MOLECULAR PHARMACOLOGY, 19:282-290

Effects of Lanthanides on Muscarinic Acetylcholine Receptor Function

ESAM EL-FAKAHANY AND ELLIOTT RICHELSON

Departments of Psychiatry and Pharmacology, Mayo Foundation, Rochester, Minnesota 55901

Received June 23, 1980; Accepted October 20, 1980

SUMMARY

EL-FAKAHANY, E., AND E. RICHELSON. Effects of lanthanides on muscarinic acetylcholine receptor function. *Mol. Pharmacol.* 19:282-290 (1981).

Incubation of mouse neuroblastoma cells (clone N1E-115) with the lanthanides terbium, europium, neodymium, and lanthanum resulted in a concentration-dependent reduction in the maximal cyclic GMP response to full agonists of the muscarinic acetylcholine receptor, and an increase in the response to partial agonists. At 1 mM concentration these lanthanides also caused a significant decrease in the EC_{50} of the agonists, which was accompanied by a significant reduction in the binding affinity of the ^3H-labeled antagonist quinuclidinyl benzilate (QNB), as determined for both mouse neuroblastoma cells and rat brain homogenates. However, this concentration of the lanthanides did not cause a significant change in the agonist equilibrium dissociation constant. The rank order of potency of the lanthanides for their effects on the EC_{50} for agonists and on the affinity of the antagonist was $Tb^{3+} > Eu^{3+} > Nd^{3+} > La^{3+}$. Calcium ions mimicked the effects of lanthanides on the EC_{50} but had opposite effects on maximal responses. In addition, high calcium concentrations decreased the effects of Tb^{3+} on the EC_{50} and on [^3H]QNB binding with no change in their effect on maximal cyclic GMP responses. We conclude that these lanthanides increase the efficacy of agonists, probably by interacting with Ca^{2+} binding sites on a factor which couples the receptor to the calcium channels; and that the lanthanides reduce maximal cyclic GMP responses to agonists by interacting with the calcium channels.

INTRODUCTION

Activation of muscarinic acetylcholine receptors of mouse neuroblastoma clone N1E-115 cells (1) in the stationary phase of growth leads to the elevation of intracellular cyclic GMP levels (2, 3) and to a hyperpolarization of the cell membrane (4). The activation of muscarinic receptor-coupled calcium channels probably is responsible for transmitting the signal from the agonist-activated receptor to the uncoupled guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.] (5), which is present mostly in the cytoplasm of these cells (6). This receptor-mediated cyclic GMP response is rapidly regulated by a desensitization process (7) which is markedly temperature-dependent (8), reversible, and appears to involve the inactivation of calcium channels (5). This rapid desensitization is followed by a loss of muscarinic receptor binding sites only with prolonged (>1 hr) incubation of cells with agonist (9).

Muscarinic receptor-mediated cyclic GMP formation is also regulated by these cells with respect to cell division (10). Thus, log phase cells do not respond to receptor activation despite the presence of the same number of receptors as are found for stationary phase cells. In part, this lack of response is related to the low levels of guanylate cyclase in log phase cells, but data suggest that the development of functional muscarinic receptors is dependent upon other components of the system which are yet to be defined fully.

Because of the role of calcium in the receptor-mediated response and the fact that lanthanide ions have very high affinity for superficial calcium binding sites in various biological systems (for review see ref. 11), we investigated the effects of some lanthanides, namely, terbium, europium, neodymium, and lanthanum, on muscarinic receptor-mediated cyclic GMP formation and the binding properties of these receptors. This paper presents evidence that the lanthanides interact at a coupling site between the muscarinic receptor and calcium channels and at calcium channels.

MATERIALS AND METHODS

Cell culture conditions. Mouse neuroblastoma clone N1E-115 cells (subculture 9-18) were grown in tissue culture flasks (75 cm^2/250 ml; Falcon Plastics, Cockeysville, Md.), in 20 ml of Dulbecco's modified Eagle's medium (Grand Island Biological Company, Grand Island, N. Y., catalogue no. 430-2100) supplemented with 10%

This work was supported by the Mayo Foundation and by United States Public Health Service Grant MH 27692.

0026-895X/81/020282-09$02.00/0

(v/v) newborn calf serum (Grand Island Biological Company) (Medium I). The cells were incubated at 37° in an atmosphere consisting of 10% CO_2 and 90% humidified air. Subculture was achieved by incubation of cells 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 days 3 and 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, and the experiments were carried out after at least 12 days from subculture (10). Cells were negative for *Mycoplasma* (pleuropneumonia-like organisms) by bacteriological criteria.

Assay of muscarinic receptor-mediated cyclic GMP formation. The details of assaying the relative changes in cyclic GMP formation using a radioactively labeled precursor and intact cells have been described elsewhere (3). 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 Hepes,[1] 20 (phosphate was omitted to prevent precipitation of the lanthanides as their phosphates). The pH was adjusted to 6.8 (to avoid precipitation of the lanthanides as hydroxides), 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 (1 μM 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, FB16-24 TC, Bellco Glass, Vineland, N. J.) in 240-μl aliquots. 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 agonists were 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 were 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 GTP. 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 80–85%, 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 and with rat brain homogenates. The assay procedure for the muscarinic receptor antagonist [^{3}H]QNB binding to intact mouse neuroblastoma cells and to crude rat brain homogenates was a modification of the procedure reported by Yamamura and Snyder (13). Mouse neuroblastoma cells were harvested as described above, suspended in Medium III (pH 6.8), and then frozen and thawed once (a procedure which reduced nonspecific binding to cells). Rat brain homogenates were prepared by homogenizing whole rat brains in 20 volumes of Medium III (Polytron, setting 5 for 15 sec; Brinkmann Instruments, Rexdale, Ontario, Canada), centrifuged at $10{,}000 \times g$ for 10 min at 5° (Sorvall RC-5B centrifuge; DuPont Instruments, Newtown, Conn.), and resuspended in Medium III to give approximately 1% (w/v) suspension. For the binding assay, the tissue was incubated for 45 min at 37° in 2 ml of Medium III containing (−)-[^{3}H]QNB (40.2 Ci/mmole) (0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 nM final concentration in duplicate), with or without 10^{-7} M dexetimide, in the presence or absence of the lanthanides. 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. The amount of specific binding was obtained by subtracting the amount bound in the presence of dexetimide (nonspecific binding) from the amount bound in its absence (total binding). Nonspecific binding was a linear function of the ligand concentration. In general, duplicates varied from their means by less than 10%.

