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# Muscarinic Responses and Binding in a Murine Neuroblastoma Clone (N1E-115): Cyclic GMP Formation is Mediated by a Low Affinity Agonist-Receptor Conformation and Cyclic AMP Reduction Is Mediated by a High Affinity Agonist-Receptor Conformation

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**SUMMARY**

Murine neuroblastoma cells (clone N1E-115) possess two subtypes of the muscarinic receptor each of which separately mediates a cyclic nucleotide response. The formation of cyclic GMP is postulated to involve a low affinity agonist-receptor conformation, whereas the reduction of prostaglandin $E_1$-stimulated cyclic AMP formation appears to involve a high affinity conformation. Further evidence supporting this hypothesis was obtained in experiments measuring the equilibrium dissociation constants for the full agonist carbachol by the method of partial receptor inactivation. Quinuclidinyl benzilate (QNB) was employed to occlude muscarinic receptors; measurements with [$^3$H]QNB ensured that the amount of QNB appearing in the assay after washout had only a minimal effect on the determination of the equilibrium dissociation constants. Carbachol mediated cyclic GMP formation with an equilibrium dissociation constant ($K_D$) of 325 $\mu$M and cyclic AMP reductions with a $K_D$ value of 13 $\mu$M. These $K_D$ values are similar to but somewhat higher than those determined by direct binding at 15°, and they are strong evidence in support of the view that a low affinity conformation mediates cyclic GMP formation, whereas a high affinity conformation mediates cyclic AMP reductions.

Murine neuroblastoma cells (clone N1E-115) have been widely used in a variety of neurobiologic studies. In our laboratory they have been employed in studies of neural receptors, notably to discern the molecular pharmacologic details of muscarinic receptor coupling (1). There are two binding subtypes of the muscarinic receptor in these cells and several biochemical responses elicited by the receptor. Two of these responses are the elevation of cyclic GMP levels and the inhibition of hormone-induced cyclic AMP formation. Studies of the effects of pirenzepine on the two responses to a full agonist like carbachol have clearly shown that two different receptor-effector complexes are involved in the two responses, whereas the binding studies indicate that partial agonists, which mediate only cyclic AMP reductions, cannot induce or reveal a detectable level of a low affinity agonist-receptor conformation (2). To show conclusively that a low affinity conformation mediates cyclic GMP formation, it is necessary to measure the equilibrium dissociation constant for a full agonist in this assay. Our previous work employed the alkylating agents phenoxybenzamine and dibenamine (3); the rather high agonist $K_D$ value (400 $\mu$M) obtained led us to suspect that these agents may have alkylated non-receptor sites (for example, the effector). Propylbenzilylcholine mustard is presumably a more specific alkylating agent; however, this drug can be expected to alkylate non-receptor sites as well. Furchgott and Bursztyn (4) used an irreversible (alkylating) agent in their experiments demonstrating the feasibility of their method of determining agonist $K_D$ values, but there is no theoretical reason why a high affinity reversible blocker would not work, as long as the assumption holds that "the fraction of the receptors inactivated neither decreases nor increases significantly." Rang (5) showed that it was theoretically possible for high affinity reversible muscarinic antagonists to affect the dose-response curve to a quick-acting agonist in

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**ABBREVIATIONS:** $EC_{50}$, concentration of agonist mediating 50% of maximal response; $K_D$, equilibrium dissociation constant; $K_H$, equilibrium dissociation constant for the high affinity agonist-receptor complex; $K_L$, equilibrium dissociation constant for the low affinity agonist-receptor complex; [$^3$H]NMS, tritiated *N*-methylscopolamine; PBS, phosphate-buffered saline; $PGE_1$, prostaglandin $E_1$; (±)QNB, racemic quinuclidinyl benzilate; [$^3$H]-(−)-QNB, tritiated L-(−)-quinuclidinyl benzilate.

