MOLECULAR PHARMACOLOGY, 28:400–409

Agonist Interactions with Cardiac Muscarinic Receptors

Effects of Mg^{2+}, Guanine Nucleotides, and Monovalent Cations

KATHRYN K. MCMAHON[1] AND M. MARLENE HOSEY[2]

Department of Biological Chemistry and Structure, University of Health Sciences/The Chicago Medical School, North Chicago, Illinois 60064

Received April 10, 1985; Accepted August 20, 1985

SUMMARY

Analysis of [^{3}H]quinuclidinyl benzilate/acetylcholine competition curves indicated that the agonist acetylcholine bound with three different affinities to chick heart muscarinic receptors. The estimated K_D values for acetylcholine were 2.7, 240, and 4000 nM. Mg^{2+} increased and guanosine 5′-(β,γ-imino)triphosphate (Gpp(NH)p) decreased the proportion of the receptors in the highest affinity state without altering the K_D values. Monovalent cations increased the K_D values of the three affinity states and obscured the detection of the highest affinity state. The nature of the three affinity states and the sites of action of Mg^{2+}, guanine nucleotides, and monovalent cations were probed with three experimental protocols. Treatments with *N*-ethylmaleimide or pertussis toxin eliminated both the highest affinity state and the sensitivity to Gpp(NH)p. In contrast, partial effects of Mg^{2+} were retained after either of these treatments. The effects of monovalent cations on the affinity of the receptor for agonists were unaffected by both treatments. Solubilization of the receptors with digitonin-cholate yielded preparations displaying only the low affinity state for agonist. Agonist binding to the solubilized receptors was insensitive to Mg^{2+} and guanine nucleotides but retained sensitivity to monovalent cations. The results indicate that chick heart muscarinic receptors can exist *in vitro* in three agonist affinity states and that the entire population of receptors can be interconverted from one state to another by Mg^{2+} and guanine nucleotides. Guanine nucleotides presumably act via the inhibitory guanine nucleotide-binding regulatory (N_i) protein, whereas there appear to be at least two distinct sites of action of Mg^{2+}. One site is associated with N_i. Another is distinguishable from N_i but does not appear to be on the receptor itself. The effect of monovalent cations on the interaction of agonists with cardiac muscarinic receptors is qualitatively different and mediated at distinct sites from the effects of Mg^{2+} and guanine nucleotides.

INTRODUCTION

Extensive studies of many receptor systems have shown that the affinity of receptors for agonists may be influenced by the coupling of the receptors to effector systems. The most widely studied examples are receptors which couple to the adenylate cyclase system. It is generally held that association of receptors with N proteins of the adenylate cyclase system results in high affinity agonist binding (1, 2). This situation is enhanced in the presence of Mg^{2+} and is reversed when guanine nucleotides associate with the N protein (1, 2). Thus, in these systems, two affinity states for agonists are observed.

A somewhat more complex situation has been observed for certain muscarinic cholinergic receptors. In neuronal tissue, three affinity states for agonists are observed (3). The physiological significance of these three affinity states has not been established. However, in brain, there is evidence that muscarinic receptors couple to several effector systems (4–6). Thus, the three affinity states for agonists could be an expression of receptor coupling to different effector systems. In the mammalian heart (7–9) and the embryonic chick heart (10), it has also been shown that the muscarinic receptors can exhibit three affinity states for agonists. In the present study we show that the receptors in all of the three affinity states are interconvertible, and we consider the influence of ions and guanine nucleotides on the number and affinity of the receptors in each of the three affinity states. In order to gain an understanding of the physiological significance of the different affinity states, we probed the sites of

This work was supported by grants from the Chicago Heart Association and the American Heart Association.

[1] Senior Research Fellow of the Chicago Heart Association. Present address: Department of Pharmacology, University of South Carolina School of Medicine, Columbia, SC 29208.

[2] Established Investigator of the American Heart Association.

0026-895X/85/050400-10$02.00/0

action of guanine nucleotides, Mg^{2+}, and monovalent cations using several biochemical approaches.

EXPERIMENTAL PROCEDURES

Materials. Chicks were hatched from fertilized White Leghorn eggs (SPAFAS, Roanoke, IL). [^{3}H]QNB[3] (31–34 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). NEM was from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was prepared from *Bordetella pertussis* as described in Ref. 11. Digitonin (Lot 3773093) was from Gallard-Schlesinger (New York, NY). All other reagents were from sources as previously described (10, 12, 15).

Receptor preparations. Hearts were removed from newborn (0–2 weeks old) chicks and processed as described (12). For the detergent solubilization and pertussis toxin studies, crude membranes were prepared by two centrifugations at 30,000 × *g* for 40 min. For solubilization, the crude membrane pellets (1–2 mg of protein/ml) were incubated in 0.4% digitonin:0.08% cholate for 30 min at 4° according to standard procedures (13). The suspension was centrifuged at 150,000 × *g* for 1 hr and the supernatant was used as the source of solubilized receptors. Pertussis toxin treatments were as described in Ref. 14. In brief, newborn chicks were injected with 1 μg of pertussis toxin/40 g of body weight and sacrificed 48 hr later; then, crude cardiac membranes were made as described above. The 1 μg of toxin/40 g of body weight dose is the maximal sublethal dose which can be given for 48 hr (14).

Experiments with NEM were performed with cardiac homogenates or crude membranes. We found the effects of NEM pretreatment to be similar regardless of whether homogenates or purified membranes (15) were used as the receptor source. NEM treatment was for 30 min at 4° as previously described (15), except that the buffer used was 10 mM sodium-potassium phosphate (buffer A: 8 mM Na_2HPO_4/2 mM KH_2PO_4, pH 7.4). The treatments were terminated by addition of dithiothreitol equimolar to the NEM and centrifugation as described (15). In some experiments, after adding the dithiothreitol, membranes were diluted 50-fold in buffer before centrifugation. The results were the same whether or not the dilution step was included.

Ligand binding assay. The experiments for competition by agonists for the antagonist ligand [^{3}H]QNB were performed as previously documented (12) using buffer A in the presence of 1 mM EDTA, 0.05–0.1 mg of protein, and [^{3}H]QNB (75–150 pM for membrane-bound receptor assays and 2 nM for solubilized receptor assays) in a total volume of 2 ml. In addition, some assays contained, as indicated, 10 mM $MgCl_2$, 50 μM Gpp(NH)p and/or 0.2 M NH_4Cl. When buffer A (8 mM Na_2HPO_4/2 mM KH_2PO_4, pH 7.4) was replaced with 10 mM histidine, pH 7.4, qualitatively similar results were obtained for the effects of Mg^{2+} (data not shown), Gpp(NH)p, and monovalent cations (12, 15). All assays containing acetylcholine were performed with 10^{-5} M eserine salicylate present. All assays were performed using a rapid filtration assay; solubilized receptors were detected using polyethylenimine-soaked filters (16). IC_{50} values were calculated from Hill plots of the data. All data are means ± SE from at least three experiments. Statistical analyses of IC_{50} values and Hill coefficients were performed using either the Student's *t* test or a paired *t* test. Computer-assisted analyses of [^{3}H]QNB/agonist competition curves were performed using the LIGAND (17) program. All analyses were performed by allowing LIGAND to simultaneously fit the data obtained from at lest four separate experiments. Thus, the data shown represent the best fit of each group of data. The K_D values for [^{3}H]QNB are slightly affected by Mg^{2+}, guanine nucleotides, and monovalent cations (12, 18). Therefore the K_D values for [^{3}H]QNB that were used in the computer analyses were those appropriate for the experimental conditions (see figure legends). The best fit of the data to a one-, two-, or three-state model was determined by LIGAND by analysis of variance and the *F*-test (17). Extensive discussions of the LIGAND program and its use can be found in Refs. 17 and 19.

