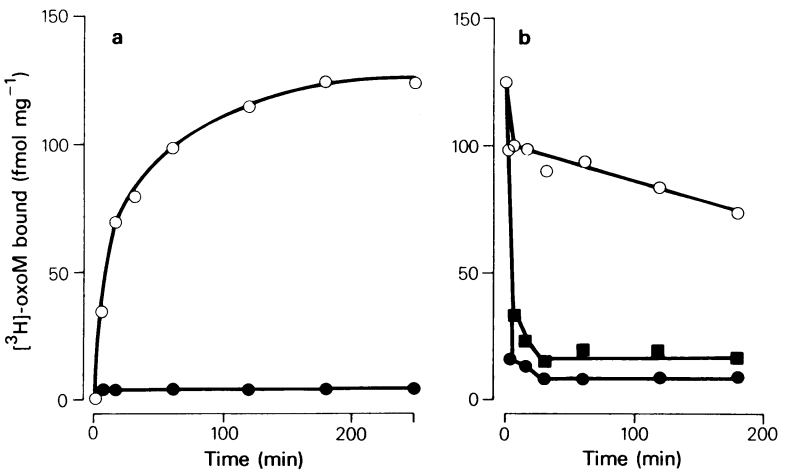

Figure 1 (a) Time-course binding of [^{3}H]-oxotremorine-M ([^{3}H]-oxoM) (3 nM) to KCl/pyrophosphate (PPi)-extracted EDTA-pretreated membranes from rat myocardium in the presence of 1 mM Mg^{2+} at 0°C and in the absence of (○) or presence (●) of guanylylimidodiphosphate (GppNHp) (100 μM). (b) Dissociation of [^{3}H]-oxoM from KCl/PPi-extracted EDTA-pretreated membranes from rat myocardium prelabelled with [^{3}H]-oxoM (3 nM) in the presence of 1 mM Mg^{2+} for 180 min at 0°C. Membranes were supplemented as follows: (○) 1 μM unlabelled oxoM (■) 100 μM GppNHp; (●) 1 μM oxoM + 100 μM GppNHp. s.e.means were ca. 5% of mean values. The concentration of [^{3}H]-NMS (10 nM) binding sites was 200 fmol mg^{-1} protein.

GTP analogue GppNHp (100 μM). (3) The dissociation time course of [^{3}H]-oxoM was biphasic, a minor initial component being succeeded by a very slow phase such that after 2 h only ca. 30% of the ^{3}H ligand had dissociated. (4) In the presence of GppNHp (100 μM) the dissociation rate constant was accelerated to such an extent that almost all of the specifically bound [^{3}H]-oxoM dissociated within 5 min; 100 μM GppNHp alone was sufficient to give rapid dissociation of ca. 90% of the binding of 3 nM [^{3}H]-oxoM. Comparison of Figures 1(a) and (b) shows that GppNHp is more effective at inhibiting [^{3}H]-oxotremorine-M binding than in promoting dissociation of bound [^{3}H]-oxo-M. This is a reproducible finding for which there is no established explanation. The effects of GppNHp on [^{3}H]-oxoM binding were much more dramatic at 0 °C than at 30 °C. (Hulme *et al.*, 1983b). A number of measurements showed that specific binding of 10 nM [^{3}H]-oxoM to these membrane preparations at 0 °C in the presence of 1 mM Mg^{2+} attained 40–60% of the level given by a near-saturating concentration (10 nM) of the antagonist [^{3}H]-NMS.

Solubilization of myocardial muscarinic receptors

Solubilization of membranes (5 mg ml^{-1}) prelabelled with [^{3}H]-oxoM and [^{3}H]-NMS was accomplished by stirring with increasing concentrations of digitonin at 0 °C for 15 min, the supernatant being separated from undissolved material by centrifugation at 140,000 *g* for 30 min. Assay of the supernatant by gel filtration showed the presence of bound [^{3}H]-oxoM and [^{3}H]-NMS (Figure 2). There was evidence to suggest that retention of [^{3}H]-oxoM binding was more sensitive than [^{3}H]-NMS binding to an increasing digitonin:protein ratio. Optimal recovery of bound [^{3}H]-oxoM was obtained at digitonin:protein ratios of 1:1 to 2:1, and approached 100% in the best cases, while recovery of bound [^{3}H]-NMS was in the range of 80–90%. These detergent concentrations solubilized 20–40% of the total membrane protein.

