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Phenoxybenzamine and Dibenamine Interactions with Calcium Channel Effectors of the Muscarinic Receptor

ESAM EL-FAKAHANY AND ELLIOTT RICHELSON

Departments of Psychiatry and Pharmacology, Mayo Foundation, Rochester, Minnesota 55905

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SUMMARY

Phenoxybenzamine and dibenamine were more effective at blocking muscarinic acetylcholine receptor-mediated cyclic GMP formation in mouse neuroblastoma cells (clone N1E-115) than at blocking radiolabeled antagonist binding to the muscarinic receptors of these cells. High calcium concentrations antagonized the effects of these 2-halogenoethylamines on the cyclic GMP response but not on the receptor binding. In addition, the apparent equilibrium dissociation constant for carbachol and the muscarinic receptor determined in the biological assay with the use of the 2-halogenoethylamines was several-fold greater than that determined by radioligand binding assays. These results suggest that phenoxybenzamine and dibenamine interact with calcium channels in addition to muscarinic receptors, and on the basis of these and other results, we propose a scheme for the interactions of agonists and antagonists with muscarinic receptors and their effectors (calcium channels).

INTRODUCTION

2-Halogenoethylamines represent a unique pharmacological class of irreversible antagonists having diverse effects. It is well documented that they inhibit responses induced by activation of muscarinic acetylcholine (1–3), *alpha*-adrenergic (4, 5), histamine H_1 (6), 5-hydroxytryptamine (7), dopamine (8), and opiate receptors (9). They also antagonize responses due to nonreceptor stimulants such as calcium, potassium, and barium (5, 10, 11).

The antagonistic action of 2-halogenoethylamines at various receptor systems would appear more plausible on the basis of their inhibitory effect on a common effector rather than on exclusive reliance on their tenuous molecular complementarity with obviously diverse receptor sites. To obtain data in support of this hypothesis, we investigated the effects of these agents on both muscarinic receptor-mediated cyclic GMP formation and muscarinic ligand binding by intact mouse neuroblastoma clone N1E-115 cells.

MATERIALS AND METHODS

Cell culture conditions. Mouse neuroblastoma clone N1E-115 cells (subculture 9–20) were grown in tissue culture flasks (150 cm^2/1000 ml; Corning Glassworks, Corning, N. Y.), in 40 ml of Dulbecco's modified Eagle's medium (Grand Island Biological Company, Grand Island, N. Y., Catalogue No. 430-2100) supplemented with 10% (v/v) newborn calf serum (Grand Island Biological Company) (Medium I) at 37° in an atmosphere consisting of 10% CO_2 and 90% humidified air. The cells were subcultured by incubation in a modified Puck's D_1 solution (12) (Medium II), resuspension in Medium I, and distribution into flasks (approximately 5×10^5 cells per flask). The culture medium was changed on day 5 and every day thereafter by adding 10 ml of fresh Medium I and removing 10 ml of medium. The medium was changed nearly 24 hr before harvesting the cells for assay. Cells used for binding assays were at least 1 week post-subculture; cells for the cyclic GMP experiments, at least 12 days post-subculture (13).

Assay of muscarinic receptor-mediated cyclic GMP formation. Relative changes in cyclic GMP formation were assayed using a radioactively labeled precursor and intact cells. The details of the method were described elsewhere (14). Briefly stated, the cells were harvested for assay by aspirating Medium I and incubating for 5 min with Medium II, followed by low-speed centrifugation ($250 \times g$) for 90 sec at 5°. The pellet was washed twice with a buffer having the following composition (millimolar) (Medium III): NaCl, 110; KCl, 5.3; $CaCl_2$, 1.8 (unless otherwise stated); $MgCl_2$, 1.0; glucose, 25; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20. The pH was adjusted to 7.3 and sucrose was added to adjust osmolality to 335–340 mOsm. The cells were resuspended in 2 ml of Medium III, incubated with [^{3}H]guanine, 10 μCi/ml (1μM final concentration), and rotated at 37° for 45 min at 80 rpm (Gyratory Shaker, New Brunswick Scientific Company, Edison, N. J.). The cell suspension was diluted with Medium III and distributed into the wells of multiwell trays (Disposo trays, FB16-24TC, Bellco Glass, Vineland, N. J.) in 240-μl ali-

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quots. After incubation of cells at 37° for 15 min in a shaker bath (Model 25, GCA/Precision Scientific Company, Chicago, Ill.) at 80 oscillations/min, the cells were treated as described under Results. The carbachol was added in 30 μl of Medium III for 30 sec and the reaction was terminated by the addition of 30 μl of 50% (w/v) trichloroacetic acid. After adding to each well 0.5 nCi of ^{14}C-labeled cyclic GMP as an internal standard, the contents of each well were passed through an AG50-W-X2 ion exchange column (0.8 × 8 cm) which had been equilibrated with 0.1 N HCl. Each well was then washed with 0.5 ml of 5% (w/v) trichloroacetic acid and the wash was transferred to the columns, which were then washed successively with 4.4 ml of 0.1 N HCl (eluate discarded), 1.0 ml of water (eluate discarded), and finally 1.4 ml of water which was collected in plastic Microfuge tubes. To this eluate, equal volumes (25 μl) of 3.2 M $ZnSO_4$ and 3.2 M Na_2CO_3 were added to precipitate any residual GDP or TP. The tubes were then centrifuged in a Microfuge (Beckman Instruments, Palo Alto, Calif.), the supernatant was transferred to 7 ml of 3a70B complete counting cocktail (Research Products International Corporation, Elk Grove Village, Ill.), and the radioactivity was determined in a Searle Isocap/300 liquid scintillation counter. Efficiency rates for counting tritium and carbon atom 14 in their respective channels were 38% and 45% on average, respectively. All samples were corrected for the recovery of ^{14}C-labeled cyclic GMP, which was usually approximately 75%, and quenching was corrected by using the external standard ratio technique. In general, duplicates differed from their means by less than 20%.