Protein assay and cell counts. Protein was determined by a modification of the procedure reported by Lowry *et al.* (14), 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, acetylcholine chloride, methacholine chloride, bethanechol, Hepes, lanthanum chloride, europium chloride, and neodymium chloride were obtained from Sigma Chemical Company, St. Louis, Mo. Terbium chloride was purchased from Ventron Corporation, Alfa Division, Danvers, Mass., and oxotremorine from Aldrich Chemical Company, Milwaukee, Wisc. [^{3}H]Guanine and ^{14}C-labeled cyclic GMP were obtained 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 lanthanides on muscarinic receptor-mediated cyclic GMP formation. While screening the effects

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QNB, quinuclidinyl benzilate.

of various calcium channel blockers on muscarinic receptor-mediated cyclic GMP formation by mouse neuroblastoma N1E-115 cells, we found that the lanthanide ions Tb^{3+} (Fig. 1), La^{3+} (Fig. 2A), Eu^{3+} (Fig. 2B), and Nd^{3+} (data not shown) caused a concentration-dependent reduction in the maximal cyclic GMP response to the full agonist carbamylcholine. In addition, there was a leftward shift of the agonist concentration-cyclic GMP response curve with a decrease in the EC_{50} (concentration producing half-maximal response) of carbamylcholine. For the different lanthanides, 1 mM was sufficient for the maximal effect on the EC_{50} of the agonist, whereas the effect on the maximal response increased by increasing the concentration of the lanthanides above 1 mM. This reduction in EC_{50} was reproducible, although its magnitude varied among different experiments. The rank order of potency for lowering the EC_{50} was $Tb^{3+} > Eu^{3+} > Nd^{3+} > La^{3+}$ (Table 1). There was no correlation between the magnitude of the shift in the EC_{50} and the reduction of the maximal response for the various lanthanides (Table 1), or for Tb^{3+} in 16 independent experiments ($r = 0.22$) (data not shown). In addition, raising the external calcium concentration also decreased the EC_{50}, and in high Ca^{2+} the effect of Tb^{3+} on carbamylcholine's EC_{50} was reduced with no change in the effect of Tb^{3+} on the maximal response (Fig. 1 and Table 2).

When acetylcholine or methacholine, both of which also act as full agonists with these cells, were used to stimulate cyclic GMP formation, we found that 1 mM Tb^{3+} had effects similar to those obtained when carbamylcholine was used as agonist (Fig. 3A and 3B). Terbium's effects persisted in the presence of the anticholinesterase agent physostigmine, indicating that the effects were not due to an inhibition of cholinesterase (Fig. 3A and 3B). However, the effects of lanthanides were more profound in the case of the partial agonist bethanechol (Fig. 3C). In some experiments, 1 mM Tb^{3+} caused an increase in the maximal response to bethanechol, making it almost equal to that of full agonists (data not shown). Interestingly, oxotremorine did not elicit any significant cyclic GMP response at concentrations up to 1 mM in control preparations, but did increase cyclic GMP levels in cells pretreated with 1 mM Tb^{3+} (Fig. 3D). In addition, we found that oxotremorine was a competitive antagonist at the muscarinic receptor of these cells as determined with the cyclic GMP assay, with an equilibrium dissociation constant of approximately 0.2 μM.[2]

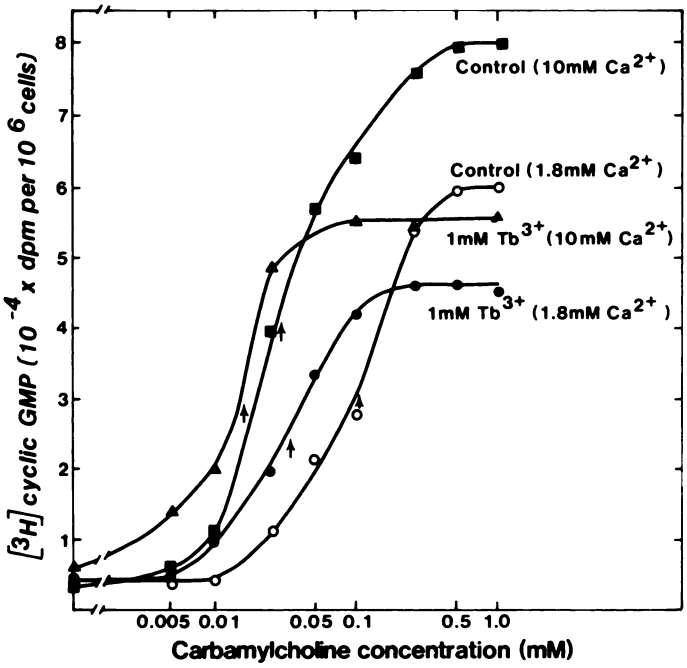

FIG. 1. *Effects of Tb^{3+} and high Ca^{2+} concentration on carbamylcholine-stimulated cyclic GMP formation by mouse neuroblastoma cells*

Clone N1E-115 cells (subculture 14, 12 days after subculture) were prepared for assay as described under Materials and Methods. The cells were suspended in Medium III containing 1.8 mM Ca^{2+} (○), 1.8 mM Ca^{+2} + 1 mM Tb^{3+} (●), 10 mM Ca^{2+} (■), or 10 mM Ca^{2+} + 1 mM Tb^{3+} (▲), incubated at 37° for 15 min, and then exposed to the indicated concentrations of carbamylcholine for 30 sec in duplicate. Each assay contained ~1 × 10^5 cells. The data shown are representative results from one of three independent experiments. The *vertical arrows* denote the EC_{50} for carbamylcholine.

Effect of lanthanides on binding of the muscarinic antagonist QNB. Since the EC_{50} effect of the lanthanides on the dose-response curves for muscarinic agonists could be due to an increase in the affinity of the muscarinic receptor sites, we studied the effects of these ions on the binding of the muscarinic receptor ligand (−)-[^{3}H]QNB. With intact mouse neuroblastoma N1E-115 cells, we found that the lanthanides decreased the apparent binding affinity of (−)-[^{3}H]QNB for the receptors with the following order of potency: $Tb^{3+} > Eu^{3+} > Nd^{3+} > La^{3+}$ (Fig. 4A and 4B; Table 3). We also found that all of these lanthanides caused an increase in the maximal binding capacity. In addition, Tb^{3+} increased to a similar extent the maximal binding and the K_D for [^{3}H]QNB and the mouse neuroblastoma receptor in a centrifugation binding assay (data not shown), ruling out the possibility that these changes resulted from an artifact of the filtration binding technique.

Similarly, the lanthanides decreased the apparent binding affinity of (−)-[^{3}H]QNB for rat brain homogenates (Fig. 5A and 5B; Table 4); however, the effect on maximal binding was absent. In rat brain homogenates, Tb^{3+} displaced the specific binding of 0.1 nM (−)-[^{3}H]QNB with an average (± standard error of the mean) IC_{50} of 0.88 ± 0.18 mM ($n = 4$). The guanine nucleotides GTP and guanyl-5-yl-imidodiphosphate [Gpp(NH)p] (10 μM) were not able to antagonize the displacement of (−)-[^{3}H]QNB binding by Tb^{3+} (data not

[2] E. Richelson, unpublished data.