the same way as do irreversible blockers. Rang (5) presented experiments demonstrating this effect and derived an equation similar to that of Furchgott and Bursztyn (4) for determining the agonist $K_D$ value, under conditions of "hemi-equilibrium." We therefore opted to use QNB to block muscarinic receptors in our system. The active (−)-enantiomer of QNB binds with high affinity ($K_D$ = 50 pM) to the N1E-115 muscarinic receptor (2, 6) and, after removal of unbound QNB, and under the restricted conditions of a response assay, its inactivation of the muscarinic receptor could be regarded as essentially irreversible. This assumption was borne out by measurements with [$^3$H]QNB under the conditions of the assay. Analysis by the method of Furchgott and Bursztyn (4) of the effects of QNB on the response curves showed that cyclic GMP formation was mediated by the low affinity muscarinic receptor subtype, whereas cyclic AMP reduction was linked to the occupancy of a subtype with higher (25-fold) affinity.

## Materials and Methods

**Cell culture.** Clone N1E-115 cells were grown as described (7) in modified Dulbecco's medium supplemented with 10% fetal bovine serum. Confluent cells were detached from flasks with Puck's $D_1$ solution, collected by low speed centrifugation, and washed in a physiological isosmolar PBS (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, 25 mM $Na_2HPO_4$, pH adjusted to 7.35 and osmolality adjusted to 340 ± 5 mOsm with sucrose). All assays were performed in this same buffer. Cells of low subculture were used to minimize variations in the results.

**Cyclic nucleotide assays and receptor binding assays.** Details of these procedures are given in previous publications (2, 7–9). For assay of cyclic GMP or cyclic AMP, the cells were prelabeled with the appropriate $^3$H-purine base or nucleoside; [$^3$H]cyclic GMP and [$^3$H]cyclic AMP were isolated using ion-exchange columns. The binding parameters for agonists or other agents in competition with [$^3$H]NMS were determined as described (2), using intact N1E-115 cells at 15°. This temperature is necessary to prevent receptor desensitization; our previous work (2, 6) has shown that agonist binding data with intact cells at 15° are similar to binding data obtained in homogenates of N1E-115 cells at 37° or brain tissue at 25°, conditions under which desensitization does not occur. The binding curves were analyzed by an iterative nonlinear computer method as described (2, 6, 9); this method allows the determination of the binding dissociation constants and capacities for the multiple muscarinic receptor subtypes to which agonists bind in N1E-115 cells.

**Partial receptor inactivation procedures.** Detached, washed, and labeled N1E-115 cells (approximately 10 × 10$^6$) were split into two batches ("control" and "QNB"); the latter cells were incubated with 0.8–1.2 nM racemic QNB in 5 ml of PBS at 37° for 20–30 min; control cells were incubated without QNB. The concentration of QNB required to reduce the maximal response was determined empirically and usually varied from 0.8 to 1.2 nM. It was noted that higher subcultures required less QNB to decrease the maximal response. Usually the labeling medium was not removed before the QNB incubation, although this did not seem to affect the results. Handling of the cells was kept to a minimum in order to preserve responsiveness, especially for the cyclic GMP assay. In particular, the length of incubation time with QNB was kept short because the cells are decreasingly responsive with extended periods in suspension. This short incubation time precluded the attainment of equilibrium with this ligand. After the QNB incubation, the cells were washed by suspension in 10 ml of PBS at 37°, sedimentation by low speed centrifugation, and resuspension in PBS. This procedure was repeated and the cells were suspended in PBS at 37° and distributed into multiwell trays for assay. Prewarmed PBS was used for washing and suspension to minimize the time for the cells to regain responsiveness after washing. Two washes were used as the best compromise to minimize handling and to maximize removal of unbound or nonspecifically bound QNB. The cells were preincubated in a shaking water bath at 37° for 10–12 min (cyclic GMP) or 6 min (cyclic AMP). The times were adjusted this way so that the total time after removal of QNB would be about the same in both assays. For the cyclic GMP assays, increasing concentrations of carbachol were incubated with the cells for 30 sec. For the cyclic AMP assays, increasing concentrations of carbachol were added sequentially to the wells, after which 10 μM $PGE_1$ was added for 10 min. After stopping the reactions with trichloroacetic acid, the radioactive cyclic nucleotides were isolated with ion exchange columns as described (7, 8).