[3] The abbreviations used are: QNB, quinuclidinyl benzilate; Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; N_i-protein, inhibitory guanine nucleotide-binding regulatory protein of the adenylate cyclase complex; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetate.

RESULTS

Effects of Mg^{2+} and guanine nucleotides on the interaction of the chick heart muscarinic receptor with agonists. Mg^{2+} caused a decrease in the IC_{50} for acetylcholine as determined by [^{3}H]QNB/acetylcholine competition stud-

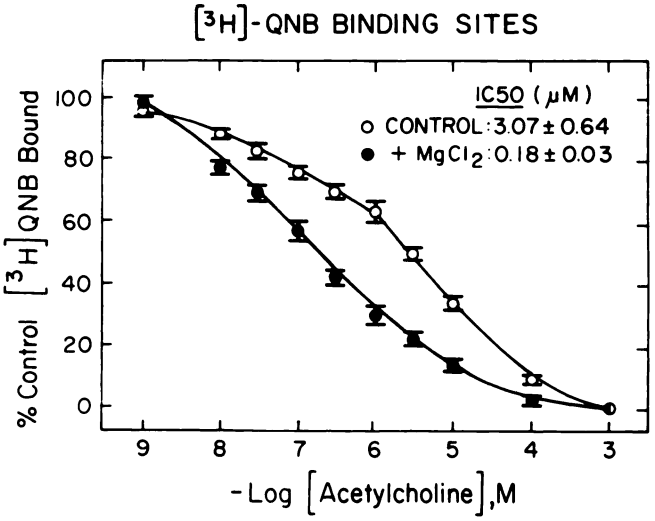

FIG. 1. *Effect of Mg^{2+} on acetylcholine competition for [^{3}H]QNB binding sites*

The assays were performed in the presence or absence of $MgCl_2$ as indicated. The [^{3}H]QNB concentration was 85 pM. Results shown are means ± SE from three separate experiments performed in duplicate.

TABLE 1

High, middle, and low agonist affinity states for acetylcholine binding to chick heart muscarinic receptors: effects of Mg^{2+}, Gpp(NH)p, and monovalent cations

Competition curves of [^{3}H]QNB/acetylcholine were generated as described in Experimental Procedures. The B_{max} for [^{3}H]QNB did not change under any of these conditions (12, 18). The K_D values for [^{3}H]QNB binding which were used for the computer fitting were those appropriate for each test condition (12, 18). The data shown represent the best fit for each case after considering a one-, two-, or three-site fit; the *p* values indicating the goodness of fit were not more than 0.0002. The K_1, K_2, and K_3 values are estimated K_D values and are given in nanomolar concentration. R_1, R_2, and R_3 are the per cent densities of the receptors in the different affinity states.

Addition to assay	K_1 (nM) R_1 (%)	K_2 (nM) R_2 (%)	K_3 (nM) R_3 (%)
None	2.7 ± 1.2 24 ± 2	240 ± 60 30 ± 2	4,000 ± 700 46 ± 1
Mg^{2+}	2.7 ± 1.2 45 ± 5	240 ± 60 36 ± 4	4,000 ± 700 19 ± 2
Mg^{2+} + Gpp(NH)p	ND[a]	240 ± 60 62 ± 5	4,000 ± 700 38 ± 3
Mg^{2+} + 0.05 M Na^+	12 ± 9 26 ± 6	240 ± 60 43 ± 7	7,000 ± 1,200 28 ± 2
Mg + 0.2 M Na^+	ND	110 ± 30 52 ± 3	5,800 ± 600 48 ± 3
Mg + 0.2 M NH_4^+	ND	1,700 ± 400 43 ± 3	31,000 ± 2,000 57 ± 3

[a] ND, not detectable.

ies (Fig. 1). Hill plots of the data in the absence and presence of Mg^{2+} yielded Hill coefficients (n_H) of 0.35 ± 0.01 and 0.45 ± 0.01, respectively. These low Hill coefficients suggested either the presence of a heterogeneous population of receptors with differing affinities for agonist or cooperative interactions between binding sites (20). We therefore analyzed the competition curves with the computerized curve-fitting program LIGAND. These analyses indicated that the data were best fit by a three-state model, in both the presence and absence of Mg^{2+} (Table 1). The computer-derived K_D estimates for acetylcholine were 2.7 nM (K_1, high), 240 nM (K_2, middle), and 4000 nM (K_3, low). Mg^{2+} had no effect on these K_D values but rather caused an increase in the proportion of receptors in the high affinity state (R_1) and a decrease in the proportion in the low affinity state (R_3) (Table 1). In the presence of both Mg^{2+} and guanine nucleotide, competition curves (not shown) were displaced 19-fold to the right (IC_{50} = 3.6 ± 0.6 μM). Computer analyses of these curves indicated that R_1 was eliminated in the presence of Gpp(NH)p and Mg^{2+}, whereas R_2 and R_3 were both increased (Table 1).

Effects of monovalent cations on agonist interactions with chick heart muscarinic receptors. Previous studies have shown that monovalent cations increase the IC_{50} values of [^{3}H]QNB/agonist competition curves (12, 15, 21, 22). In order to determine the nature of this effect, we examined the effect of monovalent cations on the number and affinity of the receptors in the multiple affinity states described above. Monovalent cations appeared to increase the K_D values for acetylcholine binding to these states (Table 1). A low concentration of Na^+ (0.05 M) increased the IC_{50} values by 3-fold (data not shown). Under these conditions all three affinity states were observed, but the K_D values were higher than those observed in control membranes (Table 1). A higher concentration of either Na^+ or NH_4^+ (0.2 M) caused 9- and 56-fold increases in IC_{50} values, respectively. Under these conditions only two affinity states were observed. The K_D values were significantly higher in the presence of 0.2 M NH_4^+ than in the presence of 0.2 Na^+. This is consistent with previous results showing that NH_4^+ is more potent than Na^+ in increasing IC_{50} values of agonist competition curves (12). The K_D values shown were obtained in assays that contained Mg^{2+}; essentially similar results were obtained in the absence of Mg^{2+} (see below for details).

Effects of NEM pretreatment on the ability of Mg^{2+} to interconvert receptor affinity states. Under certain conditions, NEM pretreatment of cardiac membranes eliminates the effects of Mg^{2+} and Gpp(NH)p, but not monovalent cations, on [^{3}H]QNB/agonist competition curves (15, 22, 23). We have found that Gpp(NH)p has *no* effect on muscarinic receptors in chick heart preparations pretreated with 1 mM NEM (15). To test further the relationship between the effects of Mg^{2+} and guanine nucleotides, it was of interest to determine the effect of NEM pretreatment on the ability of Mg^{2+} to influence the interaction of acetylcholine with the different affinity states of the chick heart muscarinic receptors.

