Ganglioside Modulation of Neural Cell Adhesion Molecule and N-cadherin-dependent Neurite Outgrowth

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Abstract. We have used monolayers of control 3T3 cells and 3T3 cells expressing transfected human neural cell adhesion molecule (NCAM) or chick N-cadherin as a culture substrate for PC12 cells. NCAM and N-cadherin in the monolayer directly promote neurite outgrowth from PC12 cells via a G-protein-dependent activation of neuronal calcium channels. In the present study we show that ganglioside GM1 does not directly activate this pathway in PC12 cells. However, the presence of GM1 (12.5–100 μg/ml) in the co-culture was associated with a potentiation of NCAM and N-cadherin-dependent neurite outgrowth. Treatment of PC12 cells with GM1 (100 μg/ml) for 90 min led to trypsin-stable increases in both β-cholera toxin binding to PC12 cells and an enhanced neurite outgrowth response to N-cadherin. The ganglioside response could be fully inhibited by treatment with pertussis toxin. These data are consistent with exogenous gangliosides enhancing neuritic growth by promoting cell adhesion molecule-induced calcium influx into neurons.

During development and after injury, neurons extend axons in a precise manner to innervate their target tissue. Our current understanding of the molecular basis of axonal growth has largely evolved from observations on neurons studied in in vitro model systems. Growth can be modulated by a wide variety of diffusible and nondiffusible molecules that can operate both as stimulatory and inhibitory cues (for reviews see Dodd and Jessell, 1988; Doherty and Walsh, 1989; Strittmatter and Fishman, 1991). Many of these factors operating in a highly orchestrated manner have the potential to contribute to axonal growth and guidance in vivo.

Several molecules in the extracellular matrix and on the surface of cells are potent inducers of neurite outgrowth. In this context, there are currently four well-defined receptor systems present on neuronal growth cones that support or mediate neuritic growth over a variety of non-neuronal cell types including astrocytes, muscle cells, and Schwann cells (reviewed in Doherty and Walsh, 1989). These are the integrins (Reichardt and Tomaselli, 1991) and the cell adhesion molecules (CAM)[1], N-cadherin (Takeichi, 1991), neural cell adhesion molecule (NCAM) (Walsh and Doherty, 1991), and L1 (Seilheimer and Schachner, 1988). Whereas the integrins are receptors for extracellular components such as laminin, N-cadherin, NCAM, and L1 primarily promote neurite outgrowth via a homophilic interaction with products of the same gene expressed in non-neuronal cells (Matsunaga et al., 1988; Doherty et al., 1990*a*, 1991*a*; Lemmon et al., 1989). However, recent evidence suggests that two of these molecules can also function as heterophilic receptors for products of distinct but related genes. For example, N-cadherin can bind to the recently described R-cadherin (Inuzuka et al., 1991) and L1 can function as a neurite outgrowth promoting receptor for the Axonin-1 glycoprotein (Kuhn et al., 1991).

Neurons derived from several brain areas show dramatic changes in their responsiveness to the above neurite outgrowth promoting molecules when isolated at differing stages of development. For example, the ability of both retinal ganglion cells (RGCs) and hippocampal neurons to respond to NCAM is downregulated during development (Doherty et al., 1990*a*; Doherty, P., S. D. Skaper, S. E. Moore, A. Leon, and F. S. Walsh, manuscript submitted for publication). In the case of retinal ganglion cells, loss of responsiveness was not associated with loss of receptor (i.e., NCAM) from the neuronal growth cone. Many other factors including posttranslational processing, isoform switching, or the expression of other molecules that interact with CAMs directly or with molecules that transduce the CAM recognition signals (see Doherty et al., 1991*a*) have the potential to modulate neurite outgrowth. In the context of the latter possibility, a consideration of the complexity, distribution and biological effects ascribed to neuronal gangliosides (e.g., see Hakamori, 1981; Hannun and Bell, 1989) is consistent with them playing such a role.

Exogenous gangliosides can stably associate with cell membranes where they can function in a similar manner to their endogenous counterparts. For example GM1 readily associates with neuronal and nonneuronal cells in a temperature, time, and saturable manner, and after incorporation into the membrane can act, like its endogenous counterpart,

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; NCAM, neural cell adhesion molecule; NGF, nerve growth factor.

as a functional receptor for cholera toxin (Moss et al., 1976; Spiegel et al., 1984). The observation that exogenous gangliosides can promote neurite outgrowth from a wide variety of neuronal cell lines and primary neurons (Roisen et al., 1991; Facci et al., 1984; Doherty et al., 1985*a*,*b*; McCaig, 1990) lends support to the general postulate that one function of endogenous gangliosides may be to modulate axonal growth during development.

