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Marked Up-Regulation of the α-Bungarotoxin Site in Adrenal Chromaffin Cells by Specific Nicotinic Antagonists

M. QUIK, S. GEERTSEN, and J. M. TRIFARÓ

Department of Pharmacology, McGill University, Montreal, Quebec H3G 1Y6 (M.Q., S.G.) and Department of Pharmacology, University of Ottawa, Ottawa, Ontario K1H 8M5, (J.M.T.), Canada

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SUMMARY

The effect of nicotinic antagonists was studied on various parameters of adrenal medullary chromaffin cells in culture. Incubation of the cells in culture with *d*-tubocurarine or mecamylamine for 0.5–6 days resulted in up to an 8-fold increase in the binding of α-bungarotoxin (α-BGT) to the cells; other ganglionic blockers, on the other hand, such as hexamethonium and dihydro-β-erythroidine, had no effect. This enhanced binding was due to an increase in the number of α-BGT sites with little change in affinity of the ligand for the receptor. The nature of the increase in the number of toxin-binding sites in chromaffin cells observed after treatment with *d*-tubocurarine or mecamylamine was subsequently investigated. A direct interaction of the drug with the α-BGT recognition site was not required for the increase in receptor number to occur; mecamylamine did not affect binding of α-BGT to chromaffin cells in culture in competition binding experiments, although *d*-tubocurarine did compete with α-BGT for binding to its recognition site. The reversal of the antagonist-induced increase in the α-BGT-binding sites by nicotine and carbachol suggested it was mediated through an interaction at an acetylcholine receptor recognition site. The depolarizing agent veratridine greatly attenuated the increase in the number of toxin-binding sites in response to antagonists; this effect of veratridine could be reversed by tetrodotoxin. These latter findings indicate that neuronal excitability can influence the observed increase in the number of α-BGT sites after exposure of the cultures to nicotinic antagonists. The antagonist-induced increase in the α-BGT sites in the cells was not associated with an increased functional responsiveness of the cells to acetylcholine. The present results demonstrate that the number of α-BGT-binding sites in adrenal medullary chromaffin cells can increase dramatically in response to some, but not other, nicotinic antagonists by an interaction at a nicotinic acetylcholine recognition site. The differential effect of antagonists at the nicotinic-like α-BGT site and the functional nicotinic receptor suggests these two parameters are distinct. The unusually large alteration in receptor number may be related to the unique localization of the adrenal medulla and could infer that these receptors have a role in this tissue.

Extensive work with different neurotransmitter systems has shown that manipulation of the cellular environment may affect receptor-mediated function and/or receptor binding parameters (1). Decreased neuronal activity produced either by receptor blockade or neuronal denervation can result in an enhanced receptor responsiveness. In contrast, administration of agonist compounds or increased neuronal activity may lead to a decrease in receptor sensitivity.

Because of the relative ease with which the external environment can be manipulated and the possibility of elucidating the molecular mechanisms involved in super- and subsensitivity, studies involving receptor regulation have frequently been done using cells in culture. Experiments to study the factors controlling nicotinic acetylcholine receptors in muscle cells in culture have shown that the receptors, measured using α-BGT, are decreased in the presence of the agonist carbachol but unaffected by the antagonist *d*-tubocurarine (2, 3). The decrease in the number of α-BGT-binding sites in muscle cells is paralleled with a decrease in receptor sensitivity to applied acetylcholine. As well, in neuronal cells in culture, there also appears to be a parallel regulation of the nicotinic α-BGT-binding site and cholinergic sensitivity. Exposure of neuronal cells to cholinergic agonists resulted in a down-regulation of the α-BGT receptors, whereas nicotinic antagonists did not affect the toxin-binding sites (4–6); a decrease in sensitivity of the cells to acetylcholine is also observed after incubation of cultured neuronal cells with the agonist carbachol, whereas nicotinic antagonists had no effect (7).

Although there appear to be similarities in the changes in functional responses and nicotinic α-BGT sites after exposure to cholinergic drugs in neuronal cells in culture, the nature of the nicotinic α-BGT-binding site in nervous tissue is far from clear as α-BGT frequently fails to block nicotinic responses (8, 9). As well, the localization of the α-BGT sites in neuronal tissues does not necessarily coincide with that of the nicotinic

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ABBREVIATIONS: α-BGT, α-bungarotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

acetylcholine receptor population (10–13) and, moreover, purification studies have demonstrated that the nicotinic acetylcholine-binding component and the α-BGT-binding site in nervous tissue are distinct molecules and can be separated from each other (14–18). Furthermore, in neuronal cells in culture, experimental situations have arisen in which the α-BGT-binding site and the functional nicotinic receptor appear to be regulated independently (5, 19). These observations could infer that the nicotinic-like α-BGT receptor in nervous tissue is involved in functions which are distinct from those currently associated with nicotinic receptor-mediated activities.

In the present work, the effect of cholinergic antagonists on the α-BGT-binding sites in adrenal medullary chromaffin cells in culture is investigated. In these cells, in contrast to muscle cells and other neuronal cells in culture, long-term application of antagonist results in a dramatic increase (800%) in the α-BGT sites, whereas receptor sensitivity to applied acetylcholine is unaffected. The differential effects of antagonists on the nicotinic-like α-BGT-binding sites and the functional nicotinic receptor suggest that these molecular entities are distinct. The adrenal medulla is unique as compared to other neuronal tissues in its localization, as it is surrounded by adrenal cortex; possibly it is this association with cortical tissue which is involved in the extensive up-regulation of the α-BGT site by antagonists in these cells as compared to other neuronal cells in culture.