Muscarinic receptor binding assay with mouse neuroblastoma cells. The assay procedure for binding of the muscarinic receptor antagonist [^{3}H]QNB[1] to intact mouse neuroblastoma cells was as described before (13). Mouse neuroblastoma cells were harvested as described above, suspended in Medium III (pH 7.3), and then frozen and thawed once (a procedure which reduced nonspecific binding to cells). For the binding assay the tissue was incubated for 45 min at 37° in 2 ml of Medium III containing six to eight concentrations of (−)-[^{3}H]QNB (40.2 Ci/mmole) in duplicate, with or without 1 μM atropine (for nonspecific binding) in the presence or absence of the halogenoethylamines. The assay was terminated by filtering the suspension through Whatman GF/B filters (2.4 cm, Whatman, England) under vacuum followed by rapidly washing the filters four times with ice-cold 0.9% (w/v) NaCl solution. After 5–10 min, the filters were placed into scintillation vials to which were added 7 ml of scintillation cocktail. Radioactivity was determined at least 8–12 hr later.

To determine the IC_{50} values for halogenoethylamines and carbachol, cells were incubated in duplicate with a fixed concentration of [^{3}H]QNB (0.5 nM) and increasing concentrations of the displacing compound in the absence or presence of 1 μM atropine. In general, duplicates varied from the mean by less than 10%.

Data analysis. All experimental data were fit to the following logistic function (15):

$$E = \frac{M[A]^P}{[A]^P + K^P} \quad (1)$$

where E is the observed effect, which in our studies is either percentage of control maximal cyclic GMP responses or percentage of control radioligand binding; M is the maximal effect; $[A]$ is the concentration of agonist or competitor; K is the concentration of agonist causing 50% of maximal cyclic GMP response or concentration of a competitor causing 50% inhibition of specific radioligand binding; and P is the slope factor, the absolute value of which is equivalent to the Hill coefficient ("pseudo-Hill coefficient" when radioligand A is being displaced by compound B).

Fitting this function to the data by the least-squares method of Waud and Parker (15) was achieved with the use of a Hewlett-Packard 9845B desktop computer. Graphs were generated by this computer and plotted on a Hewlett-Packard 9872B plotter.

Protein assay and cell counts. Protein was determined by a modification of the procedure reported by Lowry *et al.* (16), with bovine serum albumin as standard. Cell counts were obtained with an electronic cell counter (Model Z_F, Coulter Electronics, Hialeah, Fla.).

Chemicals. Carbamylcholine hydrochloride and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were obtained from Sigma Chemical Company (St. Louis, Mo.); phenoxybenzamine hydrochloride and dibenamine hydrochloride were purchased from Smith Kline & French Laboratories (Philadelphia, Pa.); [^{3}H]guanine and ^{14}C-labeled cyclic GMP from Amersham/Searle Corporation (Arlington Heights, Ill.); and (−)-[^{3}H]QNB (40.2 Ci/mmole) from New England Nuclear Corporation (Boston, Mass.).

RESULTS

Effect of phenoxybenzamine and dibenamine on muscarinic receptor-mediated cyclic GMP formation. Incubation of mouse neuroblastoma clone N1E-115 cells with 0.2 μM phenoxybenzamine or 5 μM dibenamine for 30 min in buffer containing 1.8 mM Ca^{2+} resulted in a significant reduction in maximal cyclic GMP formation induced by the muscarinic receptor agonist carbachol (Fig. 1A) without any significant change in the time course of cyclic GMP formation (data not shown). This reduction in the maximal response was accompanied by a shift of the concentration-cyclic GMP formation curve to the right with a concomitant increase in the agonist's EC_{50} (concentration producing 50% of the maximal response).

From these data we calculated IC_{50} values (concentration of antagonist that inhibits by 50% specific binding or maximal response) for the haloalkylamines realizing that it is a time-dependent parameter, according to the formula (17)

$$\frac{M}{M'} = \left(1 + \frac{[I]}{IC_{50}}\right) \quad (2)$$

where M is the maximal response or binding in absence of antagonists, M' is the maximal response or binding in presence of antagonists, and $[I]$ is the concentration of antagonist.

We found these IC_{50} values to be 0.1 μM and 2.2 μM for phenoxybenzamine and dibenamine, respectively (Table 1). We also determined the apparent percentage of receptors inactivated by the antagonists under these condi-

[1] The abbreviation used is: QNB, quinuclidinyl benzilate.

