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Binding and Hydrodynamic Properties of Muscarinic Receptor Subtypes Solubilized in 3-(3-Cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate

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SUMMARY

The muscarinic receptor from the cerebral cortex, heart, and lacrimal gland can be solubilized in the zwitterionic detergent 3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate (CHAPSO) with retention of high affinity [^{3}H]*N*-methylscopolamine binding. However, in this detergent there are significant differences in the binding properties of the receptors, compared with those observed in membranes and digitonin solution. Some agents retain a degree of selectivity. In the heart and cortex, agonists can bind with high affinity to a receptor-GTP-binding protein complex. A second, lower affinity, agonist binding state is also present, which resembles a class of sites seen in membranes but not in digitonin solution. The high affinity agonist binding state has been resolved from the lower affinity state on sucrose density gradient centrifugation. Hydrodynamic analysis suggests that the high affinity state is approximately 110,000 Da larger than the lower affinity state. The binding properties of the receptor in CHAPSO can be altered to those seen in digitonin by exchanging detergents after CHAPSO solubilization.

Recent work in molecular biology has resulted in the sequencing of cDNA clones encoding five different muscarinic receptor subtypes (1–6). Despite this sequence data, the molecular basis for the pharmacological differences observed between different muscarinic receptor subtypes is far from clear. Pharmacological studies using pirenzepine (7), AFDX-116 (8), and other antagonists have shown the presence of at least three receptor subtypes, "neuronal" with a high affinity for pirenzepine, "cardiac" with a low affinity for pirenzepine and high affinity for AFDX-116, and "glandular" with intermediate affinity for pirenzepine (see Ref. 9 for a review of nomenclature). The receptors so far cloned and expressed do not completely match this pharmacology and, furthermore, their postulated ligand binding sites show a strong sequence conservation. It is, thus, still an open question as to how much receptor selectivity is due to the receptor per se and how much is due to the local membrane environment. With agonist binding the situation is even more complicated, with three agonist binding states having been described (SH, H, and L sites; see Ref. 10 for review). It is clear that the receptor can form complexes with other components, particularly G protein, but the exact nature of these complexes and their contribution to pharmacological diversity is not clear.

One approach that has been used to tackle these questions is to solubilize the receptor in detergent to allow biochemical characterization of the states so produced, particularly with regard to their association with other protein, and to correlate this with the binding properties. A start has been made on this work with several detergents, including digitonin (e.g., Refs. 11–13) and CHAPS (14, 15). In this paper, we present data on the muscarinic receptor solubilized in CHAPSO, a novel zwitterionic detergent. We show that antagonist binding properties are modified but selectivity between receptor subtypes is maintained. We also characterize a high affinity agonist binding state, which probably represents a receptor-G protein complex.

Materials and Methods

EDTA-washed rat cerebral cortical membranes were prepared as previously described by Hulme *et al.* (16). The preparation of myocardial membranes was as previously described by Berrie *et al.* (11), except that the treatment with potassium chloride and disodium pyrophosphate was omitted. This did not affect the yield or stability of the solubilized receptors in these experiments. The lacrimal membranes

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ABBREVIATIONS: G protein, GTP-binding protein; CHAPSO, 3-(3-chlolamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate; CHAPS, 3-(3-chlolamidopropyl)dimethylammonio-1-propane sulfonate; NMS, *N*-methylscopolamine; ABT, *o*-aminobenzhydryloxytropane; OxoM, Oxotremorine-M; Gpp(NH)p, guanylylimidodiphosphate.

were prepared in an analogous fashion to those from the cortex. After preparation, membrane pellets were frozen and stored at −20°.

To solubilize the tissues, the membrane pellets were thawed, resuspended at 5 mg/ml in 20 mM Tris, pH 8.0, 1 mM $MgCl_2$, and stirred with 0.5% CHAPSO for 15 min at 4°, followed by centrifugation at 140,000 × *g* for 40 min. All further experimental procedures were carried out in this buffer, unless otherwise specified. Binding assays were carried out as previously described, at 4° (12, 16). In competition experiments, preparations were preincubated for 24 hr with the competing ligand before addition of [^{3}H]NMS for an additional 24 hr (4 days in the case of gallamine). This was because several ligands bind much more slowly than NMS so that, if they were added simultaneously, the NMS would occupy the receptors before any significant amounts of competing ligand could bind. Hence, true equilibrium would be determined by NMS off-rate, and incubations would be excessively long. Under certain conditions (gel filtration; see Results), the lacrimal receptor aggregated. To test whether the receptor remained truly soluble throughout the course of a binding experiment, a sample of the solubilized preparation was spun at 100,000 × *g*-hr 3 days after initial solubilization. Over 90% of the binding activity remained in the supernatant, showing that the receptor remained in solution over this time period. Gel filtration and sucrose density gradients were carried out as described by Berrie *et al.* (11), and calculations of hydrodynamic parameters according to the method of Haga *et al.* (17). Detergent exchange was performed on 2-ml G50-F columns that were preequilibrated with the detergent solution into which transfer was to take place. Samples were applied as 0.2-ml aliquots, washed onto the column with 0.4 ml of buffer, and then eluted with an additional 0.5 ml of the buffer.

Muscarinic receptor was purified according to the method of Wheatley *et al.* (18). Briefly, a digitonin extract of cortical membrane was subjected to DEAE-Sephacel ion exchange chromatography, followed by two rounds of uptake and elution on an ABT-Sepharose affinity gel.

Materials were obtained from sources described previously (12). AFDX-116 was a gift of Dr. Karl Thomae GmbH. Hexahydrosiladifenidol was a gift from Professor G. Lambrecht (University of Frankfurt).

Results

Solubilization of muscarinic receptors with CHAPSO. The antagonist NMS binds to all subclasses of muscarinic receptors with high affinity and so can be used to monitor total receptor number. Low concentrations of the agonist OxoM bind with high affinity to receptors complexed to G proteins and, thus, it can be used to determine the number of coupled receptors. Table 1 shows the recoveries of [^{3}H]NMS and [^{3}H]OxoM binding (labeling after solubilization), using a CHAPSO concentration of 0.5%. In preliminary experiments, this concentration was found to be optimal (data not shown). It can be seen that there was a 30% recovery of [^{3}H]OxoM binding in both the solubilized heart and cortex preparations, indicating that receptor-G protein interaction was still possible. No [^{3}H]OxoM binding was found in the soluble lacrimal preparation. However, it was also impossible to label a significant percentage of the sites with [^{3}H]OxoM in membranes in this tissue. The recovery of [^{3}H]NMS binding in the solubilized heart and lacrimal preparations was also about 30%, but only 10% in the cortex. The reasons for this low yield are not known. Given the molecular heterogeneity of cortical receptors, it is possible that not all subtypes are solubilized efficiently, perhaps because some have different lipid requirements from other receptors. About 50% of the binding sites initially present in the membranes were apparently inactivated. When the receptors were prelabeled with [^{3}H]NMS or [^{3}H]OxoM and solubilized, there was little difference in the yields.

TABLE 1

Distribution of binding sites in the supernatant and pellet following solubilization

Typical receptor binding capacities of untreated membranes, estimated with 10 nM[^{3}H]NMS, were 250, 1100, and 100 fmol/mg of protein for heart, cortex, and lacrimal gland, respectively. The following were the corresponding figures for the binding of [^{3}H]oxoM (10 nM) to membranes: 100, 200, and 0 fmol/mg of protein; for the binding of [^{3}H]NMS (10 nM) to soluble receptors: 200, 250, and 80 fmol/mg of protein; and for the binding of [^{3}H]oxoM (10 nM) to soluble receptors: 80, 100, and 0 fmol/mg of protein. The yield of protein solubilized by CHAPSO was 43 ± 5% and was independent of the tissue. Values are expressed as a percentage of control values (untreated membranes) (mean ± standard error from three experiments).

