0026-895X/86/030193-07$02.00/0

MOLECULAR PHARMACOLOGY, 30:193-199

Effect of Solubilization on the Distinct Binding Properties of Muscarine Receptors from Rabbit Hippocampus and Brainstem

DONNA D. FLYNN and LINCOLN T. POTTER

Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

Received August 13, 1985; Accepted June 9, 1986

SUMMARY

The binding of carbachol, quinuclidinyl benzilate, pirenzepine, and scopolamine to muscarine receptors from the rabbit hippocampus and brainstem was examined in membranes and in digitonin solution, in order to determine whether the dispersion of receptor molecules altered the distinct ligand-binding profiles of the receptors of these tissues. The modification of receptor-effector complexes showing high affinity for carbachol in membranes, with guanyl 5′-yl imidodiphosphate (GppNHp), *N*-ethylmaleimide (NEM), and ethylenediaminetetraacetate (EDTA), did not remove receptor heterogeneity. In addition to receptor heterogeneity, GppNHp-sensitive high affinity agonist binding to membranes was NEM sensitive in the brainstem but NEM insensitive in the hippocampus. Solubilization with digitonin in the presence of Mn^{2+} ions yielded GppNHp-sensitive, high affinity agonist-binding complexes from the brainstem but not the hippocampus. More extensive dissociation was achieved with solubilization in EDTA and NEM, and resulted in receptor populations which showed markedly lower and slightly different affinities for carbachol. However, the selectivities and affinities for three antagonists were little changed in solution from those found in membranes. These results support the view that there are differences in muscarine receptors as well as different receptor complexes with guanine nucleotide-binding regulatory proteins. The selective binding of carbachol appears largely dependent upon the association of receptors with other molecules, including effector molecules, whereas antagonists appear to recognize receptors irrespective of associated molecules.

Muscarine receptors in membranes are known to be heterogeneous with respect to their affinities for certain antagonists (1–3) and agonists (4–7), the sensitivity of their high affinity agonist-binding states to NEM (7–11), guanine nucleotides (12), and pertussis toxin (13), locations (14, 15), mechanisms (16, 17) and functions (18, 19). Marked differences in the binding of agonists and antagonists to hippocampal and brainstem membranes persist even in the presence of GppNHp, NEM, and EDTA (7), which are believed to dissociate complexes of receptors with guanine nucleotide-binding regulatory proteins (20). In contrast, soluble muscarine receptors appear identical with respect to size, isoelectric point, and reactivity to antibodies (21, 22). Thus, it is possible that a single receptor protein assumes heterogeneous conformations in membranes because of different associated molecules. Studies of soluble receptors are clearly necessary to establish whether there is only one or several receptors. Upon solubilization in detergents, some muscarine receptors retain guanine nucleotide-sensitive high affinity binding of agonists (23, 24). Thus, receptor-effector complexes persist in solution and must be dissociated in order to distinguish different receptors from distinct receptor-nucleotide protein complexes. With purification, muscarine receptors lose sensitivity to guanine nucleotides, but those from the heart retain at least two affinity states for agonists (25). Receptors in the heart (26), the hippocampus, and brainstem (7) are also known to have allosteric sites for agonists. Thus, the presence of mixed agonist affinities in one receptor preparation in solution (25, 27) does not constitute adequate evidence for distinct receptors. Conversely, isolation of receptor populations with distinct agonist affinities (either from different tissues or by purification techniques) would suggest separate receptors. Upon solubilization, muscarine receptors with different affinities for pirenzepine have also been described (27–29). However, it has not yet been shown that these different affinities persist when receptor-effector complexes are dissociated.

To examine further the question of whether there are muscarine receptors with distinct affinity profiles, we have compared the binding properties of hippocampal and brainstem receptors in membranes and in solution, with particular attention to conditions which demonstrably decrease high affinity agonist binding. The results show that the hippocampus and brainstem have receptors with different affinities for pirenzepine, scopolamine, and QNB, even in solutions containing

This work was supported by grants from the United States Public Health Service (NS19065) and the National Science Foundation.

ABBREVIATIONS: NEM, *N*-ethylmaleimide; GppNHp, guanyl 5′-yl imidodiphosphate; EDTA, ethylenediaminetetraacetate; QNB, quinuclidinyl benzilate; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

GppNHp, or NEM plus EDTA. A small difference also appears to persist for carbachol. This evidence favors the view that there are pharmacologically distinct receptors as well as different receptor-effector complexes.

Experimental Procedures

Materials. (−)-[^{3}H]QNB (phenyl-4-^{3}H; 30–40 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA. Protamine sulfate was from Calbiochem-Behring, La Jolla, CA, and pirenzepine was a gift from Boehringer Ingelheim, Ltd., Ridgefield, CT. Carbachol, NEM, digitonin, scopolamine, PMSF, GppNHp, polyethylene glycol (M_r 8000) and all buffers were obtained from Sigma Chemical Co., St. Louis, MO. The Triton X-100-based phosphor used for liquid scintillation spectrometry (Liquiscint) was obtained from National Diagnostics Co., Sommerville, NJ.

Preparation of membranes. Adult albino rabbits weighing 1.6–2.7 kg were anesthetized with diethyl ether and exsanguinated. The "brainstem" was isolated by a coronal cut at the rostral boundary of the superior colliculi, and by removal of the cerebellum; the sample used included the medulla, pons, and lower midbrain. The hippocampi (0.4–0.7 g) and brainstem (0.8–1.2 g) were homogenized with a Brinkman Polytron P10 blender (setting 6/10 for 30 sec) in 19 ml/g of ice-cold 50 mM sodium phosphate buffer containing 10 mM Na_3EDTA, pH 7.4, and homogenates were left on ice for 30 min to allow dissociation of endogenous agonist-receptor complexes (7). In all cases, PMSF was added to the buffer immediately before homogenization (1 ml of 0.1 M PMSF in isopropanol/liter).

