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Regulation of α-Bungarotoxin Sites in Chromaffin Cells in Culture by Nicotinic Receptor Ligands, K^+, and cAMP

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SUMMARY

Previous work had shown that incubation with the nicotinic antagonist *d*-tubocurarine resulted in a marked increase in α-bungarotoxin (α-BGT) binding in adrenal medullary chromaffin cells in culture; the possible molecular mechanisms involved in up-regulating the α-BGT sites were investigated. To determine whether changes in the extracellular K^+ concentration could influence the number of toxin binding sites, the chromaffin cells were incubated in the presence of 2–50 mM K^+; this resulted in an increase in α-BGT binding similar to that observed with the nicotinic antagonist. This enhanced binding was maximal with 20 mM K^+ and was not due simply to a generalized ion effect, inasmuch as incubation of the cells with a concentration of Na^+ of equivalent osmolarity did not alter α-BGT binding. Carbachol and the agonist nicotine completely prevented the K^+-induced increase in the binding sites. In contrast to the marked up-regulation of the nicotinic α-BGT sites by K^+, this agent did not increase the acetylcholine-induced release of [^{3}H]noradrenaline from chromaffin cells in culture, further supporting the contention that the nicotinic α-BGT site and the functional nicotinic receptor are distinct. The increases in toxin binding due to K^+ and *d*-tubocurarine were partially additive, suggesting that *d*-tubocurarine and K^+ may share a common pathway, but only to a small degree. The calcium channel agonist BAY K 8644 and antagonist D600 had no effect on α-BGT binding either alone or in the presence of K^+ or *d*-tubocurarine. On the other hand, forskolin, an activator of adenylate cyclase, and dibutyryl cAMP, an analog of cAMP, partially prevented the K^+ and the *d*-tubocurarine-induced increases in toxin binding. These results suggest an involvement of cAMP in both the nicotinic antagonist-induced and K^+-induced up-regulation of the sites. The observation that several mechanisms exist for the fine regulation of the nicotinic α-BGT binding sites in adrenal chromaffin cells could imply that this nicotinic receptor population plays a role in this tissue.

α-BGT is an excellent marker for the nAChR at the neuromuscular junction and in electroplax. This is contrast to the situation in mammalian nervous tissues. Although α-BGT binds with high affinity to a cholinergic receptor with the characteristics of a nicotinic receptor ligand (1, 2), the site to which it binds appears to be distinct from the nAChR involved in synaptic events in many neuronal tissues. This is evidenced from the results of functional studies, which showed that α-BGT did not affect nicotinic sensitivity at many neuronal synapses (2, 3), and receptor characterization work, which provided evidence that the neuronal nicotinic receptor and the α-BGT site have different molecular structures (4–6). As well, ultrastructural localization studies (7–11) and experiments involving receptor regulation (12–14) suggest these two components may be distinct.

Work with adrenal medullary cells or PC12 cells, which are derived from a chromaffin cell tumor, also indicate the functional nicotinic receptor and the nicotinic α-BGT site may be distinct in this tissue of neural origin. Although α-BGT bound in a saturable manner to a site with nicotinic characteristics (4, 15–17), the toxin did not block nicotinic receptor-mediated function in chromaffin cells or PC12 cells (4, 16, 18–21). Furthermore, antibodies against the eel acetylcholine receptor blocked nicotinic sensitivity but did not recognize the α-BGT binding component in PC12 cells (4). More recent work has shown that the functional acetylcholine receptor in PC12 cells reacts with a monoclonal antibody to brain nicotinic receptors, which have been shown to be distinct from the α-BGT site (22). Additionally, experiments concerned with receptor regulation demonstrated that the α-BGT site and the nAChR are not necessarily altered in parallel in response to varying experimental manipulations (13, 14, 17, 21, 23). All the above observations indicate a dissociation between the functional nicotinic receptor and the nicotinic α-BGT binding site in adrenal medullary chromaffin cells and PC12 cells, in line with the results obtained in other neuronal tissues.

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ABBREVIATIONS: α-BGT, α-bungarotoxin; DBcAMP, dibutyryl cAMP; nAChR, nicotinic acetylcholine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

Previous studies from our laboratory (23) have shown that the nicotinic antagonist *d*-tubocurarine, as well as mecamylamine, resulted in a marked (5–10-fold) up-regulation of the nicotinic α-BGT binding sites in adrenal medullary chromaffin cells in culture. In other neuronal preparations, only a minimal increase in the α-BGT sites was observed in response to nicotinic antagonists (24–26). In view of the magnitude of the increase in adrenal chromaffin cells, these cells appeared to be an ideal system to examine the molecular mechanisms involved in the up-regulation of the α-BGT binding sites. K^+ has previously been shown to result in an increase in α-BGT binding in cultured neuronal cells (25). To determine whether the nicotinic antagonist-induced increase in the α-BGT sites might share some common pathway with the K^+-induced increase, we investigated the effect of K^+ on α-BGT binding and on the *d*-tubocurarine-induced increase in toxin binding sites. Adenylate cyclase activation can induce a down-regulation of several receptor systems (27) and, in addition, can result in an inhibition of nicotinic-mediated cellular processes in various cell types (28, 29). In light of these observations, the effects of an adenylate cyclase activator and an analog of cAMP were investigated on toxin binding to chromaffin cells. Alterations in Ca^{2+} flux and/or mobilization is also known to be involved in receptor regulation. For instance, blockade of Ca^{2+} channels produces an increase in α-BGT receptors in chick retina cultures (30) and in muscarinic acetylcholine receptors in a neuroblastoma cell line (31); therefore, the effect of a Ca^{2+} channel agonist and antagonist was determined. The results obtained suggest that several cellular mechanisms are involved in the regulation of the nicotinic α-BGT sites.