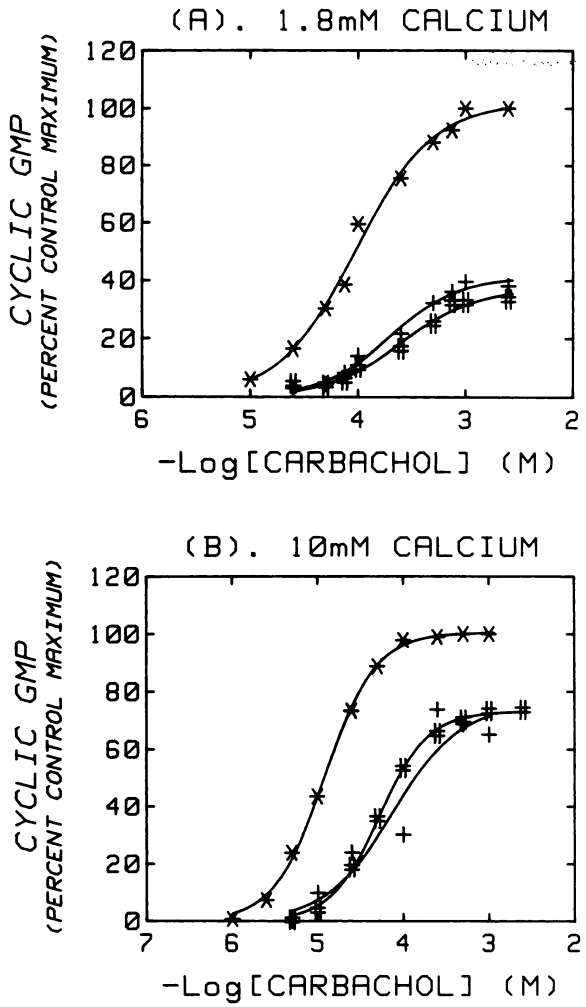


FIG. 1. *Effect of phenoxybenzamine and dibenamine on muscarinic receptor-mediated cyclic GMP formation in mouse neuroblastoma clone N1E-115 cells*

Cells were prepared for assay as described under Materials and Methods, then incubated in 1.8 mM Ca^{2+} (A), or 10 mM Ca^{2+} (B) without antagonists (•), with 0.2 μM phenoxybenzamine (+), or with 5 μM dibenamine (#) for 30 min. ^{3}H-labeled cyclic GMP was determined after cells were then stimulated with the indicated concentrations of carbachol for 30 sec. Each *point* is the average of data pooled from three to nine experiments. Data are presented as percentages of maximal carbachol-induced cyclic GMP formation in control cells. The standard error was around 20% of the mean.

tions, according to the following formula which was derived by Furchgott (18):

$$\frac{1}{[A]} = \frac{1}{q} \cdot \frac{1}{[A']} + \frac{(1-q)}{q} \cdot \frac{1}{K_A} \qquad (3)$$

where $[A]$ is the concentration of free agonist in region of the receptors and in absence of antagonists, $[A']$ is the concentration of free agonist in region of the receptors producing a response equal to $[A]$ in the presence of antagonists, q is the fraction of receptors remaining in active form after irreversible inactivation of $1 - q$ by antagonists, and K_A is the equilibrium dissociation constant of the agonist-receptor complex.

With the use of this formula, it appeared that in these experiments phenoxybenzamine and dibenamine inactivated 79% and 81% of the receptors, respectively (Table 2). In addition, from the plot of $1/[A]$ against $1/[A']$, the apparent equilibrium dissociation constant (K_A) for carbachol (estimated from the slope and the intercept of the resulting straight lines) was 400 μM ± 99 (mean ± SEM, $n = 12$) using pooled data from experiments with either phenoxybenzamine or dibenamine.

When the same experiments were carried out in buffer containing 10 mM Ca^{2+}, both antagonists were less effective at antagonizing carbachol-induced cyclic GMP formation (Fig. 1B), resulting in IC_{50} values of 0.47 μM and 14.1 μM for phenoxybenzamine and dibenamine, respectively (Table 1). However, the apparent percentage of receptors inactivated by the antagonists was not significantly changed by raising the external calcium concentration (Table 2). In addition, the apparent equilibrium dissociation constant for carbachol in high calcium concentration was 153 ± 44 ($n = 8$), a value which is significantly different ($p < 0.0125$) from that obtained in low-calcium buffer (see above).

The slope factors or Hill coefficients for carbachol at 1.8 mM Ca^{2+} (Fig. 1A) were 1.37 ± 0.14, 1.30 ± 0.11, and 1.40 ± 0.15 (mean ± SEM) for carbachol alone, carbachol with dibenamine, and carbachol with phenoxybenzamine, respectively. These values were significantly greater than 1 ($p < 0.025$ in each case). At 10 mM Ca^{2+} (Fig. 1B) the Hill coefficients were 2.45 ± 0.40, 1.73 ± 0.18, and 1.62 ± 0.25 for carbachol alone, carbachol with dibenamine, and carbachol with phenoxybenzamine, respectively. The slope factors for carbachol at the higher calcium concentration were significantly greater than those found at the lower calcium concentration, either in the absence of antagonists ($p < 0.01$) or in the presence of dibenamine ($p < 0.025$) but not in the presence of phenoxybenzamine (probably because of the smaller number of determinations in this case).

