Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 314-321

REGULATION OF ANTAGONIST BINDING TO CARDIAC MUSCARINIC RECEPTORS

M. Marlene Hosey

University of Health Sciences/The Chicago Medical School
Department of Biochemistry, North Chicago, IL 60064

Received June 3, 1982

The regulation of ligand binding to the muscarinic acetylcholine receptor in developing chick heart has been studied using the radiolabeled antagonist [^{3}H]quinuclidinyl benzilate (QNB). In assays containing only buffer and a source of receptor protein, the antagonist radioligand bound to a single, high affinity state of the receptor. If Mg^{2+} and EDTA were added, [^{3}H]QNB bound to a single, low affinity state. The guanine nucleotide analog, guanylylimidodiphosphate [Gpp(NH)p], reversed the effect of Mg^{2+}/EDTA so that [^{3}H]QNB again bound only to a single, high affinity state. Sodium could also reverse the effect of Mg^{2+}/EDTA on antagonist binding but the effects of sodium and Gpp(NH)p on [^{3}H]QNB binding were not additive.

INTRODUCTION

The cardiac muscarinic acetylcholine receptor (mAChR) is coupled to adenylate cyclase in an inhibitory manner (1,2). Guanine nucleotides enhance cholinergic inhibition of cardiac adenylate cyclase (2) and modulate the affinity of cardiac muscarinic receptors for agonists (3-14). Several studies have shown that there are multiple affinity states of the muscarinic receptor which are recognized by cholinergic agonists (3, 6, 10-13, 15). A high affinity state of the receptor for agonists is converted by guanine nucleotides to a state having low affinity for agonists (3-14). Two groups (10, 16, 17) recently demonstrated the existence of a heterogenous population of cholinergic antagonist binding states in rat and frog cardiac muscle. Furthermore, they showed that guanine nucleotides convert the heterogenous states for antagonist in these tissues to a single high affinity state (10, 16, 17). We have studied the effects of guanine nucleotides and ions on the properties of the chick heart mAChR. We have found conditions whereby the affinity of

The abbreviations used are QNB, quinuclidinyl benzilate; Gpp(NH)p, guanylylimodiphosphate; G-protein, GDP/GTP binding protein of adenylate cyclase complex.

0006-291X/82/130314-08$01.00/0

the muscarinic receptor for antagonists can be modulated by guanine nucleotides, magnesium and sodium ions.

METHODS

EXPERIMENTAL PROCEDURES

Fertilized White Leghorn eggs were obtained from SPAFAS, Roanoke, IL and incubated in a Humidaire egg incubator at 37° C. l-[^{3}H]QNB was purchased from Amersham/Searle; atropine sulfate, acetylcholine iodide, eserine salicylate were from Sigma. Guanylylimidodiphosphate [Gpp(NH)p] was purchased from P-L Biochemicals. Ultra-pure sucrose was obtained from Schwarz/Mann. All other reagents were purchased from commercial sources and were of the highest purity available.

Tissue preparation. Hearts were removed from newborn chicks (0-7 days old) or 10-day embryos and homogenates prepared as previously described (18). All tissues were diluted in assay buffer (50 mM Tris-HCl, pH 7.4) immediately prior to use. The final dilution of homogenates in the assay was 2000-5000 fold. After preparation, tissues were used immediately or stored in liquid N_2 prior to use.

Assay of [^{3}H]QNB binding: [^{3}H]QNB binding was assayed according to Hosey and Fields (18) except that the buffer used was 50 mM Tris-HCl, pH 7.4 and protein used was 50-100 µg of homogenates. Other additions are noted in the figure legends. Non-specific binding was defined as that obtained in the presence of 2 µM atropine sulfate and ranged from 6-15% of the total radioactivity bound. The data were analyzed as described (18) and/or with a computer program, LIGAND (19), which is a general non-linear and curve-fitting program that uses a weighted least squares analysis to analyze receptor binding data. Data from multiple experiments are expressed as means ± S.E.M.