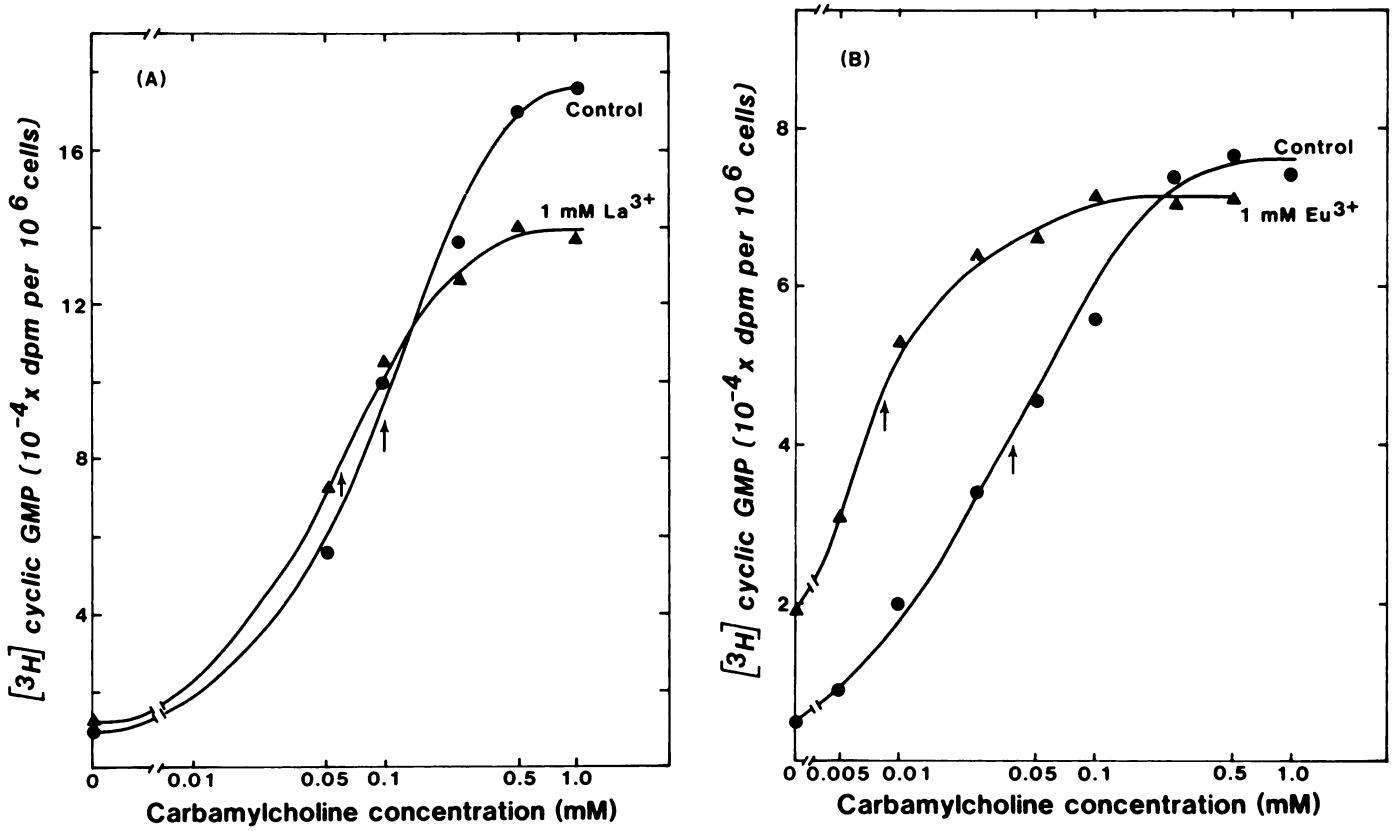

FIG. 2. *Effect of La^{3+} and Eu^{3+} on carbamylcholine-stimulated cyclic GMP formation by mouse neuroblastoma cells*

Clone N1E-115 cells were prepared for assay as described above, incubated in the absence (●) or presence (▲) of 1 mM La^{3+} (A) or Eu^{3+} (B) for 15 min and then stimulated with the indicated concentrations of carbamylcholine for 30 sec in duplicate. The *vertical arrows* denote the EC_{50} for carbamylcholine.

A. Subculture 13, 16 days after subculture. Each assay contained ~1.2 × 10^5 cells and the data are representative results from one of three experiments.

B. Subculture 15, 15 days after subculture. Each assay contained ~0.8 × 10^5 cells and the data are representative results from one of three experiments.

shown). However, increasing the calcium concentration resulted in an increase in the IC_{50} of Tb^{3+} (Fig. 6). In addition, changing the calcium concentration in the range from 1.8 to 50 mM did not result in any significant change in (−)-[^{3}H]QNB binding (see legend to Fig. 6).

In the presence of 1 mM Tb^{3+}, QNB was less potent at blocking the muscarinic receptor-mediated cyclic GMP response by mouse neuroblastoma cells (Fig. 7). In addition, we found that this concentration of Tb^{3+} approximately doubled the apparent equilibrium dissociation constant of QNB (from 70 pM to 130 pM), which was determined with the use of the cyclic GMP assay at concentrations of QNB that caused a parallel shift in the dose-response curves for carbamylcholine (data not shown). The decreased ability of QNB to antagonize the response to carbamylcholine in the presence of Tb^{3+} is consistent with the binding data showing a decreased affinity of QNB for the receptors.

Effect of lanthanides on agonist equilibrium dissociation constant. The equilibrium dissociation constant for carbamylcholine-stimulated cyclic GMP formation was

TABLE 1

Effect of lanthanides on EC_{50} and maximal responses of carbamylcholine-stimulated cyclic GMP formation in mouse neuroblastoma cells

The numbers in parentheses denote the number of independent observations. The data are presented as means ± standard error of the mean.

	% Control EC_{50}	% Control maximal cyclic GMP response
1 mM Tb^{3+}	26 ± 3 (16)	65 ± 3 (16)
1 mM Eu^{3+}	31 ± 6 (3)	62 ± 4 (3)
1 mM La^{3+}	64 ± 13 (3)	74 ± 3 (3)
1 mM Nd^{3+}	85 ± 9 (4)	58 ± 2 (4)

TABLE 2

High calcium concentrations and the effect of 1 mM Tb^{3+} on EC_{50} and maximal responses of carbamylcholine-stimulated cyclic GMP formation in mouse neuroblastoma cells

Data are from experiments in which the effect of 1 mM Tb^{3+} was determined at both calcium concentrations simultaneously. The numbers in parentheses denote the number of independent observations. The data are presented as means ± standard error of the mean.

% Control EC_{50}		% Control maximal cyclic GMP response	
1.8 mM Ca^{2+}	10 mM Ca^{2+}	1.8 mM Ca^{2+}	10 mM Ca^{2+}
16 ± 7 (3)	41 ± 3 (3)[a]	59 ± 6 (3)	54 ± 7 (3)[b]

[a] Significantly diferent from the effect in 1.8 mM Ca^{2+}: $p < 0.01$.
[b] Not significantly different from the effect in 1.8 mM Ca^{2+}: $p > 0.05$.