The biochemical mechanisms underlying ganglioside induction and/or modulation of neurite outgrowth are not known. However, ganglioside-induced neuritogenesis from mouse Neuro-2A cells is known to be dependent on extracellular calcium (Spoerri et al., 1990; Wu et al., 1990), and exogenous GM1 can interact with nerve growth factor (NGF) to recruit a Ca^{2+}-dependent protein kinase in PC12 cells (Hillbush and Levine, 1991). This latter response was abolished by removal of extracellular calcium from the medium, or by treatment with an L-type calcium channel antagonist. These data suggest that gangliosides might stimulate neurite outgrowth by promoting calcium influx into cells.

We have recently provided evidence that NCAM and N-cadherin stimulate neurite outgrowth from PC12 cells via G-protein dependent activation of L- and N-type neuronal calcium channels (Doherty et al., 1991*a*) and that a similar, if not identical, mechanism can account for NCAM-dependent neurite outgrowth from primary neurons (Doherty, P., S. D. Skaper, S. E. Moore, A. Leon, and F. S. Walsh, manuscript submitted for publication). In the present study we have investigated whether exogenous gangliosides can modulate this physiologically relevant calcium-dependent response. Our results show that GM1 can become stably associated with PC12 cells, although this by itself has no direct effect on cell morphology. In contrast, cell-associated GM1 was found to significantly enhance both NCAM- and N-cadherin–dependent neurite outgrowth. These data suggest that exogenous gangliosides may promote axonal growth in vivo by promoting CAM-induced calcium influx into neurons. Likewise endogenous gangliosides may affect growth during development by modulating signals arising from cell–cell interactions mediated by CAMs.

Materials and Methods

Cell Culture

Parental and CAM-transfected clones of mouse 3T3 cells (Doherty et al., 1991*a*) were routinely maintained on culture dishes in DME containing 10% FCS and kept at 37°C in 8% CO_2. Monolayers for co-culture were established by seeding 80,000 cells per chamber well of eight-chamber tissue culture slides (Lab-Tek Division, Miles Laboratories, Naperville, IL) (Doherty et al., 1990*a*). PC12 cells were routinely grown in chemically defined SATO medium (Doherty et al., 1988*a*) on a collagen-coated substratum. For establishment of co-cultures, PC12 cells were dissociated in versene solution (Gibco Laboratories, Grand Island, NY) followed by incubation at 37°C for ~10 min with 0.05% (wt/vol) trypsin in the same buffer. A single-cell suspension was obtained by trituration (19-gauge syringe needle) of pelleted trypsinized cells resuspended in SATO medium. For co-culture experiments 1–1.5 × 10^3 PC12 cells were added to each chamber of an eight-chamber slide. Co-cultures were maintained for 20–48 h in SATO medium supplemented as described below. Cultures were then carefully fixed for 30 min with 4% paraformaldehyde.

Antibody Reagents and Immunocytochemistry

The Thy-1 antigen was recognized by the OX-7 mAb used at a 1:50 dilution of tissue culture supernatant. Cultures were processed for immunocytochemistry by sequential incubation with OX-7, biotinylated anti-mouse immunoglobulin and Texas red–conjugated streptavidin (Amersham International, Amersham, UK) (both diluted at 1:500) as previously described (Doherty et al., 1991*a*).

Ganglioside Treatment of Cells

Gangliosides were purified from bovine brain according to the method of Tettamanti et al., (1973). GM1 was of the highest grade available (>99% pure) and was also free from peptide and glycoprotein contamination (Ghidoni et al., 1980).

GM1 was taken to dryness under a stream of nitrogen from a chloroform–methanol (2:1, vol/vol) solution and directly dissolved in either culture medium (SATO) or DME as indicated in the text. In one set of experiments, GM1 was added to cells throughout the total period of co-culture, in other experiments monolayer cultures of PC12 cells were treated with either GM1 in SATO for 16 h or GM1 in DME for 90 min as described in the text. The latter treatment has been shown to result in the saturable incorporation of the order of 230 pmole of GM1 per 1 mg of protein, or 2.8 × 10^6 molecules of exogenous GM1 into the plasma membrane of individual PC12 cells (Hilbush and Levine, 1991).

