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Cholinergic Function and α-Bungarotoxin Binding in PC12 Cells

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SUMMARY

The cell line PC12, derived from an adrenal chromaffin cell tumor, expresses both ganglionic (C6) acetylcholine receptors (nAChR) and an α-bungarotoxin (BGT) binding protein of unknown function. We measured nicotinic Na^+ fluxes of 180–260 nmol/mg protein·min and 0.35–0.8 pmol [^{125}I]BGT binding sites/mg protein; 45–65% of the [^{125}I]BGT binding was to intracellular sites. We blocked ganglionic Na^+ fluxes with reversible and irreversible inhibitors and tested whether a residual BGT-sensitive flux could be identified. No such flux was detected. These experiments place an upper limit on the amount of an undetected Na^+ flux such that we question whether the BGT binding protein could act as a functional nAChR. Na^+ flux and [^{125}I]BGT binding were irreversibly inactivated by the affinity-directed antagonist 4-(*N*-maleimido)benzyltrimethylammonium bromide (MBTA), and the appearance of new nAChRs and BGT binding proteins was monitored. New ganglionic nAChRs appeared at a rate of 0.029 hr^{-1}, corresponding to a steady state turnover $t_{½}$ of 24 hr. BGT binding protein was synthesized more rapidly (K = 0.11 hr^{-1}, $t_{½}$ = 6.5 hr). When protein synthesis was simultaneously blocked with cycloheximide, insertion of BGT binding protein into the plasma membrane decreased to 11% of control values. Cycloheximide also induced a biphasic decline in intracellular BGT binding sites. Incubation of PC12 cells in 5 mM carbamylcholine for varying intervals resulted in a rapid 30% loss of Na^+ flux activity. In contrast, the concentration of BGT binding protein did not change.

The availability of elapid α-neurotoxins that bind to nAChRs with high affinity and almost absolute specificity has been perhaps the primary reason that progress in biochemical characterization of these receptors has progressed so rapidly.

In contrast, much less is known about a second class of nAChRs known to exist in the vertebrate autonomic nervous system. This state of affairs has arisen largely because the neurotoxins that have proved so useful in characterizing and isolating neuromuscular junction nAChRs do not bind to these neuronal nAChRs (1–3).

There are, however, proteins in the autonomic nervous system (1, 2, 4–6) and in the brain (7–10) that are bound by BGT and related neurotoxins used to identify neuromuscular junction nAChRs. Early efforts to characterize these BGT binding sites documented that their properties closely resembled those of nAChRs and, indeed, identified them as nAChRs. Subsequent experiments documented that although these nicotinic α-toxins would bind to sites in the autonomic nervous system, they would not block ganglionic (C6) conductances (1, 2, 4–6, 10–12). It is now generally agreed that both ganglionic nAChRs and BGT binding sites are biochemically separate entities that coexist in the same cells in the autonomic nervous system and that ganglionic nAChRs are responsible for most if not all constituent cholinergic conductances. It is unknown whether ganglionic nAChRs and BGT binding sites are colocalized in the brain. Clearly, BGT binding sites are not colocalized with a third nAChR-like molecule, the high affinity agonist site identified by [^{3}H]acetylcholine and [^{3}H]nicotine binding sites (13–16).

In this manuscript, we extend our previous studies on neuronal BGT binding proteins by examining the BGT binding protein known to be present in the cell line, PC12 (1). PC12 cells are a clonal cell line obtained from a rat adrenal chromaffin cell tumor (17). Adrenal chromaffin cells are of neural crest origin, and PC12 cells share many properties with both their parent chromaffin cells and sympathetic neurons in general. PC12 cells express both a BGT binding protein (1) and a nAChR that has been characterized using a Na^+ flux assay (1, 18–20). These nAChRs are of the ganglionic or C6 subtype present in the autonomic nervous system (12, 21, 22). PC12 cells consequently provide an ideal opportunity to understand the physiological role of neuronal BGT binding proteins and their relationship with ganglionic nAChRs.

Methods

A subclone of the PC12 cell line first isolated by Greene and Tischler (17) was obtained from Dr. Christiane Richter-Landsberg. Cells were grown in RPMI 1640 media containing 2 mM glutamine, 10% horse serum, and 5% fetal calf serum. For use, cells were plated onto 60 × 15 mm culture dishes that had been pretreated with polylysine (1 mg/ml, 40 min at room temperature). After 30 min, most cells were attached

ABBREVIATIONS: nAchR, acetylcholine receptor; BGT, α-bungarotoxin; MBTA, 4-(*N*-maleinido)benzyltrinethylammonium bromide; DTT, dithiothreitol.

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to the plate, and the cells were typically used within 3 hr, usually within 1 hr.

[^{125}I]BGT was purchased from New England Nuclear Corporation. α-Bungarotoxin was purchased from Sigma Chemical Company and, based on the outcome of experiments described in this manuscript, was judged to be free of contaminating toxins capable of blocking ganglionic fluxes. Mecamylamine was a gift from Dr. B. J. Morley. Dihydro-β-erythroidine was a gift from Dr. R. J. Bradley. MBTA was synthesized as described (23).

