Neuronal Acetylcholine Receptors That Bind α-Bungarotoxin Mediate Neurite Retraction in a Calcium-Dependent Manner

Phyllis C. Pugh and Darwin K. Berg

Department of Biology, University of California at San Diego, La Jolla, California 92093-0357

Neuronal membrane components that bind α-bungarotoxin with high affinity have only recently been shown unambiguously to function as nicotinic receptors. Activation of the receptors increases intracellular levels of free calcium in neurons. In the chick ciliary ganglion, where the receptors have been studied in some detail, they have been shown to have a predominantly nonsynaptic location on neurons and may be concentrated on pseudodendrites emerging from the somata. This has raised questions about the physiological significance of the receptors for the neurons. Here we show that activation of the receptors on isolated ciliary ganglion neurons in cell culture produces neurite retraction. Focal application of either nicotine or ACh at low concentrations induces the retraction, and α-bungarotoxin blocks the effect. The retraction requires external calcium and is confined to the individual neurite stimulated with agonist. Brief exposure to elevated concentrations of K+ also induces neurite retraction, and both the K+-induced and the nicotine-induced retractions can be prevented by the calcium channel blocker ω-conotoxin. The results suggest that activation of the α-bungarotoxin-binding nicotinic receptors on neurites triggers activation of voltage-gated calcium channels presumably by depolarizing the membrane, and that together they permit sufficient calcium to enter the neurite to prevent further outgrowth and induce retraction.

[Key words: nicotinic receptors, neuronal ACh receptors, α-bungarotoxin, neurite extension, growth cones, intracellular calcium, ciliary ganglion neurons]

Nicotinic ACh receptors (AChRs) are abundant in the nervous system and comprise a family of receptor subtypes (for review, see Sargent, 1993). Speculations about their likely physiological roles have been heavily influenced by the much-studied muscle AChR. Located in the postsynaptic membrane at the neuromuscular junction, muscle AChRs function as cation-selective ligand-gated ion channels responsible for fast, excitatory synaptic input from the motor nerve terminal. Numerous examples of nicotinic responses have been observed in neurons as well.

Only at preganglionic neuron synapses on autonomic neurons and at motoneuron synapses on Renshaw cells, however, is there good evidence that neuronal AChRs mediate fast, excitatory synaptic transmission. This has led to speculation that some neuronal AChR subtypes may serve other functions such as that of modulating neuronal signaling or regulating aspects of neuronal development.

One class of neuronal AChRs that has spawned numerous questions about function is the species that binds the snake neurotoxin α-bungarotoxin (αBgt). Numerous attempts over a number of years failed to demonstrate a function for such receptors (αBgt-AChRs). New impetus for the analysis of αBgt-AChRs was provided by the cloning of the AChR α7 gene. Not only was the α7 gene product shown to be a subunit of at least some αBgt-AChRs (Schoepfer et al., 1990; Keyser et al., 1993), but it was also found to produce functional ligand-gated ion channels, apparently as a homomer, when expressed in Xenopus oocytes (Couturier et al., 1990; Seguela et al., 1993). Subsequent experiments have provided evidence that native αBgt-AChRs on neurons do function as ligand-gated ion channels (Alkondon et al., 1992; Zorumski et al., 1992; Z.-w. Zhang and D. K. Berg, unpublished observations). Previous failures to detect an electrophysiological response for the receptors probably resulted from their rapid rate of agonist-induced desensitization and from their being overshadowed by the responses of other AChR subtypes on the neurons.

The physiological role of αBgt-AChRs in vivo remains unresolved. In the chick ciliary ganglion, where αBgt-AChRs have been studied in some detail, the receptors are located predominantly in nonsynaptic membrane on the neurons both in the embryo and in the adult (Jacob and Berg, 1983; Loring et al., 1985). Few, if any, αBgt-AChRs are concentrated in the postsynaptic membrane, making it unlikely that the receptors contribute directly to synaptic transmission. Instead, other AChRs, distinguished by their ability to bind the monoclonal antibody mAb 35 and their inability to bind αBgt, are primarily found in the postsynaptic membrane and appear to be responsible for nicotinic transmission through the ganglion (Jacob et al., 1984; Loring et al., 1984; Loring and Zigmond, 1987). These largely synaptic receptors are referred to here as mAb 35-AChRs and, as a population, contain the α3, β4, and α5 gene products (Vernallis et al., 1993). They do not contain detectable amounts of the α7 gene product. In contrast, αBgt-AChRs on the neurons contain the α7 gene product but not the α3, β4, or α5 gene products, indicating that αBgt-AChRs and mAb 35-AChRs are clearly distinct receptor species. Ciliary ganglion αBgt-AChRs also lack the α8 gene product (Vernallis et al., 1993) found in some forms of brain and retina αBgt-AChRs (Schoepfer et al., 1990; Keyser et al., 1993).

Recently it has been shown that activation of αBgt-AChRs on chick ciliary ganglion neurons can produce a marked elevation in the level of intracellular free calcium (Vijayaraghavan et al., 1992). Receptors made by expressing the chick and rat α7 genes in *Xenopus* oocytes are highly permeable to calcium (Galzi et al., 1992; Seguela et al., 1993). If the same is true of native αBgt-AChRs, the receptors would have the potential of influencing a vast array of calcium-related events in the neurons.

One function that may be regulated by αBgt-AChRs is neurite extension. Numerous studies have indicated that conditions which change intracellular calcium levels in the growth cone can dramatically alter the growth pattern of the neurite (for review, see Kater and Mills, 1991). Nicotinic antagonists have been shown to enhance the number and mean length of neurites from retinal ganglion cells in culture, implicating nicotinic receptors in the regulation of neurite outgrowth (Lipton et al., 1988). In the case of the pheochromocytoma cell line PC12, αBgt has been shown to promote the extension of neurites (Quik et al., 1990). Since PC12 cells can synthesize and release ACh under some conditions (Greene and Rein, 1977), the αBgt effects may result from its serving as an AChR antagonist.