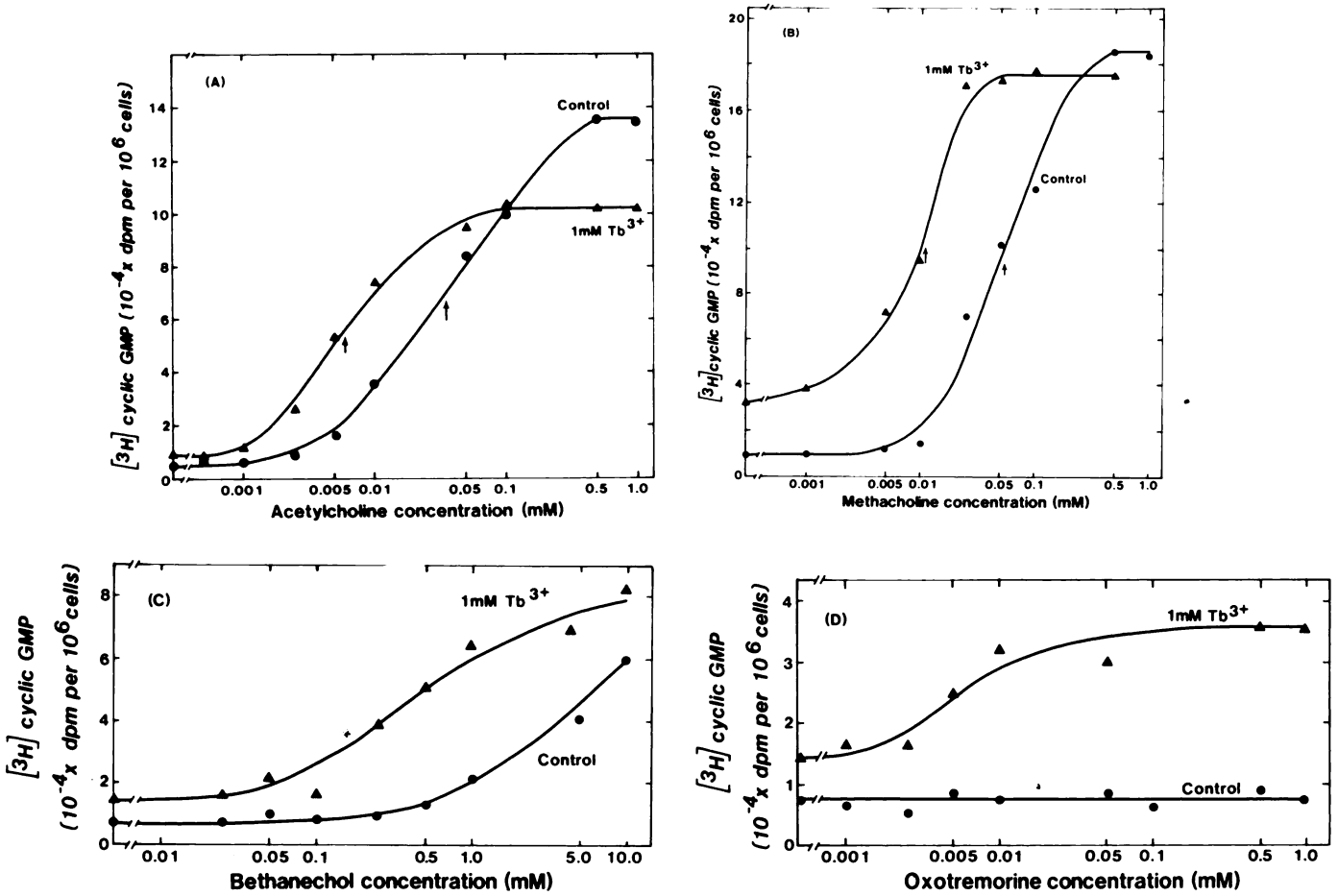

FIG. 3. *Effect of Tb^{3+} on cyclic GMP response to different muscarinic receptor ligands*

Mouse neuroblastoma cells (clone N1E-115) were prepared for assay as described above, incubated in Medium III in the absence (●) or presence (▲) of 1 mM Tb^{3+} for 15 min, and then stimulated in duplicate with the indicated concentrations of acetylcholine (A), methacholine (B), bethanechol (C), or oxotremorine (D) for 30 sec. In A and B, 0.1 mM physostigmine was present during the incubation. Data are representative results from one of three independent experiments for A, B, C, and D. The *vertical arrows* denote the EC_{50} for the agonists.

A and C. Subculture 15, 14 days after subculture, ~0.8×10^5 cells/assay.

B. Subculture 14, 15 days after subculture, ~0.8×10^5 cells/assay.

D. Subculture 16, 19 days after subculture, ~1×10^5 cells/assay.

determined according to the technique described by Furchgott (15) with the use of the irreversible receptor antagonist dibenamine [*N*-(2-chloroethyl)dibenzylate]. Control cells (no terbium) incubated with increasing concentrations of dibenamine for 15 min first showed a parallel shift of the agonist concentration-cyclic GMP response curve to the right, followed by, at higher concentrations of dibenamine, a progressive reduction in the maximal cyclic GMP response and a progressive increase in the EC_{50} for carbamylcholine (data not shown). The maximal response to carbamylcholine was reduced approximately 50% by incubation of cells with 10 μM dibenamine for 15 min (Fig. 8). From such experiments, we derived for carbamylcholine an equilibrium dissociation constant of 280 ± 180 μM (± SEM, $n = 8$). Since in these experiments the EC_{50} was 46 ± 8 μM, this half-maximal response to carbamylcholine occurred at 15% receptor occupancy, indicating that spare receptors were present for the carbamylcholine-cyclic GMP response.

In the presence of 1 mM Tb^{3+}, cells treated with 10 μM dibenamine for 15 min showed only a slight shift of the concentration-response curve to the right (Fig. 8), with little or no reduction in the maximal response. At 25 μM dibenamine, a further parallel shift of the curve was seen, without a reduction of the maximum, whereas higher concentrations of dibenamine caused a progressive reduction in the maximal response, accompanied by an increase in the EC_{50} (data not shown). These data suggest that Tb^{3+} may protect the receptors against inactivation by dibenamine, probably by a mechanism similar to that for the effect of Tb^{3+} on QNB affinity.