Membranes were sedimented by centrifugation at 48,000 × g_{max} for 10 min, and were gently resuspended with the blender to the original weight of the homogenates in one of the two buffers used for assays. Assays were performed with 0.1 ml of suspension, thus utilizing unselected membranes from 5 mg of tissue. The recovery of binding sites for ^{3}H-QNB from homogenates exceeded 98%.

Assays of receptors in membranes. Freshly prepared membranes were incubated at 37° for 45 min in 10 ml of 1.0 nM ^{3}H-QNB, in one of two assay buffers. This large volume and high concentration of QNB were necessary to prevent ligand depletion, which can greatly distort affinity profiles (30). Triplicate assays without and with 1 μM (±)-QNB served to establish total and nonspecific binding, and the difference was taken as specific binding to receptors. For competition studies, various concentrations of counterligands were included, and the percentage inhibition of specific QNB binding was taken as the percentage occupation of receptors by the counterligand used. Membranes with bound ^{3}H-QNB were recovered by filtering suspensions at reduced pressure on 2.4-cm-diameter Reeve Angel 934H glass fiber filters. Each assay tube and filter was rinsed three times with 4 ml of ice-cold assay buffer within 10 sec. Filters were placed in counting vials, dried in an oven at 60°, and covered with 4 ml of phosphor, and radioactivity was counted at an efficiency of 37–38%.

To assess the binding of carbachol to the high affinity state of each receptor, and the ability of GppNHp or NEM to interconvert these sites to those having lower affinity, assays were carried out in 20 mM Tris-HCl buffer containing 1 mM $MnCl_2$ at pH 7.4. Similar results could be obtained in Hepes buffer. To stabilize receptors in their lowest agonist-affinity states, assays were carried out in 50 mM sodium phosphate buffer containing 1 mM EDTA and 0.1 mM NEM, at pH 7.4 (7).

To measure the association rate for ^{3}H-QNB, binding was initiated by injecting 0.1 ml of membrane suspension into 9.9 ml of ^{3}H-QNB at 37°, as for competition experiments, followed by 1 ml of 10 μM (±)-QNB after 15–135 sec, and immediate filtration. To measure the dissociation rate, samples were equilibrated as for competition assays, dissociation was initiated by the addition of 0.1 ml of 100 μM (±)-QNB, and samples were filtered after further incubation for periods up to 5 hr.

In preliminary experiments the following points were established. Recovery of membrane-bound ^{3}H-QNB on filters was indistinguishable from recovery by ultracentrifugation. No delay was necessary between adding phosphor and counting. No quenching of internal standards was found with filters. The same binding curves were obtained for carbachol when the incubation time was 45 min or 2 hr. Depletion of the free radioligand never exceeded 5%. Since K_d values for ^{3}H-QNB were found to be 4–8 pM and the final free radioligand concentration was no less than 0.95 nM, receptor occupation always exceeded 99%. Average receptor levels in the hippocampus and brainstem were 73 and 28 pmol/g of tissue, respectively. By comparison, nonspecific binding was equivalent to 2–3 pmol/g.

Preparation of soluble receptors. Membranes were prepared as described above. For experiments in which NEM was used, membranes were pretreated with ice-cold 0.1 mM NEM for 30 min by resuspension and recentrifugation in assay buffer containing NEM. For all experiments membrane proteins were then dissolved by resuspension of membranes in ice-cold assay buffer containing 1% digitonin (20-ml suspension for membranes from 1 g of tissue). After 30–60 min with occasional swirling, most insoluble material was removed by recentrifugation. Assays were performed with 0.1 ml of the resultant supernatant fluid, thus utilizing membrane proteins from 5 mg of tissue.

Assays of receptors in solution. Freshly prepared membrane protein was incubated at 37° for 15 min with 1.9 ml of buffer and a final concentration of 1.0 nM ^{3}H-QNB. The final concentration of digitonin was 0.05%. The same buffers, counterligands, and procedures for determining the kinetics of binding were used as described for membranes, except that NEM was not present during assays. After incubation, receptors with bound ^{3}H-QNB were precipitated by the sequential addition of 0.2 ml of 5% (w/v) protamine sulfate and 2.0 ml of 25% (w/v) polyethylene glycol, and chilling of the samples on ice for 3–5 min (31). Precipitated protein was recovered by filtration at reduced pressure on glass fiber filters and rinsed three times with 4 ml of ice-cold 10% polyethylene glycol in buffer. Filters were dried and radioactivity was measured as described above.

In preliminary experiments the following points were established. Centrifugation at 100,000 × g for 1 hr did not sediment soluble receptors. An average of 36% of hippocampal and 66% of brainstem receptors were solubilized in PO_4/EDTA/digitonin, whereas 10% and 26% were solubilized in Tris/Mn^{2+}/digitonin, respectively. Depletion of the free radioligand never exceeded 10%. Since K_d values for ^{3}H-QNB were found to be 6–22 pM and the final free radioligand concentration was no less than 0.9 nM, receptor occupation always exceeded 97%. Nonspecific binding in the presence of 1 μM (±)-QNB was less than 10% of specific binding in ^{3}H-QNB alone.

Data analyses. Since binding curves for carbachol, pirenzepine, and scopolamine conformed closely to mass action curves for a single type of site in the hippocampus and in the brainstem, when assays were performed in EDTA, no computerized analysis was necessary to analyze the present results. An estimate of the dissociation constant for each of these receptor ligands was made according to the equation (32): apparent $K_d \equiv IC_{50} \div 1 + ([QNB]/K_d$ for QNB). The K_d values for QNB were determined for the receptors in each tissue by kinetic analyses.