Materials and Methods

Adrenal medullary chromaffin cell cultures. Bovine adrenal glands were removed and placed in sterile cold Locke's solution. The cortex was removed from the medulla, the medulla was perfused, and the chromaffin cells were isolated as previously described (20). They were then plated on collagen-coated plastic dishes (10^6 cells/35-mm dish) in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal calf serum, ascorbic acid (0.1 mM), glucose (1 mg/ml), HEPES buffer (3.6 μg/ml), and the following antibiotics: penicillin (100 μg/ml), streptomycin (100 μg/ml), tetracycline (5 μg/ml), gentamicin (10 μg/ml), and mycostatin (25 units/ml). Arabinosylcytosine (10^{-5} M) and 5-fluorodeoxyuridine (10^{-5} M) were also included in the medium to prevent fibroblast proliferation by inhibiting cell division. Cultures were maintained at 37° in a humidified incubator under a CO_2 + air (5:95) atmosphere. The culture medium was changed every 2–3 days and the cultures were inspected by phase contrast optics.

Treatment of adrenal medullary chromaffin cells in culture with various drugs. In experiments in which chromaffin cells were incubated with drugs for various lengths of time (hours to days), the experimental protocol was arranged such that the binding of ^{125}I-α-BGT was assayed on the same number of days after the initial plating of the cells (day 0); this was done to minimize the possibility that drug effects were actually effects due to variations in the days of culturing of the cells. Thus, in the time course experiments, for cultures incubated with drug for 6 days, the antagonist treatment was usually initiated 6 days after plating, whereas, for cultures incubated with antagonist for 1 day, the drug was added 11 days after plating. Thus, all cultures were the same age on the day of the receptor binding assay.

Binding of ^{125}I-α-BGT to chromaffin cell membranes. Adrenal medullary chromaffin cells were isolated from bovine adrenal glands according to the method of Trifaró and Lee (20). The cell pellet was resuspended in 10 mM Tris-HCl, pH 7.4 (2–10 × 10^6 cells/100 μl) and homogenized with a Polytron at setting 7 for 10 sec. After centrifugation at 45,000 × *g* for 10 min, the membrane pellet obtained was resuspended as above. Binding of ^{125}I-α-BGT to the adrenal medullary membranes was determined as previously outlined (21).

Binding of ^{125}I-α-BGT to chromaffin cell cultures. After 7–14 days in culture, the binding of ^{125}I-α-BGT to the cells in culture was measured as described (21). Previous work had shown that ^{125}I-α-BGT bound to a receptor in chromaffin cells in culture with the characteristics of a nicotinic receptor ligand (21). In experiments in which the cultures were pretreated with the antagonists, the cultures were extensively washed with Locke's solution in an attempt to remove as much residual drug as possible. This involved changing the original culture medium with 2 ml of Locke's solution three times over a 30-min period. This washing procedure probably removed most residual drug since the saturation curve done after antagonist treatment of the cells did not exhibit any change in the K_d as compared to the control situation (see Fig. 2). If residual drug had remained, the K_d would have been increased after treatment of the cells with the drug. The cells were then incubated for 60 min at 37° with two further changes of Locke's solution. To start the ^{125}I-α-BGT binding assay, cultures (10^6 cells/dish containing 0.8 ml of Locke's solution) were preincubated for 1 hr at 37° in the absence or presence of *d*-tubocurarine (3 × 10^{-4} M); a 20- to 90-min incubation (37°) was then started with the addition of 20 μl of ^{125}I-α-BGT to the culture dish. Earlier work (21) had shown that binding of the radioligand to the receptors had reached a plateau by 60 min and, for this reason, the 90-min incubation time was routinely used in all but one experiment (see Fig. 4) as indicated in the figure legends. At the end of this time, the medium bathing the cells was carefully removed and the cells were subsequently washed six times with 2-ml aliquots of Locke's solution over a 1-hr period to remove any unbound radiolabeled α-BGT. The cells were then resuspended in 1 ml of Locke's solution with a rubber policeman and the radioactivity in the sample was determined in a gamma counter.

Nonspecific binding to the cultures was defined as that occurring in the presence of 3 × 10^{-4} M *d*-tubocurarine. At a concentration of 1.5 nM ^{125}I-α-BGT, the nonspecific binding was approximately 10–20% of the total. The composition of the Locke's solution (in mM) was: NaCl, 154; KCl, 2.6; K_2HPO_4, 2.15; KH_2PO_4, 0.85; $MgCl_2$, 1.2; $CaCl_2$, 2.2; dextrose, 10, adjusted to pH 7.2 using NaOH.

A comparison of the results obtained from the binding assay done with intact chromaffin cells in culture and the binding assay done with chromaffin cell membranes showed that the results as expressed per million cells was roughly similar under the two conditions. The number of ^{125}I-α-BGT receptor sites in control cells in culture using 1.0 nM radioactivity was approximately 0.65 fmol/culture dish, averaged over several experiments. Each culture dish contained 10^6 cells. With noncultured cell membranes using 1.0 nM ^{125}I-α-BGT, the control binding was 1.0 fmol/10^6 cells. Thus, binding to cells in culture or noncultured cell membranes is similar with respect to the number of sites observed per million cells.