Materials and Methods

Adrenal medullary chromaffin cell cultures. Bovine adrenal glands, obtained from the Crabtree Slaughterhouse (Vanier, Ontario Canada) were placed in cold sterile Locke's solution. The cortex was removed and the medulla was perfused with Ca^{2+}/Mg^{2+}-free Locke's solution. The chromaffin cells were isolated and purified as previously described by Trifaró and Lee (18). Cells were plated on collagen-coated culture dishes; there were 10^6 cells/culture dish for the ^{125}I-α-BGT binding assays and 2.5×10^5 cells/well of a 24-well culture plate for the [^{3}H]noradrenaline release studies. The media bathing the cells was Dulbecco's modified Eagle's medium enriched with 10% fetal calf serum, ascorbic acid (0.1 mM), glucose (1 mg/ml), and HEPES buffer (3.6 μg/ml). The media also contained the following antibiotics; penicillin (100 μg/ml), streptomycin (100 μg/ml), tetracycline (5 μg/ml), gentamicin (10 μg/ml), and mycostatin (25 units/ml). To inhibit fibroblast proliferation, 10^{-5} M 5-fluorodeoxyuridine and 10^{-5} M cytosine arabinoside, which inhibits cell division, were also added. Cultures were stored in a humified incubator at 37°, under a CO_2 and air (5:95) atmosphere. The culture medium was changed every 3–4 days.

^{125}I-α-BGT binding to chromaffin cells in culture. The binding of α-BGT to 7- to 14-day-old chromaffin cells in culture was determined as previously described (23). During the last 3–4 days in culture, the cells were incubated with various drugs as indicated in the figure legends. To remove any residual drugs before the α-BGT binding assay, the medium was removed and the cells were washed extensively with Locke's buffer. This involved three washes with 2 ml of Locke's buffer over a period of 30 min, with two additional washes with 2 ml of Locke's buffer, each with a 30-min incubation at 37°. This was followed by a 60-min preincubation of the cell cultures at 37° in the presence or absence of 10^{-4} M *d*-tubocurarine. ^{125}I-α-BGT (1.5 nM) was then added to each plate for a 90-min incubation at 37° (0.1 to 10 nM ^{125}I-α-BGT was used for the ^{125}I-α-BGT saturation curve). The cells were subsequently washed six times with 2-ml aliquots of Locke's buffer over a 60-min period to remove excess radiolabeled α-BGT. The cells were resuspended in 1 ml of Locke's buffer using a rubber policeman and the radioactivity was measured in a γ-counter.

DNA determination. To determine the DNA content of the chromaffin cell samples, 0.5 ml of 1.8 N trichloroacetic acid was added to the cells that had previously been suspended in 1 ml of Locke's buffer. The samples were centrifuged at $7800 \times g$ for 10 min; all additional centrifugations were carried out in this manner. The pellet was then washed three times with 1 ml 0.6 N trichloroacetic acid and twice with 0.1 M potassium acetate in absolute ethanol. One ml of absolute ethanol was added to the pellets, before a 15 min period at 60°. The samples were centrifuged, the supernatant was discarded, and the pellet was dried at 37° for 60 min. The pellets were then resuspended in 250 μl of 1.0 N perchloric acid and heated at 75° for 30 min. After centrifugation, 100-μl aliquots were removed and the DNA content was determined according to the method of Setaro and Morley (32).

[^{3}H]Noradrenaline uptake and release studies. Seven- to 9-day-old chromaffin cell cultures were incubated in the absence or presence of an additional 20 mM K^+ for 3 days. On the day of the experiment, each culture well was washed as described for the ^{125}I-α-BGT binding assay. The cells were then incubated for 1 hr at 37° with 250 μl of amino acid-free Dulbecco's modified Eagle's medium containing 0.25 μCi [^{3}H]noradrenaline ($\simeq 0.2 \times 10^{-7}$ M). After the loading phase, cultures were washed seven times with regular Locke's buffer over a 60-min period. Basal release was determined for a 3-min incubation period before stimulated release; release of [^{3}H]noradrenaline in the presence of 10^{-4} M acetylcholine was measured over three consecutive 3-min stimulation periods, followed by one more 3-min period of nonstimulated release of the radiolabeled compound. Ice-cold perchloric acid (0.4 N) was added to lyse the cells and determine intracellular [^{3}H]noradrenaline content. Total uptake of [^{3}H]noradrenaline is represented by the sum of radioactivity measured in the perchloric acid extract and the radioactivity secreted under basal and stimulating conditions. Release was expressed as a percentage of total [^{3}H]noradrenaline originally taken up per 2.5×10^5 cells.

Statistical comparisons were done using the Student's *t*-test.

Materials. ^{125}I-α-BGT (10–20 μCi/μg) and [^{3}H]noradrenaline (43.7 Ci/mmol) were obtained from New England Nuclear (Boston, MA); *d*-tubocurarine, nicotine, carbachol, forskolin, and D600 (methoxyverapamil) from Sigma Chemical Co. (St. Louis, MO); and DBcAMP from Boehringer (Mannheim West Germany). BAY K 8644 was a generous gift from Miles Institute (New Haven, CT).