β-cholera Toxin Binding to Cells

Ganglioside treated PC12 cells were plated into individual wells of a polylysine-coated 96-well microtiter plate and fixed with 4% paraformaldehyde at the times indicated in the text. Nonspecific protein binding sites were blocked by incubation with PBS containing gelatin (0.5%). Cells were then incubated with HRP-conjugated β-cholera toxin (List Biological Laboratories, Inc., Campbell, CA) at 2 μg/ml for 60 min at room temperature. The cultures were washed four times with PBS and incubated with 50 μl of 0.2% (wt/vol) *o*-phenylenediamine in 0.02% (vol/vol) H_2O_2 in citrate buffer. After ~5–10 min the conversion of *o*-phenylenediamine to its oxidized product was stopped by the addition of 50 μl of 4.5 M H_2SO_4 and optical density determined at 492 nm using a 96-well microtiter plate reader. Control experiments showed a linear relationship between reaction product against cell number over the range tested (0–4 × 10^3). The binding of β-cholera toxin to PC12 cells could also be completely inhibited (>98%) by the presence of free GM1 (10 μg/ml) during the incubation.

Other Reagents

Pertussis toxin was a gift from Dr. J. Kenimer (Food and Drug Administration, Bethesda, MD) and was used as previously described (Doherty et al., 1991*a*). Pertussis toxin had no effect on NCAM or N-cadherin expression in the monolayers or on PC12 cells (our own unpublished observation).

Image Analysis

All images were detected by immunofluorescence microscopy using a low light-sensitive video camera (model 4722–5000; Cohu Inc., San Diego, CA) and analyzed using a Sight Systems Image Manager (Sight Systems, Newbury, England). Cultures were scanned in a systematic manner, and the length of the longest process per PC12 cell was measured as previously described (Doherty et al., 1990*b*). For computational ease, cells with no obvious process were included by assigning an arbitrary value of 2 μm. This did not significantly affect the results.

Results

GM1 Potentiates CAM-dependent Neurite Outgrowth

Culture of both primary neurons and PC12 cells on monolayers of 3T3 fibroblasts expressing transfected NCAM or N-cadherin is associated with a direct induction of neurite growth by the transgene product (e.g., see Doherty et al., 1990*a*,*b*; 1991*a*,*b*). Fig. 1 shows examples of PC12 cells cultured for ~40 h on monolayers of parental and N-cadherin expressing 3T3 cells in the presence and absence of 50 μg/ml GM1. On parental 3T3 monolayers, PC12 cells rarely extend neurites and GM1 has no obvious effect on PC12 cell morphology (Fig. 1, *A* and *B*). In contrast the majority of PC12