It was shown by the use of [$^3$H](−)-QNB that the assumptions regarding pseudo-irreversible blockade and minimal effects of residual unbound QNB were justified. One nM [$^3$H](−)-QNB was incubated with N1E-115 cells under the conditions described above. Immediately after the two washes the total cell-associated [$^3$H](−)-QNB was 84 fmol/10$^6$ cells. Although we did not measure what fraction was nonspecific, sister cells were shown in a separate assay to have 66 fmol/10$^6$ cells maximal specific muscarinic receptor binding. One nM [$^3$H](−)-QNB would occupy 95% of these sites. Thus, we calculated that the fraction of nonspecific binding in this experiment was about 25%. The amount of [$^3$H]QNB removed after two washes and that remaining in the medium was also measured. More than 90% of the unbound [$^3$H]QNB was removed by this procedure. At 25–30 min after washing, which was the time frame of the response assays, the concentration of unbound [$^3$H]QNB was 4.6 ± 0.1 and 5.4 ± 0.1 pM (mean of five determinations ± SE), respectively. Since, in the response assays, racemic QNB was used, only about half this amount, that corresponding to the active (−)-isomer, would be active in those experiments. This amount of unbound (−)-QNB, if assumed to be in competition with carbachol during the assay, would cause only a small (5%) increase in the $K_D$ determined for carbachol, and this error would be the same in both response assays. Another consideration is the small increase in the unbound QNB resulting in the 5-min differential between assay of the beginning wells and assay of the ending wells in a particular experiment. However, even assuming that this arose from receptor dissociation, our calculations show that, in the typical experiment, this effect would amount to a minor (about 12%) overestimation of the $K_D$, and again this would equally affect the determinations of $K_D$ values for carbachol in the two assays. Therefore, our measurements and calculations indicated that, under restricted conditions, QNB can be used for determinations of agonist $K_D$ values by the method of partial receptor inactivation.

The response curves for untreated and QNB-treated cells were fitted with a logistic model by a least squares computer method (10), producing the best fit values of $EC_{50}$, maximal response, and an exponent describing the steepness of the response curve. These values were then used to calculate accurately the concentrations of carbachol which elicited a given response level in control ($A$, μM) and QNB-treated cells ($A'$, μM); only data in the linear regions of the response curves were used. The inverses of the values were plotted ($1/A$ versus $1/A'$), and the slope and intercept were determined by the method of least squares. The fraction of unoccluded receptors ($Q$) is the inverse of the slope (4). The fractional occupancy of the blocking agent would therefore be $1-Q$. The $K_D$ for the agonist is calculated by the following equation (4):

$$K_D = (\text{Slope} - 1)/\text{Intercept}$$

## Results

Occlusion of the N1E-115 muscarinic receptor with 0.8–1.0 nM (±)-QNB [which contains 0.4–0.5 nM concentration of the active enantiomer, (−)-QNB] caused a reduction in the level of the cyclic GMP response to carbachol that was accompanied by a slight shift of the response curve to the right (Fig. 1). A calculation using the previously determined $K_D$ value for (−)-