Results

Effect of high K^+ and *d*-tubocurarine on α-BGT binding to chromaffin cells in culture. Previous work had shown that the nicotinic antagonist *d*-tubocurarine resulted in a marked increase in ^{125}I-α-BGT binding to adrenal medullary chromaffin cells in culture. To determine whether other agents such as K^+ could increase the α-BGT sites, cells were incubated for 3 days in the absence or presence of 10^{-4} M *d*-tubocurarine or 50 mM KCl. The addition of 10^{-4} M *d*-tubocurarine resulted in a significant increase in α-BGT binding (Fig. 1), as had previously been demonstrated. K^+ resulted in a somewhat larger increase in α-BGT binding (from 0.24 ± 0.06 fmol/culture dish to 2.74 ± 0.19 fmol/culture dish). On the other hand, addition of NaCl of equivalent ionic strength did not produce any change in binding. To characterize the K^+-induced up-regulation of α-BGT binding, a dose-response curve was obtained. Adrenal medullary chromaffin cells in culture were incubated for 3 days with an extra 0–50 mM K^+. The osmolarity of the medium was not adjusted when this additional K^+ was added. The results, depicted in Fig. 2, show that in this case a maximal increase occurs with approximately 20 mM K^+. The DNA content of the samples was determined. There was no difference in the DNA

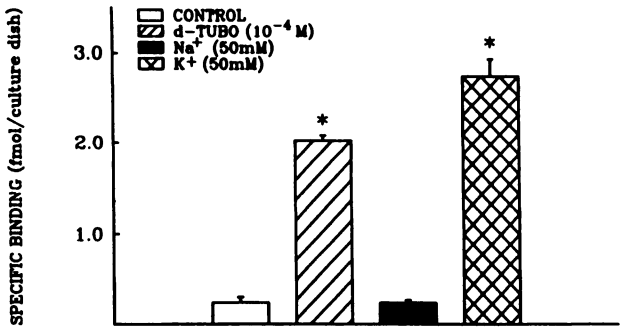

Fig. 1. The effect of high K^+ and *d*-tubocurarine on the specific binding of α-BGT to chromaffin cells in culture. Adrenal medullary chromaffin cells were plated as described in Materials and Methods. The cells were incubated for 3 days in the absence or presence of 10^{-4} M *d*-tubocurarine (*d-TUBO*), 50 mM NaCl (*Na^+*), or 50 mM KCl (*K^+*). Specific binding of α-BGT is expressed as fmol/culture dish. Each *bar* depicts the mean ± standard error of five or six culture dishes. Significance of difference from control cultures is indicated by *, $p < 0.001$.

content per sample for any of the concentrations of K^+ used, except for a slight decrease with 50 mM K^+ (Fig. 2A).

To determine whether the increased α-BGT binding seen after treatment with K^+ was due to an increase in the maximal number of binding sites (B_{max}) and/or to an increase in affinity of the receptor for the toxin (K_d), a saturation curve was obtained comparing the binding of ^{125}I-α-BGT in control cultures to that in cultures that had been treated for 3 days in the presence of 20 mM K^+. The saturation studies were done using a 90-min incubation period; previous work had shown that ^{125}I-α-BGT binding to the cells has plateaued at this time (17). Dissociation studies (data not shown) indicated that the toxin bound reversibly to the receptor in chromaffin cells in culture with a half life of approximately 6 hr; this is of a similar magnitude (that is, hours) as has been observed for the dissociation of α-BGT from brain membranes (1). Because the binding was reversible and the experiments were done when the binding had reached a plateau with respect to time, saturation and Scatchard analysis could be done. The saturation curve, depicted in Fig. 3A, shows that toxin binding plateaus at approximately 2 nM for both control and K^+-treated cultures. A Scatchard plot (Fig. 3B) yielded B_{max} values of 0.07 and 0.14 fmol/μg of DNA and K_d values of 0.96 and 0.66 nM for control and treated cultures, respectively, using linear regression analysis. Although chronic treatment of the cells with K^+ produced a marked increase in α-BGT binding in all experiments, the magnitude of the increase varied among experiments, despite the fact that experimentals and controls were always compared within a given culture set. The reasons for this are not clear; however, it may arise as a result of variability in the source of the cells (different groups of animals) from culture to culture, as well as differences in batches of fetal calf serum, medium, and other materials used in the culture process. With regard to the variations obtained, the increase in the α-BGT sites after incubation with K^+ ranged from 2- to 8-fold.

Regulation of the K^+-induced up-regulation of α-BGT binding sites by nicotinic agonists. Chromaffin cells were cultured and incubated for 3 days in the absence or presence of the nicotinic agonists carbachol (10^{-3} M) or nicotine (10^{-4} M), either alone or in combination with 20 mM K^+. Nicotine, but not carbachol, resulted in a significant decrease in ^{125}I-α-BGT binding (Fig. 4). In the majority of experiments involving