The equilibrium dissociation constant for carbamylcholine in the presence of 1 mM Tb^{3+} was 156 ± 50 μM ($n = 4$), a value which was not significantly different ($p > 0.3$) from that obtained in control preparations (see above) and which cannot account for the shift of the dose-response curve caused by Tb^{3+}. However, one should be cautious in interpreting these data because of the large variation in the values of the agonist equilibrium dissociation constant. When we used phenoxybenzamine for similar experiments, there was also a large scattering in the values of the dissociation constant. We also found that dibenamine essentially decreased the maximal binding of [^{3}H]QNB to mouse neuroblastoma cells in a noncompetitive and irreversible manner and that there was a marked difference between the concen-

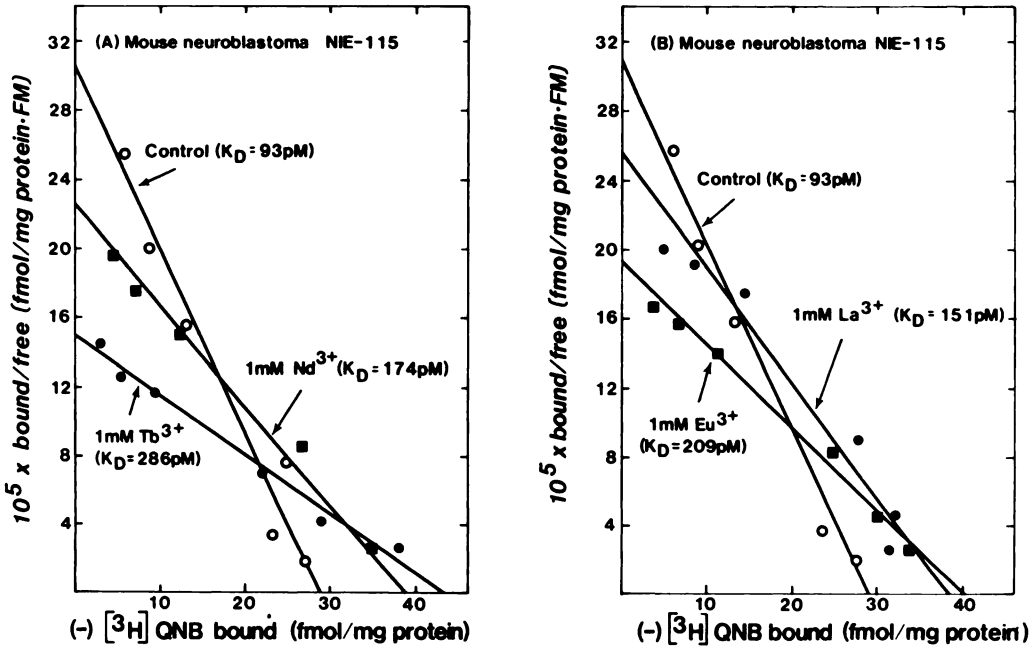

FIG. 4. *Effects of lanthanides on (–)-[^{3}H]QNB binding to intact mouse neuroblastoma cells (clone N1E-115): Scatchard analyses*
The binding assays were carried out as described under Materials and Methods. Each assay tube contained 0.48 mg of protein. The lines are the best fit as determined by linear regression analysis. Data in A and B are from the same experiment but are presented separately for increased clarity.

trations of dibenamine and phenoxybenzamine that inhibited by 50% the maximal [^{3}H]QNB binding and maximal carbamylcholine-stimulated cyclic GMP formation.[3]

DISCUSSION

The lanthanide ions have been useful agents for studying biological responses which are dependent on calcium influx through cellular membranes (16). The muscarinic receptor-mediated cyclic GMP response in mouse neuroblastoma N1E-115 cells is calcium-dependent (3) and likely involves the activation of calcium channels (5). In the present work, we used the lanthanides to study muscarinic receptor function and found that at 1 mM they caused approximately 25% reduction of the maximal cyclic GMP response to carbamylcholine, although 2 mM La^{3+} was reported to abolish completely the calcium currents in these same cells (17). Such a discrepancy has also been shown for the lanthanide inhibition of ^{45}Ca uptake by vascular smooth muscle, an effect which did not correlate clearly with lanthanide's ability to block tension responses to agents that increased calcium influx (18). Thus, it cannot be assumed that all responses dependent upon calcium uptake are susceptible to, or even accessible to, lanthanide ions to the same degree. In addition, more than one type of calcium channel (19) or calcium binding site may exist.

We found it very interesting to observe that for full agonists the lanthanides shifted the agonist concentration-cyclic GMP response curve to the left with a concomitant decrease in the agonist EC_{50} and that for partial agonists they increased their efficacy. These observations could reflect an increase in the number or affinity of the muscarinic receptor binding sites or an increase in intrinsic efficacy of the agonist-receptor complex due to improved coupling between the receptor and calcium channel, or to decreasing the rate of desensitization during exposure to the agonist, a process that we believe is due to inactivation of calcium channels (5). However, we found no evidence for a change in the equilibrium dissociation constant for carbamylcholine in the presence of Tb^{3+}, and in addition the lanthanides caused an apparent decrease in the binding affinity of [^{3}H]QNB for intact mouse neuroblastoma cells and for rat brain homogenates. We also found that 1 mM Tb^{3+} had no effect on desensitization produced by carbamylcholine or on resensitization of cyclic GMP responses.[4]

The binding data with mouse neuroblastoma cells was corroborated by pharmacological studies showing a decreased potency of QNB in the presence of Tb^{3+}. There was a good correlation between the effect of the lanthanides on [^{3}H]QNB binding in both mouse neuroblastoma cells and rat brain homogenates (r = 0.954). However, the lanthanides significantly increased the maximal binding capacity in the former but not in the latter. Perhaps this difference reflects the degree of coupling of the receptor to an effector (e.g., calcium channel) which would be greater in intact cells. In fact, homogenized cells did not show as great an increase in B_{max} caused by Tb^{3+} as did intact cells (data not shown). However, this modest increase in receptor number cannot explain the shift of the concentration-cyclic GMP response curves alone.

The effects of terbium and probably all of the lanthanides on the EC_{50} for agonists and on [^{3}H]QNB binding are probably due to an interaction with some calcium binding site, since these effects were reduced by high

[3] E. El-Fakahany and E. Richelson, manuscript in preparation.

[4] E. El-Fakahany and E. Richelson, unpublished data.

TABLE 3

Effects of lanthanides on (−)-[^{3}H]QNB binding to mouse neuroblastoma N1E-115 cells

The numbers in parentheses denote the number of independent observations. The data are presented as means ± standard error of the mean.

Condition	B_{max}	K_D
	fmoles/mg protein	*pM*
Control	29 ± 2 (6)	88 ± 3 (6)
+1 mM Tb^{3+}	41 ± 2 (6)[a]	278 ± 31 (6)[a]
+1 mM La^{3+}	37 ± 1 (4)[b]	140 ± 8 (4)[a]
+1 mMNd^{3+}	38 ± 2 (4)[c]	150 ± 19 (4)[c]
+1 mM Eu^{3+}	40 ± 2 (4)[c]	188 ± 15 (4)[a]

[a] Significantly different from control: $p < 0.0005$.
[b] Significantly different from control: $p < 0.005$.
[c] Significantly different from control: $p < 0.0025$.

TABLE 4

Effects of lanthanides on (−) [^{3}H]QNB binding to rat brain homogenates

The numbers in parentheses denote the number of independent observations. The data are presented as means ± standard error of the mean.