[^{125}I]BGT binding experiments. Cells were plated onto 60 × 15 mm culture dishes that had been pretreated with polylysine as described. Culture medium was removed from the dishes and replaced with buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose, 25 mM HEPES, adjusted to pH 7.4 with Tris base (HEPES buffer). [^{125}I]BGT binding to PC12 extracellular sites was examined by incubating the cells with 10^{-8} M [^{125}I]BGT for 40 min at room temperature in HEPES buffer. Cells were then exhaustively (6 × 1 ml) rinsed with buffer to remove unbound toxin, and the cells were solubilized in 2 ml of 0.5 M NaOH and counted. Control experiments were done similarly, but the cells were first incubated with 1.25×10^{-6} M unlabeled BGT for 30 min at room temperature. Preliminary experiments established that under these conditions all accessible BGT sites were saturated.

To assay solubilized BGT binding sites, cells were first plated on polylysine-coated dishes as before. Cells were removed from the dishes by trituration, then pelleted by low speed (500 × *g*) centrifugation. The pellet was then solubilized in buffer containing 1% Triton X-100, 5 mM EGTA, 5 mM EDTA, 1 μg/ml pepstatin, 10 mM sodium phosphate, pH 7.4 (1% Triton), for 30 min at 4°C. The solution was then centrifuged at 30,000 × *g* and the supernatant assayed using a Sephadex CM-50 pasteur pipette column assay described previously (9). Intracellular BGT sites were assayed in a similar manner after extracellular sites were saturated with 5×10^{-7} M unlabeled BGT for 40 min at room temperature. Protein assays were done by the method of Lowry (24).

Na^+ flux experiments. Na^+ flux experiments were done using methods described by Stallcup (18). Cells were plated on polylysine dishes as before and incubated in HEPES buffer for 30 min at room temperature before use. Flux measurements were initiated by addition of HEPES buffer containing 0.7–1.2 μCi/ml [^{22}Na], 2 mM carbamylcholine chloride, and 5 mM ouabain. After incubation for 1 min at room temperature, the dishes were rapidly rinsed in three beakers containing 800 ml ice cold HEPES buffer. The cells were digested in 2 ml of 0.5 M NaOH for 12 hr, counted, and assayed for protein.

The amount of carbamylcholine-stimulated Na^+ flux observed ranged from 190 to 260 mmol/mg protein · min, in good agreement with that reported by Stallcup (18). The fluxes varied slightly depending on variables not controlled for, including ambient temperature and the interval between last feeding and last replating, etc.; thus control experiments were done with each group of culture dishes. Control values obtained in the absence of carbamylcholine were 31 ± 4 nmol/mg · min and were identical to values obtained with high concentrations of cholinergic inhibitors such as mecamylamine and *d*-tubocurarine. These observations suggested that muscarinic AChRs do not contribute to Na^+ fluxes in PC12 cells.

Blockade of Na^+ flux and [^{125}I]BGT binding with MBTA. All steps were done in HEPES buffer at room temperature. Cells were plated on polylysine-coated dishes as before. In experiments where the toxin site was protected by BGT, cells were first incubated in 10^{-8} M BGT, cells were first incubated in 10^{-8} M BGT for 40 min at room temperature, then washed (6 × 1 ml) to remove unbound BGT.

Reaction conditions were essentially those of LePrince (19). Cells were incubated in 10^{-3} M DTT for 30 min, then washed (6 × 1 ml). Cells were then reacted with 10^{-5} M MBTA for 10 min and washed as before. Preliminary experiments established that this concentration of MBTA produced a maximal effect in blocking both Na^+ flux and [^{125}I]BGT binding. Cells were then incubated in 10^{-4} M 5,5′-dithiobis(2-nitrobenzoate) (DTNB) for 20 min and again washed. The DTNB step reoxidizes the disulfide bond reduced by DTT and reverses receptor inactivation induced by reduction of this bond. In experiments where the BGT site was protected with BGT, cells were then incubated in 0.14 M carbamylcholine for 5 hr at 37°C to dissociate BGT from its site. Cells were then exhaustively washed (10 × 1 ml) and incubated before use in buffer for 30 min at room temperature to allow recovery from desensitization. Control experiments established that approximately 60% of carbamylcholine-stimulated Na^+ flux was recovered with this protocol if the MBTA step was omitted. The loss of Na^+ flux activity due to the carbamylcholine incubation step was presumed to be largely a consequence of down-regulation (see Results).

The appearance of new ganglionic nAChRs and BGT binding proteins was examined after blocking existing plasma membrane AChRs and BGT binding proteins with MBTA. Cells were then placed in culture medium and returned to the incubator for prescribed intervals. The amounts of Na^+ flux and [^{125}I]BGT binding were then determined as described. In experiments where the effect of cycloheximide on extracellular BGT binding sites was examined, cycloheximide (25 μg/ml) was added concurrently with MBTA. The effect of cycloheximide on intracellular BGT sites was examined by adding cycloheximide to the culture medium and incubating at 37°C for prescribed intervals. Forty minutes before the end of the incubation period 10^{-3} M DTT was added to the medium. Immediately after incubation, extracellular sites were blocked with 10^{-5} M MBTA. Cells were then solubilized in 1% Triton and assayed as described previously.