An off-rate experiment analogous to that shown in Figure 1b but using a 0.8% digitonin supernatant from membranes prelabelled with [^{3}H]-oxoM in the presence of 1 mM Mg^{2+} is shown in Figure 3. The off-rate in the absence of GppNHp was again very slow, so that after 150 min only 15% of the bound ligand had dissociated. In contrast over 50% of the binding had dissociated after 5 min if GppNHp was present. As was observed with membranes, GppNHp alone did not give as rapid or complete dissociation as the combination of GppNHp and unlabelled oxotremorine-M. The dissociation rates were some-

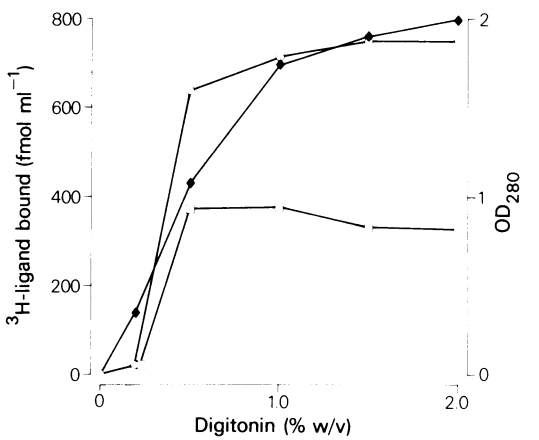

Figure 2 Digitonin solubilization of bound [^{3}H]-oxotremorine-M ([^{3}H]-oxoM) (□) and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS)(○) from KCl/PPi-extracted EDTA-pretreated rat myocardial membranes pre-labelled with 10 nM [^{3}H]-oxoM or [^{3}H]-NMS for 2 h at 0 °C in the presence of 1 mM Mg^{2+}. Solubilization was by stirring with indicated concentrations of digitonin for 15 min at 0 °C. Optical density $(OD)_{280}$ (◆) was measured on each supernatant. Membrane binding of [^{3}H]-NMS (10 nM) and [^{3}H]-oxoM (10 nM) was equivalent to 897 fmol ml^{-1} and 358 fmol ml^{-1}, respectively.

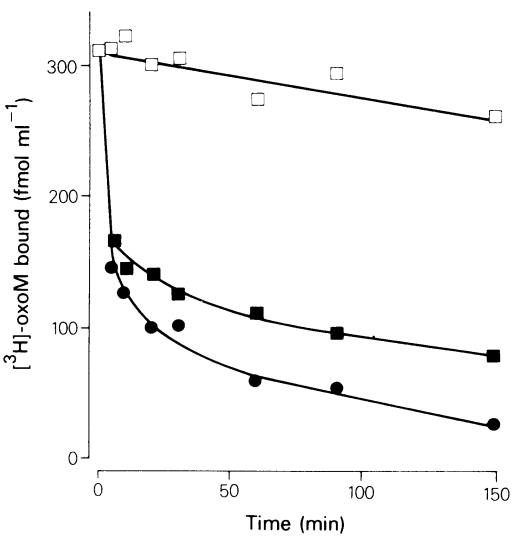

Figure 3 Dissociation of [^{3}H]-oxotremorine-M ([^{3}H]-oxoM) from solubilized preformed muscaranic acetylcholine receptor (mAChR)-[^{3}H]-oxoM complex. KCl/PPi-extracted EDTA-pretreated myocardial membranes were incubated with 3 nM [^{3}H]-oxoM in the presence of 1 mM Mg^{2+} for 2 h at 0 °C, and solubilized with 0.8% digitonin. [^{3}H]-oxoM binding to the supernatant was measured under the following conditions: (□) 1 μM unlabelled oxoM; (■) 100 μM guanylylimidodiphosphate (GppNHp); (●) 1 μM oxoM + 100 μM GppNHp.