Electron microscopic studies with HRP-conjugated αBgt suggest that αBgt-AChRs on chick ciliary ganglion neurons are concentrated preferentially on pseudodendrites extending from the somata (Jacob and Berg, 1983). Given the location of the receptors and their ability to elevate intracellular levels of free calcium, it seemed possible that αBgt-AChRs could regulate neuronal morphology. We report here that activation of αBgt-AChRs on ciliary ganglion neurons in dissociated cell culture inhibits neurite extension and induces a partial retraction of the processes in a calcium-dependent manner. Further, experiments with calcium channel blockers suggest that the retraction depends at least in part on functional voltage-gated calcium channels, presumably recruited by activation of the αBgt-AChRs.

Materials and Methods

Ciliary ganglion cultures. Freshly dissected ciliary ganglia from 8 d chick embryos were dissociated with trypsin as previously described (Nishi and Berg, 1981) and plated on 35 mm dishes coated either with 0.5 mg of poly-D-lysine alone or with poly-D-lysine plus 15 mg of laminin. The culture medium consisted of Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum and 3% (v/v) embryonic chick eye extract (Nishi and Berg, 1981). Cell cultures were maintained overnight (14–30 hr) in a humidified incubator with 5% CO_2, 95% air at 37°C before being taken for analysis.

Neurite measurements. For measurement of neurite length, cultures were perfused at 37°C with culture medium containing 10 mM HEPES, pH 7.4, instead of bicarbonate for buffer, and cells were viewed with phase-contrast optics at either 320× or 400× magnification. Neurite lengths were quantified using a reticulated eyepiece to measure the distance from the origin of the neurite at the soma perimeter to the most distal edge of the growth cone, defined as either the outer edge of the lamellapodia or the farthest reaching filopodium. Only data from neurons having an unbranched neurite were used for the compilations. Photomicrographs were taken with Kodak T-max 100 film at 400× with phase-contrast optics using a Leitz Diavert microscope.

Stimulation paradigms. AChR agonists in perfusion medium (vehicle) were applied to neurites by pressure from micropipettes positioned 5–15 μm from the growing tip of the neurite. The protocol usually consisted of three 10 sec pulses 6 min apart ($3 \times 10''$, 6'). In some cases the protocol was changed to deliver five 2 sec pulses of agonist 3 min apart ($5 \times 2''$, 3'). For long-term nicotine application, the initial culture volume was reduced to 0.5 ml and the cells were perfused with 1 μM nicotine at a rate of at least 2.5 ml/min for 3 min and then 0.17 ml/min for the remainder of the 72 min observation time. Agonists included nicotine alone or ACh in the presence of atropine. When K^+ was used to stimulate, it was substituted for nicotine in the micropipette (final concen-

tration of 25 mM); the stimulation protocol involved three 10 sec pulses 6 min apart. Neurites were monitored at regular intervals for approximately 1 hr after applying agonist or until complete retraction had occurred and no regrowth was observed for at least 5 min.

When AChR antagonists were employed, they were present in the perfusion medium prior to and during tests with agonist, but were not present in the micropipette with the nicotine. αBgt was incubated with the cells at 50 nM for at least 60 min before perfusing the cells at 10 nM and stimulating with agonist. Methyllycaconitine (MLA) was present at 50 nM both before (at least 15 min) and during the tests with nicotine. d-Tubocurarine (dTC) was present at 0.1 mM both before (at least 10 min) and during the tests. When ACh was substituted for nicotine as agonist, atropine was added to the perfusion medium at 0.25 μM 5 min before and during the test to block activation of muscarinic receptors and was present in the micropipette as well. The minus Ca^{2+} condition was obtained by including 1 mM EDTA and 10 mM BAPTA in the micropipette with the nicotine. The calcium channel blockers ω-conotoxin (ω-Ctx; 2 μM) and nifedipine (10 μM), when present, were added to the perfusion medium for 30 and 15 min, respectively, prior to testing.

Statistics. Statistics were computed using STATVIEW 512+ (BrainPower, Inc.). Significance values were determined using a three-way ANOVA, including a negative control (vehicle), a positive control (agonist), and the experimental condition to be compared. In some cases a two-tailed t test was applied instead of the ANOVA, as indicated.

Materials. White Leghorn chick embryos were obtained locally and maintained at 37°C in a humidified incubator. Tissue extracts and culture media were prepared as described previously (Nishi and Berg, 1981). αBgt was purified from *Bungarus multicinctus* venom (Ravdin and Berg, 1979). MLA was the kind gift of Dr. M. H. Benn (University of Calgary). Eagle's minimal essential medium powder was purchased from GIBCO; fetal bovine serum, from Whittaker; ACh, atropine, and ω-conotoxin GVIA, from Research Biochemicals Inc.; laminin from Upstate Biotechnology Inc.; and poly-D-lysine, nicotine, dTC, nifedipine, EDTA, HEPES, and BAPTA, from Sigma.

Results

Neurite extension and growth cone morphology in culture. Ciliary ganglion neurons from 8 d chick embryos were used to test the effects of αBgt-AChRs on neurites in cell culture. The neurons are known to have the receptors at this time (Smith et al., 1985) and can readily survive and extend processes in culture (Helfand et al., 1976; Nishi and Berg, 1981). The neurons were plated at low density and examined after relatively short periods of time (14–30 hr) so that cells would remain isolated from each other for the duration of the experiment. Usually polylysine was used as the substratum to facilitate cellular adhesion to the surface while permitting easy visualization of growth cones.