	NMS		OxoM	
	Supernatant	Pellet	Supernatant	Pellet
	%			
Heart	31 ± 1	29 ± 6	36 ± 9	31 ± 17
Cortex	12 ± 1	34 ± 6	32 ± 3	30 ± 6
Lacrimal	33 ± 3	40 ± 2	ND[a]	ND

[a] ND, not detected.

Hydrodynamic characterization of the solubilized muscarinic receptors. The nature of the solubilized sites was investigated by sucrose density gradient centrifugation and gel filtration. There was essentially no difference in the behavior of receptors solubilized from either the heart or the cortex (Table 2). Sucrose density gradient centrifugation was carried out in H_2O and D_2O to obtain the sedimentation coefficients ($s_{20,w}$) for [^{3}H]OxoM and [^{3}H]NMS-liganded species. As can be seen in Fig. 1A, the [^{3}H]OxoM-labeled peak migrated as a single, approximately symmetrical, band with an $s_{20,w}$ of 10.5 (Table 2). The half-width of this peak was 1.6 ± 0.1 times the half-width of the catalase standard. In contrast, the [^{3}H]NMS-labeled peak had an $s_{20,w}$ of 7.1 and was much wider than the [^{3}H]OxoM peak, due to the presence of a leading shoulder in a position corresponding to the [^{3}H]OxoM band. This was seen even in the presence of 100 μM Gpp(NH)p, where the NMS-labeled peak was not significantly narrowed. This must imply that Gpp(NH)p fails to cause complete dissociation of receptors from G proteins, even though there is functional uncoupling (see Discussion). On gel filtration (Fig. 1B), both peaks were sharp and symmetrical, with the [^{3}H]OxoM-liganded species having a slightly higher Stokes radius than the [^{3}H]NMS-liganded species (Table 3). The lacrimal receptor proved to be more difficult to analyze by a hydrodynamic approach because of apparent aggregation. With this soluble receptor species, it was necessary to include 3 mg/ml phosphatidylcholine in the buffers for both sucrose density gradient centrifugation and gel filtration and, even under these conditions, it was not possible to obtain an accurate *s* value in D_2O because the bound [^{3}H]NMS was distributed in a very broad peak. However, in H_2O sucrose density gradient centrifugation and gel filtration, the binding activity tended to comigrate with the heart and cortical [^{3}H]NMS-liganded receptors, indicating that it was of a similar molecular weight.

The data from these studies were used to compute molecular weights of the various molecules (Table 3). The [^{3}H]OxoM binding species had an apparent molecular weight of 295,000 ± 25,000; 110,000 greater than the [^{3}H]NMS binding species (M_r 185,000 ± 25,000). It was not possible to calculate the amount of bound detergent with confidence, because the partial specific volume of CHAPSO is 0.79, which is close to the range seen with most proteins (0.76–0.71) (11, 17).

TABLE 2

Hydrodynamic parameters of receptor solubilized from heart, cortex, and lacrimal glands

$S_{20,H}$ and $s_{20,D}$ are sedimentation coefficients calculated from the position of molecular weight standards in H_2O and D_2O, respectively. For calculation of V,$s_{20,w}$, and molecular weights, data from heart and cortex were pooled.

	$s_{20,H}$	$s_{20,D}$	V	$s_{20,w}$	Stokes radius	Molecular weight
					Å	
OxoM						
Heart	5.6 ± 0.2	4.8 ± 0.1	0.76 ± 0.02	10.5	5.8 ± 0.1	295,000 ± 25,000[a]
Cortex	5.8 ± 0.1	4.4 ± 0.1			6.0 ± 0.1	
NMS						
Heart	4.2 ± 0.1	3.7 ± 0.2	0.73 ± 0.05	7.1	5.7 ± 0.1	185,000 ± 25,000[a]
Cortex	4.0 ± 0.1	3.5 ± 0.1			5.6 ± 0.1	
Lacrimal	3.8 ± 0.2	ND[b]			5.7 ± 0.1	

[a] Four experiments.
[b] ND, not determined.

The profile obtained with [^{3}H]NMS bound to the cortical receptor was compared with that seen when purified cortical receptor was centrifuged through a sucrose gradient. Because the purified cortical receptor is prepared in digitonin-containing buffer, it was necessary to transfer this into a 3 mg/ml phosphatidylcholine/0.5% CHAPSO mixture by gel filtration (see below). The gradient also contained a 3 mg/ml phosphatidylcholine supplement. A direct comparison with the receptor solubilized from a crude cortical homogenate was then possible (Fig. 2). The purified receptor migrated as a sharp symmetrical peak; it was about 30% narrower than the receptor solubilized from the crude homogenate and there was no trace of a leading edge. The purified receptor appeared to sediment about half a fraction further than the receptor solubilized from the crude homogenate. This is consistent with a molecular weight difference of about 30,000. It should be noted that the presence of phospholipid causes a decrease in the observed sedimentation coefficient in H_2O for the nonpurified cortical receptor; under these conditions it has an estimated $s_{20,w}$ value of 6.0 (cf. Table 2). The overall yield of receptor was also doubled by this addition. This emphasizes the importance of phospholipid to the properties of the receptor solubilized in CHAPSO.

Kinetics of [^{3}H]NMS and [^{3}H]OxoM binding to the solubilized receptors. The time course of [^{3}H]NMS and [^{3}H]OxoM binding to solubilized receptors from cortex, heart, and lacrimal gland was examined. The kinetics of the binding of 10 nM [^{3}H]NMS to the soluble cortical receptor (Fig. 3A) could be described by a single exponential function, with a half-time of about 25 min. The binding was stable for at least 4 days. Using 0.3 nM [^{3}H]NMS, the half-time for binding was doubled. The binding of 10 nM [^{3}H]NMS to the solubilized lacrimal receptor was considerably slower than to the cortical receptor ($t_{1/2}$ = 200 min). Gpp(NH)p) (100 μM) had very little effect on this rate in either the cortical or lacrimal receptors. A somewhat different behavior was found for the solubilized heart preparation (Fig. 3B). The amount of [^{3}H]NMS bound reached a plateau after 2 days and then began to decline at a rate of about 10 fmol/ml/day. Addition of 100 μM Gpp(NH)p resulted in the maximal level of binding being reached within 6 hr, which then decayed at the same rate as that found in the absence of nucleotide. The data are consistent with [^{3}H]NMS binding to the cardiac receptors with a $t_{1/2}$ of 500 min, which is reduced to about 60 min in the presence of Gpp(NH)p. In addition, the solubilized cardiac receptor is unstable, with the activity decaying with a $t_{1/2}$ of about 3.3 days. The apparent lower level of peak binding seen in the absence of Gpp(NH)p may be due to the instability of the preparation at longer incubation times. The on-rate of 10 nM [^{3}H]OxoM to both heart and cortical receptors had a $t_{1/2}$ of 160 min; the binding to the cortical receptor was stable for up to 3 days, but in the heart there was instability comparable to that seen with [^{3}H]NMS ($t_{1/2}$ of 3 days).

In the solubilized cortex (Fig. 4A), the [^{3}H]NMS off-rate could be fitted to a single exponential, with a $t_{1/2}$ of 7.5 days; Gpp(NH)p did not significantly decrease this. A similar value was found for the solubilized lacrimal receptor. In the solubilized heart, it was necessary to take into account the instability of the preparation; when this was done, the $t_{1/2}$ for dissociation was 2.6 days. The preequilibrium dissociation of 10 nM [^{3}H]NMS from the solubilized cortex was examined by labeling material for only 12 min with radioligand before addition of 50 μM atropine. The half-time for dissociation under these conditions was reduced to 50 ± 12 hr (two experiments).