Effect of phenoxybenzamine and dibenamine on [^{3}H]QNB binding. Preincubation of mouse neuroblastoma clone N1E-115 cells for 30 min with increasing concentrations of phenoxybenzamine or dibenamine resulted in a gradual reduction in the specific binding of

TABLE 1

Effect of calcium on the IC_{50} values (micromolar) of phenoxybenzamine and dibenamine in cyclic GMP and [^{3}H]QNB binding assays

Condition	[^{3}H]QNB binding		Cyclic GMP	
	Phenoxybenzamine	Dibenamine	Phenoxybenzamine	Dibenamine
1.8 mM Ca^{2+}	0.45 ± 0.08 (6)[a]	6.57 ± 0.85 (8)	0.1 ± 0.01 (5)[b]	2.16 ± 0.32 (7)[b]
10 mM Ca^{2+}	0.65 ± 0.22 (3)[c]	10.9 ± 3.3 (3)[c]	0.47 ± 0.08 (3)[d]	14.1 ± 2.6 (5)[d]

[a] The numbers in parentheses represent the number of independent observations.
[b] Significantly different from values obtained in binding experiments ($p < 0.00025$).
[c] Not significantly different from values obtained in low calcium ($p > 0.05$).
[d] Significantly different from values obtained in low calcium ($p < 0.00025$).

TABLE 2

Effect of calcium on the percentage of receptors inactivated by 0.2 μM phenoxybenzamine or 5 μM dibenamine as calculated from cyclic GMP and [^{3}H]QNB binding assays

Condition	[^{3}H]QNB binding		Cyclic GMP	
	Phenoxybenzamine	Dibenamine	Phenoxybenzamine	Dibenamine
1.8 mM Ca^{2+}	28.4 ± 2.5 (3)[a]	40.8 ± 4.2 (5)	79.3 ± 6.1 (5)[b]	80.8 ± 4.6 (7)[b]
10 mM Ca^{2+}	27.1 ± 7.4 (3)[c]	35.0 ± 8.8 (3)[c]	75.2 ± 9.6 (3)[c]	74.8 ± 5.5 (5)[c]

[a] The numbers in parentheses indicate the number of independent experiments.
[b] Significantly different from values obtained in binding experiments ($p < 0.00025$).
[c] Not significantly different from values obtained in low calcium ($p > 0.1$).

[^{3}H]QNB (Fig. 2) with slope factors of 1.04 ± 0.19 and 0.89 ± 0.11 for phenoxybenzamine and dibenamine, respectively, values which were not significantly different from 1 ($p > 0.15$ in each case). The IC_{50} values (concentration producing 50% inhibition of specific binding) were 0.37 ± 0.03 μM and 8 ± 0.6 μM (mean ± SEM) for phenoxybenzamine and dibenamine, respectively.

When we used six to eight concentrations of [^{3}H]QNB in the absence of the irreversible antagonists or in the presence of 0.2 μM phenoxybenzamine or 5 μM dibenamine, we were able to determine maximal binding capacity and equilibrium dissociation constants of [^{3}H]QNB in the absence and the presence of the antagonists. At these concentrations of the antagonists, there was no significant change in the apparent K_D values of [^{3}H]QNB (Table 3), $p > 0.1$ in all cases when compared with control, whereas there was a reduction in the apparent number of receptors (Table 2). In addition, increasing Ca^{2+} concentration did not have any significant effect on the apparent dissociation constant for [^{3}H]QNB (Table 3).

The IC_{50} values for the haloalkylamines were calculated from Eq. 2 using maximal specific binding in the absence and the presence of the haloalkylamines. From these experiments and the experiments using one concentration of [^{3}H]QNB and several concentrations of the haloalkylamines, we calculated IC_{50} values of 0.45 μM and 6.57 μM for phenoxybenzamine and dibenamine, respectively, for inhibition of [^{3}H]QNB binding (Table 1). The percentage of inactivated receptors (Table 2) or the inhibition constants for [^{3}H]QNB binding (Table 1) did not change by raising the external calcium concentration.

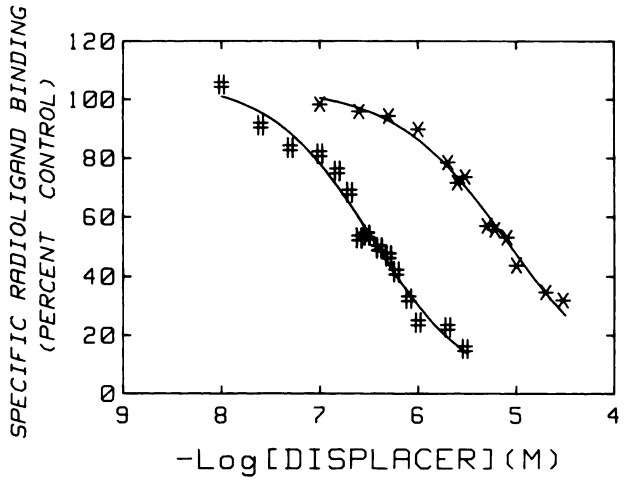


FIG. 2. *Effect of phenoxybenzamine and dibenamine on [^{3}H]QNB binding in mouse neuroblastoma clone N1E-115 cells*

Cells were prepared for assay as described under Materials and Methods, and then incubated with 0.5 nM [^{3}H]QNB in the presence of increasing concentrations of phenoxybenzamine (*) or dibenamine (•). Data are presented as percentages of the specific [^{3}H]QNB binding in the absence of displacers. Each *point* is the average obtained from three experiments. The standard error was less than 10% of the mean.