Results

Fig. 1 shows binding curves for carbachol to muscarine receptors from the brainstem, in membranes, and in solution, in Tris/Mn^{2+} buffer. Without GppNHp or NEM, the binding curves were clearly flatter than expected for a single population of sites. Addition of either or both of these agents caused a steepening of each binding curve, with no loss in the total number of binding sites. The final binding curve in solution fit a mass action curve suggesting a single population of binding sites, whereas the membrane binding data deviated slightly from a curve calculated for a single class of sites. These data emphasize the near-homogeneity of brainstem receptors (1, 7), their ability to exist in two affinity states, both in membranes

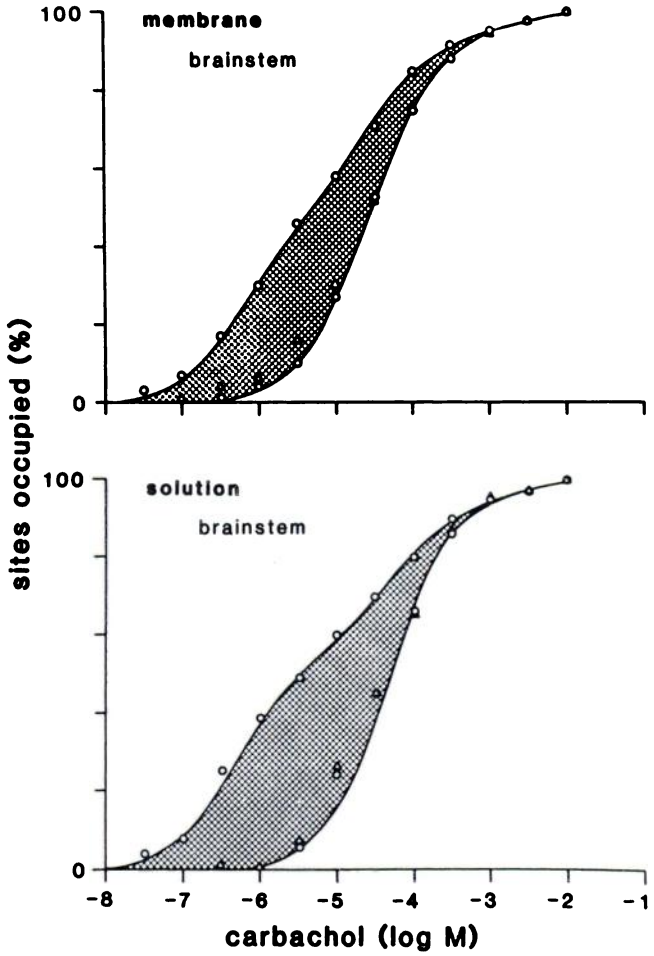

Fig. 1. Effect of GppNHp and of NEM on the affinity of brainstem receptors for carbachol in Tris/Mn^{2+} buffer. In this and subsequent figures *points* are mean values for specific binding from two to six separate experiments, *curves* were drawn by eye except where data fit a single mass action curve, and results obtained with membranes are shown above results obtained in solution. Here the left-hand points were obtained with buffer alone; for the right-hand points: ○, obtained with 0.1 mM NEM; △, obtained with 0.1 mM GppNHp. The right-hand points in solution are fit to a single mass action curve. The figure demonstrates the presence of GppNHp- and NEM-sensitive high affinity binding in solution as well as in membranes.

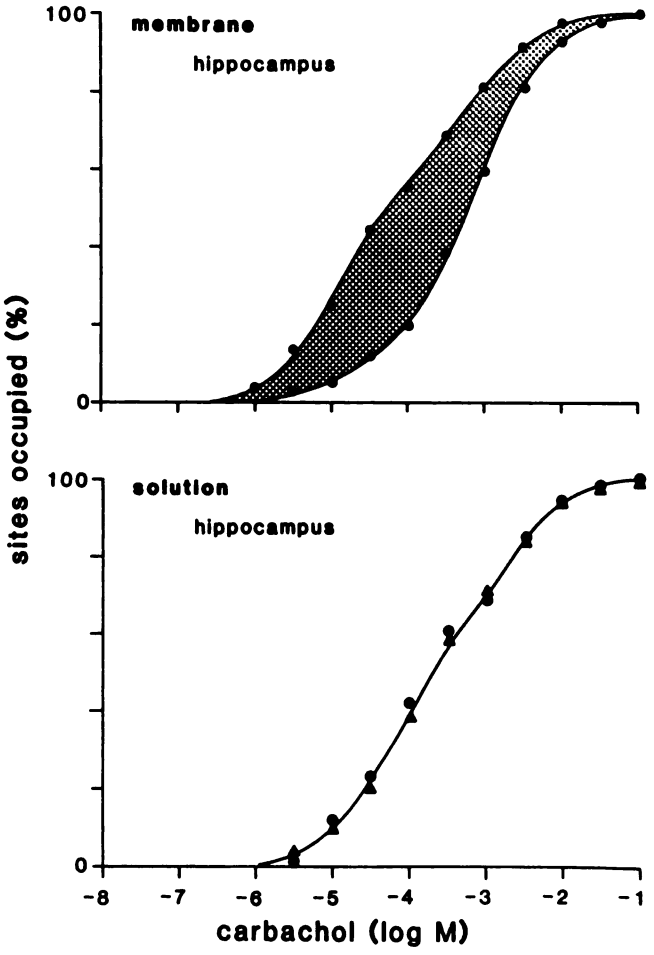

Fig. 2. Effect of GppNHp on the affinity of hippocampal receptors for carbachol in Tris/Mn^{2+} buffer. All results were obtained with receptors treated with 0.1 mM NEM. In the *upper panel* the left-hand curve was obtained without GppNHp and the right-hand curve with 0.1 mM GppNHp. In the *lower panel* results were the same without (●) and with (▲) 0.1 mM GppNHp. The figure demonstrates the presence of GppNHp-sensitive high affinity binding only in membranes.

(1, 7) and in solution (27, 28), and the fact that either GppNHp or NEM can be used to interconvert higher to lower affinity. Thus, solubilization does not disrupt complexes of brainstem receptors with guanine nucleotide-binding protein, in Tris/Mn^{2+} buffer.