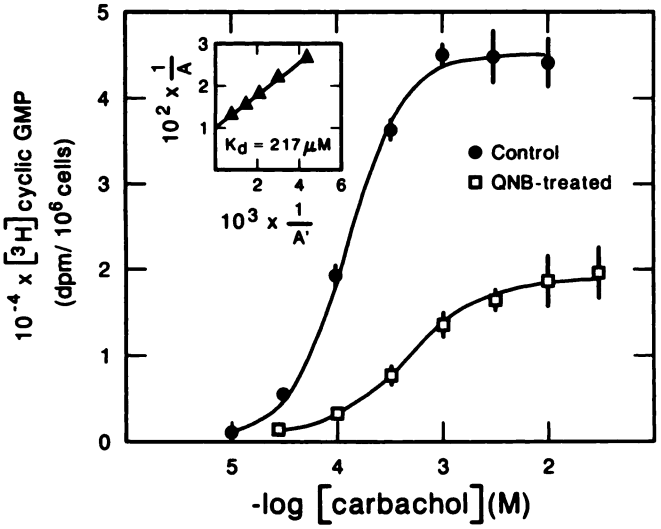


**Fig. 1.** Effect of occlusion of muscarinic receptors with QNB on the concentration-response curve for carbachol in the cyclic GMP response. One nM (±)-QNB was incubated with N1E-115 cells (subculture 9) for 30 min at 37°, after which the unbound QNB was removed by washing and the cells were assayed in the carbachol-cyclic GMP response. Data shown for control (●) and QNB-treated (□) cells were analyzed by the method of Furchgott and Bursztyn (4). The *inset* shows the plot which determined the $K_D$ value of 217 μM.

QNB (6) showed that this concentration of (−)-QNB would occupy 88–91% of the muscarinic receptors at equilibrium; however, the actual occupancy would be somewhat less than this because of the short time of incubation (30 min) with (−)QNB. The actual occupancy of (−)-QNB calculated from the slope of the transformed data in Fig. 1 (*inset*) was 72%. This occupancy gives a calculated $K_D$ value for (−)-QNB of 0.19 nM, which, despite being an overestimate, is an approximate agreement with the known value of 0.05 nM (6). Calculation of the equilibrium dissociation constant for carbachol by the method of Furchgott and Bursztyn (4) for the data shown in Fig. 1 yielded a value of 217 μM (Fig. 1, *inset*). This value is similar to but somewhat lower than that previously obtained using alkylating agents (3). The average (±SE) $K_D$ for carbachol determined in four such experiments with cells of subcultures 8–9 was 325 ± 60 μM (Table 1). The average (±SE) occupancy by QNB in these experiments was 68 ± 2%.

This method of using (±)-QNB to occlude receptors was employed to determine the $K_D$ value of carbachol in the cyclic AMP response. Significantly different $K_D$ values were obtained (an example is shown in Fig. 2). The $K_D$ for the data in Fig. 2 (*inset*) was 15 μM, and the average of three experiments was 13 ± 3 μM (Table 1). This $K_D$ was significantly different from that obtained in the cyclic GMP assay ($p < 0.005$). The average (±SE) QNB occupancy in these experiments was 65 ± 8%. For this comparison of the $K_D$ values in the two responses, sister cells of subcultures 8–9 were used. We did not study higher subculture cells extensively, but, in one cyclic GMP assay of subculture 14 cells, a $K_D$ of 590 μM was obtained, whereas with sister cells in two cyclic AMP assays, $K_D$ values of 25 μM and 21 μM were obtained.

The $K_D$ values determined by the method of partial receptor inactivation were higher than those previously determined in low subculture cells by direct binding assays at 15° (2). Therefore we measured direct binding constants for carbachol by competition binding with [$^3$H]NMS in sister cells to those used in the above experiments. The combined data from six experiments are shown in Fig. 3. The equilibrium dissociation constants obtained this way were 7 ± 1 μM ($K_H$) and 100 ± 5 μM ($K_L$) (average ± SE; $n = 6$) (Table 1), values that were somewhat higher than those previously obtained (2), but more similar to the values measured in the present work by the method of partial receptor inactivation. The fraction of the

TABLE 1

**Muscarinic responses and binding parameters determined for carbachol in two cyclic nucleotide responses in N1E-115 cells**