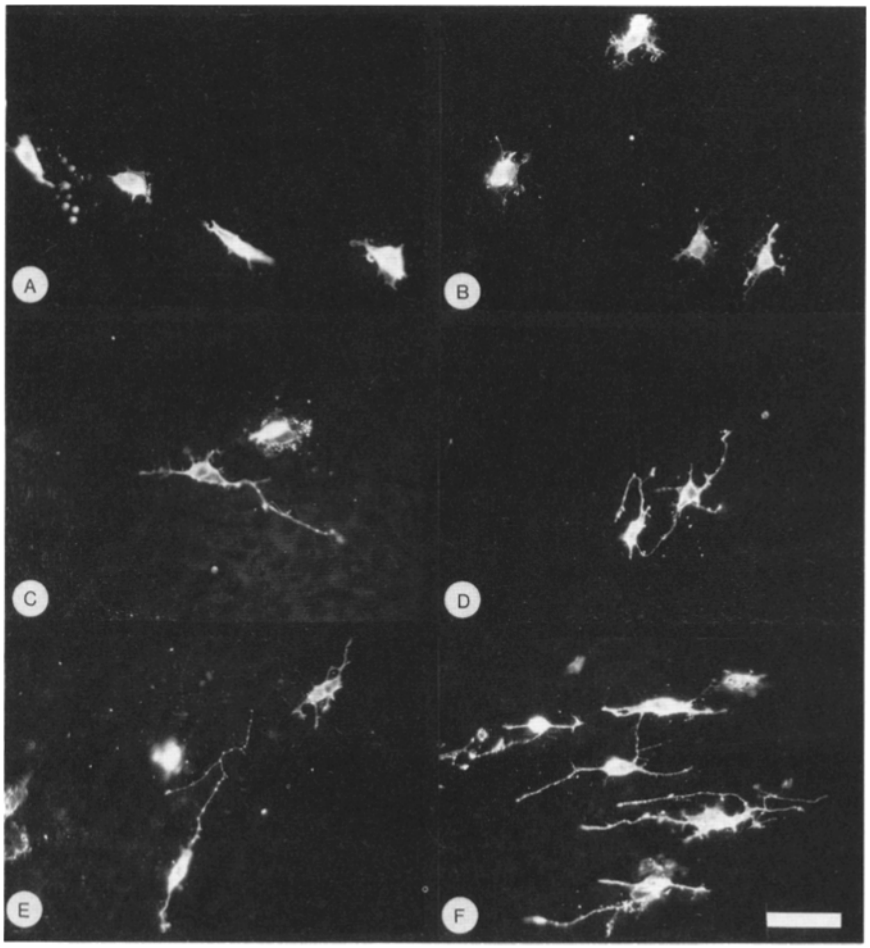

Figure 1. GM1 effects on PC12 cell morphology on control and N-cadherin expressing 3T3 cell monolayers. PC12 cells, specifically identified by immunostaining with a mouse anti-rat Thy-1 mAb, were grown for 40 h on a substratum of confluent 3T3 cells (*A* and *B*), 3T3 cells expressing transfected N-cadherin (*C*, *D*, *E*, and *F*) in control media (*A*, *C*, and *D*) or media supplemented with 50 μg/ml GM1 (*B*, *E* and *F*). Bar, 50 μm.

cells cultured on N-cadherin expressing monolayers extended one or more primary neurites and treatment of these co-cultures with GM1 was associated with an enhancement of neurite outgrowth (Fig. 1, *C–F*). Similar responses were observed when PC12 cells were cultured on NCAM-expressing monolayers (data not shown). These data suggest that GM1 has no direct effect on PC12 cell morphology, although GM1 can potentiate the neurite outgrowth activity of both NCAM and N-cadherin.

To quantitate these effects, the length of the longest PC12 cell neurite was measured after ~40 h of co-culture with the results from one experiment shown in Fig. 2. GM1 (50 μg/ml) had no significant effect on the length of the longest neurite for PC12 cells cultured on parental 3T3 cell monolayers. However, GM1 potentiated ($P < 0.005$) the response induced by both NCAM and N-cadherin. In the example shown, the mean length of PC12 cell neurites on N-cadherin expressing monolayers was 254% of the control value and this increased to 424% for GM1-treated cultures. In these same cultures, GM1 had very little effect on the mean number of neurites per PC12 cell, the value increasing from 1.5 ± 0.15 ($n = 50$) on N-cadherin alone to 1.84 ± 0.15 ($n = 62$, both values mean ± SEM) on N-cadherin plus GM1. GM1 did not obviously alter the polarity of PC12 cells by, for example, promoting the growth of one neurite at the expense of others.

In a series of five independent experiments, GM1 (50–100 μg/ml) was consistently found to have no effect on the length of the longest neurite for PC12 cells cultured on parental 3T3 monolayers (pooled value in the presence of GM1 being 114.2 ± 13.3% of respective control). Neurite lengths on N-cadherin expressing monolayers were 221 ± 13.7% ($n = 5$) of the value found on control 3T3 monolayers and this was significantly ($P < 0.01$) increased to 329 ± 31.0% ($n = 5$) on treatment with GM1 (50–100 μg/ml). The effects of GM1 on the percentage of cells with a neurite >20 μm in length was also measured. 16.7 ± 3.3% ($n = 4$) of PC12 cells had neurites >20 μm on parental 3T3 monolayers and GM1 increased this by a relatively small amount to 22.4 ± 5.1% ($n = 4$). In contrast 56.7 ± 4.7% ($n = 5$) extended a neurite >20 μm on N-cadherin–expressing monolayers and this was increased to 76.2 ± 5.0% ($n = 5$) after treatment with GM1 (all values mean ± SE for the given number of independent experiments).

The relationship between GM1 concentration and its ability to modulate N-cadherin–dependent neurite outgrowth is shown in Fig. 3. On parental 3T3 monolayers GM1 (at up to 100 μg/ml) has no effect on the average length of PC12 cell neurites. In contrast, N-cadherin–dependent neurite outgrowth was potentiated in a dose-dependent manner with a significant ($P < 0.05$) effect first measured at 25 μg/ml. In a further two experiments, 200 μg/ml was found to be no more effective than 100 μg/ml GM1 suggesting the latter to be the maximal or near maximal response.