Results

Table I shows the amount of [^{125}I]BGT binding to intact PC12 cells and to PC12 cells that were solubilized with the nonionic detergent Triton X-100. Since cells are known to be impermeable to BGT, incubation with [^{125}I]BGT in the absence of detergents would be expected to saturate only cell surface sites. If additional intracellular sites are present, then solubilization of the cells with detergents would result in additional BGT binding sites being detected. The approximately two-fold increase in BGT sites observed on solubilization suggests that a significant fraction of the BGT binding sites in PC12 cells are intracellular. To further substantiate this conclusion, additional experiments were conducted were extracellular BGT binding sites were first saturated with unlabeled BGT. The cells were washed to remove unbound BGT then solubilized with 1% Triton, and the amount of BGT binding liberated by solubilization was determined. These experiments, which directly measure intracellular BGT sites, agreed very closely with the previous experiments where intracellular sites were calculated as the difference between BGT binding to extracellular sites and to sites solubilized by 1% Triton.

Although the experiments described in Table 1 utilized unlabeled BGT to block extracellular sites, an alternative approach, described in Fig. 3A, was to block extracellular sites with MBTA. The concentration of intracellular sites measured with MBTA was essentially identical to that measured with unlabeled BGT, and, because the block of extracellular sites by

TABLE 1
Binding of [^{125}I]BGT to PC12 cellular compartments

	[^{125}I]BGT binding
	fmol/mg protein
Cell surface sites	268
Sites solubilized by 1% Triton	510
Intracellular sites solubilized by 1% Triton after saturation of cell surface sites with 5×10^{-7} M unlabeled BGT	249

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MBTA was irreversible, this approach is advantageous for some experiments.

As was the case for Na^+ flux, the amount of [^{125}I]BGT binding in PC12 cells varies depending on factors not corrected for, including interval between last refeeding and replating, growth density, etc. Actual amounts of [^{125}I]BGT binding were found to range from 0.35 to 0.8 pmol/mg protein, with 45–65% of this associated with intracellular sites. In general, cells with higher concentrations of BGT sites tended to have a higher proportion of intracellular sites.

Previous investigations had established that BGT did not significantly block PC12 Na^+ flux (1, 2, 22). However, on reviewing the experimental data in these reports, we felt that it could not be unequivocally concluded that the BGT binding protein made no contribution to nicotinic Na^+ flux. In particular, a small (<10%) contribution to the total cholinergic flux mediated by the BGT binding protein might not have been detected in previous investigations. To address this possibility, our strategy was to block ganglionic flux with specific inhibitors, much as one might use quinuclidinyl benzilate to block muscarinic receptors in a tissue that displayed both muscarinic nicotinic responses.

A difficulty in this approach is that both ganglionic nAChRs and BGT binding proteins share a nicotinic pharmacology in regard to their binding sites, and thus the choice of suitable antagonists was not obvious. Based on a survey of the literature and our own preliminary experiments, we concluded that mecamylamine, hexamethonium, and *d*-tubocurarine were the most suitable candidates. We also tested a fourth ligand, dihydro-β-erythroidine, which was of interest because it was reported to be the only nicotinic antagonist found to be effective in blocking high affinity [^{3}H]acetylcholine binding to a putative central nAChR (15).

Figure 1 shows the concentration dependence of inhibition of Na^+ flux by the nicotinic antagonists mecamylamine, hexamethonium, *d*-tubocurarine, and dihydro-β-erythroidine. A concentration of antagonist was then selected that was sufficient to inhibit 85–90% of the total carbamylcholine-stimulated conductance. Na^+ fluxes were compared in the presence and absence of BGT. These experiments sought to determine if, under conditions where ganglionic nAChRs were largely inhibited, some component of the Na^+ flux could be identified that was potently blocked by BGT. Table 2 summarizes these experiments. No BGT-blockade component of Na^+ flux was observed in the presence of any of the four inhibitors tested.

We also tested the ability of these ligands to compete with [^{125}I]BGT binding to PC12 BGT binding sites. Based on the resultant dose-response curves (data not shown but in close agreement with Refs. 1 and 9), mecamylamine, hexamethonium, and *d*-tubocurarine were found to be 1,000-, 7-, and 4-fold more potent in blocking Na^+ flux than [^{125}I] BGT binding. Although dihydro-β-erythroidine was found to be an effective ganglionic blocker, it was an even better ($K_i = 4 \times 10^{-6}$ M) inhibitor of PC12 [^{125}I]BGT binding.

Cholinergic antagonists are known to often act both as competitive inhibitors of agonist binding and also as channel blockers. It was not possible to assess the channel blocking effects of antagonists acting on a putative BGT binding protein mediated Na^+ flux, since the very existence of such a flux has not been demonstrated. Thus, our inability to detect BGT binding protein-mediated Na^+ flux could simply reflect that each antagonist studied was as effective in blocking BGT binding protein-mediated Na^+ flux as it was in blocking flux mediated by ganglionic nAChRs.