Fig. 2 shows the affinities of hippocampal receptors for carbachol, in membranes, and in solution, in Tris/Mn^{2+} buffer containing 0.1 mM NEM. NEM was used to uncouple high affinity binding to receptors in the hippocampus having properties like those in the brainstem. Without GppNHp the binding curves were flatter than expected for a single population of sites. With GppNHp, the binding curve for receptors in membranes shifted to the right and steepened, approaching a single mass action curve. Thus, in membranes, some hippocampal receptors can form an NEM-insensitive, GppNHp-sensitive complex, presumably with a guanine nucleotide-binding protein. In contrast, no effect of GppNHp was found in solution, in confirmation of results by Berrie *et al.* (28). The fact that the binding curve remained flat in solution in Tris/Mn^{2+} may result from solubilization of receptor complexes which are no longer sensitive to GppNHp, or relatively selective solubilization of a small population of receptors known to be present in the hippocampus, which has properties like those in the brainstem (7).

Fig. 3 restates the right-hand curves shown in Figs. 1 and 2, in order to compare directly the properties of the low affinity states of muscarine receptors in the hippocampus and brainstem. It is clear that, although there is a loss of affinity for carbachol upon solubilization, the carbachol affinities of most of the receptors in these two tissues remain different after solubilization, even after attempts to dissociate receptor-effector complexes.

Fig. 4 shows the effect of using NEM and EDTA instead of GppNHp to stabilize both hippocampal and brainstem receptors in their lowest affinity states for agonists. Solubilization markedly decreased the affinity of each receptor for carbachol and the selectivity of carbachol for receptors characteristic of the brainstem. The binding profiles in solution could be fitted to single mass action curves, and their IC_{50} values differed by a factor of 9. A difference of 3.6 is expected on the basis of the different affinities of hippocampal and brainstem receptors for

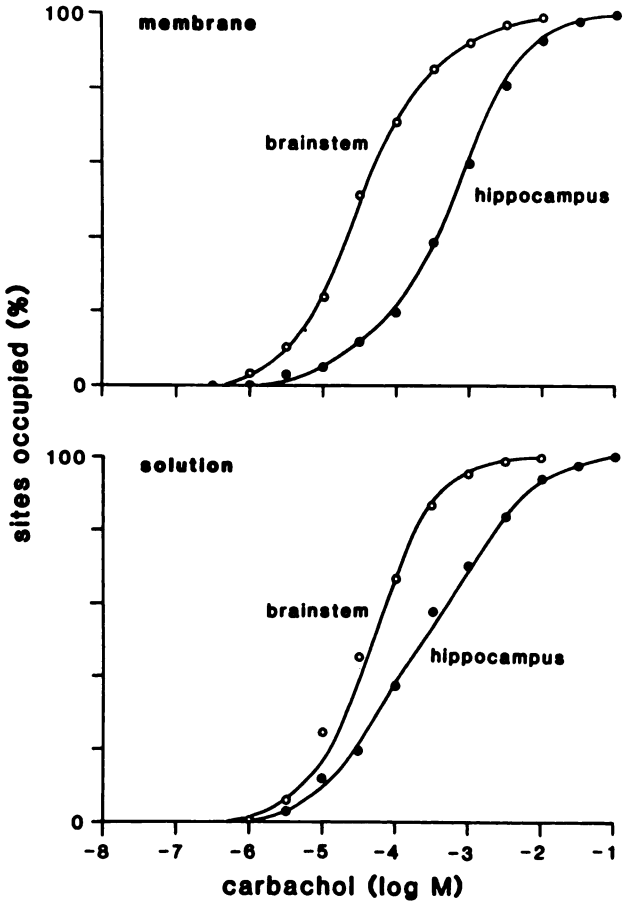

Fig. 3. This figure restates the right-hand curves found in Figs. 1 and 2, in order to compare directly the different properties of hippocampal and brainstem receptors in their lower affinity states in Tris/Mn^{2+} buffer.

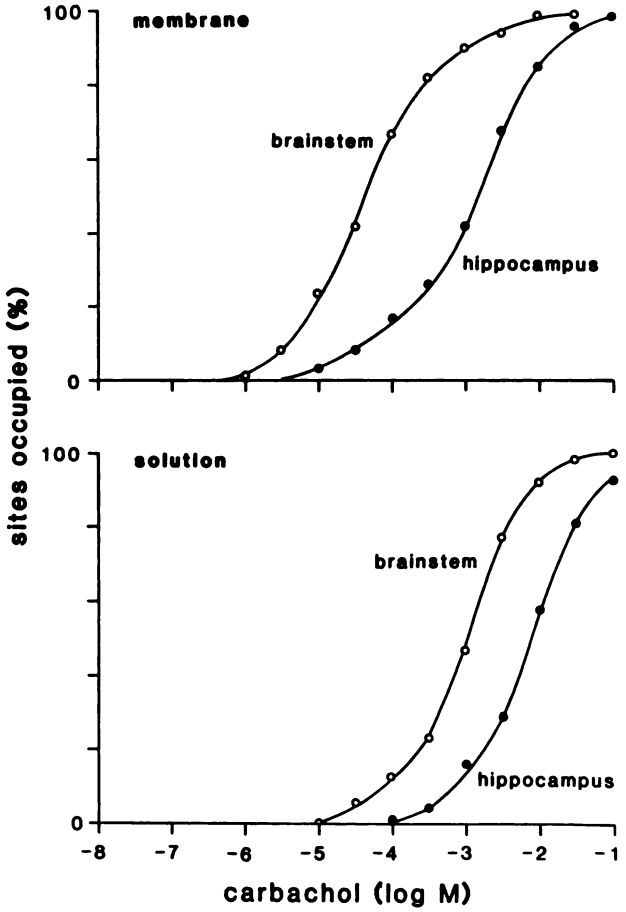

Fig. 4. Effect of EDTA and NEM on the affinity of receptors for carbachol. All results were obtained in the presence of 1 mM EDTA with receptors treated with 0.1 mM NEM. The figure demonstrates that solubilization results in a large loss in the affinity of carbachol for each receptor population and a decrease in selectivity between populations. The *curves* for soluble receptors represent single classes of sites. IC_{50} values are given in the text.