The dissociation rates for [^{3}H]OxoM were faster than for [^{3}H]NMS. In the solubilized heart (Fig. 4B), the off-rate could be fitted to a single exponential, with a $t_{1/2}$ of 17 hr. Addition of Gpp(NH)p dramatically enhanced the dissociation of 70% of the [^{3}H]OxoM bound ($t_{1/2} \ll 1$ min); the remainder dissociated with a $t_{1/2}$ of about 2 hr. A very similar situation was found with the solubilized cortical receptor, with Gpp(NH)p enhancing the [^{3}H]OxoM off-rate by over 1000-fold.

Binding of muscarinic ligands to receptors solubilized in CHAPSO. Because it was possible to label the muscarinic receptors with [^{3}H]NMS in all three tissues after solubilization, the affinity for a range of ligands could be determined by competition experiments. It was also possible to measure the direct binding of [^{3}H]OxoM in the heart and cortex. The results are summarized in Table 3. The binding of [^{3}H]NMS and [^{3}H]OxoM to the solubilized heart receptor is illustrated by way of example (Fig. 5). At the concentrations used, both ligands bound predominantly to a uniform population of sites; for [^{3}H]OxoM this had a K_d of 1.6 nM and a capacity of 394 fmol ml^{-1} and with [^{3}H]NMS the K_d was 2.5 nM and the capacity was 598 fmol ml^{-1}. The [^{3}H]NMS dissociation constant was about 10-fold weaker than that found in membranes but was similar to the value reported for digitonin solution (11). Similar results were found for the solubilized cortical receptor (Table 3). However, in the solubilized lacrimal gland, binding of [^{3}H]NMS was about 4-fold weaker than binding to receptors from the other two tissues. Atropine bound to all three receptor preparations with high affinity, but it did show a modest selectivity in the order of cortical receptors > heart receptor > lacrimal receptor.

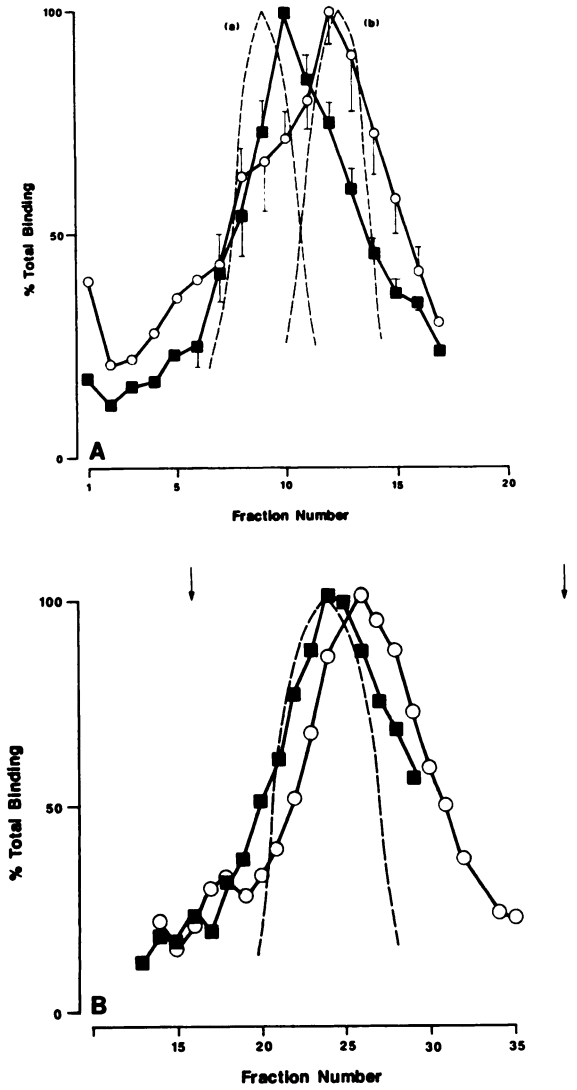

Fig. 1. A, Sucrose density gradient centrifugation in D_2O of [^{3}H]OxoM- and [^{3}H]NMS-labeled receptors (data pooled from heart and cortical receptors.) The gradient limits were 5 to 20% and contained 0.5% CHAPSO, 10 nM concentrations of the radioligand, and, in the case of [^{3}H]NMS, 100 μM Gpp(NH)p. ■, [^{3}H]OxoM (peak binding = 50 fmol ml^{-1}); ○, [^{3}H]NMS + 100 μM Gpp(NH)p (peak binding = 200 fmol ml^{-1}). *Broken lines* indicate molecular weight standards (*a*, catalase; *b*, lactate dehydrogenase). β-Galactosidase and malate dehydrogenase were also included as standards (not shown). B, Sepharose 6B gel filtration of [^{3}H]NMS- and [^{3}H]OxoM-labeled receptors from heart, in 0.5% CHAPSO. ■, [^{3}H]OxoM (peak binding = 50 fmol ml^{-1}; ○, [^{3}H]NMS + 100 μM Gpp(NH)p (peak binding = 100 fmol ml^{-1}). The *broken line* indicates β-galactosidase molecular weight standard (catalase and lactate dehydrogenase standards not shown). The *arrows* mark the positions of the excluded and included volumes.

The greatest selectivity was seen with hexahydrosiladifenidol (Fig. 6). This bound to the solubilized lacrimal receptor with a K_d of 20 nM, making it 6 times more potent as a ligand in this preparation compared with the soluble cortex and 20 times more potent when compared with the soluble heart.

Inhibition curves for pirenzepine are shown in Fig. 7. In the soluble cortex, it could apparently discriminate between (a major) high and (a minor) low affinity populations. The data were compatible with the presence of two sites, with dissociation constants of 20 nM and 1 μM, respectively. By contrast, in the soluble heart and lacrimal gland preparations, just one site was identified, with a K_d close to 1 μM. This is similar to the situation seen in cortical and heart membranes, in which there is a component that binds pirenzepine with a dissociation constant of about 1–5 μM, but in the cortex there is an additional high affinity site with a K_d of 10 nM. The lacrimal receptor in membranes behaves rather differently compared with in CHAPSO solution; it typically has a dissociation constant of about 100 nM (7, 19). In contrast to pirenzepine, AFDX-116 showed little selectivity between the CHAPSO-solubilized receptors (Table 3); in membranes it is cardioselective (8, 19).

Gallamine binding to muscarinic receptors in membranes shows negative heterotropic cooperativity with conventional muscarinic ligands and, in addition, dramatically slows the kinetics of NMS binding (20). The slowing of NMS binding was also noted in all three CHAPSO-solubilized tissue preparations, with the normal 24-hr incubation period with [^{3}H]NMS not being sufficient for equilibrium to be reached. The binding to the three solubilized receptor preparations after 4 days, estimated by [^{3}H]NMS competition experiments, is shown in Fig. 8. The binding to the heart receptor clearly showed evidence of negative cooperativity; there was a fraction of about 12% of the specific [^{3}H]NMS bound that could not be inhibited by gallamine. Gallamine had a K_d of 1 μM when binding to the unliganded receptor and 10 μM for binding to the receptor-[^{3}H]NMS complex. For the soluble cortical and lacrimal receptors, the binding curves were flat (Hill coefficients of 0.58 for cortical receptor and 0.52 for lacrimal receptor). However, they could not be fitted to a model involving negative cooperativity. This may be because the systems were still not in equilibrium at the high gallamine concentrations, where the kinetic slowing of [^{3}H]NMS binding due to the allosteric interactions becomes increasingly important. Consequently, in Table 3 only the IC_{50} and n_H values are presented. Obviously, gallamine was very much less potent in the lacrimal glands than in the cortex.