Condition	B_{max}	K_D
	pmoles/mg protein	
Control	1.1 ± 0.1 (6)	93 ± 6 (6)
+ 1 mM Tb^{3+}	1.2 ± 0.1 (4)	282 ± 28 (4)[a]
+1 mM La^{3+}	1.2 ± 0.1 (5)	171 ± 18 (5)[b]
+1 mM Nd^{3+}	1.2 ± 0.1 (5)	225 ± 23 (5)[a]
+1 mM Eu^{3+}	1.2 ± 0.1 (5)	252 ± 22 (5)[a]

[a] Significantly different from control: $p < 0.0005$.
[b] Significantly different from control: $p < 0.0025$.

calcium concentrations. On the other hand, the effects of the lanthanide on [^{3}H]QNB binding were not reduced in the presence of guanine nucleotides which have been reported to antagonize the inhibitory effects of calcium and manganese on ligand binding to brain *alpha*$_2$-noradrenergic receptors (20). The rank orders of potency of the lanthanides for their effects on the EC_{50} for agonists and [^{3}H]QNB binding were essentially identical, suggesting a common site of action. In addition, these rank orders correlate inversely with the ionic radius (A) of these ions: Tb^{3+}, 0.92; Eu^{3+}, 0.95; Nd^{3+}, 0.98; and La^{3+}, 1.03 (21). Thus, Tb^{3+} has the highest surface charge density. In contrast, calcium has an ionic radius of 1.0 A and only two positive charges.

Previous studies have shown that a wide range of cations decrease the affinity of rat brain muscarinic receptors for both agonists and antagonists, and that La^{3+} had greater effects on the affinity than could be accounted for by an increase in the ionic strength alone (22, 23). In addition, studies on the partially purified nicotinic

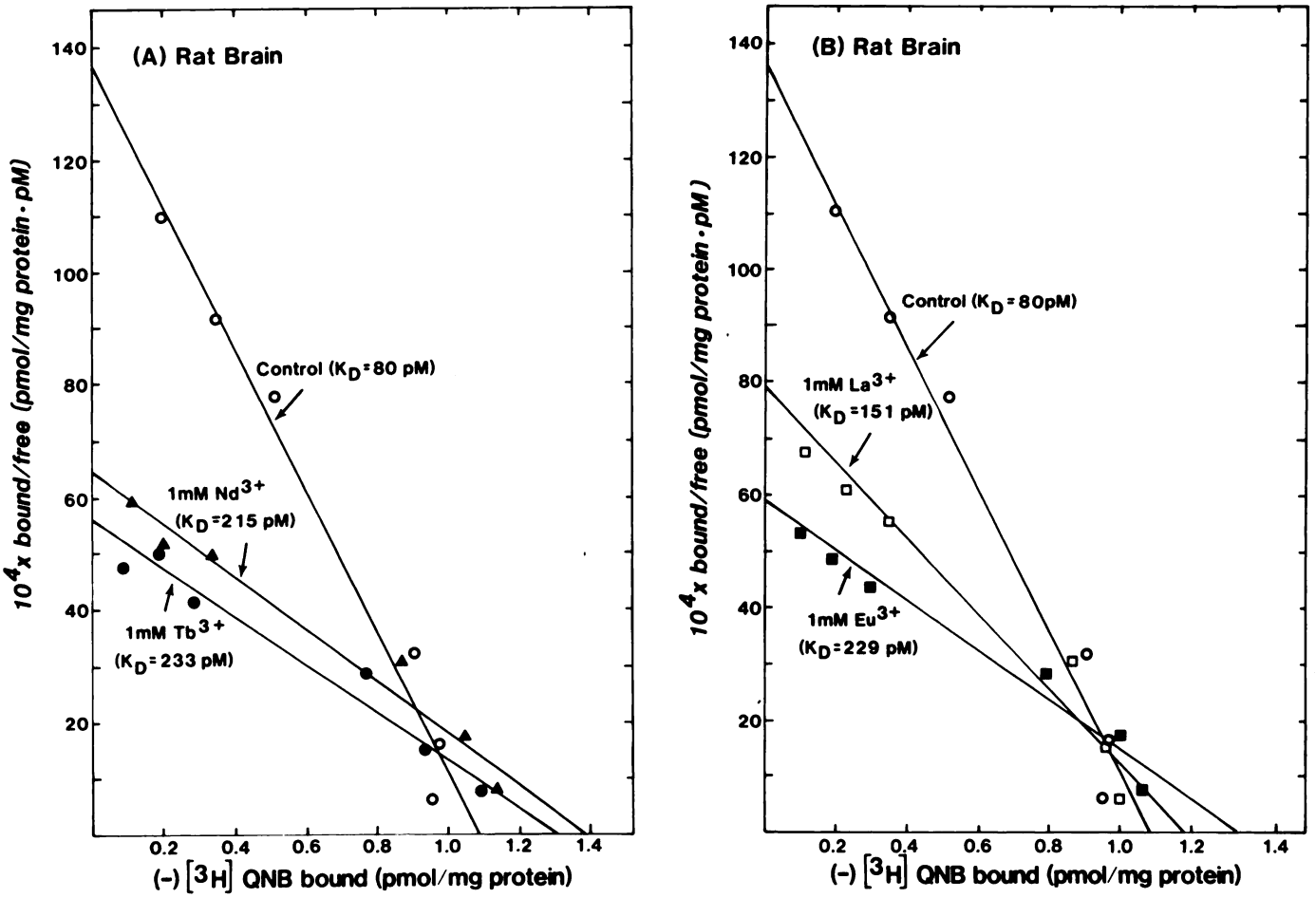

FIG. 5. *Effects of lanthanides on (−)-[^{3}H]QNB binding to rat brain homogenates: Scatchard analyses*

The binding assay was carried out as described under Materials and Methods with 0.059 mg of protein per assay. The lines were drawn using a linear regression analysis. Data in A and B are from the same experiment.

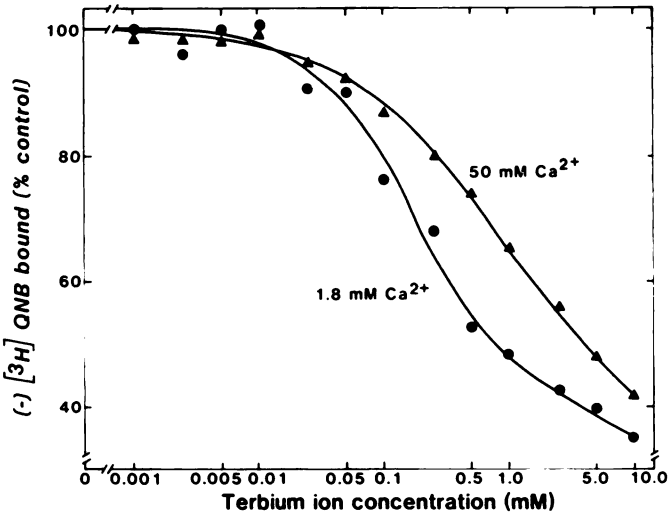

FIG. 6. *Effect of increased calcium concentration on the inhibition by Tb^{3+} of (−)-[^{3}H]QNB binding to rat brain homogenates*

The brain homogenate was prepared as described under Materials and Methods. The final concentration of (−)-[^{3}H]QNB was 0.1 nM in Medium III, containing 1.8 mM Ca^{2+} (●) or 50 mM Ca^{2+} (▲) and the indicated concentrations of Tb^{3+}. Each *point* is the mean of duplicate samples and control binding, the mean of six. Results are presented as percentage of specific binding, and nonspecific binding (determined in the presence of 1 μM atropine) was approximately 2% of total binding. Values for disintegrations per minute bound in the absence of Tb^{3+} were 4760 and 4370 in 1.8 and 50 mM Ca^{2+}, respectively. Each assay contained 0.085 mg of protein. The data are representative results from one of two experiments.

acetylcholine receptor from *Torpedo ocellata* have shown the presence of two types of terbium binding sites, one of which interacts with calcium and agonists of this receptor but not with antagonists (24).