Noradrenaline release studies. Release of ^{3}H-noradrenaline from chromaffin cells in culture was done as previously described (20). The dishes were incubated (37°) for 5 min with 1 ml of amino acid-free Dulbecco's modified Eagle's medium containing 10^{-7} M ^{3}H-noradrenaline (22). After this loading phase, each dish was incubated with six changes of 1-ml regular Locke's solution over a 60-min period. The resting or basal release of the ^{3}H-noradrenaline was then determined over two 3-min periods; subsequent to this, stimulated ^{3}H-noradrenaline release in the presence of 10^{-4} M acetylcholine was determined over three 3-min periods, followed by one more 3-min period of nonstimulated ^{3}H-noradrenaline release. The radioactivity in the fractions (1 ml) was measured in a liquid scintillation spectrometer.

DNA extraction and determination. To extract DNA from chromaffin cells, samples previously scraped from plates and suspended in 1 ml of Locke's solution were washed three times with 0.6 N trichloroacetic acid. For the first wash, 500 μl of 1.8 N trichloroacetic acid were added to each 1-ml sample resulting in a final trichloroacetic acid concentration of 0.6 N. Samples were then centrifuged in an Eppendorf microcentrifuge at 7800 × *g* for 10 min. All subsequent centrifugations were performed in this manner; the supernatants were discarded and the pellets were washed twice with 1 ml of potassium acetate in absolute ethanol and placed in a 60° oven for 15 min. Following centrifugation, the supernatant was discarded and the pellets were incubated at 37°

for 30–60 min to allow residual alcohol to evaporate. The pellets were resuspended in 250 μl of 1 N perchloric acid and placed in a 75° oven for 30 min. After centrifugation. 100-μl aliquots were removed, and DNA was determined according to the method of Setaro and Morley (23).

Statistical comparisons were made using Student's *t* test.

Materials. The ^{125}I-α-BGT (10–20 μCi/μg) and ^{3}H-noradrenaline (38.7 Ci/mmol) were purchased from New England Nuclear (Boston, MA); *d*-tubocurarine, carbachol, nicotine, and tetrodotoxin were from Sigma Chemical Co. (St. Louis, MO); atropine sulfate was from Nutritional Biochemicals Corp. (Cleveland, OH); and veratridine was from K&K (ICN) (New York, NY). Mecamylamine hydrochloride and dihydro-β-erythroidine hydrobromide were a generous gift from Merck, Sharp and Dohme (Kirkland, Quebec) and hexamethonium bromide was from Poulenc Ltd., (Montreal, Quebec).

Results

Effect of treatment of chromaffin cells in culture with nicotinic antagonists on α-BGT binding to the cells. Adrenal medullary chromaffin cells were cultured as described in Materials and Methods; after at least 1 week in culture, the nicotinic antagonists *d*-tubocurarine, mecamylamine, hexamethonium, or dihydro-β-erythroidine were added to the medium bathing the cells. The effect of time of treatment of the cells with the various antagonists is depicted in Fig. 1A; α-BGT binding to the cells was assessed 0.5–6 days after addition of either 10^{-6} or 10^{-4} M *d*-tubocurarine or mecamylamine, 10^{-4} M hexamethonium, or 10^{-4} dihydro-β-erythroidine to the culture medium. To ensure the residual drug was removed prior to the toxin binding assay, the cells were extensively washed with Locke's solution as described in Materials and Methods. An increase in the binding of α-BGT is already observed after 12 hr, with a maximal increase of 700% with the higher concentration of *d*-tubocurarine and mecamylamine. The effect of varying drug concentration on the increase in α-BGT binding is shown in Fig. 1B. A 2- to 3-fold increase in the number of ^{125}I-α-BGT-binding sites can be observed with 10^{-6} M *d*-tubocurarine or 10^{-6} M mecamylamine; these concentrations are well within the range used to study effects on nicotinic receptors (4, 5, 7, 20). With *d*-tubocurarine a maximal increase (800%) occurs at 10^{-4} M with no further increase at higher concentrations of the drug; for mecamylamine, a maximal increase was observed at 10^{-5} M drug. No increase in radiolabeled toxin binding was observed with concentrations of hexamethonium up to 10^{-3} M or dihydro-β-erythroidine up to 10^{-4} M; the muscarinic cholinergic antagonist atropine also did not increase the α-BGT-binding sites in chromaffin cells in culture (data not shown).

To determine whether the increase in binding of radiolabeled α-BGT was due to a change in the maximal number of binding sites (B_{max}) and/or to a change in affinity of the ligand for the receptor (K_d), saturation curves were done of the binding of ^{125}I-α-BGT in control cultures as compared to cultures that had been pretreated for 3 days with 10^{-4} M *d*-tubocurarine. After the initial washing of the cultures to remove residual *d*-tubocurarine as described in Materials and Methods, the binding assay was done using a range of ^{125}I-α-BGT concentrations of 0.1–4.8 nM. The saturation curve in Fig. 2A shows the binding plateaus at about 1 nM and 3 nM in the control and treated cultures, respectively. A Scatchard plot (Fig. 2B) for control and treated cultures, respectively, yielded B_{max} values of 3.65 and 10.7 fmol/10^6 cells and K_d values of 0.67 and 0.52 nM using