The direct binding of [^{3}H]OxoM to the solubilized heart receptor (Fig. 5) has been described above, and the binding to the solubilized cortical receptor was very similar (Table 3). About 40% of the total number of muscarinic sites present could be directly labeled with [^{3}H]OxoM. This direct binding was partially sensitive to guanine nucleotides; addition of 100 μM Gpp(NH)p caused a reduction of 78 ± 2% of [^{3}H]OxoM binding in the solubilized heart and 69 ± 4% in the solubilized cortex. This behavior suggests that this state is due to the formation of receptor-G protein complexes and corresponds to the SH states found in heart and cortical membranes. The high and low affinity OxoM binding sites were investigated by OxoM/[^{3}H]NMS competition experiments. In the absence of Gpp(NH)p, the inhibition curves in all three solubilized preparations had Hill slopes significantly less than unity. Fig. 9 illustrates a representative experiment with the solubilized heart receptor. The curve could be resolved into two components. After correction for NMS occupancy, 30% had a K_d of 5 nM (in reasonable agreement with direct binding studies) whereas the remainder had a K_d of 80 nM. The solubilized cortical receptor had very similar OxoM binding properties (Table 3). Gpp(NH)p (100 μM) had no effect on the low affinity agonist binding state, although in Fig. 6 it reduced the high affinity state to undetectable levels. The affinity constant of the low affinity state seen in CHAPSO solution is similar to

TABLE 3

Log affinity constants for ligands binding to the solubilized heart, cortical, and lacrimal gland receptors

Values were obtained by correcting IC_{50} values (obtained by competition experiments against [^{3}H]NMS) for [^{3}H]NMS occupancy. The values for NMS and the super high affinity OxoM states were obtained by the direct binding of [^{3}H]NMS and [^{3}H]OxoM. Hill coefficients were not significantly different from 1, except where two-site fits are presented, and the figures in parentheses in these cases represent the percentage of the second site present. In the case of gallamine in the heart, the data were fitted to a model incorporating negative cooperativity, where K_1 represents binding of gallamine to the receptor and K_2' the binding of gallamine to a receptor-NMS complex (20). The values for gallamine in the cortex and lacrimal gland are −log (IC_{50}) values with Hill coefficients (n_H). SH, super high affinity site; H, high affinity site; L, low affinity site. Values are mean of two to seven experiments.

		Log affinity constants		
		Heart	Cortex	Lacrimal gland
NMS		9.4 ± 0.1	9.55 ± 0.25	8.9 ± 0.1
Atropine		8.6 ± 0.1	9.2 ± 0.1	8.4 ± 0.1
Pirenzepine	H	ND[a]	7.3 ± 0.2	ND
	L	5.7 ± 0.2	5.6 ± 0.1 (66 ± 5%)	6.0 ± 0.1
AFDX-116		4.3 ± 0.2	4.6 ± 0.1	4.2 ± 0.1
Hexahydrosiladifenidol		6.3 ± 0.2	6.9 ± 0.1	7.7 ± 0.2
Gallamine		K_1 = 6.0 ± 0.15	5.0 ± 0.1	3.5 ± 0.14
		K_2' = 5.0 ± 0.16	n_H = 0.58 ± 0.02	n_H = 0.52 ± 0.01
	SH	8.4 ± 0.25	8.5 ± 0.25	ND
OxoM	H	6.8 ± 0.25 (65 ± 6%)	7.1 ± 0.25 (65 ± 7%)	6.9 ± 0.7
	L	ND	ND	4.3 ± 0.1 (77 ± 4%)
	SH	ND	ND	ND
OxoM + Gpp(NH)p	H	6.6 ± 0.3	6.9 ± 0.4	6.2 ± 0.4
	L	ND	ND	4.2 ± 0.2 (66 ± 8%)
	SH	7.2 ± 0.1	7.0 ± 0.1	ND
Carbachol	H	5.3 ± 0.1 (84 ± 2%)	5.6 ± 0.2 (42 ± 3%)	5.5 ± 0.2
	L	ND	ND	3.65 ± 0.25 (44 ± 16%)
Carbachol + Gpp(NH)p	SH	ND	7.2 ± 0.3	ND
	H	6.4 ± 0.25	5.8 ± 0.1 (81 ± 10%)	ND
McN-A-343		5.0 ± 0.1	5.3 ± 0.1	4.8 ± 0.05

[a] ND, not determined.

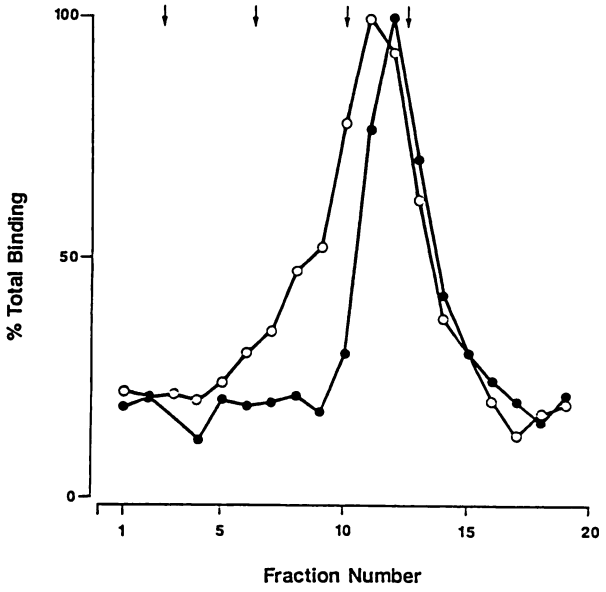

Fig. 2. Sucrose density gradient centrifugation in H_2O of [^{3}H]NMS-labeled purified (●) and nonpurified (○) cortical receptors. The gradient limits were 5 to 20% and were run in the presence of 0.5% CHAPSO, 3 mg/ml phosphatidylcholine, and 10 nM [^{3}H]NMS. Peak binding to purified receptor, 500 fmol/ml; peak binding to nonpurified receptor, 490 fmol/ml. *Arrows* indicate positions of β-galactosidase, catalase, lactate dehydrogenase, and malate dehydrogenase marker enzymes.

that of the H state found in membranes. The solubilized lacrimal gland contrasted with the solubilized heart and cortex (Table 3); the highest affinity binding had a K_d of 125 nM with about 80% of the binding having a K_d of 50 μM. Neither of these states was affected by Gpp(NH)p.

The binding of carbachol was similar to that of OxoM (Table 3). The solubilized heart and cortex both showed two affinity states for carbachol. Gpp(NH)p completely abolished the high affinity state in the solubilized heart and greatly reduced its levels in the soluble cortex. In the soluble lacrimal gland, two affinity states for carbachol could also be detected, but these were of lower affinity than the corresponding states in the solubilized heart and cortex. The agonist McN-A-343 had a monophasic binding curve in all three soluble preparations. This binding was Gpp(NH)p insensitive. It showed a modest selectivity for the soluble cortical receptor.