The molecular specificity of the ganglioside effect was also tested. In two independent experiments, asialo GM1 (tested

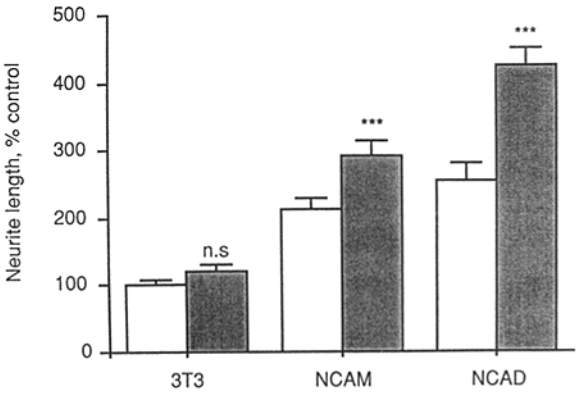

Figure 2. Effects of GM1 on neurite lengths for PC12 cells cultured on control, NCAM and N-cadherin-expressing monolayers. PC12 cells were cultured for 40 h in SATO (□) or SATO supplemented with 50 μg/ml GM1 (▨) on monolayers of control 3T3 cells, or 3T3 cells expressing NCAM or N-cadherin (*NCAD*). Co-cultures were fixed and the length of the longest neurite on Thy-1-stained PC12 cells was determined. The results show mean neurite length per cell (±1 SEM) for 90–120 PC12 cells sampled in replicate cultures. n.s., Not statistically different from growth in the absence of GM1; ***, $P < 0.0025$.

at up to 200 μg/ml) failed to significantly affect PC12 cell morphology on parental and N-cadherin-expressing monolayers. The oligosaccharide portion of GM1 (over a 1–100 μg/ml range) was also tested, but again was ineffective in modulating CAM-dependent neurite outgrowth. These data suggest that GM1 has to be available in its intact form to modulate N-cadherin-dependent neurite outgrowth. In preliminary experiments GT1b, GM3, and GD1a were found to have similar effects to GM1. These results are in line with those reported by Byrne et al. (1983) for ganglioside-induced neuritogenesis from Neuro 2A, but in contrast with studies on primary neurons where higher order gangliosides (e.g., GT1b) had more dramatic effects in neurite outgrowth (Doherty et al., 1985*b*).

PC12 Cell-associated GM1 Has Neuromodulatory Activity

As discussed above (see introduction) exogenous GM1 can become stably associated with the cell membranes of both neuronal and nonneuronal cells. To determine if PC12 cell-associated GM1 can modulate CAM-dependent neurite outgrowth, cultures of PC12 cells were treated for 16 h (in normal SATO media) with 6.25, 25, or 100 μg/ml GM1. PC12 cells were then dissociated as normal (with trypsin, see Materials and Methods) and cultured on confluent monolayers of parental and N-cadherin-expressing 3T3 cells. After 20 h, the cultures were fixed and the absolute length of the longest neurite and percentage of cells with a neurite >20 μm determined. Pretreatment with GM1 again has no effect on either parameter for PC12 cells cultured on parental 3T3 cells (e.g., the values for 0 and 100 μg/ml GM1 were 12.3 ± 1.0 [153] and 11.5 ± 0.9 [155]) μm for neurite length and 15.7 and 18.7% for percentage of cells with a neurite >20 μm, respectively). However, GM1 pretreated PC12 cells responded substantially better on the N-cadherin monolayer than control PC12 cells as indexed by both the mean length of the longest neurite per cell or the percentage of cells with a neurite >20 μm (Fig. 4). In these experiments, the normal dose-response relationship (Fig. 3) was not observed and 6 μg/ml GM1 was as effective as 100 μg/ml GM1. These data are explicable by the time, temperature, and saturable incorporation of GM1 into neuronal membranes (see Hilbush and Levine, 1991).

GM1 is more readily incorporated into neuronal cells in

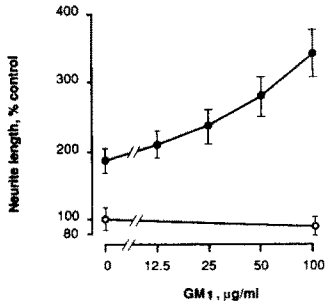

Figure 3. GM1 potentiates N-cadherin-induced neurite outgrowth in a dose-dependent manner. PC12 cells were cultured for 40 h on control (○) or N-cadherin (●) expressing monolayers in the presence of the given concentration of GM1. Mean neurite lengths were determined as in Fig. 2 and each value is the mean ± SEM for ~100 PC12 cells sampled in replicate cultures. Statistical significance ($P < 0.05$) for the GM1 effect was first observed at 25 μg/ml.