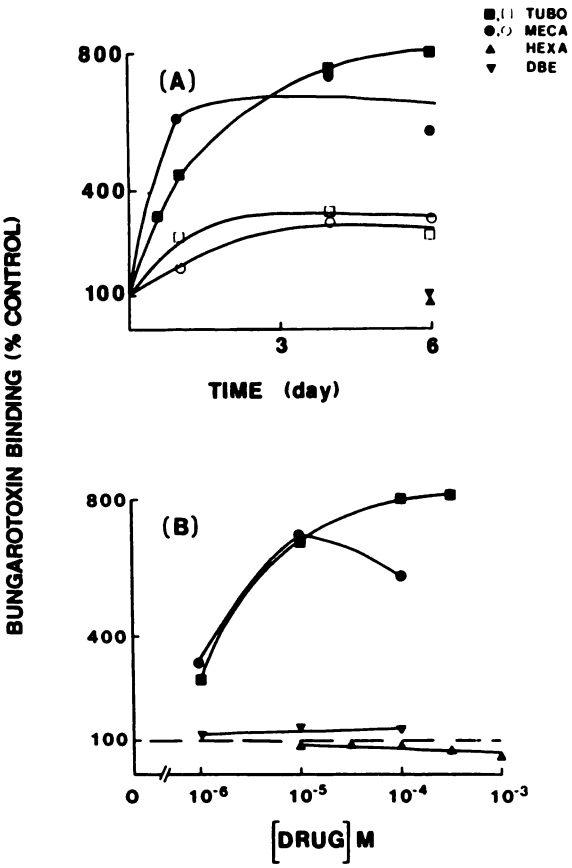

Fig. 1. The effect of treatment of chromaffin cells in culture with nicotinic antagonists on α-BGT binding to the cells. Chromaffin cells were plated and cultured as described and subsequently incubated in the absence or presence of nicotinic antagonists as indicated. In A, the cells were incubated with the antagonists for 0.5–6 days. The concentrations of antagonists were: 10^{-4} M *d*-tubocurarine (*TUBO*) (■), 10^{-6} M *d*-tubocurarine (□), 10^{-4} M mecamylamine (*MECA*) (●), 10^{-6} M mecamylamine (○), 10^{-4} M hexamethonium (*HEXA*) (▲), and 10^{-4} M dihydro-β-erythroidine (*DBE*) (▼). In B, the effect of pretreatment of the cells for 6 days with varying concentrations of the same antagonists is shown. Binding of ^{125}I-α-BGT to cultures not treated with drugs was 0.81 ± 0.06 fmol/culture dish (n = 35). Each value represents the mean of three to six culture dishes.

linear regression analysis. Whether Scatchard analysis is a valid method to determine the binding parameters for α-BGT binding may be questioned since the binding may not be at equilibrium under the present conditions; however, as Scatchard plots are widely used by others studying α-BGT binding, they have been included for comparison.

The effect of nicotinic agonists on the antagonist-induced increase in the number of α-BGT-binding sites in chromaffin cells in culture. To determine whether the *d*-tubocurarine-induced increase in α-BGT binding to the cells in culture could be reversed by agonists, the cells were incubated without or with *d*-tubocurarine in the absence or presence of 10^{-4} M nicotine or 10^{-3} M carbachol (Fig. 3). At the lower concentrations of *d*-tubocurarine (10^{-6} M and 10^{-5} M), both agonists were extremely effective in preventing the increase in the α-BGT sites. In fact, carbachol appears to completely abolish ^{125}I-α-BGT binding in the presence of 1 μM *d*-tubocurarine; the reasons for this are not clear. At the highest concentration of *d*-tubocurarine (10^{-4} M) used, nicotine still significantly reduced (45%) the number of α-BGT-binding sites.

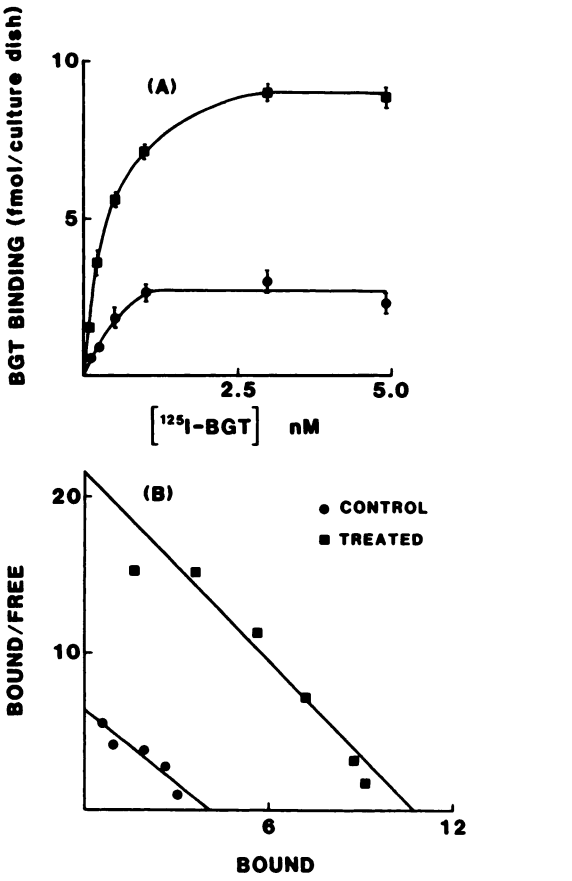

Fig. 2. Saturation curve (A) and Scatchard plot (B) of α-BGT binding to chromaffin cells which were cultured under control conditions (●) or cultured in the presence of 10^{-4} M *d*-tubocurarine (*TREATED*, ■) for a 3-day period. Binding of radiolabeled toxin was done as described with a 90-min incubation period. The concentration range of ^{125}I-α-BGT used was 0.10–4.8 nM. Each value represents the mean ± standard error of three to five culture dishes. The results are representative of two other experiments. For the Scatchard plot, bound/free (*B/F*) on the *y* axis is expressed as fmol/culture dish/nM and bound on the *x* axis as fmol/culture dish.