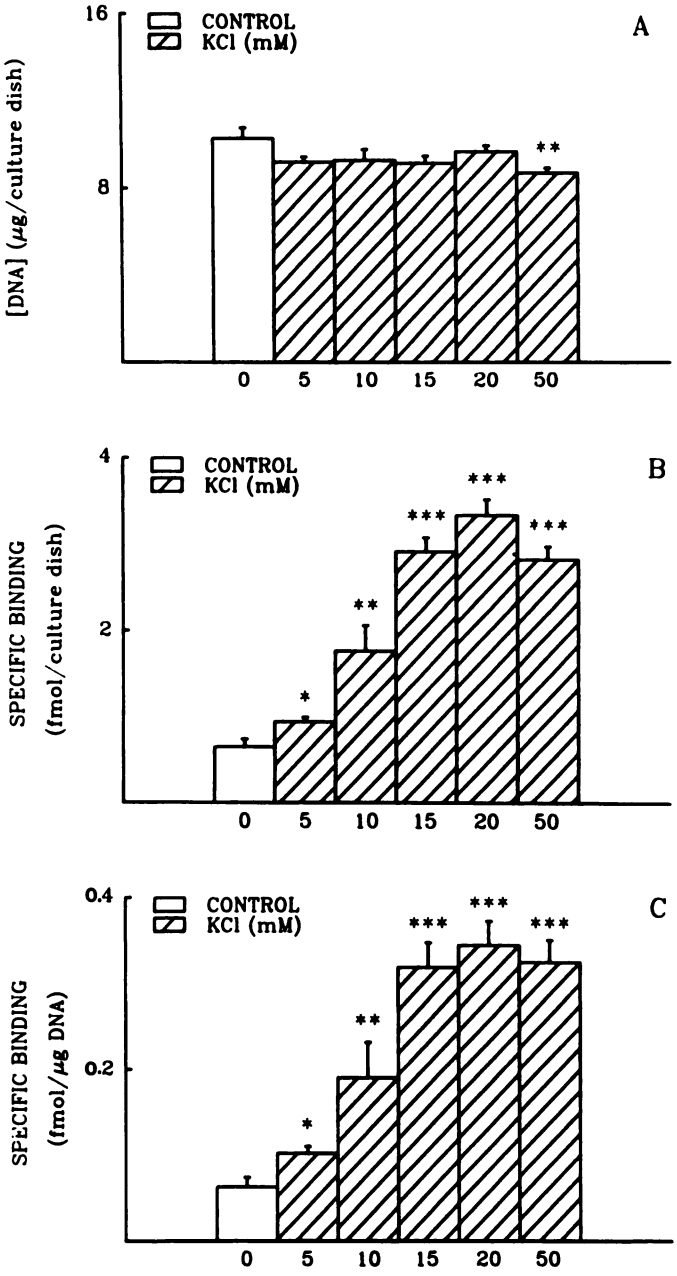

Fig. 2. The effect of varying concentrations of K^+ on specific α-BGT binding to, and DNA content of, adrenal medullary chromaffin cells in culture. Cells were incubated for 3 days with KCl; the concentrations indicated represent the additional amount of KCl added to the culture media, which already contained 2 mM KCl. In A, each *bar* represents the mean ± standard error of the DNA content (μg/culture dish) of eight or nine culture plates. Each *bar* represents the mean ± standard error of five or six culture dishes as fmol/culture dish in B and fmol/μg DNA in C. Significance of differences from control cultures is indicated by *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

nicotinic agonists, these drugs did not affect ^{125}I-α-BGT binding. However, they did occasionally result in a significant decrease in binding (approximately one in four cases), although they never initiated an increase. The reason for this variation is not clear. When 20 mM K^+ was added to the cells, binding was enhanced as compared with controls (0.34 ± 0.03 fmol/μg of DNA versus 0.06 ± 0.01 fmol/μg of DNA for control plates); both nicotinic agonists were able to prevent this K^+-induced up-regulation in α-BGT binding sites. The DNA content of the

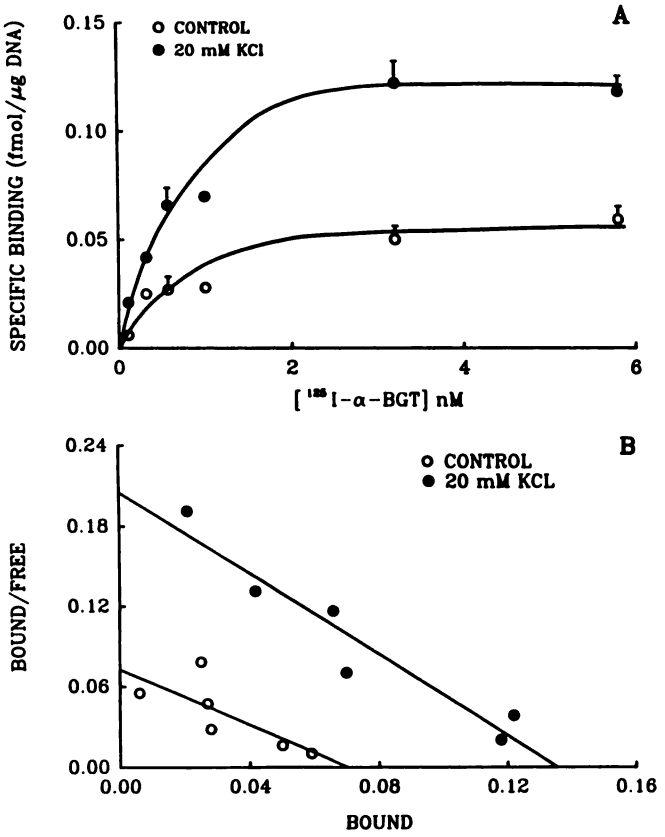

Fig. 3. Saturation curve (A) and Scatchard analysis (B) of α-BGT binding to adrenal chromaffin cells cultured in the absence or presence of elevated K^+. Cells in culture were incubated for 3 days in the absence (*CONTROL*) or presence of an additional 20 mM K^+. Binding of ^{125}I-α-BGT was determined as described in Materials and Methods. Each value represents the mean ± standard error of four or five culture dishes; the results are representative of two experiments. For the Scatchard plot, *BOUND/FREE* on the *y* axis is expressed as fmol/μg of DNA/nM and *BOUND* on the *x* axis as fmol/μg of DNA.