RESULTS

A typical experiment showing the effects of Mg^{2+} and EDTA on the binding of [^{3}H]QNB to a newborn heart homogenate is shown in Fig. 1. In the presence of only the Tris-HCl buffer, protein and [^{3}H]QNB, the ligand bound to a single affinity state of the receptor with a K_D of 14.2 pM (Fig. 1). The addition of Mg^{2+} plus EDTA to the assay resulted in the conversion of the mAChR to a state exhibiting a lower affinity for [^{3}H]QNB with a K_D of 43 pM (Fig. 1). Under these conditions, only a single low affinity state was observed (Fig. 1). This effect of Mg^{2+}/EDTA was similar in embryonic and newborn heart preparations. EDTA alone had no effect on [^{3}H]QNB binding, i.e., the results were as obtained with buffer alone (data not shown). The effect of Mg^{2+} plus EDTA on the K_D value for [^{3}H]QNB binding was not accompanied by a change in the total amount of [^{3}H]QNB bound (B_{max}) (Fig. 1).

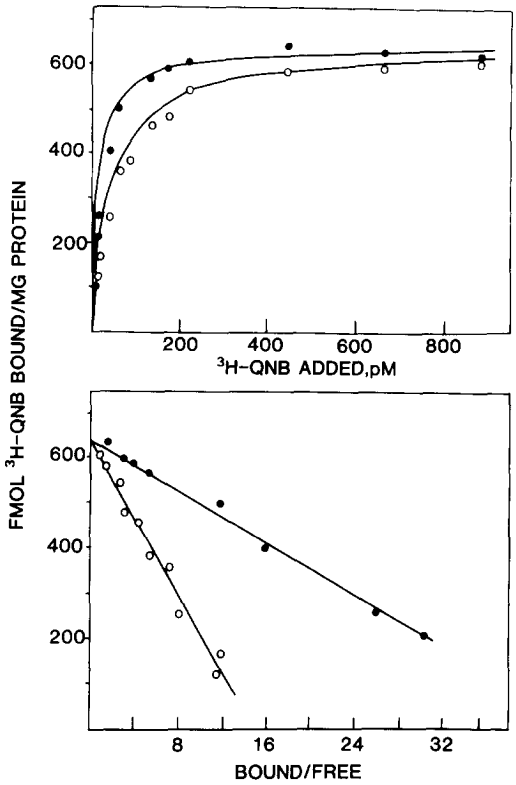

Fig. 1. Effect of Mg^{2+} and EDTA on [^{3}H]QNB binding to newborn heart homogenate. Assays contained buffer, protein (0.088 mg) and varying amounts of [^{3}H]QNB. ●—●, control; O—O, plus $MgSO_4$ (10 mM) and EDTA (1 mM). Specific radioactivity (cpms) bound ranged from 490 to 1435 (O—O) and 275 to 1296 (O—O). Top, saturation isotherm; bottom, Scatchard transformation of the data. Bound/Free = ratio of [^{3}H]QNB bound/[^{3}H]QNB free. The correlation coefficients (r) were 0.99, control and 0.94 for $MgSO_4$ plus EDTA.

An experiment showing the typical effects of Na^+, Gpp(NH)p and Na^+ plus Gpp(NH)p on [^{3}H]QNB binding to an embryonic heart preparation is shown in Fig. 2. All data was obtained in the presence of Mg^{2+}/EDTA. Na^+ partially reversed the effect of Mg^{2+}/EDTA, while Gpp(NH)p alone or Na^+ plus Gpp(NH)p converted the mAChR to a single high affinity state for the antagonist. In this experiment the K_D values (pM) were 112, 50.3, 25.9 and 25.6 for the control, Na^+, Gpp(NH)p, and Na^+ plus Gpp(NH)p, respectively. In contrast to their effects on agonist binding (4), the effects of the combination of Na^+ and Gpp(NH)p on [^{3}H]QNB binding were not greater than Gpp(NH)p alone. The effects of Gpp(NH)p and Na^+ were maximal at the concentrations used. Effects of Gpp(NH)p could be observed at concentrations as low as 1 nM, and of Na^+ as low

Table I

K_D and B_{max} Values for [^{3}H]QNB binding to Embryonic and Newborn Heart Homogenates under Various Assay Conditions