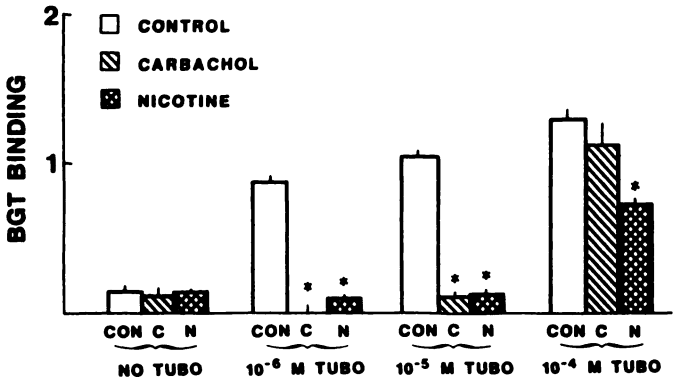

Fig. 3. The effect of nicotine and carbachol treatment on α-BGT binding to chromaffin cells in culture incubated in the absence or presence of *d*-tubocurarine. The cells were plated and cultured as previously described. They were subsequently incubated for 3 days with 10^{-3} M carbachol (*C*) or 10^{-4} M nicotine (*N*) in the absence (*NO TUBO*) or presence of the indicated concentrations of *d*-tubocurarine (*TUBO*). Each bar represents the mean ± standard error of three to five culture plates. Specific α-BGT binding is expressed as fmol/culture dish. Significance of difference from control (*CON*) culture plates is indicated by *, $p < 0.001$.

However, carbachol had only a minimal effect in blocking the *d*-tubocurarine-induced increase in α-BGT sites; an explanation for this apparent steep dose response relationship of carbachol to reverse the effect is not obvious. The ability of the agonists to reverse the *d*-tubocurarine-induced increase in the α-BGT sites could suggest that these agents inhibit the effects of *d*-tubocurarine at the receptor level; it is also possible, however, that these are two separate events, that is, *d*-tubocurarine produces an induction of the receptors whereas the agonists result in receptor down-regulation by an alternate mechanism. In contrast, both the up- and down-regulation would presumably be mediated through an interaction at nicotinic receptors. Incubation of the cells with the agonists only had no significant effect on the binding of radiolabeled α-BGT to the cells.

Because agonists per se did not affect binding in cultures incubated in the absence of *d*-tubocurarine, it is unlikely that the large reduction in the *d*-tubocurarine-induced increase in the α-BGT sites in the presence of agonist is due to a detrimental effect on the cells. However, to minimize this possibility, the DNA content of the cultures was measured under the various conditions. The DNA content of the cells cultured in the absence of drugs was 4.4 ± 0.3 μg/10^6 cells ($n = 5$); this was not significantly different under any of the conditions tested.

The effect of nicotinic antagonists on α-BGT binding to chromaffin cells in culture or chromaffin cell membranes in direct competition binding experiments. To determine whether the selective effect of some, but not all, nicotinic receptor antagonists in inducing the α-BGT-binding sites in culture was related to a direct interaction at the α-BGT site, the short-term or immediate effect of these agents was subsequently tested on radiolabeled toxin binding. As the cells in culture were not pretreated with the antagonists, the washing steps were not necessary. Instead, the antagonists were present throughout the 60-min preincubation period as well as during the 90-min incubation period with toxin. Fig. 4A demonstrates the effect of varying drug concentration on radiolabeled toxin binding. In the presence of *d*-tubocurarine, marked inhibition of ^{125}I-α-BGT binding occurred; hexamethonium and dihydro-β-erythroidine also exerted a small but significant inhibition, whereas mecamylamine had no appreciable effect on toxin binding. It is conceivable that the lack of any observed inhibition with mecamylamine, or the smaller extent of inhibition with hexamethonium or dihydro-β-erythroidine, occurred because α-BGT dissociates less readily from the binding site than do the drugs and, thus, with time the toxin occupies a greater proportion of the sites. For this reason, the initial 60-min preincubation period with antagonist was followed by only a 20- to 60-min incubation period with ^{125}I-α-BGT rather than the usual 90-min incubation period. However, as can be seen from Fig. 4B, the observed inhibition pattern of ^{125}I-α-BGT binding by the different drugs remained approximately the same with varying incubation time; mecamylamine still had no effect, whereas the effect of hexamethonium on α-BGT binding was only minimal or not evident.