As previously shown for the effects of NEM on the guanine nucleotide sensitivity of cardiac muscarinic receptors (23), the dose response curve for the alkylating agent to affect the Mg^{2+} response was quite steep and indicated the need for concentrations greater than 0.3 mM NEM (data not shown). However, treatment with 1 mM NEM, which completely eliminates the response to Gpp(NH)p (15), resulted in only a partial loss of the Mg^{2+} effect on the [^{3}H]QNB/acetylcholine competition curves (Fig. 2). After this treatment, the IC_{50} values for acetylcholine were 23 ± 1 μM in the absence of Mg^{2+} and

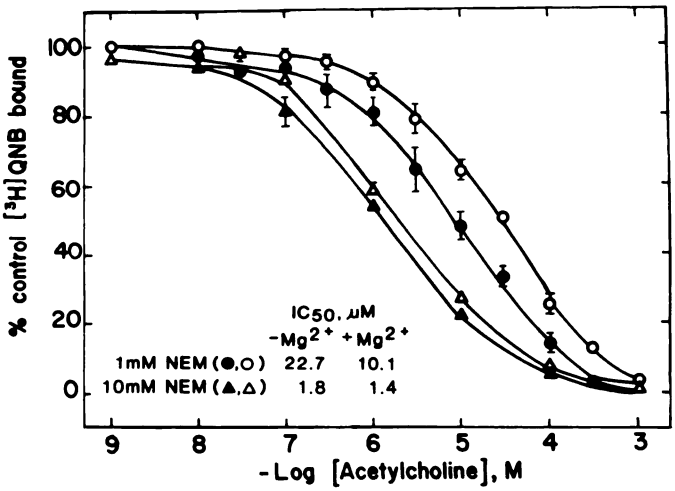

FIG. 2. *Effect of Mg^{2+} on acetylcholine/[^{3}H]QNB competition curves after pretreatment of the receptor preparations with 1 or 10 mM NEM*

Homogenates were pretreated with 1 mM (●, ○) or 10 mM (▲, △) NEM as described in the text. Assays were performed in the absence (*open symbols*) or presence (*solid symbols*) of $MgCl_2$. Other experimental conditions were as described in Experimental Procedures. The results shown are the average of three separate experiments performed in duplicate. The control curves for these experiments were identical to those shown in Fig. 1; since they would overlap with the curves for 10 mM NEM, they were omitted from the graph for the purpose of clarity.

TABLE 2

Agonist affinity states for acetylcholine binding to cardiac muscarinic receptors in NEM-pretreated preparations

Experimental conditions are described in the legend of Fig. 2. Mg^{2+} did not alter the B_{max} for [^{3}H]QNB in any experimental condition. The K_D values (picomolar concentration) for [^{3}H]QNB obtained by Scatchard analysis were: 23 for 2 mM *N*-ethylmaleimide treatment, no additions; 27 for 1 mM NEM, + Mg^{2+}; and 23 for 10 mM NEM ± Mg^{2+}. Computer fitting of the data gave preference to a two-state model versus a one-state model in all cases (p = 0.001) as described in the legend to Table 1. K_2 and K_3 are the nanomolar K_D estimates for acetylcholine obtained by LIGAND. The values of K_3 obtained after 1 mM NEM could not be constrained to the control value of 4000 nM (Table 1) and should be considered as small but significant differences caused by the NEM treatment. R_2 and R_3 are percentages of the total receptor population having K_2 or K_3, respectively. K_1 was not observed under any of these conditions.

Pretreatment	Assay conditions	K_1	K_2	R_2	K_3	R_3
1 mM	No Mg^{2+}	ND[a]	240 ± 60	26 ± 4	5700 ± 1400	75 ± 5
	+ Mg^{2+}	ND	240 ± 60	56 ± 3	5700 ± 1400	44 ± 2
10 mM	No Mg^{2+}	ND	92 ± 18	69 ± 4	3400 ± 400	31 ± 3
	+ Mg^{2+}	ND	98 ± 18	80 ± 4	4600 ± 900	20 ± 3

[a] ND, not detectable.

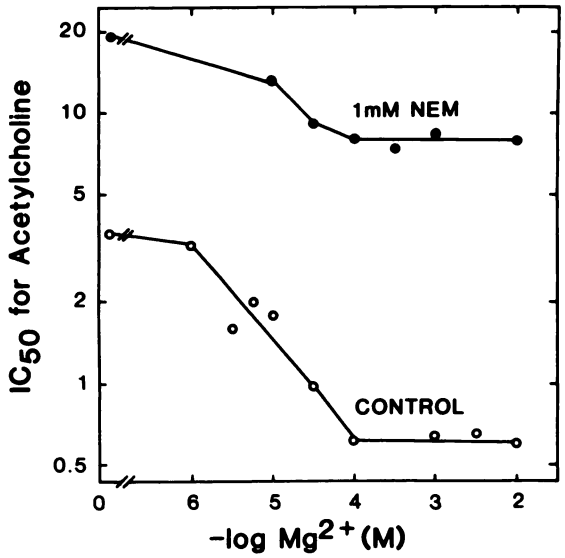

FIG. 3. *Effect of varying concentrations of Mg^{2+} on the ability of acetylcholine to compete for [^{3}H]QNB binding sites*

The concentration of free Mg^{2+} was calculated as that present in excess of 1 mM Mg·EDTA using the equation $Mg_f^{2+} = Mg_f^{2+}[1 + EDTA/(Mg^{2+} + K_{EDTA})]$ where Mg_t^{2+} is the total Mg^{2+} added to obtain the desired free Mg^{2+} concentration with EDTA (1 mM) present. K_{EDTA} is the equilibrium dissociation constant of the Mg·EDTA complex (0.4 μM) (41). Competition curves of [^{3}H]QNB/acetylcholine were generated in the presence of varying concentrations of Mg^{2+} and NEM treatments as described in the text. IC_{50} values were in micromolar concentration. Each *point* represents the mean of two to five experiments.

10 ± 1 μM in the presence of Mg^{2+}; this difference was statistically significant ($p < 0.0025$). Computerized analysis of these data indicated that the curves best fit to a two-state model with K_D values of 240 nM (K_2) and 5700 nM (K_3) (Table 2). The highest affinity state (K_1) observed in controls (Table 1) was not apparent with either NEM treatment. Mg^{2+} increased the proportion of the higher affinity receptors (R_2) but did not change either K_D value (Table 2). Pretreatment with 10 mM NEM eliminated all effects of Mg^{2+} (Fig. 2). The IC_{50} values of competition curves of 10 mM NEM-treated preparations were decreased as compared to the values from 1 mM NEM to 1.8 ± 0.1 μM and 1.4 ± 0.2 μM ($n = 4$) in the absence and presence of Mg^{2+}, respectively. Results obtained after 3 mM NEM were intermediate to those seen with 1 or 10 mM of the alkylating agent. Computerized analyses of the data obtained after the 10 mM NEM pretreatment indicated that both the affinity and proportion of receptors in the higher affinity state were increased when compared to those observed after the 1 mM NEM treatment (Table 2). The effects of NEM were specific to agonist interactions with the receptors. Neither the K_D nor the B_{max} for [^{3}H]QNB was affected by any treatment (not shown).

The concentration dependency for Mg^{2+} to influence the IC_{50} values of [^{3}H]QNB/acetylcholine competition curves was determined in native and 1 mM NEM-treated preparations (Fig. 3). In both native and NEM-treated preparations, maximal effects were obtained at 1 mM Mg^{2+} and the $K_{0.5}$ was approximately 0.02–0.03 mM.