Displacement of [^{3}H]QNB binding by carbachol. When the cells were incubated with 0.5 nM [^{3}H]QNB in Medium III containing 1.8 mM Ca^{2+}, there was a gradual reduction of the specific binding as a function of the concentration of carbachol present in the incubation medium (Fig. 3). In these experiments, carbachol had slope factors of 0.73 ± 0.03 and 0.67 ± 0.03 in 1.8 mM Ca^{2+} and 10 mM Ca^{2+}, respectively. These values were significantly different from 1 ($p < 0.0025$ in each case) and not significantly different from each other ($p > 0.05$).

DISCUSSION

Our data show that, in mouse neuroblastoma cells, phenoxybenzamine and dibenamine were more potent in inhibiting muscarinic receptor-mediated cyclic GMP formation than in inhibiting muscarinic receptor ligand binding. That is, these haloalkylamines caused more apparent loss of receptors in a biological assay than in a binding assay, which results raise some questions concerning the nature of the "receptors" inctivated by these agents. Similar results have been reported by Takeyasu *et al.* (19) in guinea pig ileum.

Activation of muscarinic receptors of this mouse neuroblastoma clone causes large increases in cyclic GMP levels (13, 14, 20) which appear to be mediated by an increase in calcium entry (21, 22). In addition, muscarinic receptor agonists are known to increase calcium influx in guinea pig ileum (23). In the present study we found that increasing the external calcium concentration increased the IC_{50} values of haloalkylamines in cyclic GMP assays but not in binding assays. These observations suggest that these antagonists interact with calcium binding sites

TABLE 3

Apparent equilibrium dissociation constant (picomolar) of [^{3}H]QNB in absence and presence of haloalkylamines

Condition	Control	Phenoxybenzamine	Dibenamine
1.8 mM Ca^{2+}	57 ± 8 (8)[a]	47 ± 5 (5)	76 ± 7 (6)
10 mM Ca^{2+}	64 ± 1 (3)	60 ± 4 (3)	76 ± 10 (3)

[a] Numbers in parentheses represent the number of independent observations.

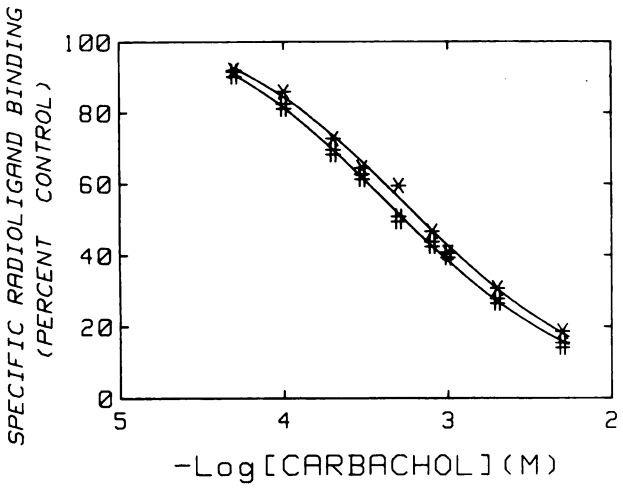


FIG. 3. *Carbachol displacement of a muscarinic radioligand from mouse neuroblastoma clone N1E-115 cells*

Cells were prepared for assay as described under Materials and Methods and were incubated with 0.5 nM [^{3}H]QNB and increasing concentrations of carbachol in 1.8 mM Ca^{2+} (•) or 10 mM Ca^{2+} (✱). Data are presented as percentages of the specific binding in the absence of carbachol. Each *point* is the mean value from four experiments. The standard error was less than 10% of the mean.

in addition to the receptors. However, an alternative explanation could be provided by the differences in the experimental conditions between binding studies and cyclic GMP assays. In binding assays, the ligand was present with the haloalkylamine for 45 min, whereas in cyclic GMP experiments the contact time was only 30 sec. There might be a slowly reversible component of haloalkylamine binding, which might manifest itself as a higher percentage inactivation of the receptors on cyclic GMP responses than in binding assays. One could also argue against this hypothesis since we do not know the stoichiometry of the agonist-receptor complex. If, for instance, there are two agonist sites per receptor complex and both are required to be occupied to give a cyclic GMP response, then occupancy of the sites by an irreversible antagonist will have a much greater effect on the response than on the binding.

There is evidence in the literature to support the hypothesis that haloalkylamines interact with calcium channels as well as receptors. Ogino (5) has reported that dibenamine blocks contractions induced by *alpha*-adrenergic agonists in rat vas deferens, and that this blockade is antagonized by increasing the external calcium concentration. In addition, methoxyverapamil, diazoxide, and local anesthetics enhance the recovery of the blocking effect of phenoxybenzamine and dibenamine on *alpha*-adrenergic receptor-mediated effects in rat vas deferens (4, 24). All of these agents are known to mediate their effects mainly through blocking calcium channels (25). Lanthanides which interact with calcium binding sites are able to abolish the effect of dibenamine on muscarinic receptor-mediated cyclic GMP formation (26). More direct evidence for the interaction of 2-halogenoethylamines with calcium channels is provided by the fact that both phenoxybenzamine and dibenamine inhibit ^{45}Ca influx in rabbit aortic strips (11). The uptake of ^{45}Ca in this clone of mouse neuroblastoma cells was inhibited by approximately 40% after treating the cells with 0.2 μM phenoxybenzamine for 30 min.[2] It is interesting that Jafferji and Michell (27) have reported that phenoxybenzamine, but not dibenamine, inhibits muscarinic receptor-mediated phosphatidylinositol turnover, which they believe controls calcium gates in cell membranes.