Many neurons extended one or more neurites ranging in length from 10 to over 200 μm. Growth cones exhibited a range of morphologies. The simplest represented neurite tips with no apparent filopodia or lamellapodia. The most complex had multiple filopodia and lamellapodia covering approximately 100 μm². In some cases, a single filopodium extended up to 20 μm beyond the end of the neurite trunk.

To determine the rate of neurite growth or retraction, neurite lengths were measured at various times over a 72 min period. Neurite length was taken to be the curvilinear distance between the point of neurite origin at the cell soma and the point of farthest extension whether that represented the distal edge of the lamellapodia or the end of a filopodium. Filopodia appeared to occur with equal frequency throughout the time period under the test conditions employed. Excluding them from the measurements, that is, taking the distalmost extension of the neurite as being the outer edge of the lamellapodia, did not change the values calculated for neurite extension or retraction in the experiments described below.

Nicotine-induced retraction of neurites. Studies with rhoda-

Table 1. Summary of neurite treatments with nicotine and vehicle

Condition	n	Relative neurite length (%)	Change in length (μm)
Control	18	116 ± 7††	10 ± 3
Vehicle	15	122 ± 8††	12 ± 4
Vehicle (5 × 2″, 3′)	8	115 ± 6†	9 ± 3
Nicotine			
0.5 μM	10	121 ± 10††	9 ± 3
1 μM	50	66 ± 5*	−13 ± 2
2 μM	14	88 ± 5*·†	−7 ± 2
10 μM	14	92 ± 5*·†	−3 ± 2
1 μM (5 × 2″, 3′)	25	75 ± 11*	−13 ± 5
1 μM (long-term)	25	113 ± 6††	4 ± 2
1 μM (+ 10 μM leak)	11	121 ± 9††	9 ± 3
Nicotine (1 μM) + blockers			
αBgt (50 → 10 nM)	21	103 ± 4††	2 ± 3
MLA (50 nM)	13	105 ± 6††	3 ± 3
dTC (100 μM) (5 × 2″, 3′)	11	115 ± 2††	9 ± 2
Nicotine (10 μM) + αBgt			
(50 → 10 nM)	14	118 ± 6‡	9 ± 3
Vehicle + αBgt (50 → 10 nM)	13	109 ± 3††	6 ± 2

Individual neurites were treated with the indicated agents and scored for retraction either as a percentage change in length or as an absolute difference in length as described in Figure 3. Values represent the mean ± SEM for the number of neurites indicated as n. The stimulation paradigm involved three 10-sec pulses of vehicle with the indicated concentrations of agonist at 6 min intervals, except in the cases designated 5 × 2″, 3′ where agonist was delivered in five 2-sec pulses at 3 min intervals, and in the case designated "long-term" where the nicotine was present throughout the stimulation (12 min) and observation (60 min) periods. In one condition [1 μM (+ 10 μM leak)] a second micropipette containing 10 μM nicotine was positioned near the growth cone for the duration of the experiment but was not used for stimulation. Statistics were computed for the relative neurite lengths, comparing the values to those obtained both with the corresponding vehicle alone and with 1 μM nicotine, except for the condition with 10 μM nicotine + αBgt which was compared with vehicle alone and 10 μM nicotine alone.

* Significantly different from vehicle ($p < 0.01$).
† Significantly different from 1 μM nicotine ($p < 0.05$).
†† Significantly different from 1 μM nicotine ($p < 0.01$).
‡ Significantly different from 10 μM nicotine ($p < 0.01$).

Figure 1. Neurite undergoing nicotine-induced retraction. Photomicrographs were taken with phase-contrast optics of the cell and its neurites before (*A*), 2 min after (*B*), 35 min after (*C*), and 62 min after (*D*) stimulation with 1 μM nicotine for three 10 sec pulses 6 min apart. The *large arrow* in *A* indicates the position of the micropipette containing nicotine; the *vertical bars* indicate the maximal extension of the neurite at the beginning of stimulation, and the *arrowheads* indicate the end of the neurite at the indicated times. The stimulated branch retracted 43% during the observation period, decreasing from an initial length of about 70 μm to a final length of about 40 μm. The unstimulated (vertical) branch remained essentially unchanged in length. Scale bar, 20 μm.

mine-labeled αBgt have previously shown that chick ciliary ganglion neurons in culture have αBgt-AChRs on their neurites as well as on their cell bodies (Ravdin et al., 1981). To determine whether AChRs might influence neurite extension, nicotinic agonists were applied directly to individual growth cones by pressure from a nearby micropipette. Stimulation paradigms usually involved applying agonist for three 10 sec periods 6 min apart. The discontinuous pattern of application was chosen to reduce possible desensitization of αBgt-AChRs on the growth cones. The physical act of applying solution from the micropipette did not alter the basal rate of neurite growth. The mean rate of neurite extension on a polylysine substratum in control cultures was 7.2 ± 2.9 μm/hr (mean ± SEM, $n = 18$), resulting in an average increase of about 20% during a 1 hr period of observation. Neurites receiving vehicle alone in the standard application paradigm displayed the same mean rate of extension and similar net elongation during the test period (Table 1).