To minimize the possibility that the observed inhibitory effects of the drugs were artifactually produced due to uptake, internalization, etc., of the drug by the cells in culture, the effect of these same antagonists was also determined on α-BGT binding to chromaffin cell membranes (Table 1). A concentration of 10^{-4} M inhibitor was used in these experiments,

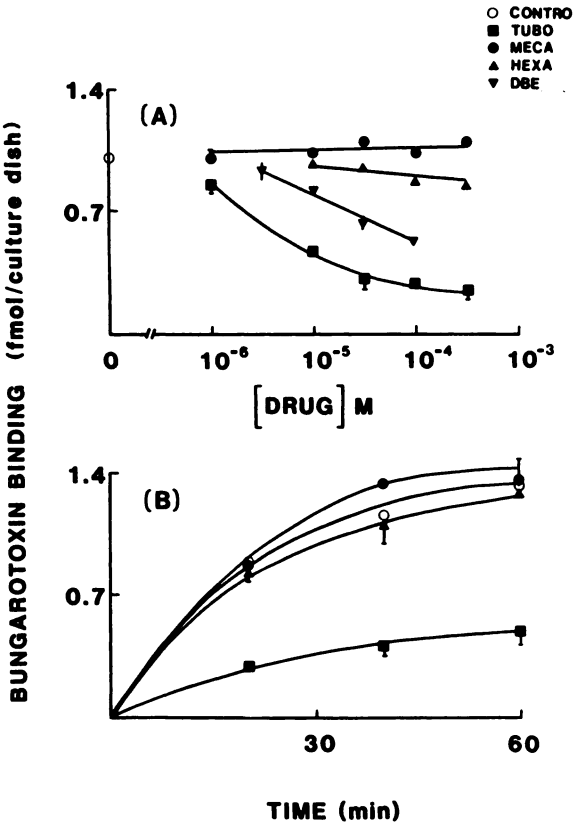

Fig. 4. Immediate effect of nicotinic antagonists on α-BGT binding to adrenal medullary chromaffin cells in culture. In A the effect of varying concentrations of antagonists on α-BGT binding is shown. Preincubation with the indicated antagonists, mecamylamine (*MECA*), hexamethonium (*HEXA*), *d*-tubocurarine (*TUBO*), and dihydro-β-erythroidine (*DBE*) was done for a 60-min period, followed by a 90-min incubation time with radiolabeled α-BGT. In B the effect of the antagonists on α-BGT binding at different times of incubation is depicted. The concentrations of the drugs used were: 10^{-4} M mecamylamine, 3×10^{-4} M hexamethonium, and 10^{-4} M *d*-tubocurarine. Each *symbol* represents the mean ± standard error of 3 to 10 culture dishes; where the standard error is not depicted it was less than 5% of the mean.

TABLE 1

The immediate effect of nicotinic antagonists on α-BGT binding to chromaffin cell membranes

The binding assay was done as previously described with a 30-min preincubation of antagonist with membranes followed by a 5-min incubation period with radiolabeled α-BGT. α-BGT binding is expressed as percentage of total binding; total binding averaged 0.64 fmol/10^6 cells over six experiments. Each value represents the mean ± standard error of the indicated number of experiments, each done in duplicate or triplicate.

Inhibitor	Concentration	Number of experiments	α-BGT binding (% total)
	M		
d-Tubocurarine	10^{-4}	3	40 ± 4
Mecamylamine	10^{-4}	4	98 ± 3
Hexamethonium	10^{-4}	3	69 ± 3
Dihydro-β-erythroidine	10^{-4}	4	81 ± 4

as this was the concentration of inhibitor generally used in the work in which the long-term effects of the drugs were studied. The extent of inhibition of binding of radiolabeled toxin to the membranes by the different antagonists was similar, although not identical, to the inhibition of binding observed with the chromaffin cells in culture. Specifically, *d*-tubocurarine resulted in the greatest inhibition of radiolabeled toxin binding. Hexamethonium and dihydro-β-erythroidine exerted an intermediate inhibitory effect on α-BGT binding, whereas mecamylamine had no significant effect.

The effect of various agents on the antagonist-induced increase in α-BGT-binding sites in chromaffin cells in culture. To determine whether impulse activity might regulate the level of α-BGT-binding sites, the depolarizing agent veratridine was added to the cells in culture concurrently with *d*-tubocurarine (10^{-4} M). After 3 days of exposure of the cells in culture to various concentrations of the drug, the cells were extensively washed as described in Materials and Methods, and α-BGT binding to the chromaffin cells was determined. Fig. 5A shows that veratridine alone resulted in a significant increase ($p < 0.05$) in the number of α-BGT sites, but only at 0.3 μM and 3 μM; thus, this increase was not consistently observed and its physiologic implications are not clear. Conversely, after treatment of the cells with *d*-tubocurarine, veratridine greatly reduced the increase in α-BGT-binding sites induced by the antagonist; veratridine also decreased the mecamylamine-induced increase in the α-BGT-binding sites (data not shown). These effects were not due to a decline in the DNA content of the cultures, indicating that the decrease in the *d*-tubocurarine-