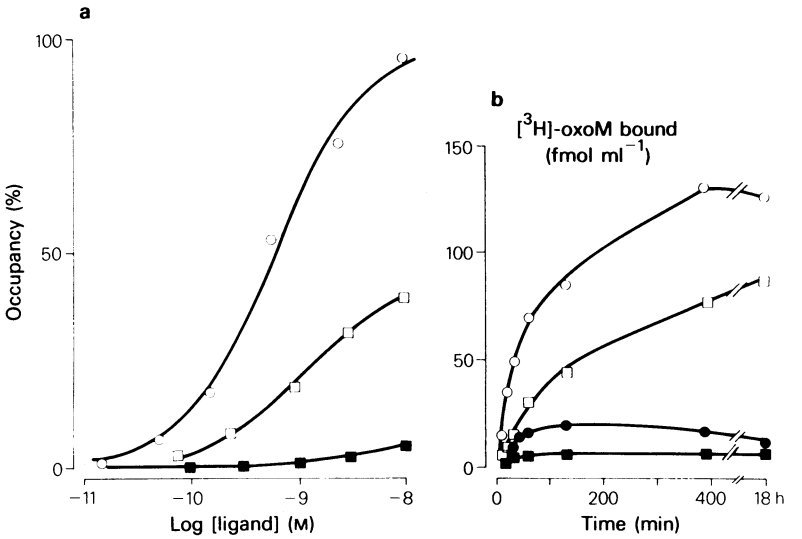

Figure 4 (a) Equilibrium binding of [^{3}H]-N-methylscopolamine([^{3}H]-NMS) (○), of [^{3}H]-oxotremorine-M ([^{3}H]-oxoM) in the absence of guanylimidodiphosphate (GppNHp) (□) and of [^{3}H]-oxoM in the presence of 100 μM GppNHp (■) to a digitonin solubilized myocardial membrane preparation at 0°C. Incubations were for 18 h at 0°C. Binding parameters were as follows: [^{3}H]-NMS (○), $B_{max} = 754 \pm 32$ fmol ml^{-1}, $K_A = 1.66 \pm 0.29 \times 10^9$ M^{-1}; [^{3}H]-oxoM (□), $B_{max} = 332 \pm 7$ fmol ml^{-1}, $K_A = 9.02 \pm 0.64 \times 10^8$ M^{-1}. Curves were normalised so that the B_{max} for [^{3}H]-NMS represents 100% occupancy. (b) Time course of binding of [^{3}H]-oxoM to a digitonin-solubilized myocardial membrane preparation at 0°C. [^{3}H]-oxoM concentration was 1 nM (□, ■) or 3 nM (○, ●) and incubations were in the absence (open symbols) or presence (closed symbols) of GppNHp (100 μM).

what slower overall in the solubilized than in the membrane-bound state. It should be noted that the gel filtration technique would not detect a component of dissociation (see open symbols in Figure 1b) sufficiently rapid to occur during passage of the supernatant through the gel.

It was not necessary to prelabel membranes in order to obtain [^{3}H]-oxoM binding to digitonin-solubilized mAChR. Figure 4 shows (a) the equilibrium binding curves for [^{3}H]-NMS and [^{3}H]-oxoM in the absence of GppNHp, and the residual slowly dissociating binding of [^{3}H]-oxoM in its presence, and (b) the association time course of [^{3}H]-oxoM (1 nM and 3 nM) in the absence and presence of GppNHp (100 μM), measured on a digitonin supernatant from myocardial membranes. The equilibrium binding curves for [^{3}H]-NMS and [^{3}H]-oxoM were both well described by simple Langmuir isotherms, yielding affinity constants of 1.7×10^9 M^{-1} and 9.0×10^8 M^{-1} respectively, values which agree closely with published estimates for membranes (Berrie *et al.*, 1979; Waelbroeck *et al.*, 1982; Hulme *et al.*, 1983b). Because of the very slow time-course of [^{3}H]-oxoM binding (Figure 4b), long (18 h) incubations were necessary to ensure achievement of equilibrium. As in the membranes, the binding of [^{3}H]-oxoM was largely suppressed by GppNHp and the resulting levels were too low to permit satisfactory fitting of the data. In the experiment shown, the yield of [^{3}H]-oxoM binding sites was 44% of the [^{3}H]-NMS binding sites; this ratio varied from 30–60%, depending on the membrane preparation, while the yield of specifically bound [^{3}H]-oxoM was also in the range of 30–60% of that obtained by solubilizing prelabelled membranes. The reason for this variation is not understood.