Ganglionic nAChRs blocked by irreversible inhibitors that bind covalently could be extensively washed such that unbound ligand would be effectively removed and thus unable to act as a channel blocker. Thus, an additional set of experiments was performed using the affinity directed antagonist MBTA (23). MBTA is known to bind to electric fish nAChRs at a sulfhydryl group closely associated with the ligand binding site (25). This sulfhydryl group normally participates in a disulfide bond that must be reduced before MBTA can bind. This reduction step inactivates ganglionic nAChRs, but activity recovers when the disulfide bond is reoxidized (19). Since MBTA irreversibly inhibited both ganglionic Na^+ flux (19) and BGT binding (26), it was necessary to develop a means of protecting the BGT site during the time the ganglionic nAChRs were exposed to MBTA. We ultimately established that protection of this site was best done with BGT itself. Figure 2 shows that the BGT could be rapidly dissociated by incubation in 0.14 M carbamylcholine at 37°C. After incubating the cells in buffer for 30 min to allow recovery from desensitization caused by the 0.14 M carbamylcholine, they were tested to determine if any BGT-sensitive Na^+ flux was observed. Table 3 shows that blockade of ganglionic nAChRs failed to unmask a BGT-blockable Na^+ flux component mediated by the BGT binding protein.

By treating PC12 cells with MBTA to abolish all Na^+ flux and [^{125}I]BGT binding, we could examine the rate at which ganglionic nAChRs and BGT binding proteins reappeared.

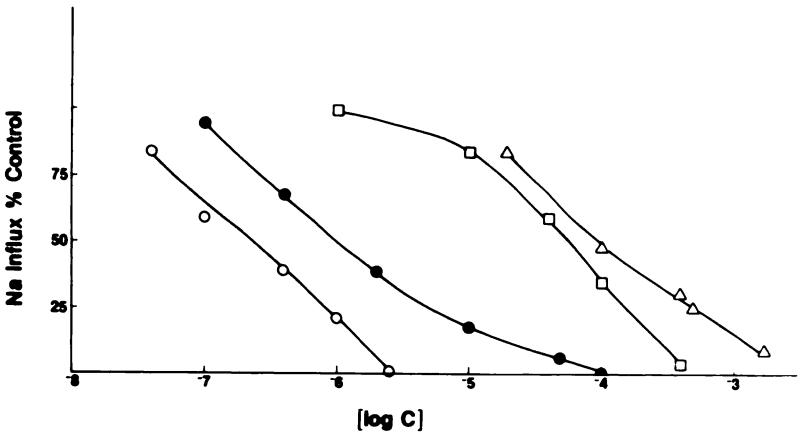

Fig. 1. Inhibition of carbamylcholine-stimulated Na^+ flux by ganglionic antagonists. Cells were plated into four to five polylysine coated dishes. One dish, containing no antagonist, was a control (100%); a second, containing no carbamylcholine, was a background (0%). Control fluxes were within the ranges described in Methods. Antagonists were added simultaneously with carbamylcholine and [^{22}Na]. ○---○, mecamylamine; ●---●, *d*-tubocurarine; □---□, dihydro-β-erythroidine; Δ---Δ, hexamethonium.

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TABLE 2

Inhibition of Na^+ flux by ganglionic inhibitors and by BGT

Inhibitor	Concentration	Na^+ flux
	M	nmol/mg · min
Hexamethonium	5×10^{-4}	42.7 ± 3.4
+ 0.5 μM BGT	5×10^{-4}	42.5 ± 3.5
Mecamylamine	1×10^{-6}	25.5 ± 6.2
+ 0.5 μM BGT	1×10^{-6}	25.5 ± 6.4
d-Tubocurarine	1×10^{-5}	21.2 ± 7.7
+ 0.5 μM BGT	1×10^{-5}	23.0 ± 0.8
Dihydro-β-erythroidine	2×10^{-4}	30.3 ± 1.4
+ 0.5 μM BGT	2×10^{-4}	28.0 ± 2.6

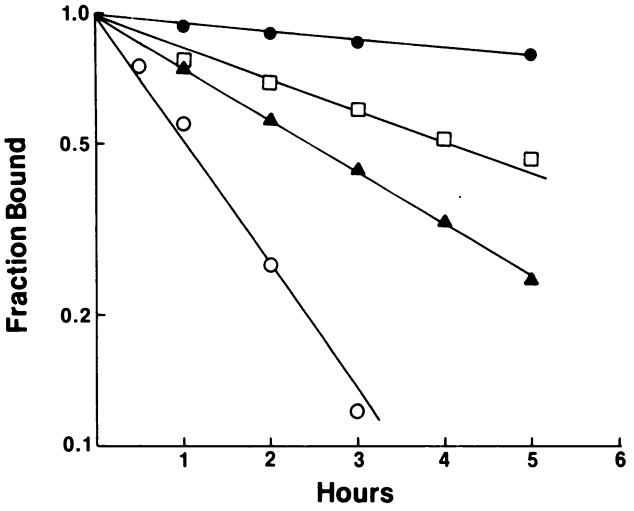

Fig. 2. Dissociation of [^{125}I]BGT bound to PC12 cells. Cells were plated on polylysine coated dishes, then incubated with [^{125}I]BGT for 40 min at room temperature. Cells were then washed (6 × 1 ml) to remove unbound [^{125}I]BGT, then incubated in carbamylcholine or NaCl under the conditions described. Dissociation of [^{125}I]BGT was determined by sampling the amount of [^{125}I] released into the buffer. ●---●, 22°C; □---□, 37° C; ▲---▲, 22°C and 0.14 M carbamylcholine; ○---○, 37°C and 0.14 M carbamylcholine.