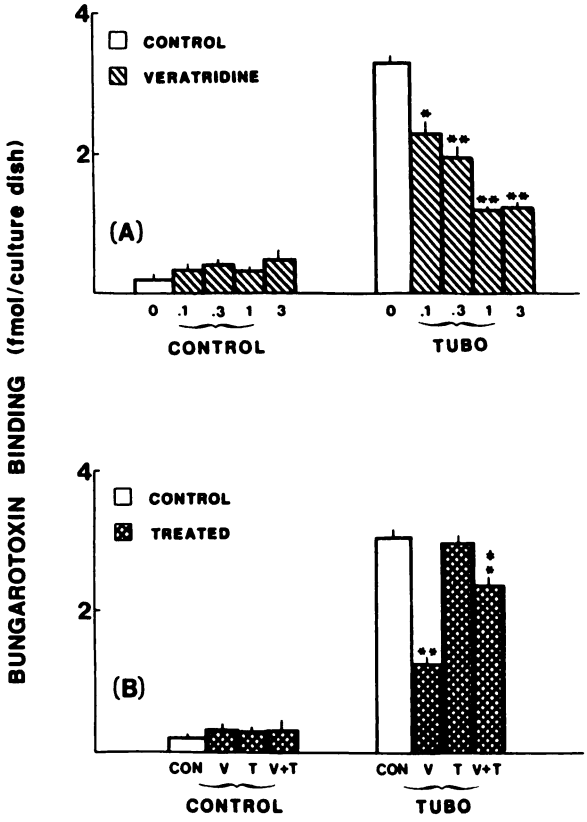

Fig. 5. The effect of veratridine and tetrodotoxin treatment on the *d*-tubocurarine-induced increase in α-BGT binding to chromaffin cells in culture. The cells were plated and cultured as previously described; they were incubated for 3 days with veratridine (*V*) and/or tetrodotoxin (*T*) in the absence (*CONTROL*) or presence of 10^{-4} M *d*-tubocurarine (*TUBO*). In A, veratridine concentrations ranging from 0.1 to 3.0 μM were used. In B, the concentrations of tetrodotoxin and veratridine were 5.0 and 1.0 μM, respectively; *CON* or *0* represents binding to the culture plates incubated in the absence of depolarizing agents. The *bars* represent the mean ± standard error of four to five culture dishes. Significance of difference from the plates incubated only in the presence of *d*-tubocurarine was: *, $p < 0.01$; **, $p < 0.001$. Significance of difference from the plates incubated in the presence of *d*-tubocurarine and veratridine was: ‡, $p < 0.001$.

induced increase in the α-BGT sites with veratridine was not due to a detrimental effect on the cells.

To determine whether the effect of veratridine could be reversed by tetrodotoxin, these two agents were added together to the cells in culture (Fig. 5B). Tetrodotoxin alone produced no significant effect on α-BGT binding as compared to control chromaffin cells in culture. In the cultures treated with antagonist, tetrodotoxin resulted in a significant ($p < 0.001$) reversal of the effect of veratridine on the *d*-tubocurarine-induced increase in the toxin-binding sites (Fig. 5B).

Effect of treatment of chromaffin cells in culture with nicotinic antagonists on the acetylcholine-stimulated release of ^{3}H-noradrenaline from the cells. After an initial 7 days in culture, 10^{-4} M *d*-tubocurarine or 10^{-4} M mecamylamine was added to the chromaffin cells. After 3 or 4 days exposure to the antagonists, the cultures were extensively washed with Locke's solution to remove any residual drug as described in Materials and Methods. The basal and acetylcholine-stimulated release of ^{3}H-noradrenaline was then determined for control and treated cultures. Experimental observations suggest that this acetylcholine-stimulated release of noradrenaline appears to be mediated through a nicotinic cholinergic receptor population (20). Fig. 6 shows that, despite the dramatic increase in the number of α-BGT sites, the functional response of the cells to acetylcholine was not affected.

The release of ^{3}H-noradrenaline in response to 56 mM K^+ was also similar in control cultures as compared to cultures pretreated with the nicotinic antagonists (data not shown).

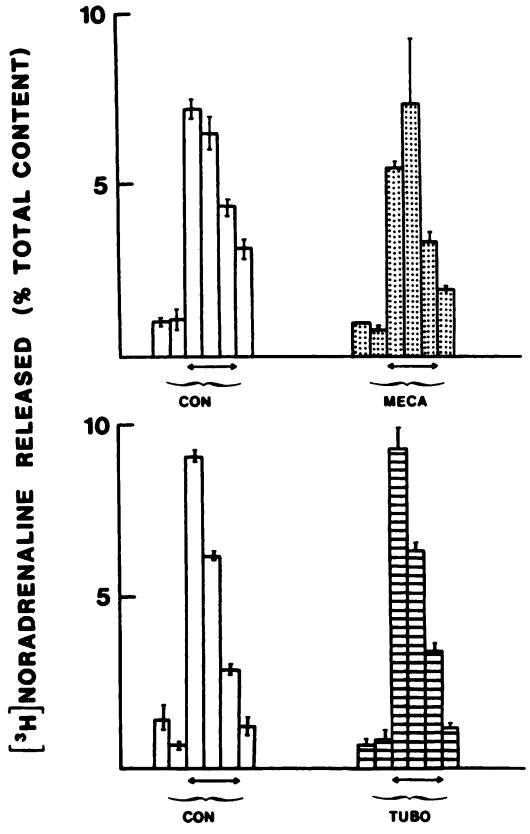

Fig. 6. Effect of treatment of chromaffin cells in culture with the nicotinic antagonists mecamylamine or *d*-tubocurarine on the acetylcholine-stimulated release of ^{3}H-noradrenaline from the cells. One-week-old cells in culture were incubated for a further 6 days in the absence (*CON*) or presence of 10^{-4} M mecamylamine (*MECA*) or 10^{-4} M *d*-tubocurarine (*TUBO*). At the end of this time, the cells were washed extensively and ^{3}H-noradrenaline release was determined as described. Each value represents the mean ± standard error of four culture dishes. The results are representative of two other experiments. The *horizontal bars* along the *x* axis indicate the periods of stimulation with acetylcholine. Each *vertical bar* represents a 3-min collection period.