Utilizing self-competition methods in which specific binding of a low concentration of the labelled ligand (1 nM) was inhibited by increasing concentrations of the unlabelled ligands, the values obtained for the oxoM affinity constant (9.1×10^8 M^{-1}, Figure 5a), and for the NMS affinity constant in the absence and presence of GppNHp (2.3×10^9 M^{-1}, Figure 5b) were very similar to those found in the direct binding studies. Inhibition of [^{3}H]-NMS binding by oxoM as previously described for membranes (Birdsall *et al.*, 1978; Berrie *et al.*, 1979) confirmed the presence of very high affinity oxoM binding sites (affinity constant 1.3×10^9 M^{-1}) amounting to 28% of the total sites labelled with 1 nM [^{3}H]-NMS in the experiment shown (Figure 5a). The remaining sites had a 30,000 fold lower affinity for oxoM (4.2×10^4 M^{-1}), essentially corresponding to the low affinity state observed by Herron *et al.* (1982) using digitonin-cholate com-

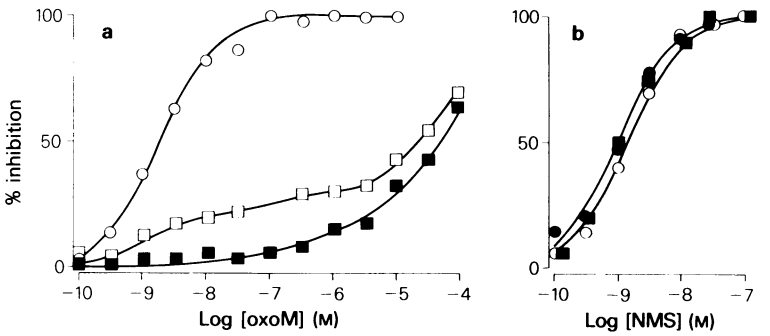

Figure 5 (a) Binding of oxotremorine-M (oxoM) to digitonin-solubilized myocardial muscarinic cholinoceptors (mAChR) (○) by competition with [^{3}H]-oxoM (0.8 nM); (□) by competition with [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (0.84 nM) in the absence of guanylylimidodiphosphate (GppNHp); (■) by competition with [^{3}H]-NMS (0.84 nM) in the presence of 100 μM GppNHp. The affinity constant for oxoM was calculated to be $9.1 \pm 1.0 \times 10^8$ M^{-1} by competition with [^{3}H]-oxoM. Two site analysis of the oxoM/[^{3}H]-NMS competition curves using the previously published model (Birdsall *et al.*, 1978) yielded the following values: in the absence of GppNHp, $K_H = 1.3 \pm 0.7 \times 10^9$ M^{-1} (28 ± 2% of total sites), $K_L = 4.2 \pm 1.0 \times 10^4$ M^{-1} (72 ± 2% of total sites); in the presence of GppNHp, $K_H = 6.5 \pm 3.5 \times 10^6$ M^{-1} (20 ± 4% of total sites) $K_L = 3.1 \pm 0.7 \times 10^4$ M^{-1} (80 ± 4% of total sites). (b) Binding of NMS to digitonin-solubilized myocardial mAChR (○) by competition with [^{3}H]-oxoM (0.8 nM; (●) by competition with [^{3}H]-NMS (0.84 nM) in the absence of GppNHp; (■) by competition with [^{3}H]-NMS in the presence of GppNHp (100 μM). Affinity constants from least-squares fitting were $1.2 \pm 0.1 \times 10^9$ M^{-1} by competition with [^{3}H]-oxoM and $2.3 \pm 0.2 \times 10^9$ M^{-1} by competition with [3H]-NMS. All incubations were for 18 h at 4 °C. Affinity constants were obtained after correction for occupancy by the ^{3}H-ligands.