TABLE 3

Na^+ flux treatment of PC12 cells with MBTA

The first three treatments were single control measurements and agreed closely with data collected in other experiments; the last two treatments were done in triplicate.

Treatment	Na^+ flux
	(nmol/mg · min)
Control[a]	214
DTT + DTNB[b]	189
DTT, MBTA, DTNB[c]	15.5
−BGT[d]	22.3 ± 4.0
+BGT[e]	19.2 ± 3.8

[a] Control flux was determined as described in Methods.
[b] Flux obtained after treating cells as described for MBTA protocol, but MBTA step was omitted.
[c] As described for MBTA blockade of Na^+ flux and [^{125}I]BGT binding.
[d] Cells were incubated in BGT to block BGT sites, reacted with MBTA as before, then incubated in 140 mM carbamylcholine chloride to remove BGT.
[e] Cells were treated as with −BGT, then incubated in 5×10^{-7} M BGT for 30 min to reblock BGT sites.

Since the amount of Na^+ flux and [^{125}I]BGT binding was found to be in an essentially steady state (when corrected for the amount of protein) we then could assume the rate of appearance to be equal to the rate of degradation and consequently estimate turnover rates. Figure 3 shows the rate of appearance of cell surface [^{125}I]BGT binding protein and, as measured by Na^+ flux, ganglionic nAChRs. We estimate the $t_{1/2}$ for the PC12 [^{125}I]BGT binding protein to be 6.5 hr and the $t_{1/2}$ for the nAChR to be 24 hr. Figure 3A also shows that when cycloheximide was added concurrently with MBTA, incorporation of cell surface BGT sites continued unabated for the first hour, then dramatically declined such that the amount of new cell surface BGT binding protein reached a maximum of 11% of control values after 4 hr. Because approximately half of BGT binding was intracellular, we also examined the effect of cycloheximide on intracellular BGT binding. The disappearance of intracellular BGT sites was found to be biphasic, composed of a faster component that involved about 10% of the sites and a slower decaying component.

In view of earlier reports of agonist-induced down-regulation of nicotinic Na^+ fluxes in PC12 cells (20), and [^{125}I]BGT binding in chick ciliary ganglion (27), we examined down-regulation of Na^+ flux and [^{125}I]BGT binding in PC12 cells. Preliminary experiments established that incubation of PC12 cells with carbamylcholine resulted in a 90% inhibition of Na^+ flux activity that could be resolved into reversible and irreversible components. Recovery from the reversible component, ascribed to desensitization, was essentially complete after the cells had been incubated in carbamylcholine-free buffer for 30 min at room temperature. Figure 4 shows that the irreversible component, regarded as down-regulation, occurred within the first hour and remained constant thereafter. [^{125}I]BGT binding to both extracellular (Fig. 4) and total (data not shown) sites was unchanged.

Discussion

The relationship between neuronal BGT binding proteins and nAChRs has in recent years been the subject of considerable controversy. Both share a nicotinic pharmacology associated with their ligand binding properties. They are sufficiently closely biochemically related that methods designed to purify muscle nAChRs can be applied almost without modification to purify neuronal BGT binding proteins (26). Both are composed of four kinds of polypeptide chains (G. Kemp, manuscript in preparation), and each contains two copies of the chain containing the toxin-binding site (26). At least one polypeptide chain from the chick brain BGT binding protein has been shown to possess sequence homology with muscle nAChRs (28), and it seems likely that neuronal BGT binding proteins and nAChRs are derived from a common ancestral gene(s).

Given the acknowledged biochemical similarities between BGT binding proteins and nAChRs, it is puzzling that the BGT binding protein is not functional in ion translocation. This is particularly true in view of the recent realization that ion channel proteins in general have conserved structures that contribute to the ion channel structure; because of their similarity to nAChRs, BGT binding proteins likely share this structure as well. Although previous studies had not demonstrated an effect of BGT on PC12 carbamylcholine-mediated ion flux, we speculated that a small contribution to nicotinic flux might have been undetected in these studies. By utilizing both reversible and irreversible inhibitors of ganglionic nAChRs, we hoped to identify a Na^+ flux component that could be attributed to the BGT binding protein. However, no such component was detected.