Neurites receiving 1 μM nicotine from the micropipette underwent a mean net retraction of approximately 35% in length during the 1 hr period of observation (Table 1). The extent of retraction varied considerably among neurites, ranging from complete retraction in about one-seventh of the cases to no
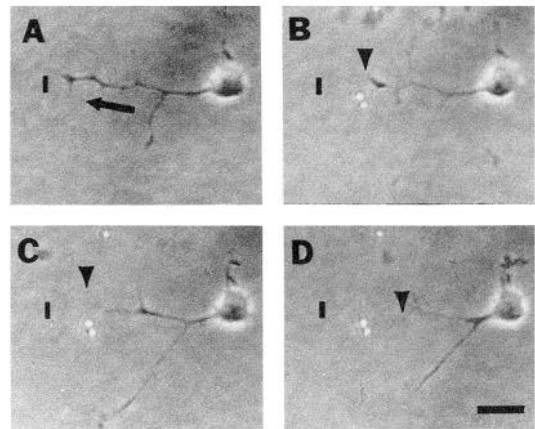
retraction or net outgrowth in about a tenth of the cases. The time course of retraction also varied greatly but usually reached a maximum within 45 min. In some cases, complete retraction occurred almost immediately upon exposure to nicotine. In the other extreme, some neurites underwent a partial retraction followed by resumption of growth at the control rate after about 50 min.

An example of an intermediate case (the most common) of nicotine-induced neurite retraction is shown in Figure 1, where photomicrographs taken at regular intervals during the observation period record the retraction of a growth cone and neurite. The neurite was bifurcated at the outset, and agonist was applied only to the tip of the horizontal branch. Over a 62 min observation period the horizontal branch receded 30 μm while the vertical branch remained essentially unchanged. In addition to illustrating the effects of nicotine, the example implies that the retraction may be a local event, being restricted to the neurite or neuritic branch that is stimulated. This was confirmed for unbranched neurites by studying neurons having multiple processes. Out of 15 such cases where the stimulated neurite retracted, only 2 of 17 unstimulated neurites extending from the same neurons did so. The frequency of retraction for the unstimulated neurites was indistinguishable from that described above for control neurites or neurites receiving vehicle alone.

Tests were usually confined to neurons with unbranched neurites. Examples of results obtained from neurites treated either with vehicle alone or with vehicle plus nicotine are shown in Figure 2, where changes in neurite length are plotted as a function of time. For neurites receiving only vehicle (Fig. 2*A*), the examples have been chosen to illustrate the most common result (triangles) along with two of the more extreme cases (circles and squares). For neurons receiving nicotine, three of the most common responses are shown (Fig. 2*B*) as well as three of the more extreme responses (Fig. 2*C*). Figure 3 compiles the results from all vehicle- and nicotine-treated neurites as histograms. Each
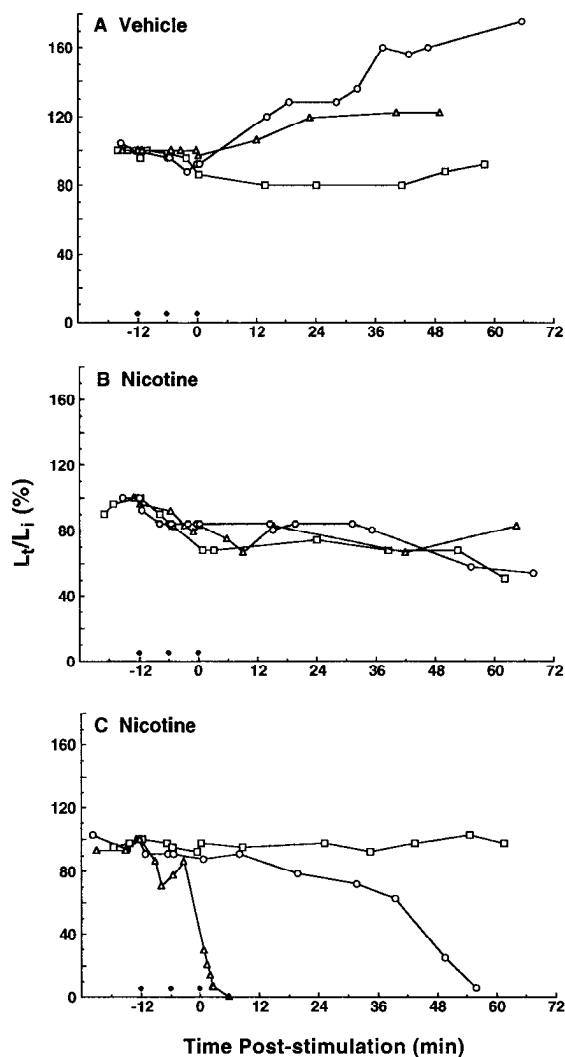
Time Post-stimulation (min)

L/L_i (%)

Figure 2. Time course of neurite retraction. Neurites were treated either with vehicle (*A*) or with vehicle containing nicotine (*B, C*) as described in Figure 1 and then measured for net length at the indicated times. For each time point, the neurite length (L_t) is expressed as a percentage of the initial length (L_i) at the beginning of the stimulation period (−12 min). Three examples of vehicle-treated neurites were chosen (*A*) to illustrate the most common result (*triangles*) as well as the rare extremes of extensive growth (*circles*) and no growth (*squares*). Three examples of nicotine-treated neurites were chosen (*B*) to illustrate the most common time course and extent of neurite retraction (*circles, triangles,* and *squares*), while three others were chosen (*C*) to illustrate the less common behaviors of complete and abrupt retraction (*triangles*), gradual retraction followed by sharp and complete retraction later in the test period (*circles*), and no retraction (*squares*). The three *solid diamonds* in each panel indicate the timing of vehicle or nicotine treatment.