^{3}H-QNB under the same conditions (see below). Hence, a small difference in the affinities of these receptors for carbachol (47 versus 22 μM) appears to remain in EDTA and NEM.

Fig. 5 shows that the rates of association of ^{3}H-QNB with all the receptors in the hippocampus and brainstem in phosphate/EDTA buffer were the same, and that solubilization had no effect on these rates (1.1 and 1.17×10^9 M^{-1} min^{-1} for receptors in membranes and in solution, respectively). In contrast, Fig. 6 shows that the rates of dissociation were different for hippocampal and brainstem receptors, both in membranes (4.3 and 9.0×10^{-3} min^{-1}) and in solution (7.2 and 25.7×10^{-3} min^{-1}); solubilization resulted in slightly more rapid rates. Both for association and dissociation, the rates were exponential (monophasic), again indicating the near-homogeneity of hippocampal and brainstem receptor populations. Kinetically determined K_d values for these populations were 4 and 8 pM in membranes, and 6 and 22 pM in solution, respectively. Thus, solubilization caused only small changes in the affinity of the antagonist, QNB, and slightly increased rather than reducing its selectivity for hippocampal receptors.

Two other antagonists, pirenzepine and scopolamine, also bound selectively to hippocampal versus brainstem receptors, both in membranes and in solution (Fig. 7). After correction for the difference in QNB affinities, apparent K_d values for pirenzepine for hippocampal and brainstem membranes were 5 and 100 nM in membranes, and 9 and 345 nM in solution, respectively. Corresponding values for scopolamine were 0.06 and 1.0 nM in membranes, and 0.11 and 1.7 nM in solution. Thus, solubilization caused only small changes in the affinity of these antagonists and, if anything, increased their selectivity for hippocampal receptors.

Discussion

We have examined the effects of two means of separating muscarine receptor molecules from other molecules, solubilization and the use of agents which uncouple receptor-effector complexes, on the remarkably distinct binding profiles of receptors from the rabbit hippocampus and brainstem. Three points may be made from this study.

First, it is apparent that the actions of digitonin, GppNHp, NEM, and EDTA do not produce pharmacologically homogeneous muscarine receptors. Instead, receptors from the hippocampus and brainstem retain different affinities for three antagonists, QNB, pirenzepine, and scopolamine, and a small but reduced affinity difference for carbachol. Several prior studies have demonstrated that binding heterogeneity for agonists remains after solubilization of muscarine receptors, reflecting the presence of GTP-sensitive receptor-effector complexes (23, 24). This study shows, in addition, that some of this heterogeneity is lost when receptor-effector complexes are dissociated

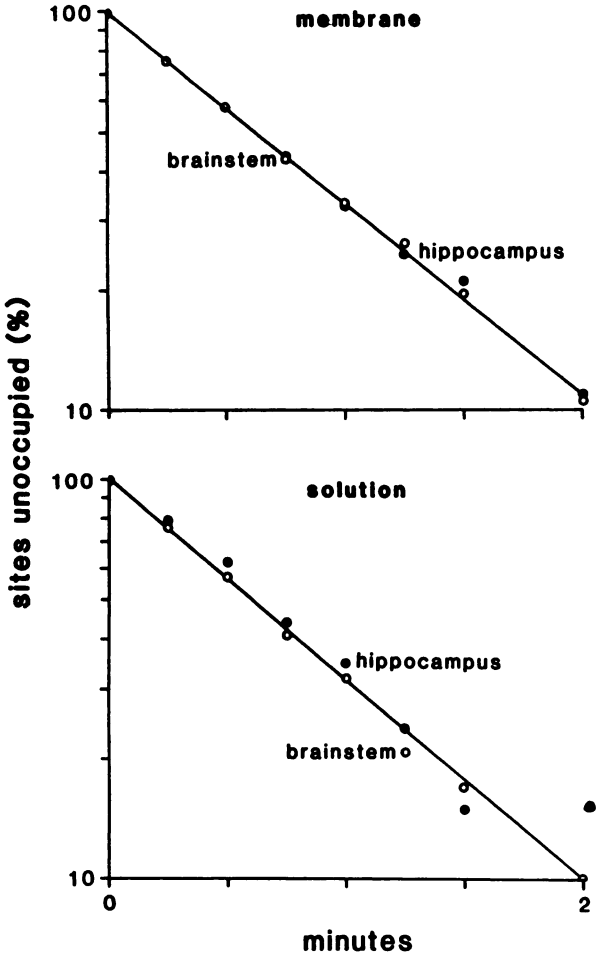

Fig. 5. Rates of association of ^{3}H-QNB in phosphate/EDTA buffer, using receptors treated with 0.1 mM NEM. Rates were monophasic and very similar in membranes and in solution. The *lines* are least squares regression lines; for each component r^2 was 0.98–1.00. Rate constants are given in the text.