Effect of pertussis toxin treatment on the ability of Mg^{2+} to interconvert receptor affinity states. Since NEM treatment may result in the alkylation of many proteins, a more specific method to perturb the system was necessary to establish the specific site(s) of action of Mg^{2+}. For receptors that attenuate adenylate cyclase, one can use pertussis toxin, which specifically inactivates the N_i protein(s) via an ADP ribosylation reaction (24). *In vivo* treatment of chicks with pertussis toxin eliminates the effects of Gpp(NH)p on cardiac muscarinic receptors (14). We therefore determined the effect of Mg^{2+} on cardiac muscarinic receptors following a similar protocol. The pertussis toxin treatment used herein causes ADP ribosylation of two peptides of 39,000 and 41,000 daltons on one-dimensional gel electrophoresis (data not shown), similar to the results reported by others (25–27). Under our conditions both peptides were modified to a similar extent (85–90%) (results not shown). Oxotremorine was used as the agonist in the competition experiments so that we could directly compare the results with our previous studies with pertussis toxin. The pertussis toxin treatment that resulted in a loss of the Gpp(NH)p effect (15) resulted in only a partial loss of the effect of Mg^{2+} on the [^{3}H]QNB/oxotremorine competition curves (Fig. 4). In control membranes, Mg^{2+} caused a 7-fold decrease in the IC_{50} value for oxotremorine whereas, after the toxin treatment, Mg^{2+} decreased the IC_{50} value by 2.4-

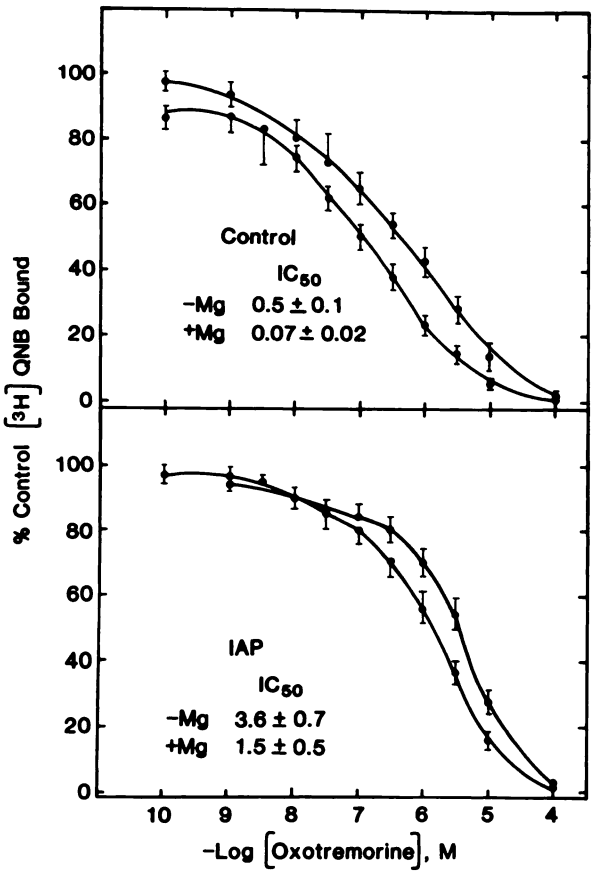

FIG. 4. *Effect of Mg^{2+} on oxotremorine/[^{3}H]QNB competition curves in control and pertussis toxin-pretreated preparations*

Assays were performed in the absence or presence of $MgSO_4$ as indicated. The [^{3}H]QNB concentration was 150 pM. Results shown are the mean ± SE of four to nine experiments performed in duplicate.

TABLE 3

Effect of Mg^{2+} on the affinity states of newborn chick heart muscarinic receptors for oxotremorine in preparations from pertussis toxin-treated chicks

Computer-assisted analyses were performed on the oxotremorine/[^{3}H]QNB competition curves described in Fig. 4. K_1 and K_2 are K_D values in nanomolar concentration. R_1 and R_2 are percentages of receptors in each affinity state. The pertussis toxin treatment did not alter [^{3}H]QNB binding; K_D values for [^{3}H]QNB used in the analyses were 33 and 19 pM in the presence and absence of Mg^{2+}, respectively. B_{max} for [^{3}H]QNB was not altered by the presence of Mg^{2+}. A two-state model was preferred over a one-state model for control membranes assayed in the absence or presence of Mg^{2+} ($p = 0.001$) and for pertussis toxin-treated membranes assayed with Mg^{2+} ($p = 0.033$). However, for the pertussis toxin-treated membranes assayed in the absence of Mg^{2+}, a two-state model was not preferred over a one-state model ($p = 0.598$).

Membrane	Mg^{2+}	K_1	R_1	K_2	R_2
Control	−	1.1 ± 0.5	38 ± 4	190 ± 10	62 ± 3
	+	3.3 ± 1.2	58 ± 5	190 ± 10	42 ± 4
Pertussis toxin treated	−	ND[a]	ND	290 ± 20	100
	+	1.3 ± 1.7	17 ± 5	260 ± 20	83 ± 4

[a] ND, not detectable.

TABLE 4

Effect of NH_4^+ on agonists binding to the cardiac muscarinic receptor: dose ratios of IC_{50} values obtained using native, NEM-, or pertussis toxin-treated preparations

Agonist/[^{3}H]QNB competition assays were performed using either acetylcholine or oxotremorine as indicated with preparations from the indicated experimental group. Each preparation was assayed in the absence and presence of 10 mM Mg^{2+} and/or 0.2 M NH_4^+. The dose ratio was calculated as the ratio of the IC_{50} values ($+NH_4^+/-NH_4^+$). The data are the mean ± SE from at least four experiments done in duplicate.

Experimental group	Mg^{2+}	Acetylcholine dose ratio	Oxotremorine dose ratio
Control	−	10 ± 3	2.7 ± 0.5
	+	55 ± 2	17 ± 2
1 mM NEM	−	8 ± 1	—[a]
	+	15 ± 2	—
10 mM NEM	−	10 ± 2	—
	+	9 ± 1	—
Pertussis toxin	−	—	2.2 ± 0.5
	+	—	4.3 ± 0.7

[a] —, not determined.

fold. The effect of Mg^{2+} after pertussis toxin treatment was statistically significant ($p < 0.02$). Analyses of these competition curves with LIGAND indicated that oxotremorine bound with two affinities in control membranes, with K_1 equaling 3 nM and K_2 equaling 190 nM (Table 3). These values are in agreement with the K_D values for oxotremorine previously estimated (10) for chick heart muscarinic receptors (see Discussion, concerning the differences in affinity states for oxotremorine and acetylcholine). As in the studies with acetylcholine (Table 1), we found that Mg^{2+} increased the proportion of receptors in the higher affinity state from 38 to 58% without significantly affecting the K_D values (Table 3). In preparations from toxin-treated animals, all of the receptors appeared to be in the low affinity state when assayed in the absence of Mg^{2+}. However, in the presence of Mg^{2+} a small percentage of receptors (17%) appeared to be in the higher affinity state.

Influence of Mg^{2+} on the effect of monovalent cations. Under certain conditions the effects of monovalent cations can be differentiated from those mediated by guanine nucleotides (15, 23). To understand further the relationship, if any, between the effects of these two modifiers, we have performed several types of experiments. First, since guanine nucleotide effects are dependent on Mg^{2+}, we have tested the dependence of the monovalent cation effect on the presence of Mg^{2+}.

The data in Table 4 show that NH_4^+ could increase IC_{50} values independent of Mg^{2+}. However, the effects of NH_4^+ appeared to be greater in the presence of Mg^{2+}. In the absence of Mg^{2+}, NH_4^+ caused a 10-fold increase in the IC_{50} value for acetylcholine. In the presence of Mg^{2+}, NH_4^+ increased the IC_{50} value for acetylcholine by 55-fold. However, the IC_{50} values obtained in the presence of NH_4^+ were the same in the absence (28 ± 6 μM) and presence (21 ± 2 μM) of Mg^{2+}. The difference was solely in the starting IC_{50} values; Mg^{2+} decreased the IC_{50} value in the control membranes by 8-fold (Fig. 1), but this effect appeared to be overcome by NH_4^+.