We estimate that the upper limit to an undetected Na^+ flux mediated by the BGT binding protein to be approximately 2

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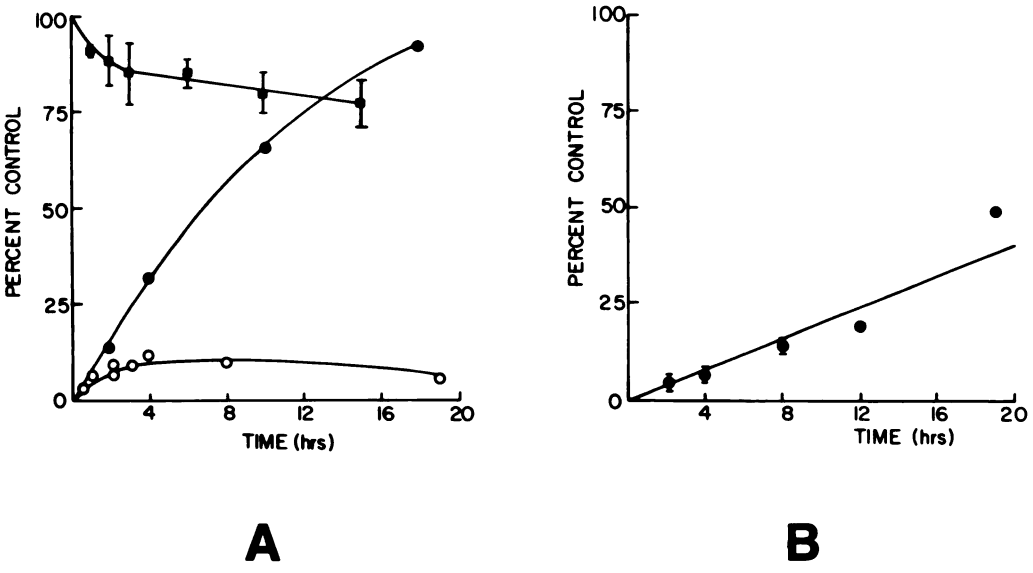

Fig. 3. A, metabolism of [^{125}I]BGT binding sites. ●----●, appearance of new extracellular sites; ○----○, appearance of new extracellular sites in the presence of 25 μg/ml cycloheximide; ■----■, intracellular sites after treatment with 25 μg/ml cycloheximide. B, reappearance of carbamylcholine-stimulated Na^+ flux after inactivation of existing nAChRs with MBTA. Data were compiled from many individual experiments, and control (100%) values were determined for each experiment. In those experiments, control extracellular binding [^{125}I]BGT binding sites ranged from 191 to 268 fmol/mg protein, and intracellular [^{125}I]BGT binding sites ranged from 209 to 490 fmol/mg protein. Control Na^+ fluxes were as described in Methods. Data points with error bars (±1 SD) were determined from three or more individual experiments.

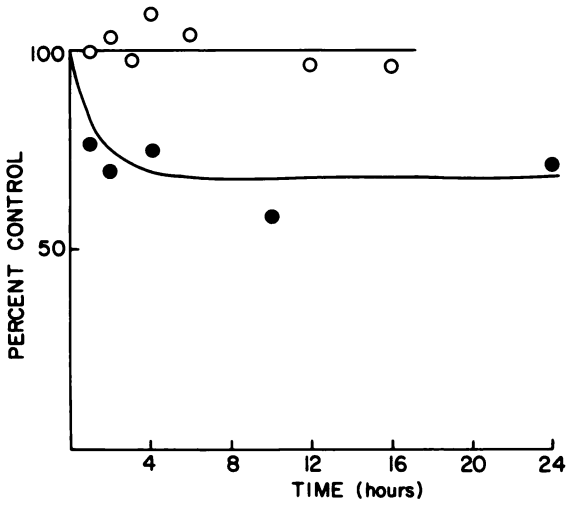

Fig. 4. Cells were incubated in medium containing 5 mM carbamylcholine at 37°C for the prescribed interval, then washed and incubated in HEPES buffer for 30 min at room temperature before the [Na^+] flux or [^{125}I] BGT binding assay. Control values were obtained by incubating cells in the absence of carbamylcholine. ●----●, Na^+ flux; ○----○, cell surface [^{125}I]BGT binding sites.

nmol/mg·min or 8 nmol/pmol [^{125}I]BGT sites. This is about 1% of the total carbamylcholine-stimulated flux and compares with previous published values of 475 nmol/pmol for BC3H-1 cells (29) and 3,200 nmol/pmol for chick myotubes (30). To propagate a transsynaptic signal, nAChRs must generate a current sufficient to activate adjacent voltage-sensitive Na^+ (and/or Ca^{2+}) channels. Given the upper limit of an undetected BGT binding protein-mediated Na^+ flux, it is possible to speculate whether this flux is sufficient to exceed this threshold. An evaluation of this matter is presented in the Appendix.

As is discussed in the Appendix, an unequivocal assessment of whether the BGT binding protein could act as a functional ion channel is not currently possible. However, our best guess at this point is that it could not.

Na^+ flux experiments have typically been done over 20–60 sec time scale, and the magnitude of the Na^+ fluxes are proportional (or nearly so) with time over this interval (18, 20). It subsequently decreases over a period of minutes, due to desensitization, such that the resultant Na^+ flux becomes that observed in the absence of agonist. Another component of carbamylcholine-stimulated Na^+ flux may occur over a millisecond time scale, as is known to occur with Torpedo nAChR (31). This flux may be physiologically significant but not detectable with the Na^+ flux assay. However, electrophysiological measurements in autonomic ganglia capable of detecting a rapidly decaying but BGT sensitive current have failed to do so (6, 12).