The effect of calcium ions on antagonist binding to muscarinic receptors seems to be of some controversy. In our experiments, raising the calcium concentration failed to affect [^{3}H]QNB binding. However, it has been reported that calcium increases (25), decreases (23), or has no effect (26) on antagonist binding in rat brain.

Studies on the binding of radiolabeled ligands to muscarinic acetylcholine receptors of mammalian brain and smooth muscle suggest the existence of multiple receptor populations which differ in their affinities for receptor agonists but not for antagonists (27). Further support for this hypothesis is the finding that guanine nucleotides and sodium ions have differential effects on the binding affinities of agonists and antagonists (28–30). To investigate the effect of the most potent lanthanide, terbium, on the equilibrium dissociation constant of the muscarinic receptor agonist carbamylcholine, we used the cyclic GMP responses and the technique described by Furchgott (15). From radioligand binding studies, it has been shown that these cells have high- and low-affinity binding sites for agonists (31). The technique that we used to estimate the agonist equilibrium dissociation constant is able to measure this parameter only for the site that mediates the cyclic GMP response, presumably the low-affinity site of the binding studies. However, Tb^{3+} (1 mM) did not significantly affect this dissociation constant, indicating that it does not affect the affinity of the receptors for the agonist, at least at the low-affinity site.

The ability of high calcium concentrations to mimick the EC_{50} effect of the lanthanides suggests that the latter shift the concentration-response relationship to lower agonist concentrations by interacting with a calcium binding site. However, high calcium concentrations increased, whereas the lanthanides decreased, the maximal response to carbamylcholine. This observation, in addition to the lack of correlation between the magnitude of the shift in the EC_{50} by the different lanthanides and their effect on the maximal response, suggests that these two effects involve two different mechanisms. This hypothesis is supported by our finding that high calcium concentrations decrease the effect of Tb^{3+} on the EC_{50} with no change in its effect on the maximal response.

In some cases, lanthanide ions are able to substitute for calcium, e.g., in inhibiting catechol-*O*-methyltransferase (32), or in activating guanylate cyclase in mouse neuroblastoma cells (6). In other systems they potentiate calcium-mediated effects; e.g., low concentrations of La^{3+} increase the twitch response to electric stimulation and increase the end-plate responses to carbamylcholine in frog neuromuscular junction (33). The EC_{50} effects of lanthanides reported here are of the latter type (potentiating), which we believe is due to an enhancement of the coupling mechanism between the receptors and calcium channels. La^{3+} is known not to penetrate the cell

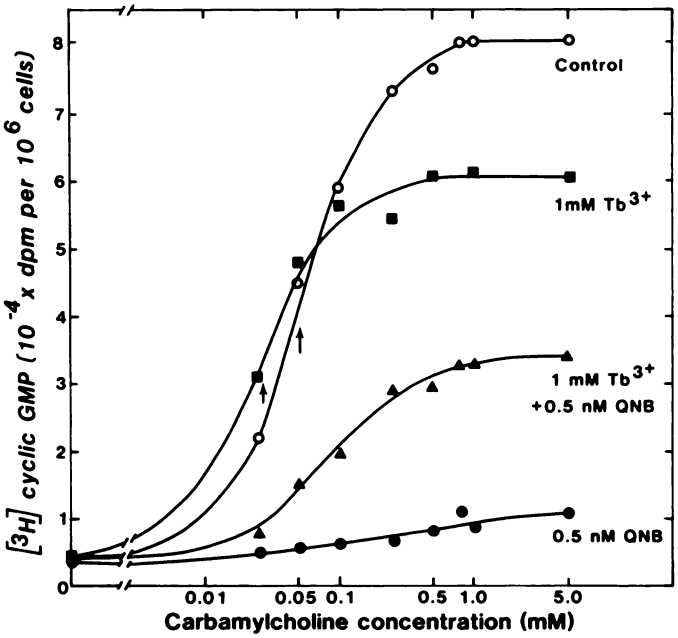

FIG. 7. *Effect of Tb^{3+} on QNB antagonism of carbamylcholine-mediated cyclic GMP formation by mouse neuroblastoma cells*

Clone N1E-115 cells (subculture 15, 13 days after subculture) were prepared for assay as described under Materials and Methods and incubated with Medium III alone (○) or with 0.5 nM QNB (●), 1 mM Tb^{3+} (■), or 1 mM Tb^{3+} + 0.5 nM QNB (▲) for 30 min. The cells were then stimulated with the indicated concentrations of carbamylcholine for 30 sec in duplicate. Each assay contained ~1 × 10^5 cells and the data are representative results from one of three experiments. The vertical arrows denote the EC_{50} for carbamylcholine. In control cells, QNB behaved like an unsurmountable antagonist, probably because the concentration used was approximately 7 times the equilibrium dissociation constant for QNB and because of the inability of carbamylcholine to displace QNB during the 30-sec incubation period of the agonist (pseudo-irreversible).

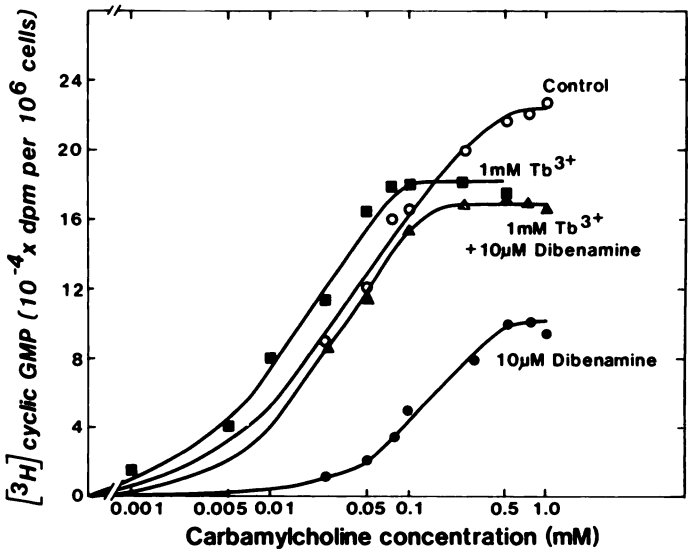

FIG. 8. *Effect of Tb^{3+} on dibenamine antagonism of carbamylcholine-stimulated cyclic GMP formation by mouse neuroblastoma cells*

Clone N1E-115 cells (subculture 12, 18 days after subculture) were prepared for assay as described under Materials and Methods. The cells were incubated with Medium III alone (○) or with 10 μM dibenamine (●), 1 mM Tb^{3+} (■), or 1 mM Tb^{3+} + 10 μM dibenamine (▲) for 15 min, then stimulated with the indicated concentrations of carbamylcholine for 30 sec in duplicate. Each assay contained approximately 1.1 × 10^5 cells and the data are representative results from one of four experiments. The basal ^{3}H-labeled cyclic GMP level of 10,000 dpm/10^6 cells was subtracted from all values.

membrane to any significant degree (34, 35), and the weak effects of the lanthanides in reducing the maximal cyclic GMP responses to muscarinic receptor agonists in this study could result from blockade of the calcium channels, noncompetitively with calcium.