Figure 3. Histogram of neurite lengths following treatments to induce retraction. Neurites were stimulated as in Figure 1 with vehicle alone (*A*), vehicle plus nicotine (*B*), or vehicle plus nicotine after blockade with αBgt (*C*). The relative change in length for each neurite was calculated by expressing the length at the sustained maximum retraction (*L*) as a percentage of the initial length (L_i) at the beginning of stimulation. When neurite length continually changed during the test period and did not undergo a sustained retraction, the relative length was calculated by expressing the final length as a percentage of the initial length (see Results). The values have been pooled into bins of 10%; for example, the 20% bin included all neurites that retracted to 11–20% of their initial lengths. The results show that nicotine treatment shifts the distribution of neurite lengths to shorter values and that αBgt treatment blocks the effect.

entry represents the relative change observed for a neurite during the test period, calculated by comparing its length at the point of maximum sustained retraction with its length at the beginning of the test period. A sustained retraction was taken to be one lasting at least 5 min. When neurite length changed continuously during the test period as in the case of vehicle-treated neurites and some agonist-treated neurites, the relative change was calculated by comparing the final length at the end of the test period
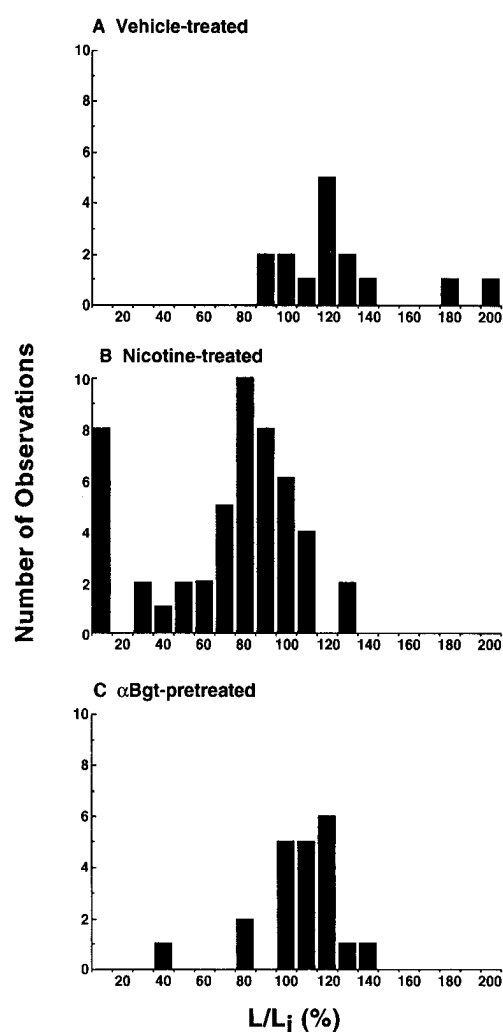
to the starting length. The point of maximum sustained retraction was used whenever possible, however, because some nicotine-treated neurites retracted but subsequently recovered partially and reinitiated growth as described above. The overall results are the same whether final lengths or maximum retracted lengths are used: those test conditions yielding values significantly different from controls do so by either method of scoring.

Varying the concentration of nicotine in the micropipette indicated that 1 μM usually produced the maximum effect. Decreasing the concentration to 0.5 μM had no effect on neurite growth, and increasing the concentration to 2 or 10 μM caused

less retraction than did 1 μM (Table 1). Changing the stimulation paradigm did not significantly enhance the effect. Applying the nicotine in five 2 sec pulses 3 min apart instead of the usual three 10 sec pulses 6 min apart did not increase the effect (Table 1). Extending the number and time period of nicotine pulses (e.g., six 10 sec pulses at 3 min intervals or thirteen 2 sec pulses at 1 min intervals) also failed to increase the extent of retraction (data not shown).

The results suggest competing processes that produce a narrow window of effective agonist concentrations. High concentrations of agonist may partially desensitize the receptors while insufficient concentrations of agonist may not reach a required threshold. Evidence that a threshold exists and that it may vary among experiments comes from the observation that for some sets of cultures the standard paradigm of challenging with nicotine at 1 μM produced little effect while 2 μM nicotine induced the usual retraction (data not shown). (This necessitated positive controls for nicotine-induced retraction being routinely conducted for all experimental conditions.) Desensitization also influences the outcome. Continuous application of 1 μM nicotine for 72 min did not induce neurite retraction (Table 1). Moreover, when high concentrations of agonist are present in the micropipette, steady leakage between stimulation pulses apparently desensitizes the receptors. This was demonstrated by showing that the standard stimulation paradigm with 1 μM nicotine was unsuccessful at inducing retraction when a second micropipette containing 10 μM nicotine was positioned near the growth cone but not used for stimulation (Table 1). Desensitization produced by such leakage probably accounts for the observation that 10 μM nicotine produces less retraction in the standard stimulation paradigm than does 1 μM.

The ability of nicotine to induce neurite retraction was not confined to cells on a substratum of poly-D-lysine. Similar results were obtained when the dishes were coated with laminin in addition to polylysine. The mean neurite length prior to nicotine application was longer for neurons grown on laminin/poly-D-lysine (99 ± 11 μm, mean ± SEM, $n = 21$) than on poly-D-lysine alone (54 ± 2 μm, $n = 350$; $p < 0.0001$, t-test). Nonetheless, application of 1 μM nicotine in the standard protocol (three 10 sec pulses, 6 min apart) readily blocked neurite extension. By the end of the 1 hr observation period, the mean length of nicotine-treated neurites was 108 ± 5% (mean ± SEM, $n = 12$) of their initial length while that of control neurites receiving vehicle alone was 168 ± 35% ($n = 9$) of their initial length. Thus, on a substratum of laminin/poly-D-lysine the nicotine-treated neurites were, on average, a third shorter than vehicle-treated neurites by the end of the test period.

