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THE DIFFERENTIAL LOSS OF [^{3}H]PIRENZEPINE VS [^{3}H](-)QUINUCLIDINYLBENZILATE BINDING TO SOLUBLE RAT BRAIN MUSCARINIC RECEPTORS INDICATES THAT PIRENZEPINE BINDS TO AN ALLOSTERIC STATE OF THE MUSCARINIC RECEPTOR

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[^{3}H]Pirenzepine ([^{3}H]PZ) and [^{3}H](-)Quinuclidinylbenzilate ([^{3}H](-)QNB) specific binding to soluble rat brain muscarinic cholinergic receptors was assessed as a function of time subsequent to receptor solubilization. The soluble brain muscarinic receptor is stable at 4°C when assayed by [^{3}H](-)QNB binding (t 1/2 = 80 hrs). In contrast the pirenzepine state of the receptor decays rapidly (t 1/2 = 3.0 hrs). Prior occupation of the receptor with [^{3}H](-)QNB or [^{3}H]PZ increases the receptor stability by two to five fold (t 1/2 QNB >1,000 hrs; t 1/2 PZ = 6.5 hrs). These data indicate that pirenzepine binds to an allosteric state of the muscarinic receptor and that caution should be employed in the assignment of receptor subtypes based solely upon the binding of ligands which recognize unique conformational states.

Recent studies have utilized the nonclassical behavior of selected drugs such as pirenzepine and McNeil A-343 to suggest that putative subtypes of muscarinic cholinergic receptors (M_1 and M_2) may be present in both central and peripheral tissues (1-7). These studies have classified the "M_1" response by physiologic and pharmacologic methods (1,3,4) as well as by utilizing the ligand [^{3}H]pirenzepine (5,6) or indirect pirenzepine binding studies (2). Recently, differential regulation of putative M_1 and M_2 high affinity binding sites with magnesium ions, N-ethylmaleimide, and guanine nucleotides in the rat heart, cerebellum, and cerebral cortex have been reported (7), suggesting that the observed differences in M_1 and M_2

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subtypes may only represent differences in allosteric regulation of ligand binding to the same receptor protein (8).

In support of this concept is an experiment by Laduron et al. in which solubilized muscarinic receptors were analyzed for high and low affinity pirenzepine sites by an indirect method. A high affinity state of the pirenzepine binding site was not detected in this study suggesting that a unique receptor configuration in the intact cell membrane or homogenate may be essential for the presence of a high affinity pirenzepine binding site (9).

In order to further characterize the solubilized muscarinic receptor, we have utilized the new ligand, [^{3}H]pirenzepine, together with our previously published methodology (8,10) to detect a high affinity pirenzepine site. From our initial results it appears that a high affinity pirenzepine state can be solubilized from a proposed M_1 tissue such as the rat cerebral cortex but that this high affinity state is rapidly lost due to a conformational change in the receptor, a change without effect on [^{3}H](-)QNB binding.

MATERIALS AND METHODS

Whole rat brain membranes were prepared as described (8). Brains were homogenized in nine volumes of 0.05 M $NaPO_4$, pH 7.4, 0.32 M sucrose, at 0-4° using a motor driven Teflon pestle homogenizer. The homogenate was subjected to 1,000xg for 10 min and the pellet discarded. The supernatant was subjected to 40,000xg for 20 minutes at 4°C. The pellet was resuspended in assay buffer and snap frozen. On the day of the experiment, the frozen samples were thawed and diluted to a volume of 20 mg protein in 5 ml with 10 mM $NaPO_4$, pH 7.4.

Solubilization of the muscarinic receptor was achieved as previously described (8,10,11). Membrane fractions were resuspended in 0.5 percent digitonin (final concentration), with 100 µM PMSF in 10 mM $NaPO_4$ buffer at pH 7.4. After 15 minutes of solubilization at 0-4° a soluble fraction was obtained, which was separated from the remaining particulate material by centrifugation at 100,000 xg for 60 minutes.

[^{3}H](-)QNB binding to the soluble receptor was measured by a rapid filtration technique (12,13). Aliquots of soluble receptor were incubated with [^{3}H](-)QNB (quinuclidinyl benzilate, 33 Ci/mmol, New England Nuclear) in a final volume of 1.0 ml of 10 mM $NaPO_4$ buffer, pH 7.4. All binding measurements were done in triplicate. Specific binding was defined by using 1 µM atropine (Sigma). Following incubation for 1.0 hour at 25°, 250 µl of 1.2 percent human gamma globulin were added to each tube followed by 6 ml of 15 percent polyethylene glycol (PEG) in 20 mM potassium phosphate buffer, pH 8. The reaction was allowed to proceed for 20 minutes on ice to precipitate solubilized receptors. The mixture was filtered through Whatman GF/B filters and filters were washed with 10 ml polyethyleneglycol (8 percent in 20 mM K_2PO_4 buffer, pH 8) (10). The filters were air dried and assayed for filter bound radioactivity by liquid scintillation spectroscopy in Formula 963 (NEN). The concentration of protein in the soluble material was estimated using standard methods (14).