Detergent exchange. The affinities of many ligands for the CHAPSO-solubilized receptors differ from those found when digitonin is used as the detergent (19, 21). To further examine the effects of detergent on the solubilized receptor, receptors were solubilized in either digitonin or CHAPSO and were then transferred to the other detergent by gel filtration. There was no significant loss of protein during this procedure. Transferring receptors solubilized in digitonin to CHAPSO caused an almost complete loss of all binding. However, if the CHAPSO solution was supplemented with lecithin (3 mg/ml), binding was preserved, suggesting that phospholipid plays an essential role in preserving binding activity in CHAPSO. The yields (relative to the CHAPSO-solubilized receptor) and affinities for NMS and OxoM are shown in Table 4. The changes in affinity constants are relatively small, although there is a reduction in NMS affinity following transfer to CHAPSO and phospholipid. [^{3}H]OxoM direct binding could not be detected under these conditions. The most striking change is the increase in capacity of NMS binding sites in the solubilized cortex following transfer to digitonin. Digitonin by itself gives about a 3-fold greater yield of cortical receptors than CHAPSO. The increase in yield in the detergent transfer experiment suggests that CHAPSO takes into solution almost as many receptors as digitonin but many of these are inactivated or at least incapable

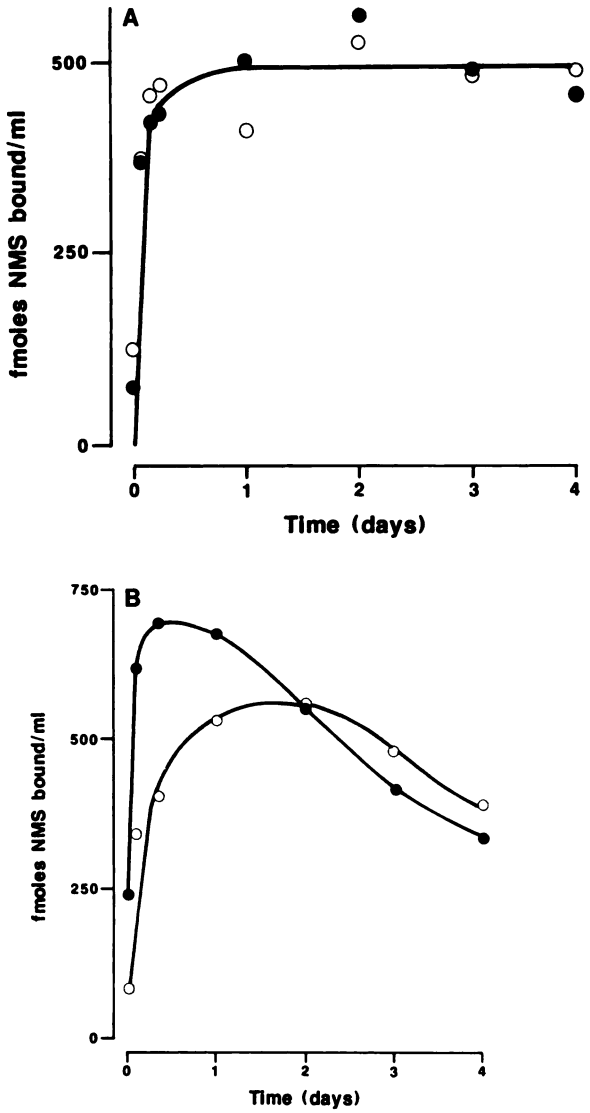

Fig. 3. A, Time course of binding of 10 nM [^{3}H]NMS to the solubilized cortical receptor at 4° in the presence (○) or absence (●) of 100 μM Gpp(NH)p. The data were fitted to an equation of the form $B = B_{max}$ (1 − e^{-kt}) where B_{max} = 495 ± 15 fmol ml^{-1} and k = 1.6 ± 0.3 hr^{-1} [absence of Gpp(NH)p] or B_{max} = 474 ± 14 fmol ml^{-1} and k = 1.8 ± 0.4 hr^{-1}. B, Time course of binding of 10 nM [^{3}H]NMS to the solubilized heart receptor at 4° in the presence (●) or absence (○) of 100 μM Gpp(NH)p. The data obtained in the presence of Gpp(NH)p were analyzed by fitting the decaying phase (i.e., from 1 to 4 days) to a monoexponential decay to estimate the rate constant for this process as 8 × 10^{-3}hr^{-1}. Using this value, the association phase of the curve (up to 1 day) was corrected for instability and was also fitted to a monoexponential, giving a rate constant of 0.7 hr^{-1} in the presence of Gpp(NH)p and 0.09 hr^{-1} in the absence of Gpp(NH)p. (For clarity, data points at ½, 1, 2, and 6 hr are not shown in the figure.)

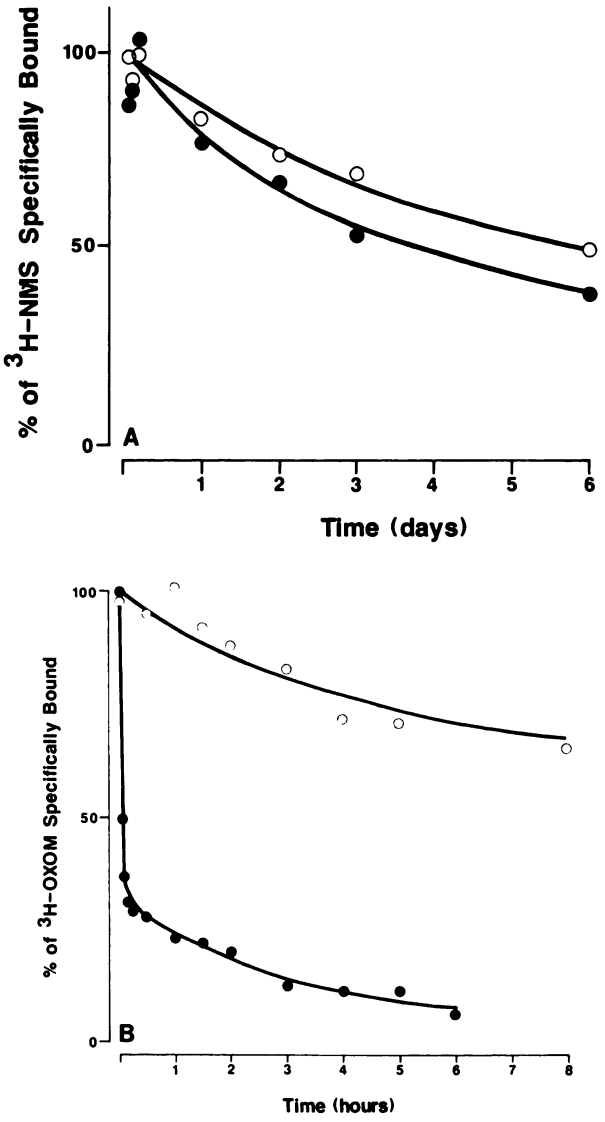

Fig. 4. A, Dissociation of [^{3}H]NMS from the solubilized cortical receptor prelabeled with 10 nM [^{3}H]NMS for 24 hr before the addition of 50 nM atropine (○) or 50 μM atropine + 100 μM Gpp(NH)p (●). The data were fitted to an equation of the form $B = B_{max}\ e^{-kt}$, where B_{max} = 475 ± 8 fmol ml^{-1} and k = 0.13 ± 0.1 days^{-1} [absence of Gpp(NH)p] or B_{max} = 446 ± 21 fmol ml^{-1} and k = 0.19 ± 0.03 days^{-1} [presence of Gpp(NH)p]. B, Dissociation of [^{3}H]OxoM from the solubilized heart receptor prelabeled with 10 nM [^{3}H]OxoM for 24 hr before the addition of 50 μM atropine (○) or 50 μM atropine plus 100 μM Gpp(NH)p (●). In the absence of Gpp(NH)p, the data were fitted to a single exponential decay, with k = 0.06 ± 0.007 hr^{-1}. In the presence of Gpp(NH)p, the data are fitted to a two-component exponential decay, where 66% of the total binding had k = 1850 ± 240 hr^{-1} and the remainder had k = 0.38 ± 0.14 hr^{-1}.

of binding low concentrations of [^{3}H]NMS. After exposure to digitonin, a proportion of these 'latent' receptors can regain their binding properties.

The affinity constants for a variety of ligands were determined after transfer from CHAPSO to digitonin (Table 5). A series of representative curves for the cortical receptor are shown in Fig. 10. The binding profiles of the various ligands are similar to those seen when the receptor is solubilized in digitonin alone (11, 13). There is a particularly striking increase in pirenzepine affinity in the heart and a decrease in low affinity OxoM binding in both heart and cortex.