αBgt-AChRs mediate nicotinic effects on neurites. The contribution of αBgt-AChRs to nicotine-induced neurite retraction was tested by incubating cells with 50 nM αBgt and then perfusing with 10 nM. The toxin is specific in this concentration range, inhibiting the response of αBgt-AChRs while not affecting mAb 35-AChRs on the neurons (Ravdin and Berg, 1979; Vijayaraghavan et al., 1992). αBgt blocked the effects of both 1 and 10 μM nicotine on neurite growth (Table 1). A second nicotinic antagonist with high affinity for αBgt-AChRs is MLA. At 50 nM the ligand should occupy most of the αBgt-AChRs on ciliary ganglion neurons while binding to very few mAb 35-AChRs (Vijayaraghavan et al., 1992). As with αBgt, MLA blocked the effects of nicotine on neurite outgrowth. dTC, a nicotinic antagonist not expected to discriminate between αBgt-AChRs

Table 2. Summary of neurite treatments with ACh ± αBgt

ACh (μM)	Agonist alone	Agonist + αBgt
50	96 ± 8 (25)*	130 ± 6 (12)†
100	88 ± 4 (20)*	95 ± 10 (14)*
200	94 ± 8 (9)*	95 ± 9 (9)*
1000	120 ± 11 (6)	ND

Individual neurites were stimulated with the indicated concentration of ACh in the standard stimulation paradigm ± αBgt and were scored as percentage of initial length as described in Table 1. Atropine at 0.25 μM was included in the perfusion and all ACh solutions. Values represent the mean ± SEM for the number of determinations indicated in parentheses. ND, Not determined.
* Significantly different from that obtained with vehicle alone (Table 1; $p < 0.05$).
† Significantly different from that obtained with the same concentration of agonist in the absence of αBgt ($p < 0.01$).

and mAb 35-AChRs, produced a slightly greater nominal blockade than did either αBgt or MLA, but the three antagonists were not significantly different in their effects either from each other or from that observed with vehicle alone (Table 1). αBgt by itself did not significantly affect neurite outgrowth (Table 1).

The presumed endogenous agonist for nicotinic receptors is ACh. To determine whether ACh induced neurite retraction and whether αBgt could block it, neurites were stimulated with several concentrations of ACh in the presence and absence of toxin. At 50 μM ACh produced a substantial retraction, and αBgt blocked it (Table 2). At higher concentrations (100 and 200 μM) the agonist also achieved retraction, but mAb 35-AChRs appeared to be responsible since αBgt did not block the effects. At 1 mM ACh did not alter neurite growth, probably because leakage of agonist from the micropipette desensitized the receptors as described above for nicotine applications. Atropine at 0.25 μM was routinely included in the micropipette along with the ACh to eliminate muscarinic receptors as possible contributors to any effects observed.

The most likely mechanism by which activation of αBgt-AChRs influences neurite outgrowth is by changing the level of intracellular free calcium. It is known that the elevation of intracellular calcium caused by αBgt-AChRs depends on extracellular calcium (Vijayaraghavan et al., 1992). Accordingly, the role of extracellular calcium in the present experiments was examined by adding calcium chelators to the calcium normally present in the micropipette used to apply nicotine. This approach minimized the exposure of the neurite to calcium-free conditions but ensured that little, if any, extracellular calcium would be available during the times in which the αBgt-AChRs were activated. Application of 1 μM nicotine by the standard protocol (three 10 sec pulses at 6 min intervals) in the presence of the calcium chelators was unable to inhibit neurite growth. The relative neurite length at the end of the test period was 125 ± 9% of the initial length (mean ± SEM, $n = 13$; $p < 0.000001$ from 1 μM nicotine). The value was indistinguishable from that obtained for unstimulated neurites or neurites challenged only with vehicle (Table 1).

Involvement of voltage-dependent Ca^{2+} channels. Recent results confirm that αBgt-AChRs function as ligand-gated ion channels and cause a rapid, transient depolarization of chick ciliary ganglion neurons (Zhang and Berg, unpublished observations). Accordingly, the ability of αBgt-AChRs to elevate intracellular calcium in the neurons may result either from the
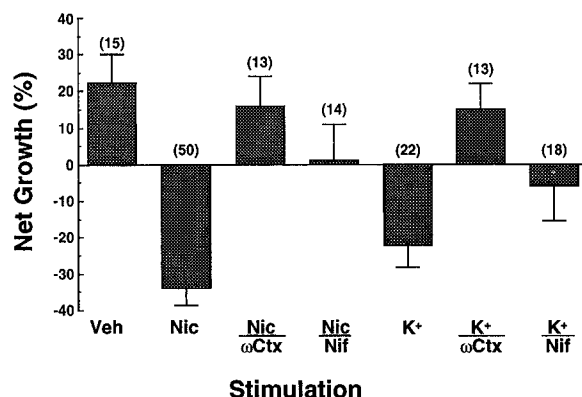
Figure 4. Effects of voltage-gated calcium channel blockers on nicotine-induced neurite retraction. Neurites were stimulated as in Figure 1 either with nicotine or with 25 mM K⁺ in the presence or absence of the indicated calcium channel blockers. The results were calculated as described in Figure 3 to obtain a mean ± SEM in each case and are shown as the net percentage difference from the starting length. The number of neurites analyzed is shown in *parentheses. Veh,* vehicle; *Nic,* nicotine; *K⁺,* potassium; *ωCtx,* ω-conotoxin; *Nif,* nifedipine. Both nicotine and K⁺ treatments resulted in neurite lengths that were significantly different from the vehicle control ($p < 0.001$) and not different from each other. Both ω-Ctx and nifedipine significantly protected against the nicotine effect, yielding values for neurite length that were significantly different from the nicotine-treated cases ($p < 0.01$) while not being significantly different from vehicle controls.

receptors being highly permeable to calcium or from their being able to activate voltage-gated calcium channels in the neurites. To investigate the contribution of voltage-dependent calcium channels to neurite retraction in the cultures, neurites were stimulated with elevated K⁺ concentrations (25 mM) from the micropipette. The brief pulses of K⁺ should depolarize the membrane and activate voltage-gated channels if present. Using the standard stimulation protocol described above for nicotine, the elevated concentration of K⁺ induced a retraction comparable to that caused by nicotine (Fig. 4).