[^{3}H]PZ binding was measured using a method modified from (6). [^{3}H]PZ (88 Ci/mmol, New England Nuclear) was incubated with soluble receptor in 1.0 ml of 10 mM $NaPO_4$ buffer, pH 7.4. Specific binding was defined with 1 μM atropine and all assays were done in triplicate. The incubation was carried out at 25° for one hour, and precipitation and filtration of the soluble receptors were performed as described for [^{3}H](-)QNB assays except that GF/B filters were pretreated with 0.1 percent polyethyleneimine (Sigma). Analysis of radioactivity was done as for [^{3}H](-)QNB binding.

RESULTS

Muscarinic receptor binding was characterized using [^{3}H]PZ and [^{3}H](-)QNB. Results from frozen membranes were similar to those previously described by one of us in fresh rat brain homogenates (5).

Our preliminary experiments indicated a marked difference in the stability of the receptor conformation required to bind [^{3}H]PZ and [^{3}H](-)QNB. In order to investigate these differences in more detail, we conducted kinetic experiments using solubilized muscarinic receptors. We followed the different receptor conformations by prelabeling the receptor in the membrane with [^{3}H]PZ or [^{3}H](-)QNB prior to detergent solubilization or by assaying the solubilized receptor for [^{3}H]PZ or [^{3}H](-)QNB specific binding.

As illustrated in Figure 1, the unoccupied detergent solubilized muscarinic receptor, assayed with [^{3}H](-)QNB, is extremely stable with a loss of only 20 percent of its activity in 24 hours at 4°C (t 1/2 = 80 hrs). As previously documented with the β-adrenergic receptor (10), the presence of ligand confers additional stability to the solubilized receptor (Figure 1). The receptor complex prelabeled with [^{3}H](-)QNB retained 100 percent of its initial activity after 24 hours (t 1/2 >1,000 hrs) (Figure 1).

In contrast to the data for [^{3}H](-)QNB, the muscarinic receptor continues to have high affinity for [^{3}H]PZ for only brief periods following solubilization from the membrane. The loss of high affinity [^{3}H]PZ binding to the soluble muscarinic receptor appeared to follow two phases (Figure 1). In the first phase (~60 percent of muscarinic sites), [^{3}H]PZ binding disappeared with a t 1/2 of 3.0 hours whereas in the slower phase (~40 percent of sites) [^{3}H]PZ binding was lost with t 1/2 of ~44 hours. Prior occupation of the muscarinic receptors with [^{3}H]PZ increased the stability of the high

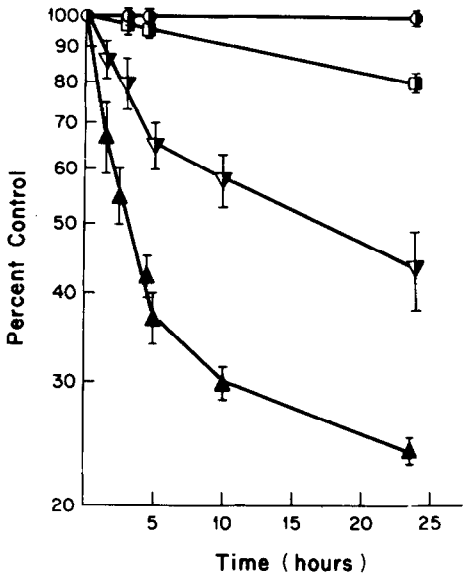

Figure 1 - The stability of the [^{3}H](-)QNB and [^{3}H]PZ binding states of the solubilized rat brain muscarinic cholinergic receptor. The loss of receptor binding as a function of time and ligand. The data presented are from six different experimental conditions:

1) Soluble unoccupied muscarinic receptors.
   a) assayed with [^{3}H](-)QNB (400 pM)(closed squares).
   b) assayed with [^{3}H]PZ (20 nM)(closed triangles).

Rat brain membranes were treated with 0.5 percent digitonin (3 mg/ml) for 15 min at 4°C, then subjected to centrifugation at 100,000 xg for 60 min. The soluble muscarinic receptors (300 µg soluble protein) were assayed in a volume of 1.0 ml of 10 mM $NaPO_4$ pH 7.4 for 1 hr at 25°C with the above ligand concentration. The reaction was terminated by the addition of 250 µl gamma globulin and 6 ml of 15 percent PEG, and after 20 min at 0°C filtered over GF/B filters and washed with 10 ml 8 percent PEG.