The equilibrium dissociation constants for carbachol were determined in either the cyclic GMP elevation or the cyclic AMP reduction response by the method of partial receptor inactivation using QNB to occupy receptors. The corresponding (control) $EC_{50}$ values for carbachol in stimulating these responses were determined in the same (or sister) cells as those used for determining the equilibrium dissociation constants. Cells were subcultures 8 and 9. The two $EC_{50}$ values are significantly different from each other (footnote *b*, $p < 0.001$), and the two direct binding equilibrium dissociation constants are also significantly different from each other (footnote *f*, $p < 0.005$). The two $K_D$ values determined after receptor inactivation are different with respect to each other (footnote *a*, $p < 0.005$). $K_H$ and $K_L$ were determined from direct binding assays (see Fig. 3). The $K_H$ is not significantly different from either the $EC_{50}$ value for mediating the cyclic AMP response or the $K_D$ determined for carbachol by receptor inactivation in the cyclic AMP response. Both the $K_L$ and the cyclic GMP $EC_{50}$ are significantly different from the $K_D$ for carbachol determined by receptor inactivation in the cyclic GMP assay (footnotes *c* and *d*, $p < 0.01$), but the $K_L$ is not significantly different from the $EC_{50}$ value in mediating cyclic GMP formation. The fraction of the receptors in the high affinity agonist-receptor conformation in the direct binding studies was 68 ± 2% ($n = 6$). Numbers in parentheses indicate the number of independent experiments performed to obtain the results.

| Response | $K_D$ from receptor inactivation | $EC_{50}$ for response | $K_D$ from direct binding |
|---|---|---|---|
| | μM | μM | μM |
| Cyclic GMP | 325 ± 60[a,c,d] (4) | 120 ± 20[b,c] (6) | $K_L$ 100 ± 5[d,f] (6) |
| Cyclic AMP | 13 ± 3[a,e] (3) | 4 ± 1[b,e] (3) | $K_H$ 7 ± 1[f] (6) |

[a] $p < 0.005$.
[b] $p < 0.001$.
[c] $p < 0.01$.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] $p < 0.005$.

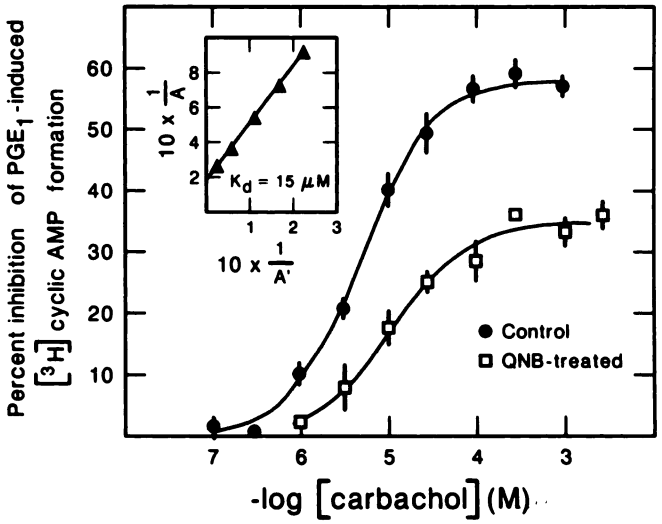


**Fig. 2.** Effect of occlusion of muscarinic receptors with (±)-QNB on the concentration-response curve for carbachol in the cyclic AMP response. Racemic QNB (1.2 nM) was used to occlude receptors. The ability of carbachol to reduce the cyclic AMP response to 10 μM $PGE_1$ was then assayed. Percentage inhibition of the $PGE_1$ response is plotted as the response of the muscarinic receptor to stimulation by carbachol. The control (●) and QNB-treated (□) data were analyzed by the method of Furchgott and Bursztyn (4). The $K_D$ value for carbachol was 15 μM (*inset*).