In conclusion, the data presented here may shed some light on the complexity of the actions of the so-called "specific" calcium channel blockers and draw attention to the fact that carefulness is required in interpreting experimental results when the lanthanides are used as probes for calcium channels. In addition, these data emphasize the importance of the combination of radioligand and pharmacological procedures for studying receptor mechanisms.

REFERENCES

1. Richelson, E. The use of cultured cells in neurobiological studies. in *International Review of Biochemistry*, (K. F. Tipton, ed.), Vol. 26. University Park Press, Baltimore, 81–120 (1979).
2. Richelson, E., and S. Divinetz-Romero. Blockade by psychotropic drugs of the muscarinic acetylcholine receptor in cultured nerve cells. *Biol. Psychiatry* **12**:771–785 (1977).
3. 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).
4. Wastek, G. J., J. R. Lopez, and E. Richelson. Demonstration of a muscarinic receptor-mediated cyclic GMP-dependent hyperpolarization of the membrane potential of mouse neuroblastoma cells using [^{3}H]tetraphenylphosphonium. *Mol. Pharmacol.* **19**:15–20 (1981).
5. 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).
6. Bartfai, T., X. O. Breakefield, and P. Greengard. Regulation of synthesis of guanosine 3′:5′-cyclic monophosphate in neuroblastoma cells. *Biochem. J.* **176**:119–127 (1978).
7. Richelson, E. Desensitization of muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells. *Nature (Lond.)* **272**:366–368 (1978).
8. El-Fakahany, E., and E. Richelson. Temperature dependence of muscarinic acetylcholine receptor activation, desensitization and resensitization. *J. Neurochem.* **34**:1288–1295 (1980).
9. Taylor, J. E., E. El-Fakahany, and E. Richelson. Long-term regulation of muscarinic acetylcholine receptors on cultured nerve cells. *Life Sci.* **25**:2181–2187 (1979).
10. 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).
11. Martin, R. B., and F. S. Richardson. Lanthanides as probes for calcium in biological systems. *Q. Rev. Biophys.* **12**:181–209 (1979).
12. Honegger, P., and E. Richelson. Biochemical differentiation of mechanically dissociated mammalian brain in aggregating cell culture. *Brain Res.* **109**: 335–354 (1976).
13. Yamamura, H., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1725–1729 (1974).
14. 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).
15. Furchgott, R. F. The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants at receptor-agonist complexes. *Adv. Drug Res.* **3**:21–55 (1966).
16. Weiss, G. B. Cellular pharmacology of lanthanum. *Annu. Rev. Pharmacol.* **14**:343–354 (1974).
17. Moolenaar, W. H., and I. Spector. The calcium action potential and a prolonged calcium dependent after-hyperpolarization in mouse neuroblastoma cells. *J. Physiol. (Lond.)* **292**:297–306 (1979).
18. Weiss, G. B., and F. R. Goodman. Interaction between several rare earth ions and calcium ion in vascular smooth muscle. *J. Pharmacol. Expl. Ther.* **195**: 557–564 (1975).
19. Study, R. E., 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).
20. U'Prichard, D. C., and S. H. Snyder. Interaction of divalent cations and guanine nucleotides at α_2-noradrenergic receptor binding sites in bovine brain mechanisms. *J. Neurochem.* **34**:385–394 (1980).
21. Shannon, R. D. Revised effective ionic radii and systematic studies of interatomic distances in halides and chalcogenides. *Acta Crystallogr. Sect. A* **32**: 751–767 (1976).
22. Birdsall, N. J. M., A. S. V. Burgen, E. C. Hulme, and J. W. Wells. Muscarinic receptors: ionic perturbation of the binding properties. *Br. J. Pharmacol.* **59**: 503P (1977).
23. 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).
24. Rubsamen, H., G. P. Hess, A. T. Eldefrawi, and M. E. Eldefrawi. Interaction between calcium and ligand-binding sites of the purified acetylcholine receptor studied by use of a fluorescent lanthanide. *Biochem. Biophys. Res. Commun.* **68**:56–63 (1976).
25. Hedlund, B., and T. Bartfai. The importance of thiol- and disulfide groups in agonist and antagonist binding to the muscarinic receptor. *Mol. Pharmacol.* **15**:531–544 (1979).
26. Aronstam, R. S., L. G. Abood, and J. Baumgold. Role of phospholipids in muscarinic binding by neural membranes. *Biochem. Pharmacol.* **26**:1689–1995 (1977).
27. Birdsall, N. J. M., and E. C. Hulme. Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem.* **27**:7–16 (1976).
28. Wei, J.-W., and P. V. Sulakhe. Agonist-antagonist interactions with rat atrial muscarinic cholinergic receptor sites: differential regulation by guanine nucleotides. *Eur. J. Pharmacol.* **58**:91–92 (1979).
29. Ehlert, F. J., W. R. Roeske, L. B. Rosenberger, and H. I. Yamamura. The influence of guanyl-5′-yl imidodiphosphate and sodium on muscarinic receptor binding in the rat brain and longitudinal muscle of the rat ileum. *Life Sci.* **26**:245–252 (1980).
30. Ehlert, F. J., H. I. Yamamura, D. J. Triggle, and W. R. Roeske. The influence of guanyl-5′-yl imidodiphosphate and sodium chloride on the binding of the muscarinic agonist, [^{3}H]cis methyldioxolane. *Eur. J. Pharmacol.* **61**:317–318 (1980).
31. 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).
32. Quiram, D. R., and R. M. Weinshilboum. Inhibition of rat liver catechol-O-methyl transferase by lanthanum, neodymium and europium. *Biochem. Pharmacol.* **25**:1727–1732 (1975).
33. Lambert, D. H., and R. L. Parsons. Influence of polyvalent cations on the activation of muscle end-plate receptors. *J. Gen. Physiol.* **56**:309–321 (1970).
34. Lesseps, R. J. Removal by phospholipase C of a layer of La staining material external to the cell membrane in embryonic chick cells. *J. Cell Biol.* **34**:173–183 (1967).
35. Langer, G. A., and J. S. Frank. Lanthanum in heart cell culture. Effect on calcium exchange correlated with its localization. *J. Cell Biol.* **54**:441–455 (1972).

Send reprint requests to: Dr. Elliott Richelson, Departments of Psychiatry and Pharmacology, Mayo Foundation, Rochester, Minn. 55901.