Discussion

This study describes the effects of CHAPSO solubilization upon the muscarinic receptor subtypes present in the heart, cortex, and lacrimal gland. There has been no previous work on the muscarinic receptor solubilized in this detergent. However, Kuno *et al.* (15) and Baron *et al.* (14) have worked with the related detergent CHAPS. Their results differ in several aspects from the findings presented here; Kuno *et al.* (15) found that the binding of carbachol was of lower affinity and Baron *et al.* (14) reported that it was not regulated by guanine nucleotides. Furthermore, they found it necessary to keep the CHAPS

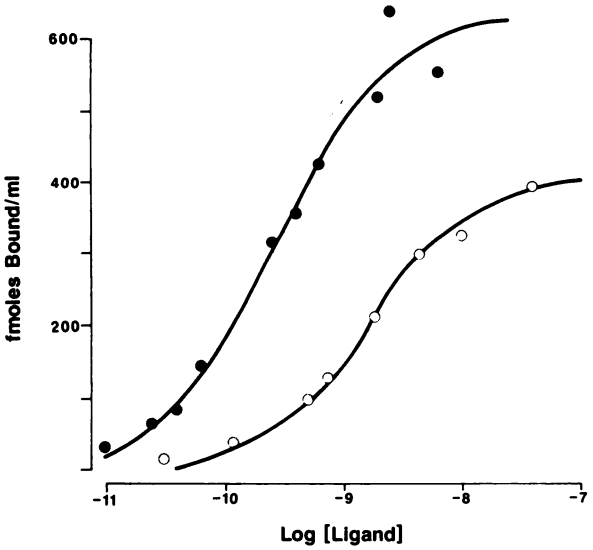

Fig. 5. Equilibrium binding of [^{3}H]NMS and [^{3}H]OxoM to solubilized heart receptor in a representative experiment. ●, NMS, K_d = 0.25 ± 0.05 nM, capacity = 598 ± 41 fmol ml^{-1}. ○, OxoM, K_d = 1.6 ± 0.05 nM, capacity = 394 ± 28 fmol ml^{-1}.

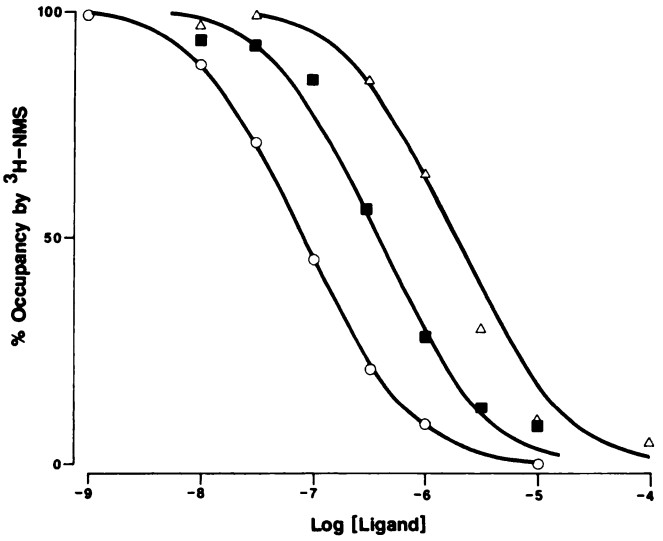

Fig. 6. Inhibition of the binding of 0.2 nM [^{3}H]NMS to solubilized lacrimal, cortical, and heart receptors by hexahydrosiladifenidol. In each case, the data fitted a simple Langmuir adsorption isotherm to give the following log (K_A) values, after correction for NMS occupancy. ○, Lacrimal, 7.4 ± 0.2; ■, cortex, 6.7 ± 0.1; △, heart, 6.0 ± 0.1.

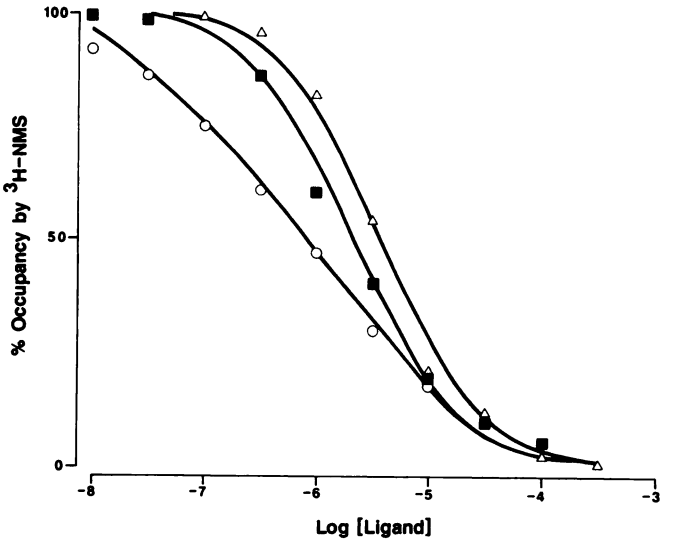

Fig. 7. Inhibition of the binding of 0.2 nM [^{3}H]NMS to solubilized cortical, lacrimal, and heart receptors by pirenzepine. In the cases of the solubilized heart and lacrimal receptors, the data fitted a simple Langmuir adsorption isotherm; in the case of the solubilized cortex, the data fitted a two-site model after correction for NMS occupancy. Fitted parameters were as follows. ○, Cortex, log K_H = 7.3 ± 0.2, log K_L = 5.7 ± 0.1, %L = 55 ± 5; ■, lacrimal, log K_A = 5.8 ± 0.1; △, heart, log K_A = 5.7 ± 0.1.

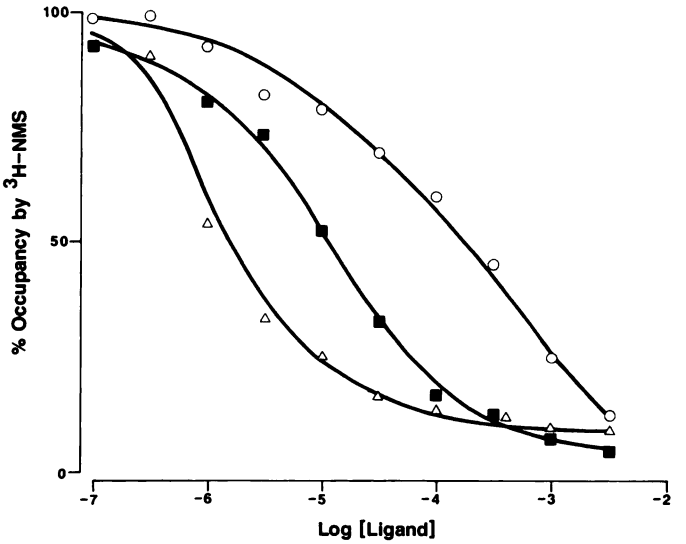

Fig. 8. Inhibition of the binding of 0.2 nM [^{3}H]NMS to solubilized heart, cortical, and lacrimal receptors by gallamine. In the case of the solubilized heart receptor, the data were fitted to a model incorporating negative cooperativity. In the case of the solubilized cortical and lacrimal receptors, the data were fitted to a model with a variable Hill coefficient (n_H) and IC_{50}. After correcting for NMS occupancy, where appropriate, the fitted parameters were as follows. △, Heart, K_1 (gallamine binding to receptor) = 1.2 ± 0.2 μM, K_2' (gallamine binding to NMS-receptor complex) = 11 ± 3 μM; ■, cortex, IC_{50} = 11 ± 2.4 μM, n_H = 0.52 ± 0.06; ○, lacrimal, IC_{50} = 170 ± 40 μM, n_H = 0.51 ± 0.05.

concentration below 0.025% to get stable specific binding, and they claimed that two peaks of L-[^{3}H]quinuclidinyl benzilate activity were found, with s values of 9.9 and 14.9 (surprisingly, the calculated partial specific volumes for these components were higher than that for CHAPS itself, the maximum that might have been expected). The disagreements between the above studies and the results presented here are unlikely to be due to intrinsic differences between the two detergents, because in our hands CHAPS behaved almost identically to CHAPSO both in binding studies and on sucrose gradients (data not shown). It is more likely to be due to the fact that the previous CHAPS studies were carried out at 25°, where receptor-G protein interactions are known to be unstable in membranes and in digitonin solution. In addition, direct binding of [^{3}H]OxoM, as used here, is a much more sensitive probe of these complexes than indirect measurement of carbachol binding.