2) Prelabeling receptors with [^{3}H](-)QNB prior to solubilization:
   a) soluble [^{3}H](-)QNB-receptor complex incubated in the presence of excess free [^{3}H](-)QNB (400 pM)(open circles)
   b) soluble [^{3}H](-)QNB-receptor complex incubated in the absence of free [^{3}H](-)QNB (closed circles)

Rat brain membranes were treated with [^{3}H](-)QNB (400 pM) for 60 min at 30°C in the presence and absence of 1.0 µM Atropine. The membrane-ligand complexes were then subjected to centrifugation (40,000 xg) for 20 min at 0°C and the pellet resuspended in 0.5 percent digitonin at a concentration of 3 mg/ml, with or without excess free [^{3}H](-)QNB (400 pM) and incubated for 15 min at 4°C. The detergent membrane mixture was centrifuged for 1 hr at 100,000 xg at 0°C and the supernatant (soluble ligand-receptor complexes) assayed by the addition of 83 µl cold gamma globulin to 333 µl soluble receptor-ligand complex. 2.0 ml 15 percent PEG was added and the mixture incubated at 0°C for 20 min followed by filtration as above.

3) Prelabeling receptors with [^{3}H]PZ prior to solubilization:
   a) soluble [^{3}H]PZ-receptor complex incubated in the presence of excess free [^{3}H]PZ (20 nM)(inverse open triangles)
   b) soluble [^{3}H]PZ-receptor complex incubated in the absence of free [^{3}H]PZ (inverse closed triangles)

Muscarinic receptors were treated as in (2) above only with 20 nM [^{3}H]PZ in place of [^{3}H](-)QNB. Each data point represents the average specific binding of triplicate determinations from four independent experiments.

Error bars denote the standard deviation for all samples. The 100 percent control for all experiments was determined with [^{3}H]-QNB (400 pM).

affinity PZ state of the receptor (t 1/2 = 6.5 hrs) (Figure 1, Table 1). However, the high affinity [^{3}H]PZ-receptor complex was always less stable than the [^{3}H](-)QNB-receptor complex by more than one order of magnitude (Figure 1, Table 1).

With both the QNB-receptor complex and the PZ-receptor complex it was the presence of bound ligand and not the ability to bind or rebind dissociated ligand that conferred the increased stability to the receptor since the presence or absence of free ligand had no effect on the stability of receptor-ligand complexes (Figure 1). The half-lives of the various forms of the receptor are summarized in Table I.

DISCUSSION

Pirenzepine is a non-classical muscarinic receptor antagonist which has been demonstrated to inhibit physiological function in a manner that is apparently selective and different from that of atropine (3,15,16,17). Pirenzepine binding to muscarinic receptors is stereoselective, saturable and

Table 1

STABILITY OF SOLUBLE MUSCARINIC CHOLINERGIC RECEPTORS

Receptor State	t 1/2 (hrs)
Free Soluble Receptor assayed with [^{3}H](-)QNB (1a)	80
Free Soluble Receptor assayed with [^{3}H]PZ (1b)	3.0
[^{3}H](-)QNB-Receptor complex with excess free [^{3}H](-)QNB (2a)	>1,000
[^{3}H](-)QNB-Receptor complex without excess free [^{3}H](-)QNB (2b)	>1,000
[^{3}H]PZ-Receptor complex with excess free [^{3}H]PZ (3a)	6.5
[^{3}H]PZ-Receptor complex without excess free [^{3}H]PZ (3b)	6.5

Data from Figure 1. The numbers in parentheses correspond to the experimental conditions outlined in the legend of Figure 1.

of high affinity (5,6). High affinity pirenzepine binding appears to a greater or lesser extent in each tissue. One criterion for the "subclassification" of muscarinic receptors as M_1 and M_2 has been that the M_1 receptor displays a high affinity for pirenzepine whereas the M_2 receptor has a lower affinity for pirenzepine while both M_1 and M_2 receptors bind QNB with equal affinity (6,18,19).

The results of the present study demonstrate for the first time that the "high" affinity pirenzepine binding form of the muscarinic receptor can be solubilized from rat brain, a putative M_1 tissue. However, despite the fact that the soluble muscarinic receptor is remarkably stable, t 1/2 >1000 hours, with regard to [^{3}H](-)QNB (a classical antagonist) binding (Figure 1, Table 1), the high affinity [^{3}H]pirenzepine binding is rapidly lost, t 1/2 = 3.0 hours (Figure 1, Table I). These data strongly suggest that high affinity pirenzepine binding is to a specific conformational state of the muscarinic receptor and that alterations in or loss of the high affinity pirenzepine conformational state have no detectable effect on classical muscarinic antagonist binding.