	Embryo		Newborn	
Addition	K_D, pM	B_{max} [c]	K_D, pM	B_{max} [c]
Control	22.50 ± 1.61 (4) [b]	396.56 ± 51.03 (4)	20.82 ± 4.47 (4)	693.06 ± 204.16 (4)
Mg^{2+}/EDTA [a]	93.44 ± 19.82 (7)	353.61 ± 39.62 (7)	49.69 ± 6.91 (8)	607.03 ± 66.53 (8)
Na^+ [a]	50.73 ± 6.43 (3)	404.21 ± 79.20 (3)	32.41 ± 4.60 (3)	697.11 ± 162.8 (3)
Gpp(NH)p [a]	30.31 ± 2.94 (3)	412.28 ± 81.18 (3)	28.96 ± 1.83 (3)	712.84 ± 194.6 (3)
Na^+ and Gpp(NH)p [a]	24.09 ± 3.54 (3)	365.81 ± 43.36 (3)	30.96 ± 6.86 (3)	702.7 ± 144.5 (3)

[a] Assays contained 10 mM $MgSO_4$ and 1 mM EDTA

[b] Mean ± SEM. Number in parentheses is number of determinations

[c] Fmol/mg protein

as 20 mM. The results were the same whether or not acetylcholinesterase (sufficient to metabolize 2 µmols acetylcholine/min)was present in the assays.

The K_D values for [^{3}H]QNB binding from many such experiments are summarized in Table 1. They indicated that the effects of ions and guanine nucleotides on [^{3}H]QNB binding are qualitatively similar in both newborn and embryonic hearts. However, the K_D values obtained with Mg^{2+}/EDTA alone appear to be higher in the embryonic hearts. Furthermore, Na^+ seemed to only partially reverse the effect of Mg^{2+}/EDTA in embryonic hearts,but was as effective as Gpp(NH)p in newborn hearts. No significant effect on B_{max} was observed under the various conditions (Table 1). The difference in receptor density in newborn and embryonic hearts that is apparent in Table 1 has been previously reported by us (18).

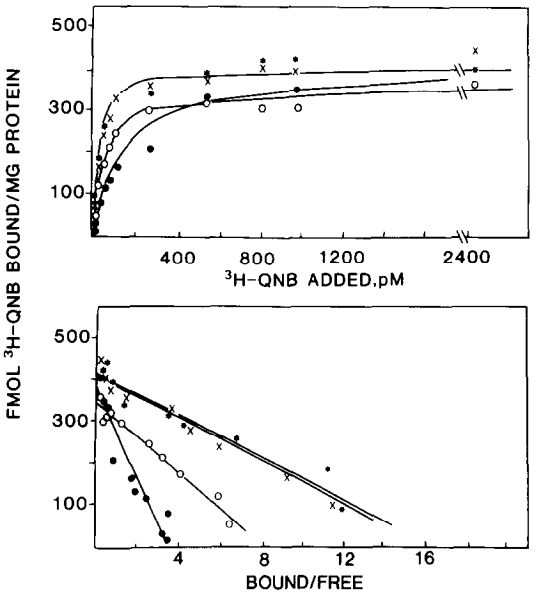

Fig. 2. Effect of Na^+ and/or Gpp(NH)p on [^{3}H]QNB binding to an embryonic chick heart homogenate. All assays contained buffer, protein (0.1 mg), $MgSO_4$, and EDTA. ●—●, control; O—O, plus Na^+ (0.1 M); X—X, plus Gpp(NH)p (10 μM); *—*, plus Na and Gpp(NH)p. Specific radioactivity (cpms) bound ranged from 26 to 1092 (●—●), 77 to 958 (O—O), 198 to 1196 (X—X), and 204 to 1174 (*—*). Top, saturation isotherm; bottom, Scatchard transformation of the data. The correlation coefficients were 0.92 (Mg/EDTA), 0.98 (Na^+), 0.97 [Gpp(NH)p], and 0.97 [Na^+ plus Gpp(NH)p].