The relationship between the effects of Mg^{2+} and monovalent cations was further investigated after modification of the membranes with NEM or with pertussis toxin. Treatment of membranes with 1 or 10 mM NEM had no effect on the ability of NH_4^+ (Table 4) or Na^+ (data not shown) to increase IC_{50} vlues when assays were conducted in the absence of Mg^{2+}. However, as described above, the 1 and 10 mM NEM treatments partially and totally eliminated the effects of Mg^{2+}, respectively (Fig. 2, Table 2). In preparations pretreated with 1 mM NEM, Mg^{2+} alone caused only a 2-fold decrease in the IC_{50} for acetylcholine (Fig. 2). Consequently, the magnitude of the NH_4^+ effect differed by 2-fold in the presence and absence of Mg^{2+}. The higher concentration (10 mM) of NEM eliminated the effects of Mg^{2+} alone (Fig. 2, Table 2). Accordingly, in these preparations the magnitude of the NH_4^+ effect was equal in the presence and absence of Mg^{2+}.

Pertussis toxin treatment did not modify the effect of monovalent cations on the cardiac muscarinic receptor (Table 4). In control or pertussis toxin-treated membranes, NH_4^+ increased the IC_{50} values for oxotremorine by 2- to 3-fold. Experiments performed with pertussis toxin-treated membranes in the presence and absence of Mg^{2+} yielded results qualitatively similar to those obtained with NEM-treated membranes; i.e., the NH_4^+ response obtained in the presence of Mg^{2+} was decreased concomitant with the pertussis toxin-induced decrease in the response to Mg^{2+} alone.

Effect of solubilization of the receptor with digitonin/cholate on the abilities of ions and guanine nucleotide to alter ligand interaction with the receptor. In order to probe further the factors which modulate the interaction of agonists with the cardiac muscarinic receptor, we determined the characteristics of ligand binding to receptors solubilized with digitonin/cholate. In order to properly analyze the interaction of agonists with solubilized receptors, it was first necessary to define the char-

acteristics of [^{3}H]QNB binding to this preparation. The solubilized preparation was enriched 4-fold in receptors and exhibited a K_D for [^{3}H]QNB that was 3- to 5-fold higher than the K_D of the membrane-bound receptor (Fig. 5). Gpp(NH)p had no effect on the K_D for [^{3}H]QNB, but Mg^{2+} decreased the K_D to 450 pM (Fig. 5). This effect of Mg^{2+} on [^{3}H]QNB binding was not extensively characterized, but it appeared to be different from that seen with the native preparation (18), as it required higher concentrations of Mg^{2+} and did not saturate even at 100 mM (data not shown).

The detergent-solubilized receptors exhibited low apparent affinity for oxotremorine (Fig. 6). The IC_{50} values

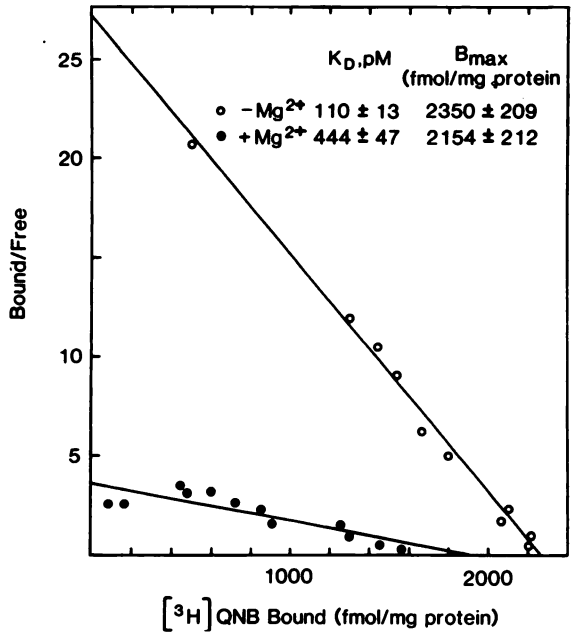

FIG. 5. *Effect of Mg^{2+} on [^{3}H]QNB binding to cardiac muscarinic receptors solubilized with digitonin/cholate*

Assays were performed in the absence or presence of $MgCl_2$ as indicated. [^{3}H]QNB concentrations were from 20 pM to 5 nM. The plotted data are from a representative experiment. The *inset* shows the mean K_D values and B_{max} obtained from experiments done in the absence (n = 13) and presence (n = 11) of Mg^{2+}.

of the [^{3}H]QNB/oxotremorine competition curves were decreased by Mg^{2+} (Fig. 6, Table 5) but were unchanged by subsequent addition of Gpp(NH)p. The competition curves were described by Hill coefficients of approximately 1.0 (Table 5). Analyses of these data by LIGAND indicated that only a single low affinity state was present. After correcting (28) for the effect of Mg^{2+} on [^{3}H]QNB binding, it was determined that Mg^{2+} had no effect on agonist interactions with the solubilized receptor. NH_4^+ increased the IC_{50} value for oxotremorine 2.4- to 3.4-fold (Fig. 6, Table 5), similar to the 2.7-fold increase observed in intact membranes (Table 5). The competition curves were again described by Hill coefficients of approximately 1 (Table 5). In contrast to lack of an effect on $K_{0.5}$ by Mg^{2+}, conversion of the IC_{50} value to a $K_{0.5}$ showed that NH_4^+ decreased the affinity of the solubilized receptor by 5-fold (Table 5).

DISCUSSION

The relevant findings of this study are that: (*1*) cardiac muscarinic receptors in the three agonist affinity states appear to be interconvertible; (*2*) the ability of guanine nucleotides and Mg^{2+} to cause interconversion of receptor affinity states appears to be due in part to association of the cardiac receptor with N_i and in part to another site of action of Mg^{2+}; and (*3*) the effects of monovalent cations on the cardiac muscarinic receptor can be distinguished from those of Mg^{2+} and guanine nucleotide both by the nature of their effect and by their sites of action. The relevance of these findings with respect to previous studies and to muscarinic receptor-effector coupling in cardiac tissue is discussed below.

Birdsall and coworkers (3) introduced evidence that certain muscarinic cholinergic receptors can exist in three different affinity states for agonists which they termed superhigh, high, and low. These affinity states were detected with neuronal muscarinic receptors via the use of nonlinear regression analysis of agonist ^{3}H-antagonist competition studies. Using this technique, methacholine (7), carbachol (8, 9), and oxotremorine (9) have been shown to bind to three affinity states of mammalian cardiac muscarinic receptors, whereas oxotremorine has

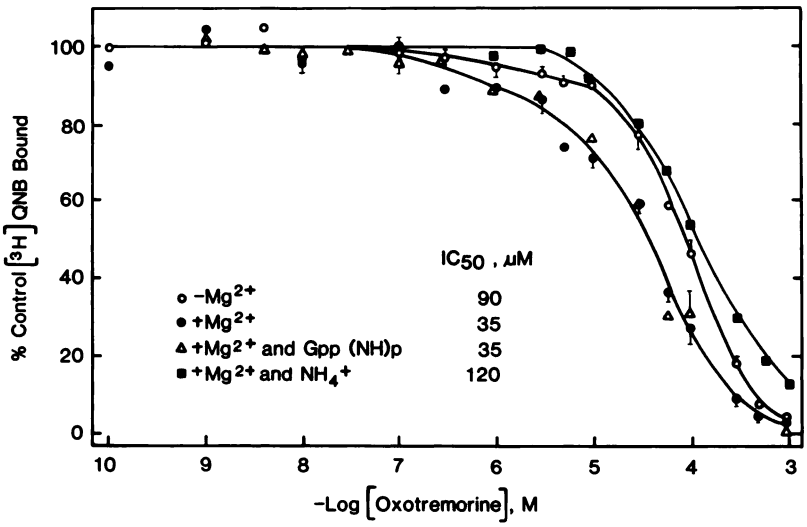

FIG. 6. *Effect of Mg^{2+}, Gpp(NH)p, and NH_4^+ on oxotremorine/[^{3}H]QNB competition curves of digitonin/cholate-solubilized cardiac muscarinic receptors*

Assays were performed in the absence and presence of Mg^{2+}, Gpp(NH)p, or NH_4^+ as indicated. The [^{3}H]QNB concentration was 2.3 nM. Results are mean ± SE of four to eight experiments performed in duplicate.