In this study, the basic pharmacology of the receptor subtypes seems to be preserved after solubilization, albeit in a modified form. The clearest indication of this is with hexahydrosiladifenidol, which retains is selectivity for the lacrimal receptor subtype. However, varying degrees of selectivity are seen with most of the other antagonists examined. For example, gallamine is most potent at the solubilized heart receptor and

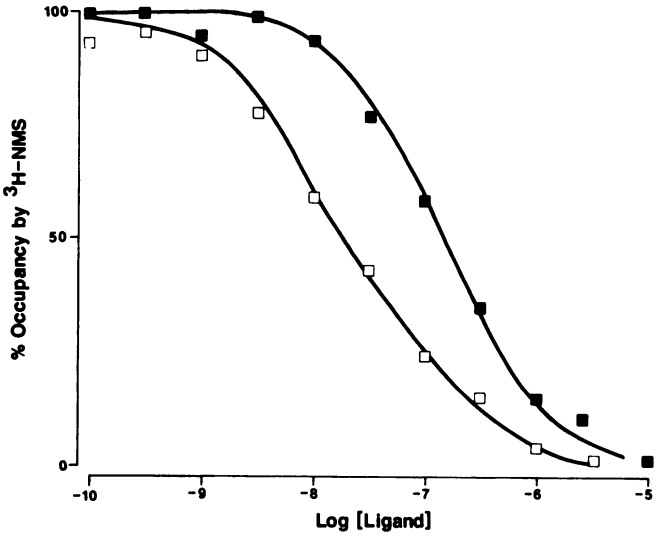

Fig. 9. Inhibition of the binding of 0.2 nM [^{3}H]NMS to solubilized heart receptor by OxoM. In the absence of 100 μM Gpp(NH)p, the data were fitted to a two-site model; in its presence the data fitted a Langmuir adsorption isotherm. After correcting for NMS occupancy, the fitted parameters were as follows. □, Absence of Gpp(NH)p, log K_H = 8.3 ± 0.1, log K_L = 6.7 ± 0.3; ■, presence of Gpp(NH)p, log K_A = 7.1 ± 0.1.

atropine is selective for the cortical receptors. The presence of two pirenzepine binding sites in the solubilized cortical preparation may indicate that this is heterogeneous; on the other hand, pirenzepine affinity can be modified by a large number of factors during the process of receptor solubilization (18, 19) and this apparent heterogeneity may arise from the operation of some of these factors on a proportion of an initially uniform receptor population. It is worth noting that gallamine continues to bind to an allosteric site after solubilization, at least in the heart, showing that its binding characteristics are also preserved in CHAPSO.

The differences between receptor subtypes are also reflected in the kinetics of ligand binding and dissociation. For example, the binding of [^{3}H]NMS is substantially faster to the soluble cortical receptor than to the heart or lacrimal receptor, whereas [^{3}H]NMS dissociation is fastest from the soluble heart receptor. This is similar to the situation seen in membranes (22, 23). The kinetic data also suggest that the mechanism of ligand binding is complicated. This can be seen by comparing the ratio k_{on}/k_{off} with dissociation constants measured at equilibrium. These numbers are not in agreement for either NMS or OxoM. There may be an isomerization of the receptor-ligand complex after binding, "locking" the ligand in place. This is supported by the fact that [^{3}H]NMS dissociation from the solubilized cortex is considerably faster when measured 12 min after addition of radioligand, compared with 24 hr. However, this still falls short of a fully quantitative description of the data. At present, we are unable to offer a complete explanation of the kinetics.

Taken together, these findings clearly indicate that the distinction between receptor subtypes is not just a product of their being confined within membranes. The almost identical behavior of the receptor subtypes during hydrodynamic studies suggests that they are all in similar environments and, hence, the basis for their pharmacological diversity must lie with differences within their primary structures. However, comparison with results obtained with digitonin shows that different detergents can produce very different binding profiles. Thus, upon solubilization with digitonin, the heart receptor binds pirenzepine with a greatly increased affinity, compared with that seen in membranes (13). This effect is totally absent with CHAPSO. Differences are also seen with other antagonists (19, 21). It seems that different detergents can cause the receptor subtypes to adopt different conformations. Furthermore, it is possible to switch between these different conformations simply by exchanging detergents by gel filtration, as clearly demonstrated in this study. It would appear that, although the local environment is not primarily responsible for receptor selectivity, it plays a major role in modulating it.

The agonist binding data have a number of features of interest. The lacrimal receptor subtype clearly stands apart from the heart and cortical receptors; carbachol and OxoM bind with much lower affinity and, at least in the case of OxoM, binding is not sensitive to guanine nucleotides. In lacrimal membranes, agonist affinities are only slightly reduced by Gpp(NH)p (24). However, in all three soluble preparations, the binding curves could be resolved into two components. The highest affinity binding site in the soluble heart and cortex resembled the SH states found in membranes (10, 16) and digitonin solution (11), and the effects of Gpp(NH)p on both equilibrium binding and kinetics of OxoM suggest that it is due to association of the receptor with a G protein. [^{3}H]OxoM binds to the cortical receptor with high affinity even if the ligand is added after solubilization; this is not the case with digitonin, where the agonist-receptor complex must be formed before the addition of detergent. A further important difference compared with digitonin is that the remaining agonist binding sites seem to correspond most closely to the H states seen in membranes and not the L states.

Several aspects of the data shed light on the physical state

TABLE 4

Effects of detergent exchange on capacities and affinities of NMS and OxoM binding

The capacity (B_{max}) values represent the percentage of change in the number of binding sites relative to that of the first detergent used to solubilize the receptor. Typically, the capacities (estimated using [^{3}H]NMS) in the heart and cortex solubilized in CHAPSO are 200 fmol mg^{-1} and (estimated using [^{3}H]oxoM) 90 fmol mg^{-1}. The capacity of the heart receptor solubilized in digitonin (estimated using [^{3}H]NMS) is typically 300 fmol mg^{-1} and, in the cortex, 1000 fmol mg of protein^{-1}. Numbers in parentheses, numbers of experiments.

	CHAPSO to digitonin				Digitonin to CHAPSO and phospholipid, NMS	
	NMS		OxoM			
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
	nM	%	nM	%	nM	%
Heart	0.42 ± 0.2 (3)	105 ± 9 (3)	1.6 ± 0.4 (3)	45 ± 5.6 (3)	3.3 ± 0.7 (4)	138 ± 42 (4)
Cortex	0.9 ± 0.1 (2)	236 ± 42 (4)	0.9 (1)	152 (1)	3.7 ± 0.8 (3)	101 ± 11.4 (3)

TABLE 5

Log affinity constants for ligands binding to the heart and cortical receptors following solubilization in CHAPSO and transfer to digitonin

With gallamine, the log (IC_{50}) values and Hill coefficients (n_H) are shown. For OxoM, the percentage of the lower affinity site is shown in parentheses. SH, super high affinity site; H, high affinity site; L, low affinity site.