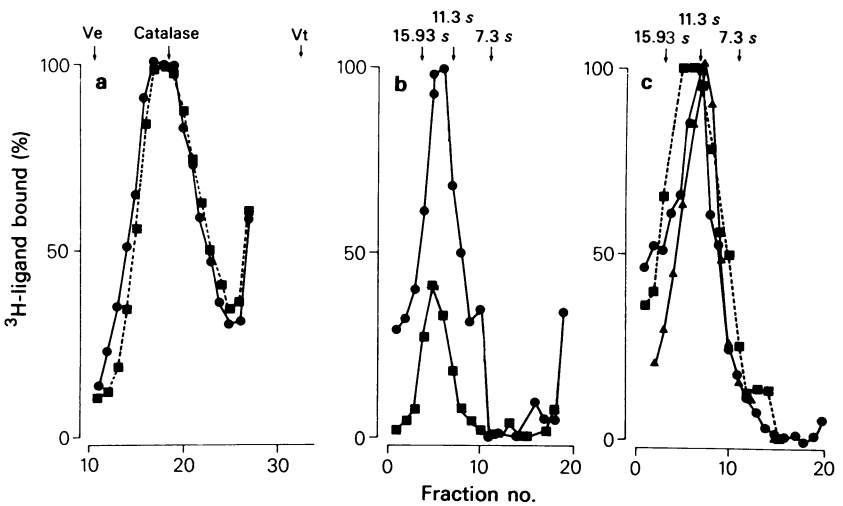

Figure 6 (a) Sepharose 6B gel filtration of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and [^{3}H]-oxotremorine-M ([^{3}H]-oxoM) labelled muscarinic cholinoceptors(mAChR) solubilized (0.8% digitonin) from membranes pre-labelled with 10 nM concentrations of the radioligands for 2 h at 0 °C in the presence of 1 mM Mg^{2+}. (●) [^{3}H]-NMS, peak binding = 67 fmol ml^{-1}, recovery 81%; (■) [^{3}H]-oxoM, peak binding 37 fmol ml^{-1}, recovery 89%. (b) Sucrose density gradient centrifugation of [^{3}H]-oxoM-labelled mAChR solubilized as in (a). (■) [^{3}H]-oxoM not present in gradient, peak binding = 40 fmol ml^{-1}, recovery 20%; (●) [^{3}H]-oxoM (10 nM) present in gradient, peak binding 91 fmol ml^{-1}, recovery 72%. (c) Sucrose density gradient centrifugation of [^{3}H]-NMS-labelled digitonin solubilized mAchR. (●) Prelabelled with [^{3}H]-NMS (10 nM) in the presence of GppNHp (100 μM), peak binding 168 fmol ml^{-1}, recovery 46%; (▲) postlabelled with [^{3}H]-NMS (10 nM) in the presence of 10 μM unlabelled oxoM, peak binding = 114 fmol ml^{-1}, recovery 55% (■) prelabelled with [^{3}H]-NMS (10 nM) in the absence of GppNHp, 10 nM [^{3}H]-NMS present in gradient, peak binding = 249 fmol ml^{-1}, recovery 90%. β-galactosidase (15.93 *s*), catalase (11.3 *s*) and LDH (7.3*s*) were used as internal markers. Other details are given in the text.

binations. Assay in the presence of GppNHp (100 μM) confirmed that the highest affinity oxoM sites were sensitive to the nucleotide, but fell short of showing conversion to the very low affinity state (Figure 5a). This aspect requires more investigation. Inhibition of [^{3}H]-oxoM binding by unlabelled NMS showed the antagonist to have a slightly lower affinity (1.2×10^9 M^{-1}) than that found in the self-competition experiment (2.3×10^9 M^{-1}), indicating that NMS may distinguish between the two agonist-binding populations in a reciprocal manner, although to a much smaller extent, than found for oxoM. As a consequence it may be that the apparent proportions of very high affinity oxoM sites estimated from the oxoM-[^{3}H]-NMS competition experiments (Figure 5a) are underestimated compared to those measured in the direct binding experiment using [^{3}H]-oxoM and [^{3}H]-NMS (Figure 4a).

Hydrodynamic properties of soluble myocardial muscarinic receptors

To confirm that the detergent-solubilized muscarinic binding sites were genuinely soluble, we have carried out a partial hydrodynamic characterization by gel filtration (Figure 6a) and sucrose density-gradient centrifugation (Figure 6b, c) using the methods described in the accompanying paper (Berrie *et al.*, 1984). Both [^{3}H]-oxoM and [^{3}H]-NMS binding activities solubilized from prelabelled membranes (with [^{3}H]-NMS labelling conducted either in the presence or absence of GppNHp) were included in a Sepharose 6B column, eluting in identical positions just marginally ahead of catalase (Stokes radius 5.2 nm, Haga *et al.*, 1977) (Figure 6a). Identical results were obtained with post-labelled supernatants (not shown) and the bound radioactive peaks were completely eliminated by labelling in the presence of QNB (1 μM). Recoveries of bound radioactivity approached 100% in each case. The Stokes radius of the digitonin-solubilized myocardial mAChR is comparable to, but perhaps slightly less than that reported for the digitonin-solubilized rat brain mAChR (6.9 nm, Berrie *et al.*, 1984).