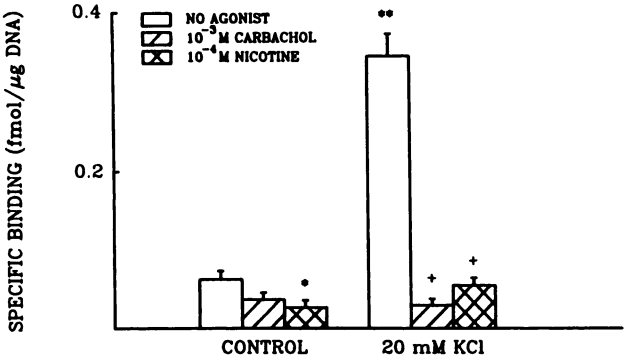

Fig. 4. The effect of nicotinic agonists on the K^+-induced up-regulation of the α-BGT sites in chromaffin cells in culture. Adrenal medullary chromaffin cells were cultured as described in Materials and Methods. After 1 week in culture, the cells were incubated for 3 days with or without 10^{-4} M nicotine or 10^{-3} M carbachol in the presence (20 mM KCl) or absence (*CONTROL*) of additional KCl. The *bars* represent the mean ± standard error of five or six culture dishes. Specific binding of α-BGT is expressed as fmol/μg of DNA. Significant differences from the *CONTROL* cultures with *NO AGONIST* are indicated by *, $p < 0.05$; **, $p < 0.001$; significant differences from cultures (*NO AGONIST*) incubated in the presence of added KCl are indicated by +, $p < 0.001$.

samples were the same as for controls, indicating that the agonist-induced reversal of the up-regulation was not due to a detrimental effect of these agents on the cells.

Combined effects of *d*-tubocurarine and K^+ on toxin binding to adrenal chromaffin cells. To determine whether the *d*-tubocurarine-induced increase in α-BGT binding might share a common pathway with that induced by K^+, the combined effect of *d*-tubocurarine and K^+ were determined on toxin binding to the cells. Cells were cultured and incubated for 3 days in the absence or presence of either 10^{-6} or 10^{-4} M *d*-tubocurarine and/or 10, 20, or 50 mM K^+. Previous results (17) had shown that *d*-tubocurarine exerted a maximal effect at 10^{-4} M. As expected, α-BGT binding was increased with both 10^{-6} and 10^{-4} M *d*-tubocurarine, as well as with 10, 20, and 50 mM K^+ (Table 1). When the two agents were added to the cells in combination, the increase in binding was partially additive in five of the six conditions, although completely additive in one treatment group (20 mM K^+ and 10^{-4} M *d*-tubocurarine). This suggests that, if there is a mechanism common to K^+ and *d*-tubocurarine in up-regulating the α-BGT sites, it is likely to be a minor component.

Effect of DBcAMP on the *d*-tubocurarine- and K^+-induced up-regulations of α-BGT binding sites in chromaffin cells in culture. Adrenal medullary chromaffin cells were cultured for 3 days in the absence or presence of *d*-tubocurarine (10^{-4} M) or K^+ (20 mM) alone or in combination with 0.1, 0.3, or 0.5 mM DBcAMP. As shown in Fig. 5, at the higher concentrations of DBcAMP there was a partial reversal

TABLE 1

The effect of elevated K^+ on the *d*-tubocurarine-induced up-regulation of the α-BGT binding sites

Bovine adrenal chromaffin cells were cultured and plated as described in Materials and Methods. In experiment A, an additional 0, 10, or 20 mM KCl was applied to the plates, which contained 0 (*none*), 10^{-6} M, or 10^{-4} M *d*-tubocurarine, (*d-Tubo*); the cells were subsequently incubated for 3 days. In experiment B, 0 or 50 mM KCl was added to the media in the absence or presence of the indicated concentrations of *d*-tubocurarine for 3 days. *n* signifies the number of plates used in the binding assays; the results represent pooled data from three separate experiments. The last two columns indicate the per cent increase in binding over control levels; *observed* is the experimentally observed per cent increase in binding, whereas *expected* is the theoretically expected increase in binding if the experimentally observed increases with KCl and *d*-tubocurarine treatment are added together. Control binding, that is, binding in the absence of added KCl and *d*-tubocurarine, was 0.20 ± 0.03 fmol/culture dish ($n = 15$).