		Log affinity constants	
		Heart	Cortex
Gallamine		IC_{50} = 6.6 ± 0.1 n_H = 0.87 ± 0.1	IC_{50} = 6.0 ± 0.1 n_H = 0.5 ± 0.06
Hexahydrosiladifenidol		7.1 ± 0.05	7.4 ± 0.9
Pirenzepine		7.5 ± 0.05	7.4 ± 0.1
OxoM	SH	8.8 ± 0.1	7.6 ± 0.4
	H/L	5.7 ± 0.3 (61 ± 3%)	5.2 ± 0.05 (75 ± 6%)

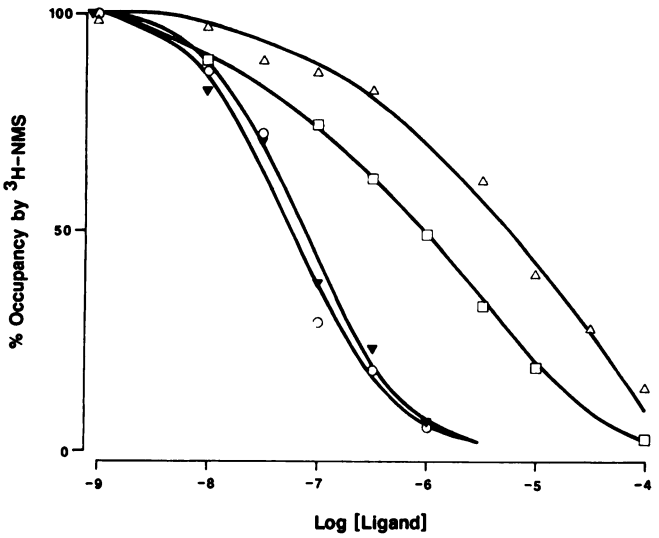

Fig. 10. Inhibition of the binding of 0.2 nM [^{3}H]NMS by various ligands to cortical receptor solubilized in 0.5% CHAPSO and transferred to 1.0% digitonin. The data for pirenzepine and hexahydrosilodifenidol were fitted to a Langmuir adsorption isotherm, that for OxoM to a two-site model, and that for gallamine to a model incorporating a variable Hill coefficient (n_H) and IC_{50}; after correcting for NMS occupancy, the fitted parameters were as follows. ▼, Pirenzepine, log K_A = 7.7 ± 0.1; ○, hexahydrosiladifenidol, log K_A = 7.6 ± 0.1; □, gallamine, IC_{50} = 1.0 ± 0.02 μM, n_H = 0.54 ± 0.1; △, oxoM, K_H = 7.0 ± 0.3, K_L = 5.1 ± 0.2, %L = 65 ± 7.

of the receptor after solubilization. Firstly, the receptor appears to be capable of coupling to a G protein, at least in the solubilized heart and cortical preparations. In most models, the binding of agonists to their receptors favors the formation of the ternary agonist-receptor-G protein complex. However, there is evidence in some systems of spontaneous coupling of receptors to G proteins in the absence of agonists (11, 25, 26). This may manifest itself in effects of guanine nucleotides on antagonist binding, and this is seen here (at least in the case of the heart receptor) as an increase in the [^{3}H]NMS association rate. The most direct evidence for the receptor subtypes forming complexes with other proteins comes from the hydrodynamic studies, where the OxoM-liganded species was shown to be about 110,000 Da larger than the NMS-liganded species. Unfortunately, the contribution of detergent to this difference cannot be assessed with confidence; however, it is clearly compatible with the notion of the receptor associating with a trimeric G protein. (The $\alpha\beta\gamma$ complex for most G proteins has a molecular weight of about 90,000.) Immunochemical evidence for the vasopressin receptor (27) and rhodopsin (28) suggests that they both associate with holomeric G proteins. A further feature of interest from the sucrose density gradient profiles is that a minor component labeled by NMS appears to run as though it were coupled to a G protein. This further supports the idea of spontaneous receptor-G protein association in the absence of agonist. This leading shoulder was not abolished by Gpp(NH)p. A possible explanation is that the receptor and G protein remain in the same detergent micelle, even when uncoupled by Gpp(NH)p. Alternatively it could represent, in part at least, functional receptor-G protein complexes "locked" together. Halothane, an agent that alters membrane properties, causes receptor-G protein complexes to lose their sensitivity to guanine nucleotides while retaining high affinity agonist binding (29). It could be that CHAPSO can promote similar states. This may explain why, in some preparations, up to 40% of the [^{3}H]OxoM binding remained in the presence of 100 μM Gpp(NH)p. This is far too large a fraction to be explained by residual binding to the lower affinity, Gpp(NH)p-insensitive site (about 10% of the total binding at 10 nM [^{3}H]OxoM). A final point of interest is that the proportion of [^{3}H]NMS-liganded receptor apparently associated with G protein is similar to the total fraction of the receptors that can show high affinity [^{3}H]OxoM binding. It would be interesting to know whether these two populations are identical, implying that over half the receptors are in a form in which they can never interact with G proteins, or whether more complicated factors govern this equilibrium.

It is impossible to say whether the receptor not coupled to G proteins is associated with other proteins, because the contribution of detergent to the measured molecular weight of 185,000 cannot be calculated. The glycosylated receptor polypeptide has a molecular weight of about 70,000 in the case of heart and cortical receptors, perhaps higher for the lacrimal receptor (30). If the receptor were the only protein in the micelle, it would be surrounded by about 180 detergent and phospholipid molecules. Modeling the receptor as a rod 70 nm long and 35 nm in diameter, it can be calculated that about 150 molecules of CHAPSO would be required to cover its surface, in good agreement with the above estimate. The role of lipids in maintaining receptor function is clearly seen in detergent-exchange experiments. Digitonin can completely substitute for phospholipids around a protein but, when it was exchanged for CHAPSO, phospholipid had to be included in order to retain any binding activity. In the solubilized cortical preparation, CHAPSO is clearly capable of solubilizing a substantial proportion of the receptors in a form that cannot bind [^{3}H]NMS with high affinity but that can be reactivated by digitonin. The molecular basis for this effect is unclear.

The comparison of the receptor solubilized from a crude cortical homogenate with purified cortical receptor demonstrates a number of points of interest. The purified receptor is free of G proteins and, when run through a sucrose gradient, appears as a narrow symmetrical peak. This provides further support for the contention that the leading edge seen with receptor solubilized from a crude cortical homogenate reflects coupling to G protein or coexistence of receptor and G protein within the same micelle. The interpretation of the slightly lower $s_{20,w}$ value is not so clear. There are several possibilities. The difference could be due to association of nonpurified receptor with an additional small protein. It could be that the phosphatidylcholine supplement is not a perfect mimic of the endogeneous lipids surrounding the cortical receptor in the

crude preparation and, hence, the difference in s value is due to the different lipid environment surrounding the two species. A third possibility is that CHAPSO selectively solubilizes a higher molecular weight muscarinic receptor subtype than is purified starting from a digitonin extract; the molecular weights of muscarinic receptor subtypes do show some differences (4, 5, 30). Unfortunately there is insufficient evidence to decide between these possibilities.

Reconstitution studies using pure proteins are in agreement with the findings of this paper. Addition of G proteins to receptors from heart or cortex increases the amount of high affinity agonist binding (31, 32) and to obtain the maximum effect all three G protein subunits are required (33). This supports the idea that the state labeled by [^{3}H]OxoM is a receptor-holomeric G protein complex. Haga *et al.* (31) report that, in addition, a fraction of their receptors show high affinity agonist binding irrespective of the absence of G proteins or presence of guanine nucleotides. This could reflect the guanine nucleotide-resistant [^{3}H]OxoM binding seen in this paper (31). We have recently confirmed that purified cortical receptors show heterogeneous agonist binding in both detergent solution and upon reconstitution (34).

In conclusion, CHAPSO solubilizes muscarinic receptor subtypes in a characteristic state, capable of interacting with G proteins and binding agonist and antagonists.

Acknowledgments

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