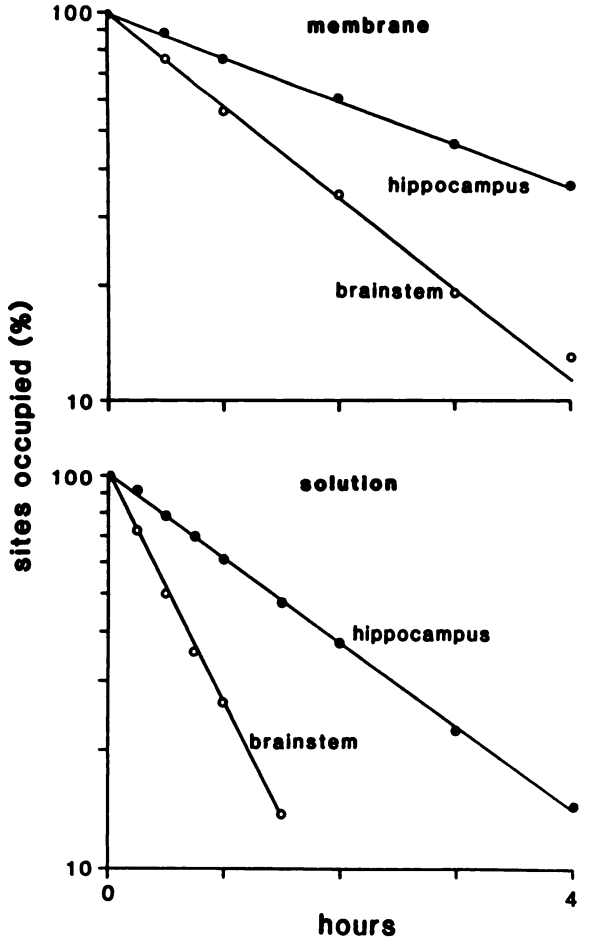

Fig. 6. Rates of dissociation of ^{3}H-QNB in phosphate-EDTA buffer, using receptors treated with 0.1 mM NEM. Rates were monophasic with r^2 values of 0.99–1.00. The figure shows that solubilization slightly increases the rates of dissociation but does not remove the distinction between hippocampal and brainstem receptors. Rate constants are given in the text.

with guanine nucleotides and/or NEM, and that most is lost upon the removal of divalent cations, which are universally required to maintain high affinity guanine nucleotide-sensitive agonist binding to receptors (20). A minor, 2-fold difference between hippocampal and brainstem receptors appears to remain in EDTA plus NEM. This could be dismissed if there were no other evidence for heterogeneity. However, prior studies have shown that muscarine receptors from different tissues retain different affinities for pirenzepine in solution (27–29). The present study has confirmed these findings and shows additionally that pirenzepine is not a unique antagonist in this regard, since both QNB and scopolamine show selectivity in solution, and that the heterogeneous binding of these three antagonists is unaffected by conditions which greatly reduce agonist selectivity. The present evidence for the heterogeneous binding of several ligands to two dispersed populations of muscarine receptors thus favors the idea that there are distinct receptors. The existence of GppNHp-sensitive, NEM-sensitive, and NEM-insensitive receptors in the brainstem and hippocampus, respectively, also favors the idea that there are distinct receptor-effector complexes. These may involve different guanine nucleotide-binding proteins (33, 34). To our knowledge this is the first demonstration of a GppNHp-sensitive, NEM-insensitive muscarine receptor in brain tissue which has a high affinity for pirenzepine. A similar receptor (having high affinity for pirenzepine which is also NEM insensitive but GppNHp-sensitive) has been demonstrated in intact 1321N1 astrocytoma cells (11, 35, 36). The reason for the difference in receptors may be minor and post-translational, such as different glycosylation (37, 38), phosphorylation, acetylation, or polymerization (39) of a single protein, or may be related to differences in primary structure. Analyses of separately purified receptors are necessary to resolve such questions.

A second conclusion of the present study is that extensive dispersion of muscarine receptors does not significantly alter the distinct affinities of hippocampal and brainstem receptors for antagonists. Some prior studies have shown abrupt increases in selectivity or affinity (40) for pirenzepine upon solubilization; these changes were not apparent in EDTA. Loss of high affinity pirenzepine-binding sites has also been observed in solution (41) and upon purification (29), but no losses were seen in the present study. The present evidence thus strongly supports the general view that antagonists recognize receptors independently of their association with other molecules, including guanine nucleotide-binding regulatory proteins.

Finally, we observed a major loss in the selective binding of

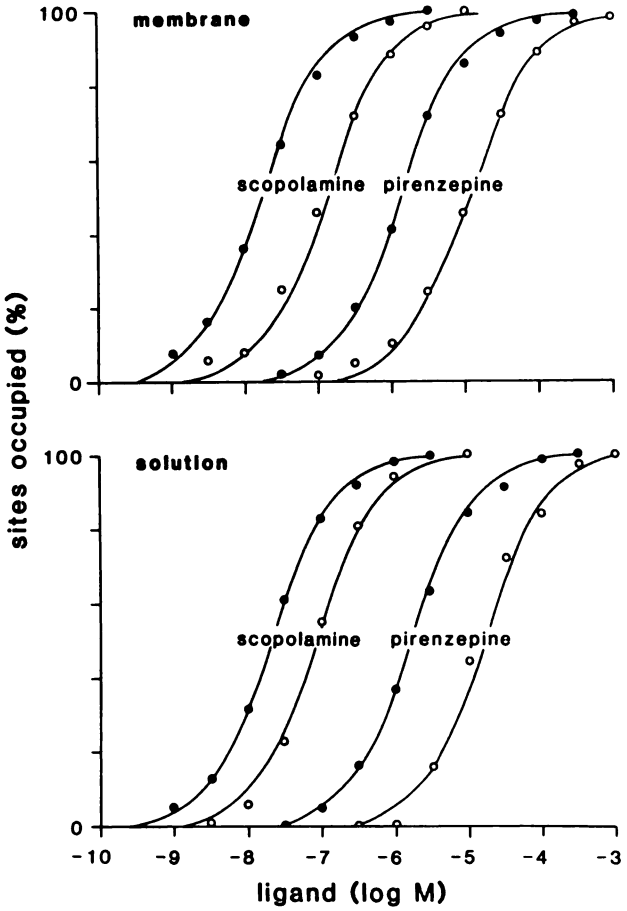

Fig. 7. Binding curves for scopolamine and pirenzepine in phosphate/EDTA buffer, using receptors treated with 0.1 mM NEM from the hippocampus (●) and brainstem (○). The figure demonstrates small decreases in affinities upon solubilization, with little change in the selectivity of these antagonists. All *curves* in this figure are single mass action curves. IC_{50} values are given in the text.