Evidence that the K⁺-induced retraction of neurites involved activation of voltage-gated calcium channels was obtained through the use of the calcium channel blockers. ω-Ctx is thought to block N-type calcium channels, while nifedipine is thought to block primarily L-type channels (for reviews, see Bean, 1989; Tsien and Tsien, 1990). ω-Ctx at 2 μM prevented the K⁺ treatment from inducing neurite retraction. The mean neurite length at the end of the observation period was 115 ± 7% ($n = 12$), a value not significantly different from that for vehicle-treated neurites. Nifedipine produced a less complete reversal of the K⁺ effect, resulting in a mean neurite length of 93 ± 8% ($n = 18$) at the end of the test period.

Both calcium channel blockers also reversed the effects of nicotine on neurite length (Fig. 4). Thus, in the presence of 2 μM ω-Ctx, neurites treated with nicotine achieved a mean final length of 116 ± 8% ($n = 13$) at the end of the test period, which was not significantly different from that seen for vehicle-treated neurites (Fig. 4). Similarly, incubation in 10 μM nifedipine allowed neurites treated with nicotine to reach a mean final length of 101 ± 19% ($n = 14$) by the end of the test period. If the two calcium channel blockers are specific under these conditions, their ability to prevent nicotine from inducing neurite retraction in culture indicates that αBgt-AChRs achieve their effects on

neurites at least in part by activating voltage-gated calcium channels.

Discussion

The major findings reported here are that activation of αBgt-AChRs can induce neurite retraction and that the retraction is likely to result from calcium influx. The conclusion that αBgt-AChRs are responsible for much of the nicotinic effect is supported by the pharmacological evidence showing that low concentrations of αBgt and MLA can each block most of the nicotine-induced neurite retraction. At the concentrations used both the αBgt and MLA would have occupied essentially all of the αBgt-AChRs while binding to few, if any, mAb 35-AChRs.

Previous studies have shown that increasing the level of intracellular free calcium can retard growth cone motility and induce neurite retraction (Kater and Mills, 1991). A similar mechanism is likely to account for the nicotine-induced neurite retraction observed here since removal of extracellular calcium blocked the effect. αBgt-AChRs when activated on ciliary ganglion neurons are known to elevate intracellular levels of free calcium (Vijayaraghavan et al., 1992). Some of the calcium increase may result from ion flux directly through the receptor channel since neuronal AChRs are, in general, permeable to calcium (Mulle et al., 1992; Vernino et al., 1992). Receptors created by expression of the α7 gene product in *Xenopus* oocytes have a particularly high permeability to calcium (Seguela et al., 1993), and chick ciliary ganglion αBgt-AChRs are known to contain α7 gene product (Vernallis et al., 1993).

Voltage-gated calcium channels represent a second pathway by which extracellular calcium could enter the cells. During embryonic development, ciliary ganglion neurons have both N- and L-type calcium channels on their presynaptic nerve terminals (Gray et al., 1990, 1992). Since activation of ciliary ganglion αBgt-AChRs can produce a rapid depolarization of the membrane as indicated above, the depolarization may activate voltage-gated calcium channels and thereby augment calcium entry. Evidence that voltage-gated calcium channels can trigger neurite retraction in the present studies is provided by the observation that brief applications of concentrated K⁺ are sufficient to mimic nicotine in reversing neurite outgrowth. Moreover, ω-Ctx blocked the effects of K⁺, implicating N-type calcium channels in the process. The fact that ω-Ctx was also able to block nicotine-induced neurite retraction provides strong evidence that αBgt-AChRs achieve their effects on the neurites in large part by activating voltage-gated channels to augment calcium influx.

Additional evidence for involvement of voltage-gated channels is provided by the finding that nifedipine can block nicotine-induced neurite retraction. Inferences about the participation of voltage-gated calcium channels, however, must be considered tentative because they depend heavily on the specificity of the calcium channel blockers. Preliminary results indicate that ω-Ctx does not block the current response resulting from activation of αBgt-AChRs on the cell soma, but the results are less clear with nifedipine (Zhang and Berg, unpublished observations). An additional complication is that conceivably αBgt-AChRs on growth cones differ in some pharmacological manner from αBgt-AChRs on the cell body, thereby limiting the value of characterizing the latter population to learn about the former. Other approaches will be needed to obtain a definitive answer about

the role of voltage-gated channels in neurite retraction mediated by αBgt-AChRs.

The narrow range of nicotine concentrations capable of inducing neurite retraction probably reflects a combination of factors. A "threshold effect" may be imposed by voltage-gated channels if their participation is required for neurite retraction. Activation of αBgt-AChRs that was insufficient to depolarize the membrane to threshold for activating the voltage-gated channels would have little effect. Activation of more than enough receptors to reach threshold may or may not induce additional retraction over that observed at threshold, depending on the calcium dependence of the process. A second consideration, operative at high agonist concentrations, is receptor desensitization. AChRs produced by expressing the $\alpha7$ gene product in *Xenopus* oocytes have a very rapid rate of agonist-induced desensitization (Couturier et al., 1990). Ciliary ganglion αBgt-AChRs may behave similarly, explaining why short pulses of agonist spaced several minutes apart were effective at inducing retraction while chronic exposure was not. As the double pipette experiments showed, even leakage of agonist from the micropipette between pulses is apparently sufficient to desensitize the receptors when a high concentration of agonist is used. Leakage-induced desensitization of the receptors, therefore, can account for the finding that a smaller effect was observed with high concentrations of agonist (e.g., 10 μM nicotine, 1 mM ACh) than with low concentrations (e.g., 1 μM nicotine, 50 μM ACh). The fact that $\alpha7$-containing AChRs not only desensitize rapidly but also prefer nicotine over ACh (Couturier et al., 1990; Vernallis et al., 1993; Zhang and Berg, unpublished observations) may explain why αBgt was unable to block the neurite retractions elicited by intermediate concentrations of ACh (100, 200 μM). Under these conditions the αBgt-AChRs were probably desensitized but sufficient mAb 35-AChRs apparently remained active to produce the response.