Expt.	Added KCl	*d*-Tubo	*n*	Increase over control	
				Observed	Expected
	mM	M		%	
A	0	None	15		
		10^{-6}	9	220 ± 31	
		10^{-4}	9	458 ± 67	
	10	None	9	243 ± 48	
		10^{-6}	9	364 ± 55	463
		10^{-4}	10	551 ± 81	701
	20	None	9	492 ± 71	
		10^{-6}	9	572 ± 93	712
		10^{-4}	10	930 ± 93	950
B	0	None	15		
		10^{-6}	10	218 ± 27	
		10^{-4}	10	430 ± 46	
	50	None	10	309 ± 54	
		10^{-6}	9	363 ± 62	527
		10^{-4}	10	510 ± 45	739

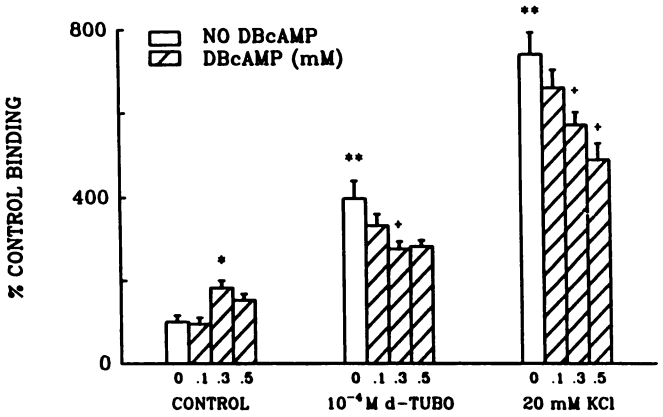

Fig. 5. The effect of DBcAMP on the *d*-tubocurarine and K^+-induced up-regulations of α-BGT binding sites in chromaffin cells in culture. Cells were incubated for 3 days in the absence (*CONTROL*) or presence of 10^{-4} M *d*-tubocurarine (*d-TUBO*) or 20 mM KCl in combination with the indicated concentrations (mM) of DBcAMP. Each *bar* represents the mean ± standard error of 3–13 culture plates; the results represent data pooled from two separate experiments. The results are expressed as percentage of control, where the control represents binding in the absence of both DBcAMP and *d*-tubocurarine; control binding was 0.033 ± 0.005 fmol/μg of DNA (n = 12). Significant differences from the *CONTROL* cultures with *NO DBcAMP* are indicated by *, $p < 0.01$; **, $p < 0.001$; significant differences from control cultures (*NO DBcAMP*) within the same treatment group are indicated by +, $p < 0.05$.

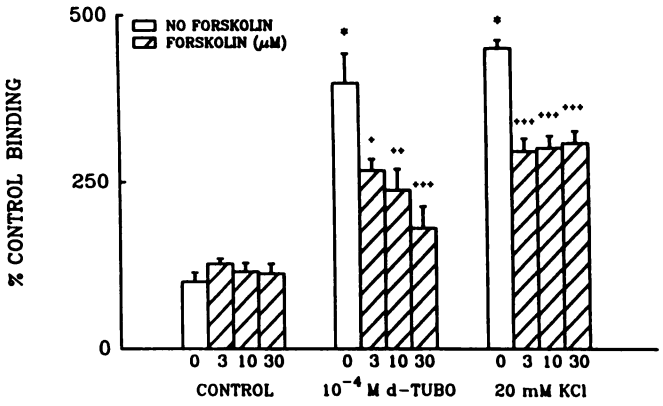

Fig. 6. The effect of forskolin on the *d*-tubocurarine and K^+-induced up-regulation of α-BGT binding sites in chromaffin cells in culture. Bovine adrenal chromaffin cells were incubated for 3 days in the absence (*CONTROL*) or presence of 10^{-4} M *d*-tubocurarine (*d-TUBO*) or 20 mM KCl in combination with the indicated concentrations (μM) of forskolin. Each *bar* represents the mean ± standard error of 9–16 culture plates and are pooled from three separate experiments. The results are expressed as percentage of control binding, where the control represents binding in the absence of both forskolin and *d*-tubocurarine or KCl; control binding was 0.13 ± 0.02 fmol/μg of DNA (n = 16). Significant differences from the *CONTROL* cultures with *NO FORSKOLIN* are indicated by *, $p < 0.001$; significant differences from control cultures (*NO FORSKOLIN*) within the same treatment group are indicated by +, $p < 0.05$; ++, $p < 0.01$, +++, $p < 0.001$.

of both the K^+- and *d*-tubocurarine-induced up-regulation of α-BGT binding sites. At 0.3 mM, DBcAMP resulted in a significant increase in toxin binding in control cultures; additional experiments are being done to determine the nature of this increase. DBcAMP did not result in any significant changes in the DNA content of the cells. In these experiments, K^+ resulted in an up-regulation of toxin binding sites significantly larger than that produced by *d*-tubocurarine, although this was not always the case (see Figs. 1 and 6). The reasons for these variations in the ratio of the increase in α-BGT binding produced by *d*-tubocurarine as compared with K^+ are not clear but may be due to differences in the cells from culture to culture. As maximal concentrations of each agent are used, the inconsistency may also reflect the fact that the two agents are exerting their effects via different mechanisms, which have differing capacities to regulate α-BGT binding sites.

Effect of forskolin on the *d*-tubocurarine- and K^+-induced up-regulations of α-BGT binding sites in adrenal chromaffin cells in culture. Chromaffin cells were incubated for 3 days in the absence or presence of 10^{-4} M *d*-tubocurarine or 20 mM KCl alone or together with 3, 10, or 30 μM forskolin. Previous work has shown these concentrations of forskolin to be effective in stimulating other adenylate cyclase-mediated processes in adrenal chromaffin cells in culture (28, 33, 34). As shown in Fig. 6, forskolin was able to partially reverse the *d*-tubocurarine- and the K^+-induced up-regulation of α-BGT binding sites. Forskolin did not result in any significant changes in the DNA content of the cells. These results show that adenylate cyclase activation can affect the antagonist- and K^+-induced up-regulation of the α-BGT sites; cAMP appears to exert a net inhibitory effect.