carbachol to brainstem versus hippocampal receptors upon solubilization of membranes in EDTA. This was due to a large loss in the affinity of hippocampal receptors, and an even greater loss in the affinity of brainstem receptors (Fig. 4). Prior investigators have noted increases (42) or decreases (43) in agonist affinities upon solubilization of receptors from a single tissue, but not the decrease of selectivity between tissues (42). Since changes in affinity and selectivity are marked for carbachol but not for antagonists upon solubilization, the basis for the selective binding of carbachol in membranes must be different from that for antagonists. It appears that the association of receptors with other molecules in membranes must be responsible for relatively high agonist affinities and selectivity. The results presented here have ruled out the possibility that selectivity is entirely due to the presence of GppNHp-, NEM-, or EDTA-sensitive receptor-effector complexes. Several remaining possibilities are that there are cooperative receptor interactions or loosely associated receptor-effector complexes (44), which are still capable of stabilizing different receptor conformations in membranes.

Acknowledgments

We thank Nick Palermo and Helene Hanchett for technical assistance.

References

1. Hammer, R., O. P. Berrie, N. J. M. Birdsall, A. S. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature (Lond.)* **283**:90–92 (1980).
2. Watson, M., H. I. Yamamura, and W. R. Roeske. A unique regulatory profile and regional distribution of ^{3}H-pirenzepine binding in the rat provide evidence for distinct M1 and M2 muscarinic receptor subtypes. *Life Sci.* **32**:3001–3011 (1983).
3. Luthin, G. R., and B. B. Wolfe. ^{3}H-Pirenzepine and ^{3}H-quinuclidinyl benzilate binding to brain muscarinic cholinergic receptors. *Mol. Pharmacol.* **26**:164–169 (1984).
4. Birdsall, N. J. M., E. L. Hulme, and A. Burgen. The character of the muscarinic receptors in different regions of the rat brain. *Proc. R. Soc. Lond. B Biol. Sci.* **207**:1–12 (1980).
5. Kloog, Y., Y. Egozi, and M. Sokolovsky. Characterization of muscarinic acetylcholine receptors from mouse brain: evidence for regional heterogeneity and isomerization. *Mol. Pharmacol.* **15**:545–559 (1979).
6. Vickroy, T. W., M. Watson, H. I. Yamamura, and W. R. Roeske. Agonist binding to multiple muscarinic receptors. *Fed. Proc.* **43**:2785–2790 (1984).
7. Potter, L. T., D. D. Flynn, H. E. Hanchett, D. L. Kalinoski, J. Luber-Narod, and D. C. Mash. Independent M1 and M2 receptors: ligands, autoradiography and functions. *Trends Pharmacol. Sci.* (suppl.) 22–31 (1984).
8. Aronstam, R. S., L. G. Abood, and W. Hoss. Influence of sulfhydryl reagents and heavy metals on the functional state of the muscarinic acetylcholine receptor in rat brain. *Mol. Pharmacol.* **14**:575–586 (1978).
9. Vickroy, T. W., H. I. Yamamura, and W. R. Roeske. Differential regulation of high-affinity agonist binding to muscarinic sites in the rat heart, cerebellum and cerebral cortex. *Biochem. Biophys. Res. Commun.* **116**:284–290 (1983).
10. Flynn, D. D., and L. T. Potter. Different effects of *N*-ethylmaleimide on M1 and M2 muscarine receptors in rat brain. *Proc. Natl. Acad. Sci. USA* **82**:580–583 (1985).
11. Martin, M. W., T. Evans, and T. K. Harden. Further evidence that muscarinic cholinergic receptors of 1321N1 astrocytoma cells couple to a guanine nucleotide protein that is not Ni. *Biochem. J.* **229**:539–544 (1985).
12. Gurwitz, D., Y. Kloog, and M. Sokolvsky. High affinity binding of ^{3}H-acetylcholine to muscarinic receptors. Regional distribution and modulation by guanine nucleotides. *Mol. Pharmacol.* **28**:297–305 (1985).
13. Hughes, A. R., M. W. Martin, and T. K. Harden. Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic receptors. *Proc. Natl. Acad. Sci. USA* **81**:5680–5684 (1984).
14. Wamsley, J. K., M. A. Zarbin, and M. J. Kuhar. Distribution of muscarinic cholinergic high and low affinity agonist binding sites: a light microscopic autoradiographic study. *Brain. Res. Bull.* **12**:233–243 (1984).
15. Mash, D. C., and L. T. Potter. Autoradiographic localization of M1 and M2 muscarine receptors in rat brain. *Neuroscience*, in press.
16. Brown, J. H., and S. L. Brown. Agonists differentiate muscarinic receptors that inhibit cyclic AMP formation from those that stimulate phosphoinositide metabolism. *J. Biol. Chem.* **259**:3777–3781 (1984).
17. Gil, D. W., and B. B. Wolfe. Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. *J. Pharmacol. Exp. Ther.* **232**:608–616 (1985).
18. Hirschowitz, B. I., R. Hammer, A. Giachetti, J. J. Kierns, and R. R. Levine (editors). Subtypes of Muscarinic Receptors. *Trends Pharmacol. Sci.* (suppl.) 1–103 (1984).
19. Levine, R. R., N. J. M. Birdsall, A. Giachetti, R. Hammer, L. I. Iversen, D. J. Jenden, and R. A. North. Subtypes of muscarinic receptors. II. *Trends Pharmacol. Sci.* (suppl.) 1–97 (1986).
20. Ross, E. M. and A. G. Gilman. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49**:533–564 (1980).
21. Venter, J. C. Muscarinic cholinergic receptor structure. *J. Biol. Chem.* **258**:4842–4848 (1983).
22. Venter, J. C., B. Eddy, L. M. Hall, and C. M. Fraser. Monoclonal antibodies detect the conservation of muscarinic cholinergic receptor structure from *Drosophila* to human brain and detect possible structural homology with α-adrenergic receptors. *Proc. Natl. Acad. Sci. USA* **81**:272–276 (1984).
23. Berrie, C. P., N. J. M. Birdsall, E. C. Hulme, M. Keen, and J. M. Stockton. Solubilization and characterization of guanine nucleotide-sensitive muscarinic agonist binding sites from rat myocardium. *Br. J. Pharmacol.* **82**:853–861 (1984).
24. Kuno, T., O. Shirakawa, and C. Tanaka. Regulation of the solubilized bovine cerebral cortex muscarinic receptor by GTP and Na^+. *Biochem. Biophys. Res. Commun.* **112**:948–953 (1983).
25. Peterson, G. L., G. S. Herron, M. Yamaki, D. S. Fullerton, and M. I. Schimerlik. Purification of the muscarinic acetylcholine receptor from porcine atria. *Proc. Natl. Acad. Sci. USA* **81**:4993–4997 (1984).
26. Dunlap, L., and J. H. Brown. Heterogeneity of binding sites on cardiac muscarinic receptors induced by the neuromuscular blocking agents gallamine and pancuronium. *Mol. Pharmacol.* **24**:15–22 (1983).
27. Luthin, G. R., and B. B. Wolfe. Characterization of ^{3}H-pirenzepine binding to muscarinic cholinergic receptors solubilized from rat brain. *J. Pharmacol. Exp. Ther.* **234**:37–44 (1985).
28. Berrie, C. P., N. J. M. Birdsall, E. C. Hulme, M. Keen, and J. M. Stockton.