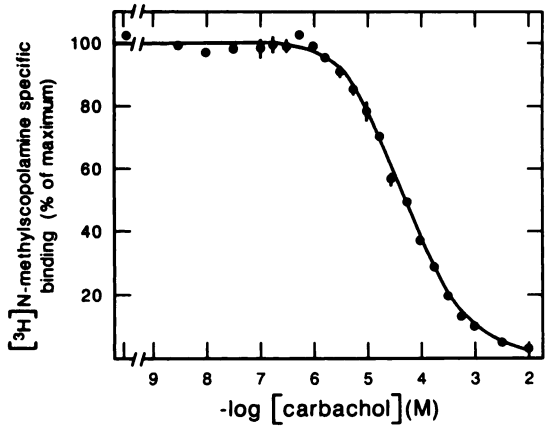


**Fig. 3.** Equilibrium binding to muscarinic receptors in intact N1E-115 cells at 15°. Sister cells to those used in the response assays were detached, washed, and suspended in PBS. Aliquots of the cell suspension were incubated at 15° with 0.5 nM [$^3$H]NMS and the indicated concentrations of carbachol for 45 min. Ten $\mu$M unlabeled NMS was in some tubes to determine nonspecific binding. The *vertical bars* (most are inside the *symbols*) show the standard error for six combined assays, each performed in triplicate. The *smooth curve* is the best fit iterative computer solution employing a two-site binding model. The averages of the individually determined binding equilibrium dissociation constants for carbachol are shown in Table 1. Of the receptors, 68 ± 2% were in the high affinity state. One hundred per cent binding represents 47 ± 5 fmol/$10^6$ cells ($n$ = 6).

receptors in the high affinity conformation in these experiments was 65 ± 8%. The high affinity equilibrium dissociation constant for carbachol determined by partial receptor occlusion with QNB was not significantly different from the value obtained by direct binding. However, the low affinity binding constant for carbachol determined by the method of partial receptor inactivation was significantly higher ($p < 0.01$) by a factor of 3 than the low affinity binding constant determined by competition binding at 15°.

The $EC_{50}$ values for carbachol in mediating the responses in these cells are also shown in Table 1; as was found previously (2, 6), the $EC_{50}$ for cyclic GMP formation was not significantly different from $K_L$, and the $EC_{50}$ for cyclic AMP reduction was not significantly different from $K_H$.

## Discussion

Using the method of partial receptor blockade we showed that the full agonist carbachol mediated the cyclic GMP response through a low affinity agonist-receptor conformation, whereas it mediated the cyclic AMP response through a high affinity conformation. Binding constants obtained in parallel competition assays were similar to those obtained by the method of receptor inactivation, except the value obtained in the response assay at 37° was 3-fold higher than that obtained in the competition binding assay, which was carried out at 15°.

With the addition of the data in the present paper to that body of data previously gathered (2, 6), the evidence for the mediation of the two cyclic nucleotide responses by separate agonist-receptor conformations in this neuron-like preparation is very strong. The data further demonstrate that it is the low affinity agonist-receptor conformation which mediates cyclic GMP formation.

We have observed that a minor fraction of receptors binding [$^3$H]QNB in intact N1E-115 cells are inaccessible to NMS (a quaternary ligand) but are accessible to atropine (2). Lee and El-Fakahany (11) have recently shown in brain homogenates that a minor population of muscarinic sites displaying low affinity for quaternary antagonists is revealed by competition with [$^3$H]QNB. It may be, therefore, that [$^3$H]QNB can gain access to some sites located in a more lipophilic domain. It is not yet known what relationship this might have to multiple agonist-binding sites. In N1E-115 cells carbachol (a quaternary agonist) binds to at least two sites whether in competition with [$^3$H]QNB or [$^3$H]NMS. If only the quaternary nature were relevant to heterogeneity, one would expect monophasic displacement of [$^3$H]NMS by carbachol. The partial agonists examined so far in N1E-115 cells displace [$^3$H]NMS monophasically (2), so that it is possible that varying lipophilicity of regions surrounding the receptor is related in some way to agonist heterogeneity in binding and responses in N1E-115 cells. One would not expect this possibility to affect the conclusions drawn from our partial occlusion studies with QNB, because this ligand appears to bind to all muscarinic receptors with the same affinity, and because the value of the agonist $K_D$ determined from the Furchgott plot does not depend on the affinity of the blocking agent, or even on the degree of blockade, so long as the maximal response to the agonist is lowered.