Effect of the Ca^{2+} channel agonist BAY K 8644 and antagonist D600 on α-BGT binding to chromaffin cells in culture. To determine whether alterations in Ca^{2+} might play a role in the up-regulation of the α-BGT binding sites, cultured chromaffin cells were incubated for 3 days in the absence or presence of either D600 (methoxyverapamil) or BAY K 8644 (1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylic acid methyl ester) alone or in combination with 20 mM K^+ or 10^{-4} M *d*-tubocurarine. Neither D600 (up to 10 μM) nor BAY K 8644 (up to 100 nM) had any effect on either the *d*-tubocurarine- or K^+-mediated increases in α-BGT binding. These concentrations of D600 and BAY K 8644 had previously been shown to be effective in altering other adrenal medullary functions (35, 36) and/or α-BGT receptor characteristics (30, 37). Experiments in the absence of Ca^{2+} or the presence of EGTA could not be done, because the cells are not viable for 3 days in culture under these conditions.

Effect of K^+ on [^{3}H]noradrenaline uptake into and release from chromaffin cells in culture. After at least 1 week in culture, cells were incubated for 3 days in the presence or absence of an additional 20 mM K^+. The effect of this treatment with K^+ on [^{3}H]noradrenaline release is illustrated in Fig. 7. Except for a small but significant decrease in [^{3}H]noradrenaline release during the second 3-min period of stimulation with 10^{-4} M acetylcholine, the pattern of release was similar for the control and K^+-treated cells in culture. To determine whether an increase in the evoked [^{3}H]noradrenaline release pattern might be detectable at submaximal concentrations of acetylcholine, release was also measured using 10^{-5} M and 3×10^{-5} M acetylcholine (data not shown); however, there were no significant increases in the treated cultures as compared with control with the lower concentrations of acetylcholine. Thus, the large increase in ^{125}I-α-BGT binding observed after treatment of the cells with elevated K^+ is not associated with a corresponding increase in nicotinic receptor-mediated [^{3}H]noradrenaline release. This further illustrates the distinctness of these two nicotinic parameters.

The effect of K^+ treatment on [^{3}H]noradrenaline uptake is presented in Table 2. Treatment with 20 mM K^+, which resulted

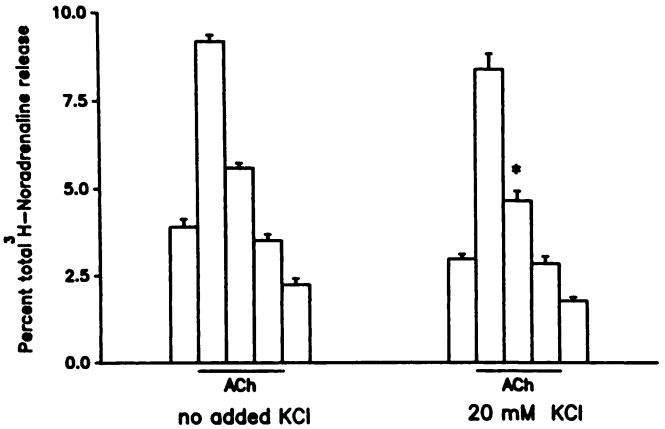

Fig. 7. The effect of elevated K^+ on [^{3}H]noradrenaline release in chromaffin cells in culture. Adrenal medullary chromaffin cells were cultured and plated as described in Materials and Methods. Groups of cells were incubated for 3 days in the absence or presence of an additional 20 mM KCl. At the end of this time, the cells were washed and the release was determined. Each value depicts the mean ± standard error for eight culture wells; the results represent data pooled from two separate experiments. The *horizontal bars* mark the period of acetylcholine (*ACh*; 10^{-4} M) mediated stimulation. Each *vertical bar* represents a 3-min collection period. Significance of difference from control cultures (*no added KCl*) at the corresponding release interval is indicated by *, $p < 0.05$.

TABLE 2

Effect of elevated K^+ on [^{3}H]noradrenaline uptake in chromaffin cells in culture

Chromaffin cells were plated and cultured as previously described. After 7–9 days in culture, cells were incubated for 3 days in the absence or presence of 20 mM KCl. At the end of this time, the cells were washed as described in Materials and Methods. Culture wells, each containing 2.5×10^5 cells, were then incubated with 0.25 μCi (in 250 μl) of [^{3}H]noradrenaline for 1 hr at 37°. Total uptake is expressed as fmol/culture well and represents the mean ± standard error of the indicated number of wells (*n*); the results represent data pooled from two separate experiments. The last column indicates the per cent uptake compared with control.

Condition	Additional KCl	*n*	Uptake	
	mM		*fmol/culture well*	*% control*
Control	0	20	251 ± 10	100 ± 4
KCl treated	20	20	248 ± 13	98 ± 5

in a marked increase in ^{125}I-α-BGT binding, did not affect uptake of [^{3}H]noradrenaline. These results involving uptake, as well as the previous data related to [^{3}H]noradrenaline release, demonstrate that K^+ does not simply result in a generalized enhancement of membrane-related events.