Kenakin and Cook (6) have reported that phenoxybenzamine is less effective in antagonizing the effects of histamine on guinea pig ileum desensitized to histamine than it is in control tissues. They concluded that this result might be due to desensitization-accompanied conformational changes in the receptor sites so that these sites are less susceptible to interaction with phenoxybenzamine. However, we have presented evidence to show that short-term desensitization of histamine- or carbachol-mediated responses in mouse neuroblastoma cells involves inactivation of calcium channels (21). It is possible that such a mechanism for desensitization also applies to the guinea pig ileum. We would further predict that desensitized calcium channels are less able to bind phenoxybenzamine and that the results of Kenakin and Cook (6) are based on this change rather than on a change in the histamine receptor.

Strange *et al.* (28) have reported the existence of two binding sites for carbachol in this clone of mouse neuroblastoma (K_A of 0.1 and 50 μM for the high- and low-affinity sites, respectively), and they believe that the low-affinity site mediates cyclic GMP responses. However, we could not resolve our binding curves for carbachol into high- and low-affinity sites despite the fact that these curves had slope factors of less than unity; and because of the value of this slope factor we could not calculate a K_i from our IC_{50} data. Nonetheless, there is a large discrepancy in the values of this dissociation constant for carbachol when determined by using these irreversible antagonists in a biological assay as compared with those determined from inhibition of [^{3}H]QNB binding (28). This discrepancy is clearly illustrated from the "receptor" occupancy curves determined according to Eq. 1 (Fig. 4), using the low-affinity binding constant (28) and the value K_A = 400 μM from the haloalkylamine experiments.

Since we think that 2-halogenoethylamines interact with both receptors and the calcium channels and are more potent at these channels, it is possible that Furchgott's analysis (18) for determining the equilibrium dissociation constant for the agonist-receptor complex (K_A) is not valid for this receptor system (and perhaps others) because of the inherent limitations of this analysis, especially since increasing the calcium concentration markedly reduced the equilibrium dissociation constant determined by this technique, but only slightly reduced the IC_{50} for carbachol determined from binding studies. In fact, the calcium channel blocker, manganese, has the same effect on carbachol's concentration-cyclic GMP response curve as does phenoxybenzamine or dibenamine, i.e., it shifts the concentration-response curve to the right while reducing the maximal response without inhibiting receptor binding (21). Using the manganese data, we computed an apparent equilibrium dissociation

[2] E. El-Fakahany and E. Richelson, unpublished observation.

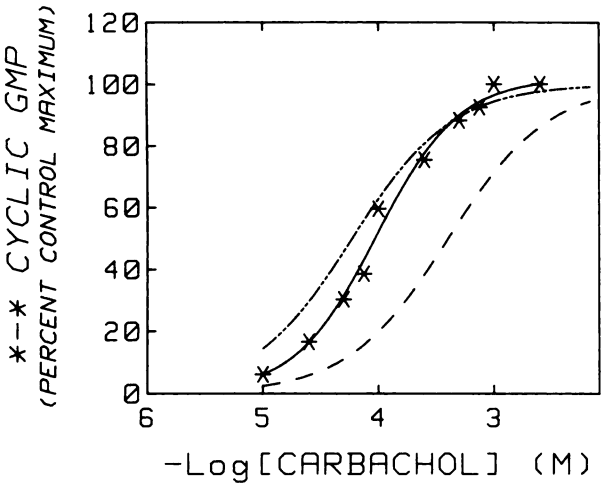


FIG. 4. *Relationship between muscarinic receptor occupancy and carbachol mediated cyclic GMP formation by mouse neuroblastoma clone N1E-115 cells*

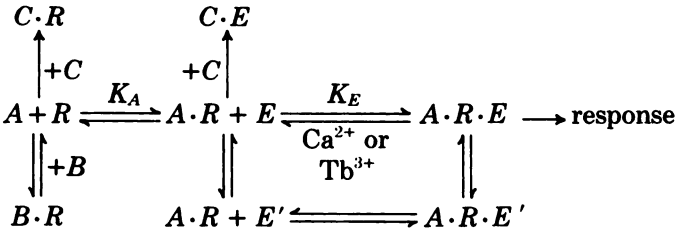
* Represents carbachol's concentration-cyclic GMP formation curve (data from the control curve of Fig. 1A); --- and ---- are agonist concentration-receptor occupancy curves obtained by using agonist equilibrium dissociation constants determined from biological assays (derived from data shown in Fig. 1A) or binding assays (derived from ref. 28), respectively.

constant of 400 μM for carbachol, a value which is identical with that reported above using the haloalkylamines. In 1.8 mM Ca^{2+} (Fig. 1), the EC_{50} of carbachol is more or less identical with the K_A determined in binding studies, but significantly less than the K_A determined in the biological assays (K_A') suggesting that there are no spare receptors per se in this system, but perhaps that there are spare effectors which could explain the rightward shift of the concentration-response curve caused by manganese and the haloalkylamines. However, in 10 mM Ca^{2+}, $EC_{50} < K_A \ll K_A'$, suggesting that under these conditions there are both spare receptors and effectors, with a lower proportion of the former than the latter, supporting the hypothesis made by Takeyasu *et al.* (19) that spare receptors are dynamic in nature.