TABLE 5

Properties of oxotremorine binding to the solubilized muscarinic receptor

The IC_{50} values and Hill coefficients (n_H) were obtained from Hill plots of the mean curves depicted in Fig. 5. The $K_{0.5}$ values were calculated using the equation $K_{0.5} = IC_{50}/(1 + L/K_Q)$ (28), where L is the [^{3}H]QNB concentration (2.3 nM) and K_Q is the K_D value for [^{3}H]QNB under each condition. K_D values for [^{3}H]QNB determined by Scatchard analysis were: 110 ± 10 pM (n = 11) in the absence of Mg^{2+}, 440 ± 50 pM (n = 11) in the presence of Mg^{2+}, and 520 ± 180 pM (n = 3) in the presence of Mg^{2+} and NH_4^+. B_{max} was not changed by Mg^{2+} or Gpp(NH)p.

Additions to assay	IC_{50} (μM)	n_H	$K_{0.5}$(μM)
None	90	1.1	4.6
Mg^{2+}, 10 mM	35	0.9	5.6
Mg^{2+} plus Gpp(NH)p	35	0.9	6.5
NH_4^+	230	1.2	25
Mg^{2+} + NH_4^+	120	1.0	26

been shown to recognize three affinity states of embryonic chick cardiac muscarinic receptors (10). Using a similar approach, we detected three affinities for acetylcholine binding to newborn chick heart muscarinic receptors. The estimated K_D values for acetylcholine were 2.7, 240, and 4000 nM, similar to those reported for neuronal muscarinic receptors (3). A larger proportion of the newborn chick heart muscarinic receptors appeared to be in the highest affinity state (24 to 45%) as compared to neuronal muscarinic receptors (5%) (3). This is in agreement with the results of others who have reported a larger proportion of the muscarinic receptor population of heart to reside in the highest affinity state than those of neuronal tissue (7–9).

It has been clearly established that Mg^{2+}, guanine nucleotides, and monovalent cations alter the interaction of cardiac muscarinic receptor with agonists (8, 12, 15, 21–23). However, the nature of these effects is not known. We further characterized the effects of these known modulators of agonist binding on the number and affinity of receptors in the three affinity states. When examined at the level of the IC_{50} values obtained from competition curves, the effects of Mg^{2+} and guanine nucleotides were to increase and decrease, respectively, the apparent affinities for agonist. Analyses of these data with LIGAND indicated that neither Mg^{2+} nor guanine nucleotides had any effect on affinity *per se* (i.e., on the K_D values), but rather that both agents modulated the proportion of receptors in the three affinity states. Therefore, the effects of Mg^{2+} and Gpp(NH)p on IC_{50} values were due to their abilities to convert receptors from one affinity state to another.

Receptors in all three of the agonist affinity states appeared to be interconvertible. Several lines of evidence support this contention. First, in native preparations Mg^{2+} increased R_1 and decreased R_2. The former finding agrees with the data of Hulme *et al.* (29), who have shown that divalent cations increase the number of sites in the superhigh affinity state that can be labeled with [^{3}H] oxotremorine-M in rat heart preparations. Second, Gpp(NH)p eliminated R_1 and increased receptors in R_2 and R_3. Uchida *et al.* (8) observed three affinity states in mammalian cardiac membranes and concluded that their highest and lowest affinity states were guanine nucleotide sensitive and interconvertible, but the middle affinity state was insensitive to nucleotide. In contrast, we found that Gpp(NH)p caused a large increase in the proportion of receptors in the middle affinity and low state. Based on our data with Mg^{2+} and guanine nucleotides, it appears that receptors in all three affinity states are interconvertible and guanine nucleotide sensitive. The most likely explanation for these differences in observations is that the present studies were performed in the presence of low salt concentrations (10 mM $NaKPO_4$), whereas in the studies in Ref. 8, 100 mM NaCl and 50 mM Tris-HCl were present. It is obvious from the data in Table 1 that analyses of curves obtained in the presence of high salt are more complex than those obtained in its absence. The most relevant point is that the receptors in the three affinity states do not reflect distinct populations of receptors, but, rather, one population of receptors that can display three different affinities for agonist depending on the experimental conditions used. Of course, we cannot rule out that the different affinity states are not due to receptors on membranes from different cell types. However, we have also demonstrated that receptors in preparations of 10-day embryonic chick heart can also exhibit three affinities for agonists (10). In these preparations there is a higher proportion of myocardial than nonmyocardial cells (30).

The sites of action of guanine nucleotide and Mg^{2+} were probed. Cardiac muscarinic receptors attenuate adenylate cyclase (31–33), presumably via the N_i protein. Therefore, the predicted sites of action of guanine nucleotides and Mg^{2+} were on N_i. Treatment with 1 mM NEM or pertussis toxin eliminated the guanine nucleotide effect and most, but not all, of the effects of Mg^{2+}. Both treatments eliminated the highest affinity state of the receptor. The pertussis toxin treatment should be specific for N_i (and perhaps other GTP-binding proteins). The NEM treatment is nonspecific, but many studies have shown it to be effective in perturbing N protein function (1). The data from the two treatments are qualitatively and quantitatively similar and consistent with the concept that the effects of guanine nucleotides and most of the effects of Mg^{2+} occur at the level of N_i. However, Mg^{2+} also may act at another site in this system. This contention is made because neither pertussis toxin nor 1 mM NEM eliminated all effects of Mg^{2+}. After either treatment, Mg^{2+} had smaller but significant effects to convert low affinity receptors to higher affinity receptors. The residual effect of Mg^{2+} was eliminated after solubilization of the receptor, suggesting perhaps that Mg^{2+} did not act on the receptor itself.

The effect of monovalent cations on cardiac muscarinic receptors differed from those of Mg^{2+} and guanine nucleotides. The monovalent cations affected both the affinity and heterogeneity of the receptor population. The effect of 50 mM Na^+ was to increase the K_D values of the three affinity states. With a higher concentration (0.2 M) of Na^+ or NH_4^+, one affinity state appeared to be eliminated. Birdsall *et al.* (34) have reported that monovalent cations alter the K_D values of the affinity states of the brain muscarinic receptor. The observation

that higher concentrations of Na^+ or NH_4^+ eliminated the highest affinity state (Table 1) differs from the observations made for the brain muscarinic receptor (34). Birdsall *et al.* (34) report that, at all concentrations of K^+ or Na^+ tested, all three affinity states are present and the fraction of receptors in the highest affinity states remains constant. It is not possible to precisely relate the affinity states observed in the presence of 0.2 M monovalent cations (R_1 and R_2) to those observed in their absence (R_1, R_2, and R_3). We suggest that the apparent loss of one affinity state could reflect a change in the affinity of the highest affinity state such that its K_D (K_1) value is no longer distinguishable by the computer program from the K_D value of the middle affinity state (K_2). This suggestion is consistent with other data showing that 50 mM Na^+ increased the K_D value for the binding of the agonist [^{3}H]oxotremorine-M to a high affinity state of rat heart muscarinic receptors without affecting the B_{max} (35). In addition, high concentrations of monovalent cations may effect receptor:N_i coupling which could lead to the loss of the high affinity state. This is suggested because the effects of guanine nucleotide are lessened in the presence of high concentrations of monovalent cations (12) and the effects of Mg^{2+} are overcome by NH_4^+ (Table 4).