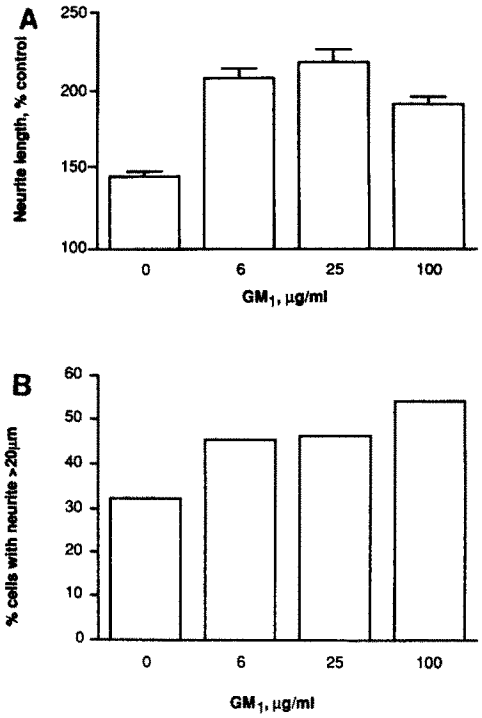

Figure 4. Cell-associated GM1 promotes N-cadherin responses. PC12 cells were incubated overnight at 37°C in SATO media supplemented with 0, 6, 25, or 100 μg/ml GM1. The cultures were washed and dissociated as normal (see Materials and Methods) and cultured on confluent monolayers of 3T3 cells or N-cadherin-expressing 3T3 cells. After 20 h the co-cultures were fixed and the mean length of the longest neurite per cell (*A*) or percentage of cells with a neurite >20 μm (*B*) determined. Mean neurite lengths are shown for growth on N-cadherin monolayers and these values are the mean ± SEM for 150–180 PC12 cells per data point. The values are expressed as a percentage of the growth measured on control 3T3 cell monolayers (measured as 12.3 ± 1.0 μm; $n = 153$). The percentage of these cells with a process >20 μm is shown, with the control value on 3T3 monolayers measured as 15.7% ($n = 153$).

Table I. Association of GM1 with PC12 Cells

	β-Cholera toxin binding	
	4 h	20 h
Control PC12	0.26 ± 0.005	0.19 ± 0.004
Trypsinized PC12	0.25 ± 0.002	–
GM1-treated PC12	0.51 ± 0.007	0.45 ± 0.013
Trypsinized GM1-treated PC12	0.44 ± 0.008	–

Cultures of PC12 cells were incubated with control media or with GM1 (100 μg/ml) in DME for 90 min at 37°C. The cultures were triturated in phosphate-buffered saline containing 0.02% EDTA and split into two aliquots. Trypsin (0.125% wt/vol) was added to one aliquot and both aliquots were shaken at 37°C for 15 min. The PC12 cells were then washed in SATO media and plated at ~5,000 cells per well onto a polylysine coated 96-well microtiter plate. The cultures were then maintained for 4–20 h in SATO media at 37°C before being fixed with 4% paraformaldehyde. The binding of β-cholera toxin (see Materials and Methods) was then determined. The results show the relative binding of β-cholera toxin ±SEM determined from seven replicate cultures for control and GM1-treated PC12 cells which were in turn treated with control or trypsin containing PBS.

the absence of serum binding proteins such as BSA (Facci et al., 1984; Hilbush and Levine, 1991). In DME, near saturable incorporation into PC12 cells can be found after treatment with ~100 μg/ml GM1 for as little as 90 min. We have assessed the relative change in GM1 levels and the biological effects in terms of neurite outgrowth after a short exposure of PC12 cells to exogenous GM1. Table I summarizes the relative binding of β-cholera toxin to control and GM1-treated (90 min, 100 μg/ml in DME) PC12 cells at both 4 and 20 h after the removal of exogenous GM1. At both time points, GM1 treated cells bound at least twice as much β-cholera toxin than untreated cells, and approximately 70% of this binding remained after treatment with trypsin (0.125% trypsin for 15 min) (see Materials and Methods).