The fact that $K_A' \gg K_A$ could be due to a reduction by the haloalkylamines in the number of effector units. Indeed, Boeynaems and Dumont (29) have predicted from their theoretical presentation of the mobile receptor hypothesis that a decrease in the effector concentration results in a decrease in the affinity of the agonist-receptor complex. Our data would therefore suggest that the muscarinic receptor in mouse neuroblastoma cells is mobile.

A Hill coefficient greater than 1 for the carbachol-mediated cyclic GMP response is to be contrasted with the ("pseudo") Hill coefficient of less than 1 for the interaction of carbachol with the muscarinic antagonist, [^{3}H]QNB. This apparent positive cooperativity for the biological response was enhanced at a high calcium concentration, suggesting a role for calcium as an allosteric activator. However, calcium was without effect in altering the Hill coefficient for the interaction between carbachol and [^{3}H]QNB.

These differences in the results between the biological response and the radioligand binding may be better understood in the context of the following hypothetical scheme which represents the interactions considered here between the agonist (A), the receptor (R), and the effector (E) (calcium channels in this case):



where A is the muscarinic agonist, B is the "pure" muscarinic antagonist (e.g., QNB, atropine), C is the antagonist of the 2-haloalkylamine type, R is the muscarinic receptor, E is the active form of calcium channel effector, E' is the desensitized effector, K_A is the equilibrium dissociation constant for the agonist-receptor complex, and K_E is the equilibrium dissociation constant for agonist-receptor-effector complex.

This model involves the formation of a ternary complex between the agonist-receptor complex and the effector, and is in general, and in part, similar to what has recently been presented for the muscarinic receptor (30) and for the *beta*-adrenergic receptor-adenylate cyclase system (31). Results from this study and others (21, 26) on the muscarinic receptors of mouse neuroblastoma clone N1E-115 are compatible with this model. Most recently (26), we presented evidence that Ca^{2+} and lanthanides (e.g., Tb^{3+}) increase the efficacy of agonists, and we postulated that this could be due to a higher affinity of the agonist-receptor complex for the effector. If calcium promotes increased efficacy of receptor-mediated cyclic GMP responses, this might account for the decreased potency of these alkylating agents and the change in the Hill coefficient of cyclic GMP dose response curves in high Ca^{2+} concentrations. Alternatively, calcium might have an effect on the rate of alkylation by these irreversible antagonists.

In our model, only agonists are capable of promoting the formation of the ternary complex, $A \cdot R \cdot E$, between the ligand, the receptor, and the effector, whereas antagonists such as QNB or atropine form only a binary complex with the receptor. Calcium and lanthanides (e.g., Tb^{3+}) act to promote the formation of the ternary complex involving the agonist, perhaps as allosteric activators. Partial agonists easily form the binary complex with the receptor, but this binary complex has less affinity for E than the binary complex formed by full agonists except in the presence of an activator (high Ca^{2+} or lanthanide concentration) (26). Once the ternary complex is formed, the biological response occurs; next follows desensitization (20), which results from an inactivation of the effector (21). Resensitization involves the recovery of the effector from its desensitized state, E', to its active state, E, in conformance with a cyclic model.[3]

Antagonists of the 2-haloalkylamine type interact both at the receptor (R) and at the effector (E), and their interaction with the effector may obviate their use to

[3] X. Liu and E. Richelson, manuscript in preparation.

calculate agonist dissociation constants from biological experiments. In conclusion, we think one should be very careful about interpreting data obtained when using 2-halogenoethylamines as antagonists for the differentiation of receptors, the establishment of the presence of spare receptors, or the determination of the agonist-receptor equilibrium dissociation constant.