The main hypothesis offered to explain these findings is that the receptor molecule is undergoing an allosteric conformational change in solution. The process of solubilization frees the receptor from conformational restraints imposed by the membrane or other membrane proteins and immediately alters its ability to bind pirenzipine with high affinity; however, if the ligand binding reaction takes place before receptor solubilization, the stabilized high affinity complex appears to be more resistant to the allosteric conformational change. Theories of the binding of QNB have emphasized that the molecule binds to a site in a region which permits it to bind with high affinity and to not recognize the specific affinity states recognized by muscarinic agonists (20). However, the simple fact that QNB can completely inhibit pirenzepine or agonist binding suggest an overlap in the recognition sites.

Biochemical and structural studies have shown the existence of only a single muscarinic receptor species with a molecular weight of 80,000 daltons

on SDS-polyacrylamide gels (8,11). When muscarinic receptors from human brain, rat brain, monkey ciliary muscle, dog heart and Drosophila heads were subjected to isoelectric focusing, a common isoelectric point was also found for every receptor (11). In that the isoelectric point (pI) of a protein is a unique parameter and isolectric focusing can detect minor differences in protein structure, these observations coupled with the finding that limited proteolysis of the muscarinic receptor from putative M_1 and M_2 tissues reveals identical tryptic fragments has lead to the concept that the muscarinic receptor protein is conserved throughout the animal kingdom (8,11), a concept strongly supported by a recent study which demonstrated complete monoclonal antibody crossreactivity with muscarinic receptors from human brain to Drosophila (11). In contrast to this data, Birdsall and Hulme (21) have described the properties of at least three putative muscarinic receptors.

Our data taken together with previously published data on pirenzepine and on muscarinic receptor structural analysis suggest that caution must be employed in the assignment of receptor subtypes (e.g., M_1 and M_2) based solely upon the binding of ligands which recognize unique allosteric states of receptors.

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REFERENCES

1. Goyal, R.K., and Rattan, S. (1978) Gastroenterol. 74. 598-619.
2. Hammer, R., Berrie, C.P., Birdsall, N.J.M., Burgen, A.S.V., and Hulme, E.C. (1980) Nature 283, 90-92.
3. Hammer, R., and Giachetti, A. (1982) Life Sci. 31, 2991-2998.
4. Roszkowski, A.P. (1961) J. Pharmacol. Exp. Ther. 132, 156-170.
5. Watson, M., Roeske, W.R., and Yamamura, H.I. (1982) Life Sci. 31, 2019-2023.
6. Watson, M., Yamamura, H.I., and Roeske, W.R. (1983) Life Sci. 32, 3011-3022.
7. Vickroy, T., Yamamura, H.I., and Roeske, W.R. (1983) Biochem. Biophys. Res. Commun. 116, 284-290.

8. Venter, J.C. (1983) J. Biol. Chem. 258, 4842-4848.
9. Laduron, P.M., Leysen, J.E., and Gorissen, H. (1981) Arch. Int. Pharmacol. Ther. 249, 319-321.
10. Strauss, W.L., Ghai, G., Fraser, C.M., and Venter, J.C. (1979) Arch. Biochem. Biophys. 196, 566-573.
11. Venter, J.C., Eddy, B., Hall, L.M., and Fraser, C.M. (1984) Proc. Natl. Acad. Sci. USA 81, 272-276.
12. Yamamura, H.I., and Snyder, S.H. (1974) Proc. Natl. Acad. Sci. USA 71, 1725-1729.
13. Fields, J.Z., Roeske, W.R., Morkin, E., and Yamamura, H.I. (1978). J. Biol. Chem. 253, 3251-3258.
14. Bohlen, P., Stern, S., Daiman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220.
15. Fuder, H., Rink, D., and Muscholl, E. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 318, 210-219.
16. Brown, D.A., Forward, A., and March, S. (1980) Br. J. Pharmacol. 71, 362-364.
17. Sustrand, K., and Severing, K.F. (1979) Pharmacol. 19, 163-164.
18. Watson, M., Roeske, W.R., Johnson, P., and Yamamura, H.I. (in press) Brain Research.
19. Yamamura, H.I., Wamsley, J.K., Deshmukh, P., and Roeske, W.R. (1983) Eur. J. Pharmacol. 91, 147-149.
20. Ehlert, F.J., Roeske, W.R., and Yamamura, H.I. (1983) Handbook of Psychopharmacology, pp.241-276, Plenum Publishing Co., New York.
21. Birdsall, N.J.M., and Hulme, E.C. (1983) TIPS 4, 459-463.