On 5–20% sucrose gradients, [^{3}H]-oxoM binding sites were recovered in a sharp peak with a sedimentation coefficient of 13.5 ± 0.2 *s* ($n = 3$) by comparison with the marker enzymes, and a recovery of ca. 20–25% (Figure 6b). This peak was completely suppressed when labelling was performed in the presence of 1 μM QNB. Recovery rose to ca. 70% when 10 nM [^{3}H]-oxoM was added to the gradient but the sedimentation coefficient was unaffected (13.3 ± 0.2 *s*, $n = 5$). When membranes were prelabelled with [^{3}H]-NMS in the presence of GppNHp (100 μM) and then solubilized, the main peak of [^{3}H]-NMS-labelled sites had a significantly lower sedimentation coefficient, 11.7 ± 0.1 *s* ($n = 6$), recovery = ca. 50% (Figure 6c). Once more the peak was completely suppressed by labelling in the presence of 1 μM QNB. A very similar profile resulted when digitonin supernatants were postlabelled with either [^{3}H]-NMS or [^{3}H]-QNB in the presence of 10 μM unlabelled oxoM to exclude binding to the high affinity agonist sites; the sedimentation coefficient was 11.7 ± 0.26 *s* ($n = 3$, recovery = 60–75%). These values are close to that determined for the digitonin-solubilized rat brain mAChR (10.9 *s*, Berrie *et al.*, 1984).

There was also evidence for labelling of a minor population of sites with a higher sedimentation coefficient; this was much more evident when membranes were labelled in the absence of GppNHp, and 10 nM [^{3}H]-NMS was included in the gradient, when the peak width was virtually doubled (dashed line, Figure 6c), and the mean sedimentation coefficient increased to 12.6 ± 0.2 *s* ($n = 5$, recovery = 75–90%). This extra component presumably corresponds to the [^{3}H]-oxoM-labelled component shown in Figure 6b, and is partly or completely suppressed by nucleotide pretreatment, or blockade of the high-affinity agonist binding sites.

Discussion

There have been numerous reports of detergent solubilization of muscarinic acetylcholine receptors with retention of high affinity binding of potent antagonists such as QNB, NMS and dexetimide (Aronstam *et al.*, 1978; Hurko, 1978; Gorissen *et al.*, 1978; Repke & Mathies, 1980; Haga, 1980a, b; Cremo *et al.*, 1981; Carson, 1982; Laduron & Ilien, 1982; Gavish & Sokolovsky, 1982; Herron *et al.*, 1982; Hulme *et al.*, 1983a; Berrie *et al.*, 1984). In contrast, most workers have described low affinity binding of potent agonists to solubilized mAChR assessed by agonist inhibition of ^{3}H-antagonist binding (Gorissen *et al.*, 1978; Carson, 1982; Gavish & Sokolovsky, 1982; Herron *et al.*, 1982). Using the same methodology, two studies have indicated that it is possible to obtain soluble preparations of brain mAChR which exhibit somewhat higher affinity for agonists, and retain some sensitivity to guanine nucleotides (Flynn *et al.*, 1982; Kuno *et al.*, 1983).

We have shown that under low ionic strength conditions, in the presence of Mg^{2+} at low temperatures, a fraction of the rat myocardial mAChR can be solubilized in good yield in a form which retains nanomolar affinity for the potent agonist [^{3}H]-oxoM, and is sensitive to guanine nucleotide. This complex has a slightly higher sedimentation coefficient (but not Stokes radius) than that obtained for the myocardial receptor labelled with [^{3}H]-NMS in the presence of GppNHp. These results almost certainly indicate

that a fraction of the myocardial mAChR binding sites retain their association with a nucleotide binding protein, and are solubilized as a complex under the conditions we have defined.

The coupled sites appear to correspond to the markedly guanine nucleotide-sensitive super high affinity population of muscarinic agonist binding sites defined in previous studies on intact membranes from both brain (Birdsall *et al.*, 1978; 1980) and myocardium (Hulme *et al.*, 1980; 1981; 1983b; Waelbroeck *et al.*, 1982). The remaining digitonin-solubilized binding sites have a strikingly (ca. 30,000 fold) lower affinity for oxoM and a slightly lower sedimentation coefficient, and must at least in part correspond to uncoupled mAChR. This enormous difference in oxoM affinity means that agonist occupancy must exert a marked stabilizing effect on the complex. It is notable that NMS retains a nanomolar affinity constant for both the low and high affinity agonist binding forms of the mAChR. The conformation of the antagonist binding site is thus relatively unaffected while that of the agonist binding site is drastically altered.

Further work can now be directed to the purification of the complex by affinity chromatography in order that its constituents be identified and reconstitution of a functional muscarinic receptor attempted.

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