The equilibrium dissociation constants for carbachol determined by direct binding in the present study are somewhat higher than those obtained previously for early subcultures (2, 9). There also appears to be a higher proportion of high affinity receptors. The reason for this is not known, but this does not invalidate the conclusion that high and low affinity complexes each separately mediate the cyclic AMP and cyclic GMP responses, respectively.

The selective inhibitory effect of pirenzepine provided the first definitive clue that different receptor conformations mediated these two cyclic nucleotide responses in N1E-115 cells (2). The finding that the cyclic GMP response was lost during sequential subculturing in parallel with the loss of the low affinity carbachol-binding site suggested that cyclic GMP was mediated by a low affinity conformation of the receptor (6). However, suggestive as that finding was, the hypothesis of "spare receptors" could still be invoked to explain those data. Another indication that the subtypes mediating the two responses did so by binding agonists with differing affinities arose from studies of the efficacies and binding characteristics of a series of 10 muscarinic agonists (2). Five of these muscarinic agonists were essentially unable to stimulate cyclic GMP. These same (partial) agonists apparently bound to a single class of high affinity sites in N1E-115 cells, whereas full agonists (which were able to mediate both cyclic nucleotide responses) were shown to distinguish between two classes of binding sites. But to show clearly which conformation mediated which response it was necessary to measure the equilibrium dissociation constants for both of these responses in a study of the type presented here.

The low affinity equilibrium dissociation constant for carbachol determined by the method of partial receptor inactivation was about 3-fold higher than that obtained by direct binding. There are several possible explanations for this. The first is related to the effects of temperature on agonist binding. The response assays are done at 37° whereas the direct binding assays with intact cells must be performed at 15°. We showed previously that the binding constants for agonists decrease with decreasing temperature (2, 6), a finding in accord with the

general thermodynamics of agonist binding (12). For the binding of carbachol to the low affinity site, the $K_L$ measured at 37° in homogenates was 1.5-fold greater than that measured at 15°. This phenomenon will account for some of the difference. The second possible reason why the low affinity dissociation constant is higher in the partial inactivation experiments is that some desensitization of the receptor may have occurred during the response assay. However, one might expect this to affect the results with control and the QNB-treated cells equally; and since the time course for desensitization is on the order of several minutes, whereas the assay takes only 30 sec, one would not expect much desensitization to occur during the assay. A third consideration is that the extensive handling of the cells may have had some effect on receptor binding characteristics.

The final conclusion to be drawn from our studies is that a connection can be made, at least partially, between the high affinity pirenzepine receptor and the low affinity agonist site. That is, pirenzepine binds with "$M_1$" type of affinity (10 nM), as determined previously (2), to the class of neural receptors at which carbachol mediates cyclic GMP formation through a low affinity conformation, as determined by the method of partial receptor inactivation. It is of interest that pirenzepine, like QNB, lowers the $V_{max}$ of the carbachol-cyclic GMP concentration-response curves (2), indicating pseudo-irreversible occlusion, or "hemi-equilibrium," as Rang (5) called it. Applying his plotting analysis method (5) to our previously published data (2) with pirenzepine, we calculated a $K_D$ value of 300 ± 29 ($n$ = 13) for carbachol, in close agreement with the value obtained with QNB, which gives added support for associating the low affinity conformation with cyclic GMP formation. However, it still remains to be determined whether there is a stoichiometric relation between agonist-binding subtypes and pirenzepine-binding subtypes, i.e., whether all or a subset of the receptors displaying low affinity for the agonist are of the $M_1$ category, and conversely. Based on the binding data with cerebral cortical tissue (13), it appears that pirenzepine recognizes an aspect of the receptor different from that feature which gives rise to the differing agonist affinities. Thus, complete overlap might not be expected.

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