DISCUSSION

The present study clearly shows conditions whereby the affinity of the chick heart muscarinic receptor for the antagonist radioligand [^{3}H]QNB is differentially regulated *in vitro* by Mg^{2+}, guanine nucleotides and sodium. In the presence of Mg^{2+},[^{3}H]QNB bound to the receptor with low affinity (Fig. 1, 2, Table 1). Others have reported effects of Mg^{2+} on the affinity of the cardiac mAChR for agonists without accompaying effects on the affinity for antagonists (9, 14, 34). While direct binding studies using [^{3}H]agonists of the mAChR have not included reports of effects of Mg^{2+}, it is clear in many other receptor-cyclase linked systems that Mg^{2+} increases receptor affinity for agonists (20-23). In some systems (23, 24), Mg^{2+} also decreases receptor affinity for antagonists. This is clearly the case for the chick heart muscarinic receptor (Figs. 1, 2, Table 1).

Gpp(NH)p increased the affinity of the chick heart muscarinic receptor for [^{3}H]QNB. Effects of guanine nucleotides on agonist binding to the mAChR

have been demonstrated previously (3-14, 16, 17). It is believed that guanine nucleotides regulate agonist binding to cyclase-linked receptor systems by dissociating receptor: G-protein complexes (25-27). Originally, guanine nucleotide effects on receptor affinities were believed to be agonist specific (28, 29). But several recent studies have shown that guanine nucleotides also affect the affinity (16, 17, 30, 31) or binding capacity (32, 33) of certain cyclase-linked receptors for antagonists (16, 17, 30, 31).

Others have observed two affinity states of mAChR for antagonists in rat and frog hearts that converted to one high affinity state in the presence Gpp(NH)p (10, 16, 17). With the chick heart, we do not see two affinity states for [^{3}H]QNB. We made a preliminary study of [^{3}H]QNB binding under our conditions to the rat heart muscarinic receptor and found essentially the same results as Hulme et al. (10, 16).

Under the conditions of the present study, Na^+ increased the affinity of the chick heart muscarinic receptor for [^{3}H]QNB. Studies with a [^{3}H]agonist of the mAChR showed that Na^+ decreases the affinity of the receptor for agonists (11). Previous studies have shown that Na^+ enhances acetylcholine mediated inhibition of cardiac adenylate cyclase (2). While Na^+ and Gpp(NH)p each affected the affinity of the receptor for [^{3}H]QNB, the effects were not additive (Fig 2, Table 1). In contrast, the effects of Na^+ and Gpp(NH)p on agonist binding are additive or syngergistic (4). The results presented in this study concerning effects of Mg^{2+}, Gpp(NH)p and Na^+ on antagonist binding to cardiac muscarinic receptors are opposite to those on agonist binding to the receptor (3-14). They suggest that care should be taken in interpreting results of agonist binding when [^{3}H]antagonist/agonist competition studies are performed, since the antagonist binds with different affinities under various experimental conditions.

Finally, the data presented herein offer a possible explanation for the wide range of dissociation constants for [^{3}H]QNB (10-300 pM) that are present in the literature (e.g. 8, 18, 35-37). The various assay conditions used by different investigators, from simple buffer systems (18, 35) to tissue culture

media (8, 36) obviously will result in large variations in the K_D values for [^{3}H]QNB binding.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the skillful technical assistance of Ms. Deborah Rymut in performing the studies described herein. She also thanks Mr. Frank Naeymi-Rad for his assistance with the computer program, and Dr. Jeremy Fields for the use of his filtration manifolds to perform the binding assays. Helpful conversations with Drs. R. D. Green, F. Marcus and R. G. Kemp are also gratefully acknowledged. This work was supported by a grant from the Chicago Heart Association and by BRSG Grant RR-5366 awarded by the Biomedical Research Support Program Division of Research Resources, National Institutes of Health.