REFERENCES

1. Kuhman-Clausen, D. A novel approach to the questions of allosteric properties or a 'receptor reserve' of drug binding sites of intestinal smooth muscle cells. *F. E. B. S. Lett.* **39**:61–66 (1974).
2. Morgenstern, R., and R. Winter. The allosteric influence of β-haloalkylamines on the acetylcholine receptor of smooth muscle. *Acta Biol. Med. Germ.* **34**: K1–K5 (1975).
3. Moran, J. F., and D. J. Triggle. Approaches to the quantitation and isolation of pharmacological receptors, in *Fundamental Concepts in Drug-Receptor Interactions* (J. F. Danielli, J. F. Moran, and D. J. Triggle, eds.). Academic Press, New York and London, 133–176 (1970).
4. Swamy, V. C., and D. J. Triggle. 2-Halogenoethylamines and the role of Ca^{2+} in adrenergic α-receptor activation in the rat vas deferens. *Eur. J. Pharmacol.* **19**:67–78 (1972).
5. Ogino, K. The irreversible inhibitory effects of phenoxybenzamine on the noradrenaline-, K-, and Ba-induced contractions of rat's isolated vas deferens, particularly in relation to Ca. *Kobe J. Med. Sci.* **22**:153–160 (1976).
6. Kenakin, T. P., and D. A. Cook. The effect of desensitization on the antagonism of the histamine response by phenoxybenzamine. *Mol. Pharmacol.* **17**: 309–313 (1980).
7. Liao, C.-S., S.-H. Lin, C.-W. Su, and C.-Y. Su. External calcium ions and the inhibitory action of dibenamine and phenoxybenzamine. *J. Formosan Med. Assoc.* **78**:706–714 (1979).
8. Walton, K., P. Liepmann, and R. J. Baldessarini. Inhibition of dopamine-stimulated adenylate cyclase activity by phenoxybenzamine. *Eur. J. Pharmacol.* **52**:231–234 (1978).
9. Kuraishi, Y., Y. Harada, M. Satoh, and H. Takagi. Antagonism by phenoxybenzamine of the analgesic effect of morphine injected into the nucleus reticularis gigantocellularis of the rat. *Neuropharmacology* **18**:107–110 (1979).
10. Singh, G. S. Inhibition of calcium induced systolic contracture of the perfused frog's heart by phenoxybenzamine. *Indian J. Physiol. Pharmacol.* **18**:372–373 (1974).
11. Shibata, S., and O. Carrier, Jr. Antagonizing action of chlorpromazine, dibenamine, and phenoxybenzamine on potassium-induced contraction. *Can. J. Physiol. Pharmacol.* **45**:587–596 (1967).
12. Honegger, P., and E. Richelson. Biochemical differentiation of mechanically dissociated mammalian brain in aggregating cell cultures. *Brain Res.* **109**: 335–354 (1976).
13. El-Fakahany, E., and E. Richelson. Regulation of muscarinic receptor-mediated cyclic GMP synthesis by cultured mouse neuroblastoma cells. *J. Neurochem.* **35**:941–948 (1980).
14. Richelson, E., F. G. Prendergast, and S. Divinetz-Romero. Muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells—ionic dependence and effects of local anesthetics. *Biochem. Pharmacol.* **27**:2039–2048 (1978).
15. Waud, D. R., and R. B. Parker. Pharmacological estimation of drug-receptor dissociation constants: statistical evaluation. II. Competitive antagonists. *J. Pharmacol. Exp. Ther.* **177**:13–24 (1971).
16. Lowry, O., N. J. Rosebrough, L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
17. Matsumoto, M., K. Sasaki, K. Somei, and M. Sato. Dose-inhibition curve and its application to the analysis of Ach-receptor activity. *Jpn. J. Physiol.* **30**: 743–750 (1980).
18. Furchgott, R. F. The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. *Adv. Drug Res.* **3**:21–55 (1966).
19. Takeyasu, K., S. Uchida, A. Wada, M. Maruno, R. T. Lai, F. Hata, and H. Yoshida. Experimental evidence and dynamic aspects of spare receptors. *Life Sci.* **25**:1761–1772 (1979).
20. El-Fakahany, E., and E. Richelson. Temperature dependence of muscarinic acetylcholine receptor activation, desensitization and resensitization. *J. Neurochem.* **34**:1288–1295 (1980).
21. El-Fakahany, E., and E. Richelson. Involvement of calcium channels in short-term desensitization of muscarinic receptor-mediated cyclic GMP formation in mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **77**:6897–6901 (1980).
22. Study, R., X. O. Breakefield, T. Bartfai, and P. Greengard. Voltage-sensitive calcium channels regulate guanosine 3′,5′-cyclic monophosphate levels in neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:6295–6299 (1978).
23. Rosenberger, L. B., M. K. Ticku, and D. J. Triggle. The effects of Ca^{2+} antagonists on mechanical responses and CA^{2+} movements in guinea pig ileal longitudinal smooth muscle. *Can. J. Physiol. Pharmacol.* **57**:333–347 (1979).
24. Nwaki, N., C. S. Lin, and V. C. Swamy. Interaction of D600 with phenoxybenzamine-induced adrenergic blockade. *Eur. J. Pharmacol.* **62**:205–207 (1980).
25. Rosenberger, L., and D. J. Triggle. Calcium translocation and specific calcium antagonists, in *Calcium and Drug Action* (G. B. Weiss, ed.). Plenum Publishing Corporation, New York, 3 (1978).
26. El-Fakahany, E., and E. Richelson. Effects of lanthanides on muscarinic acetylcholine receptor function. *Mol. Pharmacol.* **19**:282–290 (1981).
27. Jafferji, S. S., and R. H. Michell. Effects of calcium-antagonistic drugs on the stimulation by carbamylcholine and histamine of phosphatidylinositol turnover in longitudinal smooth muscle of guinea-pig ileum. *Biochem. J.* **160**: 163–169 (1976).
28. Strange, P. G, N. J. M. Birdsall, and A. S. V. Burgen. Ligand-binding properties of the muscarinic acetylcholine receptor in mouse neuroblastoma cells. *Biochem. J.* **172**:495–501 (1978).
29. Boeynaems, J. M., and J. E. Dumont. The two-step model of ligand-receptor interaction. *Mol. Cell. Endocrinol.* **7**:33–47 (1977).
30. Birdsall, N. J. M., C. P. Berrie, A. S. V. Burgen, and E. C. Hulme. Modulation of the binding properties of muscarinic receptors: evidence for receptor-effector coupling, in *Receptors for Neurotransmitters and Peptides* (G. Pepeu, M. J. Kuhar, and S. J. Enna, eds.). Raven Press, New York, 107–116 (1980).
31. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).

Send reprint requests to: Dr. Elliott Richelson, Departments of Psychiatry and Pharmacology, Mayo Foundation, Rochester, Minn. 55905.