Discussion

Changes in membrane excitability have been previously shown to affect neurotransmitter-related functions and receptor characteristics (37–39). To determine whether this might be a regulatory factor in the nicotinic antagonist-induced up-regulation of the α-BGT sites, the effect of varying K^+ concentrations was investigated. The present results indicate that K^+ was able to induce an increase in α-BGT binding sites in a dose-dependent manner. This K^+ regulation was not simply due to an increased ionic strength or osmolarity, as the addition of an equivalent concentration of Na^+ did not alter toxin binding. Elevated K^+ has been shown to have similar effects on nicotinic α-BGT binding in other tissues. Smith *et al.* (25) found that this treatment resulted in an increase in α-BGT binding sites in chick ciliary ganglion neurons in culture. As well, an increase in ^{125}I-α-BGT binding was observed in muscle cells in culture after exposure to elevated K^+ (37). Other receptors, including the muscarinic acetylcholine receptor have been shown to be similarly affected by K^+ (31). This observed increase in the number of α-BGT sites in response to K^+, although occurring in other systems as well, was initially somewhat surprising in view of our previous results with veratridine (23), which showed that veratridine reversed the antagonist-induced increases in α-BGT binding. However, recent work by Wada *et al.* (40, 41) may offer an explanation for these seemingly discrepant observations. These authors showed that, in adrenal medullary chromaffin cells, secretion of catecholamines can be elicited via several distinct mechanisms. These include 1) carbachol-evoked responses that are sodium dependent and inhibited by tetrodotoxin, 2) veratridine-evoked responses that are sodium dependent and not affected by tetrodotoxin, and 3) high K^+-evoked responses that are not sodium dependent and not inhibited by tetrodotoxin. Thus, in chromaffin cells in culture, nicotinic agonists, veratridine, and K^+ operate via different mechanisms to depolarize cells; this finding may account for the differential regulation of the α-BGT sites by these agents.

The K^+-induced increase in α-BGT binding in adrenal medullary chromaffin cells was completely prevented by nicotinic agonists such as nicotine and carbachol. Thus, these newly appearing α-BGT binding sites can be regulated by nicotinic cholinergic ligands. To determine whether the nicotinic antagonist-induced increase in α-BGT binding and the K^+-induced up-regulation of the sites might share a common pathway, the combined effects of these two agents on the binding of α-BGT to adrenal medullary cells were determined. The up-regulation of toxin binding observed in the presence of both K^+ and *d*-tubocurarine was generally less than the combined increases that occurred in the presence of either agent alone; that is, the increases were only partially additive. These findings suggest that the nicotinic antagonist and elevated K^+ appear, to a large degree, to up-regulate the nicotinic α-BGT sites via separate mechanisms, although they may also share some common features. However, there are alternate possibilities; an absence of additivity might, for example, be observed if separate regulatory pathways eventually limited the same metabolic step (that is, the rate of protein synthesis, availability of substrates and cofactors, etc.).

The adenylate cyclase system has been implicated in the regulation of a variety of nicotinic-mediated functions, including catecholamine secretion from adrenal chromaffin cells (28, 29). Also, increased cAMP levels have been shown to down-regulate opiate receptors, muscarinic acetylcholine receptors, and α_2-adrenergic receptors to varying extents in cells in culture (27); this may occur through the inhibition of the release of a factor that has the ability to up-regulate these same three receptors (42). In our system, DBcAMP and forskolin partially reversed both the *d*-tubocurarine-induced and the K^+-induced up-regulation of toxin binding sites. Thus, an enhancement of cAMP levels or an activation of adenylate cyclase appears to exert a net inhibitory effect on the induced up-regulation of the nicotinic α-BGT sites. An involvement of cAMP will be further investigated by determining whether cAMP levels are altered after treatment of the cells in culture with nicotinic agents.

The inactivation of voltage-sensitive Ca^{2+} channels has been

shown to be involved in the up-regulation of the muscarinic acetylcholine receptor in cultured neuronal cells (31). These studies suggest that Ca^{2+}-mediated mechanisms may be involved in long term receptor regulation. In adrenal chromaffin cells in culture, however, we found that the Ca^{2+} channel agonist BAY K 8644 (43) and the Ca^{2+} channel antagonist D600 had no effect on control toxin binding or on the *d*-tubocurarine and K^+-mediated increases in α-BGT binding. This suggests that Ca^{2+} channel activation and/or inactivation do not play an important role in the regulation of the α-BGT binding sites by either K^+ or nicotinic antagonists.

Noradrenaline is released from cultured chromaffin cells in response to acetylcholine acting on the functional nicotinic receptor. An increase in this population of receptors would result in an increased secretory response to applied acetylcholine. We show that, although K^+ increased the number of toxin binding sites, the uptake of noradrenaline and its subsequent release in response to applied acetylcholine was slightly reduced or not altered as compared with control cultures. This suggests that K^+ does not have a general stimulatory effect on all cellular events. This further indicates that the toxin binding site is a separate entity from the nAChR mediating noradrenaline release and that these receptors are differentially regulated. This is also inferred from the observation that both *d*-tubocurarine and a brain extract, which result, respectively, in up- and down-regulation of toxin sites in chromaffin cells, have no effect on acetylcholine-stimulated noradrenaline release (17, 23).

To conclude, these results confirm that nicotinic antagonists increase α-BGT receptors in adrenal medullary chromaffin cells and, in addition, show that K^+ can result in a marked up-regulation of the toxin binding sites, possibly by a mechanism in large part distinct from the one activated by nicotinic antagonists. Furthermore, results were presented that indicated that the adenylate cyclase system can play a role in receptor regulation. Several molecular mechanisms thus appear to be involved in the control of the nicotinic α-BGT sites in chromaffin cells in culture.

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