The GM1 pretreated cells again showed enhanced responsiveness to N-cadherin in terms of increases in mean neurite length at both 20 and 44 h of co-culture (Fig. 5). Thus, pretreatment with GM1 (100 μg/ml) for as little as 90 min can lead to stable increases in cell-bound GM1, and this is associated with a sustained increase in the ability of treated cells to respond to N-cadherin. This treatment again had no effect whatsoever on PC12 cell morphology on control 3T3 cell monolayers (see legend to Fig. 5). These data also show that the response to GM1 is essentially not reversible over this time period (~48 h). Longer term experiments are not compatible with the present experimental paradigm because of overgrowth and instability of the cellular monolayers.

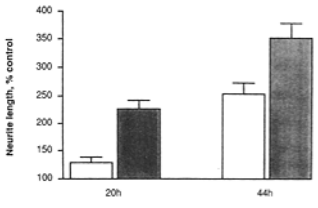

Figure 5. GM1 pretreated PC12 cells show enhanced responsiveness to N-cadherin over a 44-h period of culture. PC12 cells were pretreated with GM1 (100 μg/ml) in DME for 90 min at 37°C as described in Table I. Controlled (□) and GM1-pretreated (▩) cells were then cultured for 20–44 h on confluent monolayers of parental and N-cadherin expressing 3T3 cells. Mean neurite lengths were determined as in Fig. 2 and the data show the relative increase in growth on N-cadherin-expressing monolayers as a percentage of the growth measured on parental 3T3 cell monolayers. The latter values were 11.5 ± 0.95 (127) and 10.4 ± 0.64 (147) at 20 and 44 h for parental 3T3 monolayers in control media, and GM1 had no effect on growth in these co-cultures, measured as 10.8 ± 0.92 (138) and 9.9 ± 0.81 (141) at the same respective timepoints. All results are mean ± SEM for 120–150 PC12 cells sampled in replicate cultures.

The Effect of Pertussis Toxin on the GM1 Response

Pertussis toxin, which blocks NCAM and N-cadherin-dependent neurite outgrowth from PC12 cells and primary neurons (see introduction), was tested for its effects on the GM1 potentiation of the N-cadherin response. In the absence of pertussis toxin, N-cadherin-induced neurite outgrowth was significantly enhanced by the continued presence of GM1 (100 μg/ml) in the culture medium. In contrast, after pertussis toxin (500 ng/ml) inhibition of N-cadherin-induced neurite outgrowth (Doherty et al., 1991*a*), GM1 was no longer effective (Fig. 6). These data suggest that GM1 operates by enhancing signaling through the normal G-protein-dependent pathway that is activated by CAMs.

Discussion

Treatment of PC12 cells or chick dorsal root ganglion neurons with GM1 alone has no effect on neurite outgrowth (Ferrari et al., 1983; Doherty et al., 1985*a*,*b*). However, GM1 can promote the neurite outgrowth response from these cells that is induced by NGF treatment. In both studies there was no evidence for a direct interaction between GM1 and NGF, and in the dorsal root ganglion model, providing a program of cell survival and neurite regeneration was initiated by NGF, GM1 could operate independently of the trophic factor to increase neurite outgrowth (Doherty et al., 1985*a*,*b*). This led us to suggest that gangliosides are active at the level of signal transduction or execution, rather than signal reception.

After binding to its high affinity receptor, which has recently been identified as a tyrosine kinase (for review see Bothwell, 1991), NGF induces a very complex cascade of responses in PC12 cells which initially involves tyrosine phosphorylation of, for example, key enzymes controlling lipid derived second messenger production (Vetter et al., 1991). This ultimately results in the increased expression of several gene products, including key cell surface glycoproteins such as the low affinity NGF receptor (Doherty et al., 1988*a*) and the neural cell adhesion molecules, NCAM (Doherty et al., 1988*b*) and N-cadherin (Doherty et al., 1991*a*). In an extensive series of experiments, we found no evidence for a variety of gangliosides (GM1, GT1b, and GQ1b) modulating the NGF-induced expression of the above molecules (Doherty, P., and F. S. Walsh, unpublished observations), suggesting that if gangliosides directly modulate NGF signal transduction they must do so in a highly restrictive manner. In this context, although NGF is required for the survival of chick dorsal root ganglion neurons and promotes the transformation of PC12 cells from an adrenal to neuronal phenotype; both of these actions can essentially be viewed as being prerequisites of neurite outgrowth. The actual growth of neurites is equally dependent on the interaction of growth cone receptors (e.g., integrins, NCAM, N-cadherin, and L1) with their appropriate ligands (see introduction). It follows that GM1-induced neurite outgrowth may reflect a modulation of the signals that arise via integrins and CAMs rather than those arising from the interaction of NGF with its receptor.