It is assumed in these experiments that a Na^+ flux associated with the BGT binding protein would be blocked by BGT. We had previously demonstrated that both rat brain BGT binding protein and Narcine braziliensis nAChR will stoichiometrically bind [^{125}I]BGT when bound to α-cobratoxin-Sepharose 4B and thus contain two BGT sites per molecule (one site couples the protein to the column, the second can bind [^{125}I]BGT). We have extended this observation to the PC12 BGT binding protein (data not shown). These results argue against earlier suggestions that BGT might only occupy one site on BGT binding proteins and that occupation of this site might be insufficient to block ion flux. Occupation of two high affinity acetylcholine sites by BGT is sufficient to block NMJ nAChRs and by analogy a flux associated with BGT binding proteins as well.

However, it has recently been suggested that nAChRs contain activation sites that are distinct from those that bind BGT (32). In this scheme, BGT sites are associated with receptor inactivation and desensitization. Thus it is conceptually possible that agonists might activate conductances mediated by BGT binding proteins even after binding BGT. However, experimental observations from our studies suggest that this is not the case: 1. Table 3 shows the carbamylcholine-stimulated Na^+ flux observed after MBTA blockade of ganglionic receptors under conditions where the BGT binding protein was protected (22 ± 4.0 nmol/mg·min, 19.2 ± 3.8 when BGT sites were secondarily blocked). These fluxes are only marginally greater than obtained when the BGT binding protein was not protected (15.5 nmol/mg·min.). Furthermore, part if not all of this dif-

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ference could be attributed to synthesis of new receptors during the 5-h carbamylcholine incubation period. 2. Na^+ fluxes in the presence of saturating concentrations of ganglionic inhibitors were equal to fluxes obtained in the absence of carbamylcholine. If BGT binding proteins mediated a flux not blocked by these inhibitors, then some carbamylcholine-mediated flux might have been observed in the presence of these inhibitors. Alternatively, if an inhibitor blocked both nAChR and BGT-binding protein mediated flux but with different K_i values, then its dose-response curve might have been biphasic.

Since Robinson and McGee (20) reported agonist-induced down-regulation of PC12 ganglionic Rb^+ fluxes, we tested whether BGT binding sites would down-regulate as well. Although the concentration of BGT binding sites was unaffected by carbamylcholine incubations, we noticed that down-regulation of Na^+ flux occurred much more rapidly than Robinson and McGee had envisioned. Their first point was 12 hr, whereas we found that down-regulation was essentially complete after 1 hr.

After this portion of the experimental work in this communication was completed, Simasko *et al.* (33) characterized down-regulation of Na^+ flux in PC12 cells in some detail and reported a $t_{1/2}$ of 14.7 min in 1 mM carbamylcholine. Our results are in essential agreement with theirs, although their maximal loss of flux activity was somewhat greater than we observed (50–60% versus 30%).

Interestingly, Messing (27) reported down-regulation of BGT binding sites in chick ciliary ganglion cultures that closely resembles both the time and magnitude of the down-regulation of Na^+ flux in PC12 cells. No effort was made to determine if ciliary ganglion Na^+ fluxes were concurrently down-regulated. We did not find an analogous down-regulation of BGT sites in PC12 cells. The mechanism(s) whereby ganglionic Na^+ fluxes and neuronal BGT binding sites are down-regulated is currently unknown but is of considerable interest.

Turnover rates for muscle junctional and extrajunctional nAChRs, have been determined by labeling the receptors with [^{125}I]BGT and monitoring the appearance of [^{125}I] in the medium. These studies document the considerably greater stability of junctional nAChRs. Linden and Fambrough (34), for example, reported $t_{1/2}$ values of 22 hr for extrajunctional nAChRs and 13 days for junctional nAChRs in rat skeletal muscle in organ culture. Similar studies with neuronal BGT binding proteins have not been possible because of the relatively rapid dissociation of the toxin. By inactivating existing PC12 toxin sites and ganglionic nAChRs with MBTA, we could measure the rates at which these proteins were replaced. These experiments presume that binding MBTA to cell surface nAChRs and BGT binding proteins does not alter their rates of synthesis, an assumption we could not test. However, labeling these molecules with a small ligand like MBTA might be expected to be less perturbing than labeling them with [^{125}I]BGT, as was done in analogous experiments with muscle nAChRs (34–36).

The different rates of synthesis of the PC12 ganglionic nAChR and BGT binding protein reinforces the concept that these proteins are different molecules.