Discussion

The present results show that exposure of adrenal medullary chromaffin cells in culture to the nicotinic antagonists *d*-tubocurarine and mecamylamine results in a very dramatic increase in the binding of radiolabeled α-BGT to the cultures. Saturation analysis showed that this increase in ^{125}I-α-BGT binding was due to a change in the maximal number of binding sites with little change in the affinity of the ligand for the receptor. Moreover, this increase in the number of α-BGT sites could be reversed by nicotinic agonists, indicating that the enhancement of receptor number is probably mediated by an interaction at an acetylcholine recognition site. Whether this is occurring through a direct blocking of the effects of *d*-tubocurarine at the receptor or by some parallel mechanism of these two classes of agents at the nicotinic receptor population remains to be elucidated.

The possible mechanism whereby some, but not other, nicotinic blockers resulted in an increase in the number of α-BGT sites was subsequently investigated. To determine whether a direct interaction at the α-BGT site was important for eliciting the long-term increase, the effect of these agents was studied on the binding of radiolabeled toxin binding in competition binding experiments. *d*-Tubocurarine, which resulted in a delayed increase in the number of toxin-binding sites to the cells, inhibited the binding of α-BGT to the cells in culture; however, mecamylamine, a nicotinic antagonist which also enhanced the number of sites on a long-term basis, did not interact with the α-BGT-binding site in competition binding experiments. In contrast, the other two ganglionic blocking agents, hexamethonium and dihydro-β-erythroidine, interacted to varying extents at the α-BGT site in the short-term binding experiments, without resulting in a long-term increase in the number of α-BGT-binding sites. This would suggest that a direct interaction at the α-BGT site is not required for the long-term increase in receptor number in the cells in culture.

In contrast to the variable effect of the nicotinic blockers in inducing the increase in the α-BGT-binding sites, these same agents, *d*-tubocurarine, hexamethonium, mecamylamine, and dihydro-β-erythroidine, all effectively block nicotinic function in adrenal medulla and/or adrenal medullary chromaffin cells in culture (20, 24), indicating that they all act at the acetylcholine recognition site of the functional nicotinic receptor. This differential effect of the nicotinic antagonists in enhancing the number of α-BGT sites and blocking the functional nicotinic response could be interpreted as suggesting that the acetylcholine recognition site to which the α-BGT receptor is linked is distinct from the acetylcholine recognition site with which the functional nicotinic receptor is associated.

This conclusion is in line with earlier work which investigated the relationship between the nicotinic-like α-BGT-binding site and the physiological nicotinic receptor. The α-BGT site in chromaffin cell membranes (24) and chromaffin cells in culture (21) has been described as having the characteristics of a nicotinic receptor. However, the nature of its relationship with the functional nicotinic receptor was not obvious because

α-BGT did not affect conventional nicotinic receptor-mediated responses in adrenal medullary cells (20, 24–26). Further evidence for a dissociation of the toxin-binding site and the nicotinic receptor which mediates functional activity is also implied from the present experiments which show that, after exposure to nicotinic antagonists, the acetylcholine-stimulated ^{3}H-noradrenaline release is unaffected in the presence of a dramatic increase in the number of nicotinic α-BGT sites.

The factors which might affect the nicotinic antagonist-induced increase in α-BGT-binding sites were subsequently determined. In muscle cells, it has been shown that electrical stimulation or depolarizing agents, such as veratridine, which activate the voltage-gated sodium channel, inhibit receptor production, whereas tetrodotoxin, which blocks sodium channels and thus abolishes spontaneous electrical activity, resulted in an enhancement of receptor synthesis (27, 28). These effects on receptor synthesis in cultured muscle cells did not appear to be due to a generalized effect on protein synthesis within the cells (29). In the adrenal chromaffin cells in culture, tetrodotoxin did not affect the control levels of α-BGT sites, although veratridine significantly increased the number of sites but only at some concentrations of the drug. In contrast, after incubation of the chromaffin cells in culture with *d*-tubocurarine, veratridine greatly attenuated the increase in the number of toxin-binding sites; moreover, this effect of veratridine was partially blocked by tetrodotoxin. The reason for the incomplete reversal by tetrodotoxin is not that clear; however, it is in agreement with the results of Kilpatrick *et al.* (30), who used tetrodotoxin to reverse stimulated catecholamine secretion in adrenal medullary chromaffin cells in culture. These authors showed that, in chromaffin cells in culture, the veratridine-induced release of ^{3}H-catecholamines is reversed by tetrodotoxin (1 μM), but only by 80–90% and, as well, that acetylcholine-stimulated ^{3}H-catecholamine release from bovine chromaffin cells in culture was only inhibited 40–90% by 1 μM tetrodotoxin (20, 30). To conclude, these results with veratridine and tetrodotoxin could suggest that transmembrane sodium ion influx affects the increase in α-BGT-binding sites and, thus, that α-BGT sites are regulated by depolarization.

The present results demonstrate that some but not other nicotinic antagonists can markedly enhance the number of α-BGT-binding sites in adrenal medullary chromaffin cells in culture through an interaction at a nicotinic cholinergic receptor. The (1) differential effects of the various nicotinic antagonists in increasing the number of α-BGT sites as compared to the ability of these agents to inhibit the functional nicotinic response and (2) the observation that an 8-fold increase in α-BGT receptor number is not associated with an enhanced responsiveness of the functional nicotinic receptor both suggest that the nicotinic-like α-BGT-binding site and the functional nicotinic receptor are distinct in this tissue. The unusually large alteration in the α-BGT receptor number in response to the nicotinic antagonists in adrenal medullary cells could infer that these receptors have a functional role in this tissue.

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Send reprint requests to: Dr. M. Quik, Department of Pharmacology, McIntyre Medical Building, 3655 Drummond Street, Montreal, Quebec H3G 1Y6, Canada.