The site of action of monovalent cations can be clearly differentiated from the site(s) of action of guanine nucleotides and Mg^{2+}. We have previously suggested that NH_4^+ and guanine nucleotides act on different sites in the muscarinic receptor system (15). Others have shown that the monovalent cation and guanine nucleotide effects on platelet α_2-adregenic receptors are mediated at distinct sites (36). Further evidence for this distinction, and evidence that the effects of monovalent cations are distinct from those of Mg^{2+}, was obtained in this study. Treatment with 1 mM NEM or pertussis toxin, as well as solubilization of the receptor, caused a selective loss of the Mg^{2+} effects but did not alter the monovalent cation effect (Table 4). The results obtained with the solubilized receptors suggest that monovalent cations may act either on the receptor itself or on another protein that was also solubilized in a functional form. In addition, the effects of monovalent cations do not appear to be due to changes in ionic strength (12). Finally, the effects of NH_4^+ do not appear to be due to changes in the H^+ ion concentration caused by the dissociation of NH_4^+ to NH_3 and H^+ because other monovalent cations such as Na^+ have effects (12). The addition of 0.2 M NH_4^+ lowers the pH in our experimental system to 7.0–7.1 (data not shown); however, 0.2 M Na^+ does not alter pH, but increases the IC_{50} values (data not shown) and K_D values of the affinity states in a manner qualitatively similar to that of NH_4^+.

Based on the results of the present and previous studies, we suggest the following interpretation of the three affinity states of the cardiac muscarinic receptor. The highest affinity state of the receptor is most likely an R:N_i complex. This complex must contain N_i that is devoid of either GTP *or* GDP, because either nucleotide causes similar decreases in apparent receptor affinity (9, 23, 33). It is conceivable that Mg^{2+} promotes the formation of this state of the receptor, or stabilizes it, by causing the emptying of GDP or GTP from N_i. We suspect that *in vivo* GTP binds to N_i and is rapidly hydrolyzed to GDP, and that N_i is usually occupied by GDP. Based on this reasoning, the highest affinity state of the receptor may be largely an *in vitro* phenomenon or may form only transiently *in vivo*. Thus, although the highest affinity state may not exist *in vivo*, it is an important indicator *in vitro* of the potential for receptor-N_i interactions.

The middle affinity state of the receptor may be the most physiologically relevant state of the receptor. It most likely represents a receptor:N_i complex in which GTP or GDP is bound to N_i. The K_D values of 200–240 nM for oxotremorine and acetylcholine binding to this affinity state agree very well with the N_i values of 200 nM for muscarinic receptor-mediated inhibition of chick heart adenylate cyclase (10), and of 60 nM for muscarinic receptor-mediated inhibition of protein phosphorylation in chick heart preparations (37). Thus, it appears that the middle affinity state of the receptor could be the state which is intimately involved in the production of physiological effects mediated by activation of muscarinic receptors in cardiac tissue.

The low affinity state of the receptor may be free receptor, i.e., receptor not associated with regulatory proteins. This is suggested by the studies of the solubilized receptor, which showed only low affinity agonist binding that was unaffected by Mg^{2+} or guanine nucleotide. Most likely, the conditions used for solubilization of the receptor led to inactivation or dissociation of molecules (such as N_i) which can modulate agonist interaction with the receptor. Alternatively, the low affinity state of the receptor may be a form which is associated with effects on phosphatidylinositol turnover. Recent studies showed that high concentrations of the agonist carbachol can stimulate phosphatidylinositol turnover but that oxotremorine has a very low efficacy for this effect (38). In this regard it is interesting that, when we model oxotremorine/[^{3}H]QNB competition curves, we find little or no receptors in the low affinity state (Table 3; Ref. 10). These data may suggest that the low affinity form of the receptor is associated with phosphatidylinositol turnover and is a state to which oxotremorine binds very poorly.

Finally, it may be useful to compare briefly the results contained herein with those of others who have examined the effects of Mg^{2+}, guanine nucleotides, and NEM on other muscarinic cholinergic receptor systems. Wei and Sulakhe (22) reported tht Mg^{2+} decreased the IC_{50} values in carbachol/[^{3}H]QNB competition experiments performed with rat atrial tissue, but other reports have shown that effects of Mg^{2+} on the interaction of agonists with muscarinic receptors are not always observed (for example, see Ref. 23). Since the $K_{0.5}$ for Mg^{2+} is very low (0.02 mM), this may be due to the presence of trace amounts of Mg^{2+} or other divalent cations in assay reagents. Indeed, it has been suggested that a divalent cation may remain tightly bound to myocardial membranes (29). All of our assays contained 1 mM EDTA, which we found necessary to observe the Mg^{2+} effects.

Another difference which should be mentioned concerns the effects of NEM on cardiac and other muscarinic receptor preparations. In our system, after treatment with 1 mM NEM, R_1 was eliminated but R_2 and R_3 remained intact and were interconvertible by Mg^{2+}. Higher concentrations of NEM (i.e., 10 mM) resulted in the formation of a population of receptors (R_2) with higher affinity, suggesting that the alkylation caused a conformational change. Wei and Sulakhe (22) previously showed that a 2 mM NEM treatment of rat atrial preparations decreased the IC_{50} of carbachol/[^{3}H]QNB competition experiments similar to the data reported herein with the 10 mM NEM treatment (Fig. 2, Table 4). That the NEM appeared to be more potent in the earlier study (22) may be explained by the use of a higher temperature of incubation with the alkylating agent (25° versus 4°). Harden and coworkers also observed an increase and then a decrease in the apparent affinity of cyclase-coupled brain muscarinic receptors as the NEM concentrations were increased (39). In contrast, Vauquelin *et al.* (40) showed that NEM only increases the affinity of low affinity muscarinic receptors in rat forebrain. These muscarinic receptors do not appear to be coupled to cyclase. Taken together, the data suggest that there are two effects of NEM on muscarinic receptor systems coupled to adenylate cyclase. The first appears to be an effect on receptor:N_i interaction and the second appears to be an effect on the receptor itself. In those muscarinic receptor systems not coupled to N_i, the effect of NEM may be only to induce a conformational change in the receptor itself.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Ms. Deborah Rymut, Mrs. Judith Ptasienski, and Mr. James Stittsworth.