Solubilization and characterization of high and low affinity pirenzepine binding sites from rat cerebral cortex. *Br. J. Pharmacol.* **85**:697–703 (1985).
29. Schimerlik, M. I., S. Miller, G. L. Peterson, L. C. Rosenbaum, and M. R. Tota. Biochemical studies on muscarinic receptors in porcine atrium. *Trends Pharmacol. Sci.* (suppl.) 2–7 (1986).
30. Wells, J. W., N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Competitive binding studies with multiple sites. Effects arising from depletion of the free radioligand. *Biochim. Biophys. Acta* **632**:464–469 (1980).
31. Kalinoski, D. L. Solubilized muscarine receptor. Ph.D. thesis, University of Miami, Miami FL (1982).
32. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
33. Evans, T., M. W. Martin, A. R. Hughes, and T. K. Harden. Guanine nucleotide-sensitive high-affinity binding of carbachol to muscarinic cholinergic receptors of 1321N1 astrocytoma cells is insensitive to pertussis toxin. *Mol. Pharmacol.* **27**:32–37 (1985).
34. Florio, V. A., and P. C. Sternweiss. Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. *J. Biol. Chem.* **260**:3477–3483 (1985).
35. Evans, T., J. R. Hepler, S. B. Masters, J. H. Brown, and T. K. Harden. guanine nucleotide regulation of agonist binding to muscarinic cholinergic receptors. Relation to efficacy of agonists for stimulation of phosphoinositide breakdown and Ca^{2+}-mobilization. *Biochem. J.* **232**:751–757 (1985).
36. Evans, T., M. M. Smith, L. I. Tanner, and T. K. Harden. Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms. *Mol. Pharmacol.* **26**:395–404 (1984).
37. Rauh, J. J., M. P. Lambert, N. J. Cho, H. Chin, and W. L. Klein. Glycoprotein properties of muscarinic acetylcholine receptors from bovine cerebral cortex. *J. Neurochem.* **46**:23–32 (1986).
38. Liles, W. C., and N. M. Nathanson. Regulation of neuronal muscarinic acetylcholine receptor number by protein glycosylation. *J. Neurochem.* **46**:89–95 (1986).
39. Avissar, S., G. Amitai, and M. Sokolovsky. Oligomeric structure of muscarinic receptors is shown by photoaffinity labeling: subunit assembly may explain high- and low-affinity agonist states. *Proc. Natl. Acad. Sci. USA* **80**:156–159 (1983).
40. Berrie, C. P., N. J. M. Birdsall, E. C. Hulme, M. Keen, J. M. Stockton, and M. Wheatley. Muscarinic receptor subclasses: the binding properties of the soluble receptor binding sites. *Trends Pharmacol. Sci.* (suppl.) 8–13 (1986).
41. Roeske, W. R., and J. C. Venter. The differential loss of ^{3}H-pirenzepine *vs* ^{3}H-(−)-quinuclidinyl benzilate binding to soluble rat brain muscarinic receptors indicates that pirenzepine binds to an allosteric state of the muscarinic receptor. *Biochem. Biophys. Res. Commun.* **118**:950–957 (1984).
42. Wenger, D. A., N. Parthasarthy, and R. S. Aronstam. Regional heterogeneity of muscarinic acetylcholine receptors from rat brain is retained after detergent solubilization. *Neurosci. Lett.* **54**:65–70 (1985).
43. Baron, B., and L. G. Abood. Solubilization and characterization of muscarinic receptors from bovine brain. *Life Sci.* **35**:2407–2414 (1984).
44. Mattera, R., B. J. R. Pitts, M. L. Entman, and L. Birnbaumer. Guanine nucleotide regulation of a mammalian myocardial muscarinic receptor system. Evidence for homo- and heterotropic cooperativity in ligand binding analyzed by computer-assisted curve fitting. *J. Biol. Chem.* **260**:7410–7421 (1985).

Send reprint requests to: Dr. Donna D. Flynn, Department of Pharmacology, P. O. Box 016189, University of Miami School of Medicine, Miami, FL 33101.