Variation observed in the time course and extent of neurite retraction following application of nicotine could arise from differences in the number and distribution of αBgt-AChRs or voltage-gated ion channels among neurites. Other sources of variability may have to do with differences in the effectiveness of calcium buffering among neurites or in the efficiency of intracellular molecular mechanisms mediating calcium-dependent neurite collapse. The mechanisms have yet to be fully elucidated but are known to include calcium regulation of actin filament and/or microtubule assembly (Kater and Mills, 1991). Including filopodia in the length measurements did not enhance the variability among experiments. Filopodia seemed to occur with equal probability under all test conditions; excluding them from the measurements did not alter any of the conclusions. Reports of filopodia collapse in previous experiments may have depended on long-term elevation of intracellular free calcium (Kater and Mills, 1991) rather than the transient increases likely to have been achieved with the protocols used here.

Previous reports have indicated that cholinergic antagonists in the case of chick retinal cells (Lipton et al., 1988) and αBgt in the case of rat PC12 cells (Quik et al., 1990) can increase neurite length. Since both types of cultures can make and release ACh under appropriate conditions (Greene and Rein, 1977; Lipton, 1988), the effects can be interpreted as relieving an ongoing ACh-induced inhibition of neurite outgrowth. Indeed, the concentration of endogenous ACh in the culture medium

of chick retinal cultures was estimated to be 0.5 μM (Lipton, 1988). No significant effect of αBgt alone was observed on neurite length in the present studies. The likely reason is that extracellular ACh concentrations in the cultures used here were too low to inhibit neurite outgrowth. The cultures were plated at a low cell density and were taken at early times so that isolated neurites from a single cell could be examined without interference from neurites of other cells. Though ciliary ganglion neurons can release ACh from the tips of growing neurites (Hume et al., 1983), the sparseness of neurites in the present case plus the presence of cholinesterases in the culture medium make it highly improbable that significant amounts of ACh could have accumulated in the medium during the test period. The absence of an αBgt effect on unstimulated neurons supports this contention.

Fluorescence studies with rhodamine-labeled αBgt have shown that αBgt-AChRs are present on the neurites as well as the somata of ciliary ganglion neurons in cell culture (Ravdin et al., 1981). It is not clear, however, whether the receptors responsible for inducing neurite retraction are confined to the neurites and growth cones. Though the micropipette was positioned to optimize application of agonist to the growth cone, some agonist could have reached the soma as well. A second path that might have triggered a somatic response involves the voltage-gated channels. Activation of voltage-gated channels by αBgt-AChRs even distally on the neurites might have elicited action potentials that invaded the soma and promoted substantial calcium entry throughout the cell. An argument against the soma being the critical site at which calcium entry triggers neurite retraction is that application of nicotine to a single neurite did not necessarily induce collapse of other neurites emanating from the same cell.

One function for αBgt-AChRs *in vivo* suggested by the present results is a remodeling of dendritic morphology in the ganglion. By embryonic day 7 ciliary neurons in the ganglion have extended fine dendritic-like processes referred to as pseudodendrites and have received functional synaptic contacts from preganglionic terminals (Landmesser and Pilar, 1972, 1978). By embryonic day 10 the dendritic processes have retracted to allow the preganglionic terminals to form large calyces cupping the axon hillock region of the neurons. αBgt-AChRs are present in the ganglion by embryonic day 7 and increase in number up through hatching (Chiappinelli and Giacobini, 1978). Histochemical studies at the ultrastructural level with HRP-labeled αBgt indicate that the receptors may be most concentrated on the pseudodendrites (Jacob and Berg, 1983). It is possible that ACh released even in a diffuse manner from the preganglionic terminals may serve to activate αBgt-AChRs distally, inducing a collapse of the pseudodendrite back into the soma. Additional experiments will be required to determine whether growing neurites are influenced by αBgt-AChR activation *in vivo* where the neurons must contend with a three-dimensional matrix and substrata much more complex than presented here in cell culture.

The fact that large numbers of αBgt-AChRs remain on the neurons well into adulthood invites speculation about other possible functions. αBgt-AChRs on the cell soma may participate in a kind of nonsynaptic signaling, responding to ACh released either from other neuronal somata in the ganglion or from preganglionic terminals at nonsynaptic sites. Some activation of αBgt-AChRs may also occur by diffusion of transmitter

away from synaptic sites during periods of intense synaptic activity. The purposes of such signaling, however, can only be hypothesized at present.

A final speculation offered here is that αBgt-AChRs may have functions not dictated by a somal location. Substantial numbers of the receptors may be transported down the axon to serve as presynaptic receptors at nerve–muscle junctions in the periphery. (No effort was made in the present study to distinguish axons from dendrites in cell culture.) During development presynaptic αBgt-AChRs could provide a mechanism for terminating axonal growth at locations where the axon acquired the ability to release transmitter. In the adult the receptors might provide positive feedback for transmitter release. Activation of the receptors by ACh released either from the same terminal or from adjacent terminals would further elevate intracellular levels of calcium and thereby enhance transmitter release. The prevalence and physiological significance of presynaptic AChRs are subjects likely to attract increasing attention.

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