REFERENCES

1. Limbird, L. E. Activation and attenuation of adenylate cyclase. *Biochem. J.* **195:**1–13 (1981).
2. Cooper, D. M. F. Bimodal regulation of adenylate cyclase. *FEBS Lett.* **128:**157–163 (1982).
3. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* **14:**723–736 (1978).
4. McKinney, M., S. Stenstrom, and E. Richelson. Muscarinic responses and binding in a murine neuroblastoma clone (N1E-115). Selective loss with subculturing of the low-affinity agonist site mediating cyclic GMP formation. *Mol. Pharmacol.* **26:**156–163 (1984).
5. Smith, M. M., and T. K. Harden. Modification of receptor-mediated inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma cells by N-ethylmaleimide. *J. Pharmaco. Exp. Ther.* **228:**425–433 (1984).
6. Masters, S. B., T. K. Harden, and J. H. Brown. Relationships between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* **26:**149–155 (1984).
7. Roskoski, R. Regional distribution of choline acetyltransferase activity and multiple affinity forms of the muscarinic receptor in heart. *Adv. Exp. Med. Biol.* **161:**159–178 (1983).
8. Uchida, S., K. Matsumoto, A. Mizushima, T. Osugi, H. Higuchi, and H. Yoshida. Effects of guanine nucleotide and sulfhydryl reagents on subpopulations of muscarinic acetylchoine receptors in mammalian heart: possible evidence for interconversion of super-high and low affinity agonist binding sites. *Eur. J. Pharmacol.* **100:**291–298 (1984).
9. 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).
10. Hosey, M. M., K. K. McMahon, A. M. Danckers, C. M. O'Callahan, J. Wong, and R. D. Green. Differences in the properties of muscarinic cholinergic receptors in the developing chick myocardium. *J. Pharmacol. Exp. Ther.* **232:**795–801 (1985).
11. Burns, D., E. L. Hewlett, J. Moss, and M. Vaughan. Pertussis toxin inhibits enkaphalin stimulation of GTPase of NG108-15 cells. *J. Biol. Chem.* **258:**1435–1438 (1982).
12. Hosey, M. M. Regulation of ligand binding to cardiac muscarinic receptors by ammonium ion and guanine nucleotides. *Biochim. Biophys. Acta* **757:**119–127 (1983).
13. Cremo, C. R., G. S. Herron, and M. I. Schimerlik. Solubilization of the atrial muscarinic acetylcholine receptor: a new detergent system and rapid assays. *Anal. Biochem.* **115:**331–338 (1981).
14. McMahon, K. K., R. D. Green, and M. M. Hosey. Attenuation of chick heart adenylate cyclase by muscarinic receptors after pertussis toxin treatment. *Biochem. Biophys. Res. Commun.* **126:**622–629 (1985).
15. McMahon, K. K., and M. M. Hosey. Potentiation of monovalent cation effects on ligand binding to cardiac muscarinic receptors in N-ethylmaleimide treated membranes. *Biochem. Biophys. Res. Commun.* **111:**41–46 (1983).
16. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* **132:**74–81 (1983).
17. Munson, P. J., and D. Rodbard. LIGAND: A versatile computerized approach for characterization of ligand-binding system. *Anal. Biochem.* **107:**220–239 (1980).
18. Hosey, M. M. Regulation of antagonist binding to cardiac muscarinic receptors. *Biochem. Biophys. Res. Commun.* **107:**314–321 (1982).
19. Munson, P. M., and D. Rodbard. Computerized analysis of ligand binding data: basic principles and recent developments, in *Computers and Endocrinology, Serono Symposium, Sardinia* (D. Rodbard and G. Forte, eds.). Raven Press, New York, 1–28 (1983).
20. Weiland, G. A., and P. B. Molinoff. Quantitative analysis of drug-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29:**313–330 (1981).
21. Rosenberg, L. B., H. I. Yamamura, and W. R. Roeske. Cardiac muscarinic cholinergic receptor binding is regulated by Na^+ and guanyl nucleotides. *J. Biol. Chem.* **255:**570–577 (1980).
22. Wei, J. W., and P. V. Sulakhe. Requirement for sulfhydryl groups in the differential effects of magnesium ion and GTP on agonist binding of muscarinic cholinergic receptor sites in rat atrial membrane fraction. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **314:**51–59 (1980).
23. Harden, T. K., A. G. Scheer, and M. M. Smith. Differential modification of the interaction of cardiac muscarinic cholinergic and beta-adrenergic receptors with a guanine nucleotide binding component(s). *Mol. Pharmacol.* **21:**570–580 (1982).
24. Murayama, T., and M. Ui. Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *J. Biol. Chem.* **258:**3319–3326 (1983).
25. Malbon, C. C., P. J. Rapiejko, and J. A. Garcia-Sainz. Pertussis toxin catalyzes the ADP-ribosylation of two distinct peptides, 40 and 41 k Da in rat fat cell membranes. *FEBS Lett.* **176:**301–306 (1984).
26. Halvorsen, S. W., and N. M. Nathanson. Ontogenesis of physiological responsiveness and guanine nucleotide sensitivity of cardiac muscarinic receptors during chick embryonic development. *Biochemistry* **23:**5813–5821 (1984).
27. Malbon, C. C., T. J. Mangano, and D. C. Watkins. Heart contains two substrates (M_r = 40,000 and 41,000) for pertussis toxin-catalyzed ADP-ribosylation that co-purify with N_s. *Biochem. Biophys. Res. Commun.* **128:**809–815 (1985).
28. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22:**3099–3108 (1973).
29. Hulme, E. C., C. P. Berrie, N. J. M. Birdsall, M. Jameson, and J. M. Stockton. Regulation of muscarinic agonist binding by cations and guanine nucleotides. *Eur. J. Pharmacol.* **94:**59–72 (1983).
30. Manasek, F. J. Organization, interactions, and environment of heart cells during myocardial ontogeny, in *Handbook of physiology*, Sect. 2: *The Cardiovascular System*, Vol. I (R. M. Berne, ed.), American Physiology Society, Washington, D.C., 29–42 (1979).
31. Murad, F., Y.-M. Chi, T. W. Rall, and E. W. Sutherland. Adenyl cyclase III. The effect of catecholamines and choline esters on the formation of adenosine 3′,5′-phosphate by preparations from cardiac muscle and liver. *J. Biol. Chem.* **237:**1233–1238 (1962).
32. Jakobs, K. H., K. Aktories, and G. Schultz. GTP-dependent inhibition of cardiac adenylate cyclase by muscarinic cholinergic agonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **310:**113–119 (1979).
33. McMahon, K. K., M. M. Hosey, and R. J. Schimmel. Effects of 9-β-D-arabinofuranosyl-guanine-5′-triphosphate on adenylate cyclase: comparison with GTP and Gpp(NH)p. *J. Cyclic Nucleotide Protein Phosphor. Res.* **9:**319–329 (1983).
34. Birdsall, N. J. M., A. S. V. Burgen, E. C. Hulme, and J. W. Wells. The effects of ions on the binding of agonists and antagonists to muscarinic receptors. *Br. J. Pharmacol.* **67:**371–377 (1979).
35. Harden, T. K., R. B. Meeker, and M. W. Martin. Interaction of a radiolabeled agonist with cardiac muscarinic cholinergic receptors. *J. Pharmacol. Exp. Ther.* **227:**570–577 (1983).
36. Limbird, L. E., J. L. Speck, and S. K. Smith. Sodium ion modulates agonist

and antagonist interactions with the human platelet alpha$_2$-adrenergic receptor in membrane and solubilized preparations. *Mol. Pharmacol.* **21**:609–617 (1982).

37. Iwasa, Y., and M. M. Hosey. Cholinergic antagonism of β-adrenergic stimulation of cardiac membrane protein phosphorylation *in situ*. *J. Biol. Chem.* **258**:4571–4575 (1983).
38. 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).
39. Korn, S. J., M. W. Martin, and T. K. Harden. N-Ethylmaleimide-induced alteration in the interaction of agonists with muscarinic cholinergic receptors of rat brain. *J. Pharmacol. Exp. Ther.* **224**:118–126 (1983).
40. Vauquelin, G., C. Andre, J. P. DeBacker, P. Laduron, and A. D. Strosberg. Agonist mediated conformational changes of muscarinic receptors in rat brain. *Eur. J. Biochem.* **125**:117–124 (1982).
41. Iyengar, R., and L. Birnbaumer. Hormone receptor modulates the regulatory component of adenyl cyclase by reducing its requirement for Mg^{2+} and enhancing its extent of activation by guanine nucleotides. *Proc. Natl. Acad. Sci. USA* **79**:5179–5183 (1982).