Approximately 50% of PC12 BGT binding sites were found to be intracellular. Intracellular nAChRs in muscle cells have been described previously. Fambrough and Devreotes (35) reported that 20–25% of nAChRs on chick myotubes were intracellular. Approximately half of these receptors were associated with the Golgi apparatus and were inserted into the plasma membrane in the presence of inhibitors of protein synthesis. The remaining 50% of intracellular receptors were associated with an unidentified "hidden" pool that was not transferred to the cell surface in the presence of inhibitors of protein synthesis and that was degraded at a rate similar to cell surface receptors. Patrick *et al.* (36) similarly found two intracellular pools of nAChRs in BC3H-1 cells. One pool, constituting 14% of total nAChRs, was concluded to be a precursor of cell surface nAChRs. A second pool, 21% of total nAChRs, was also described that appeared to be neither a precursor of nor derived from cell surface nAChRs.

Our results suggest that approximately 5% of PC12 BGT sites are immediate precursors of cell surface sites, since approximately that many sites arrive on the cell surface within a 2-hr period after addition of cycloheximide. Since a similar number of sites are depleted from an intracellular pool during the same interval, it is attractive to speculate that these sites are being transferred to the cell surface, as has been shown for muscle nAChRs (35, 36). The great majority of intracellular sites reside in a pool that, in the presence of cycloheximide, cannot be transferred to the cell surface and is degraded very slowly. This apparent metabolic stability, however, may be artifactual. Cycloheximide and other inhibitors of protein synthesis have been shown to inhibit both synthesis and degradation of other proteins, presumably because the pathways for synthesis and degradation are coupled in some unknown manner (37, 38). In support of this conclusion, we measured the degradation of extracellular BGT sites in the presence of cyclonheximide and found that their turnover rate measured in this way was much slower than that predicted from the rate of synthesis data in Fig. 3A. It thus may be more appropriate to differentiate populations of intracellular BGT sites on the basis of their sensitivity to cycloheximide, rather than to attribute different turnover rates to these pools. We conclude that our results are parsimoniously summarized by assuming that BGT binding sites reside in three cellular pools analogous to those identified for nAChRs in muscle cells. Our results differ most significantly in the relative size of the "hidden" pool, which constituted approximately 90% of intracellular and 45% of total BGT sites in PC12 cells.

The functional role of intracellular pools of cell surface proteins is not well understood but is clearly of interest. It is known, for example, that ligand-occupied insulin receptors are internalized and recycled to the cell surface (38). An intracellular pool of sodium channel α-subunit has been identified in developing rat brain (39). Thus, intracellular compartments may participate in specialized regulatory processes, including perhaps modulating rapid changes in cell surface receptor density.

If BGT binding proteins do not act by translocating ions, then their function remains unknown. It has been suggested that BGT binding proteins are the neuronal equivalent of extrajunctional or neonatal muscle nAChRs. This is consistent with its invariant colocalization with ganglionic nAChRs in the autonomic nervous system. However, deafferentation of autonomic ganglia does not result in increased synthesis of BGT binding protein as might be expected by analogy with muscle nAChRs (40). It has recently been established that junctional and neonatal muscle nAChRs are composed of three common and one unique kind of polypeptide chains (41). It is currently

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unknown whether neuronal BGT binding proteins and ganglionic nAChRs share one or more polypeptide chains, but such an outcome remains an intriguing possibility.

Appendix

We have estimated the upper limit to an undetected BGT binding protein-mediated conductance to be approximately 2 nmol/mg·min. Assuming 6×10^6 cells/mg protein and a cell surface area of 350 μm^2 (42), this flux would be equivalent to a current of 1.5×10^{-15} amps/μm^2. This current density is far lower than measured estimates of the PC12 leak current (1.25×10^{-12} A/μm^2 at 50 mV) (42).

AChRs at synapses are known to be backed at very high densities, in the order of 10,000/μm^2. Although BGT binding sites have not been identified in correspondingly high packing densities, it is possible, based on available information, to estimate an upper limit to a BGT binding protein-mediated current if the protein was packed at a density of 10,000/μm^2. Based on a flux of 2 nmol/mg·min and 0.25 pmol [^{125}I]BGT binding sites/mg protein (28 BGT binding proteins/μm^2, assuming two sites per molecule), it can be calculated that BGT binding proteins packed at a density of 10,000/μm^2 could give rise to a maximum current of 5.4×10^{-13} A/μm^2, again less than the PC12 leak current. Furthermore, much of the leak current is likely to be due to Cl^- and thus will act as a buffer to resist changes in membrane potential. Rudy *et al.* (42), using a similar line of reasoning, has attributed the failure of PC12 cells to exhibit electrical excitability to an observed very low density of cell surface voltage sensitive Na^+ channels.

A somewhat more simplistic approach is to carry out a hypothetical experiment whereby muscle nAChRs at the neuromuscular junction are replaced by PC12 BGT binding proteins. One can then ask, given the upper limit to undetected fluxes measured in this manuscript, whether neuromuscular transmission would then be possible.

We have estimated, based on comparison of our data and previously published Na^+ fluxes from muscle cells (35, 36), that BGT binding proteins could at most give rise to 0.5–2% of the Na^+ fluxes measured for muscle nAChRs (see Discussion).

Since inhibition of 98–99.5% of muscle nAChRs is more than sufficient to completely block neuromuscular transmission (43), it would again appear questionable that PC12 BGT binding proteins could act as functional nAChRs.

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