REFERENCES

1. Brown, J.H. (1979) Mol. Pharmacol. 16, 841-850.
2. Rodbell, M. (1980) Nature 284, 17-22.
3. Berrie, C.P., Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C. (1979) Biochem. Biophys. Res. Commun. 87, 1000-1005.
4. Rosenberger, L.B., Yamamura, H.I. and Roeske, W.R. (1980) J. Biol. Chem. 255, 820-823.
5. Rosenberger, L.B., Roeske, W.R. and Yamamura, H.I. (1979) Eur. J. Pharmacol. 56, 179-180.
6. Ehlert, F.J., Roeske, W.R. and Yamamura, H.I. (1980) J. Supramolecular Struc. 14, 149-162.
7. Wei, J-W. and Sulakhe, P.V. (1980) Eur. J. Pharmacol. 62, 345-357.
8. Galper, J.B. and Smith, T.W. (1980) J. Biol. Chem. 255, 9571-9579.
9. Wei, J.W. and Sulakhe, P.V. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 413, 51-59.
10. Hulme, E.C., Birdsall, N.J.M., Berrie, C.P., Burgen, A.S.V. (1980) In Neutrotransmitters and their Receptors, U.Z. Littauer, Y. Dudai and I. Silman, eds., pp. 241-250.
11. Ehlert, F.J., Yamura, H.I., Triggle, D.J. and Roeske, W.R. (1980) Eur. J. Pharmacol. 61, 317-318.
12. Ehlert, F.J., Roeske, W.R., Rosenberger, L.B. and Yamamura, H.I. (1980) Life Sci. 26, 245-252.
13. Ehlert, F.J., Dumont, Y., Roeske, W.R. and Yamamura, H.I. (1980) Life Sci. 26, 961-967.
14. Mukherjee, A. and Snyder, G.D. (1981) Biochim. Biophys. Acta 674,160-165.
15. Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C. (1978) Mol. Pharmacol. 14, 723-736.
16. Hulme, E.C., Berrie, C.P., Birdsall, J.M. and Burgen, A.S.V. (1981) Eur. J. Pharmacol. 73, 137-142.
17. Burgisser, E., DeLean, A. and Lefkowitz, R.J. (1982) Proc. Natl. Acad. Sci. 79, 1732-1736.
18. Hosey, M.M. and Fields, J.Z. (1980) J. Biol. Chem. 12, 6396-6399.
19. Munson, P.M. and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
20. Williams, L.T., Mullikin, D. and Lefkowitz, R.J. (1978) J. Biol. Chem. 253, 2984-2989.
21. Bird, S.J. and Maguire, M.E. (1978) J. Biol. Chem. 253, 8826-8834.
22. U'Prichard, D.C. and Snyder, S.H. (1980) J. Neurochem. 34, 385-394.
23. Chapman, D.B. and Way, E.L. (1980) Ann. Rev. Pharmacol. Toxicol. 20, 553-579.
24. Daiguji, M., Meltzer, H.Y. and U'Prichard, D.C. (1980) Life Sci. 28, 2705-2717.
25. Limbird, L.E. (1981) Biochem. J. 195, 1-13.

26. Rodbell, M. (1980) Nature 284, 17-22.
27. Cooper, D.M.F. (1982) FEBS Lett. 128, 157-163.
28. Maguire, M.E., Van Arsdale, P.M. and Gilman, A.G. (1976) Mol. Pharmacol. 12, 335-339.
29. Lefkowitz, R.J., Mullikin, D. and Caron, M.G. (1976) J. Biol. Chem. 251, 4686-4692.
30. DeLean, A., Kilpatrick, B.F. and Caron, M.G. (1982) Endocrinology 110, 1064-1066.
31. Wolfe, B.B. and Harden, T.K. (1981) J. Cyclic Nucl. Res. 7, 303-312.
32. Koski, G., Simonds, W.F. and Klee, W.A. (1981) J. Biol. Chem. 256, 1536-1538.
33. Woodcock, E.A. and Murley, B. (1982) Biochem. Biophys. Res. Commun. 105, 252-258.
34. Gurwitz, D. and Sokolovsky, M. (1980) Biochem. Biophys. Res. Commun. 96, 1296-1304.
35. Fields, J.Z., Roeske, W.R., Morkin, E. and Yamamura, H.I. (1978) J. Biol. Chem. 253, 3251-3258.
36. Galper, J.B., Klein, W. and Catterall, W.A. (1977) J. Biol. Chem. 252, 8692-8699.
37. Wise, B.C., Shoji, M. and Kuo, J.F. (1980) Biochem. Biophys. Commun. 92, 1136-1142.