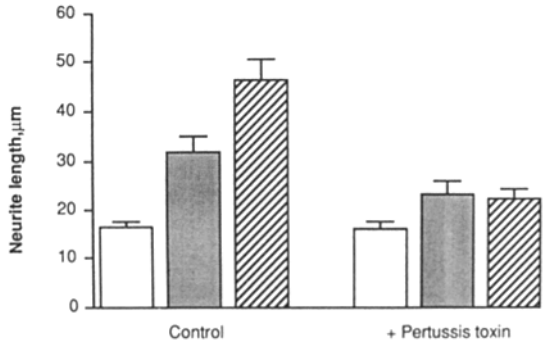

Figure 6. Pertussis toxin inhibits N-cadherin and GM1-dependent neurite outgrowth. PC12 cells were cultured for 40 h in the presence and absence of pertussis toxin (500 ng/ml) on confluent monolayers of parental (□) or N-cadherin-expressing (▦) 3T3 cells. The media was further supplemented with 100 μg/ml GM1 as shown (▨) Mean neurite lengths were determined as in Fig. 2 and each value is the mean ± 1 SEM for 80–100 PC 12 cells sampled in replicate cultures. Neither pertussis toxin nor GM1 had any significant effect on neurite length for PC12 cells grown on parental 3T3 cell monolayers.

In the present study, we have investigated the ability of exogenous gangliosides to modulate CAM-induced neurite outgrowth from PC12 cells. Our results show that GM1 has very little direct effect on neurite outgrowth from PC12 cells cultured on control 3T3 cell monolayers; however, GM1 was found to significantly potentiate the response induced by both NCAM and N-cadherin. The uptake of GM1 into the PC12 cells and/or the stable association of GM1 with the cell membrane seems to account for the response, as pretreatment with GM1 for as little as 90 min (followed by trypsinization) was sufficient to induce a maintained enhancement of N-cadherin-dependent neurite outgrowth. This observation also excludes the possibility that exogenous GM1 acts by directly binding to the monolayer cells. The observation that GM1 pretreatment substantially increases the β-cholera toxin binding capacity of PC12 cells is consistent with GM1 in the membrane directly modulating CAM-dependent neurite outgrowth. It is not, however, possible to know whether the cell-associated GM1 exists as micelles and/or inserted individual molecules. However, the fact that it is stable to trypsin suggests the latter. The lack of effect of asialo-GM1 and the oligosaccharide portion of GM1 suggests that incorporation into the membrane may be required for function and also that a metabolic product of GM1 is unlikely to be responsible for the response.

NCAM and N-cadherin promote neurite outgrowth via a G-protein-dependent activation of neuronal L- and N-type calcium channels (Doherty et al., 1991*a*). GM1 is unlikely to directly activate calcium influx via this (or another) pathway as it has no direct effect on PC12 cell neurite outgrowth. This contrasts with potassium depolarization and a calcium channel agonist which in the above co-culture model can both promote neurite outgrowth via activation of calcium channels (Saffell, J., unpublished observation). The observation that pertussis toxin can block N-cadherin-dependent neurite outgrowth (Doherty et al., 1991*a*) and the ganglioside response (this study) is consistent with GM1 operating by enhancing signaling through the normal CAM-activated pathway. GM1 could conceivably operate at any one of a number of steps in the pathway including CAM interaction with G-protein, G-protein activation of calcium channels, calcium flux through the channel, or calcium-dependent targets within the cell. However, we cannot exclude the possibility that GM1 acts upstream of this pathway for instance by directly interacting in a *cis*- or *trans*-manner with NCAM or N-cadherin or by modulating CAM–CAM binding.

Recent evidence from Hilbush and Levine (1991) complements the results of the present study and strongly suggests that GM1 may directly affect either calcium channel function or calcium-dependent responses within PC12 cells. These workers have found that GM1 has no direct effects on tyrosine hydroxylase phosphorylation; however, in the presence of NGF or K^+ depolarization, GM1 induced increases in ^{32}P incorporation into a tyrosine hydroxylase phosphopeptide. The ganglioside response was inhibited by removal of extracellular calcium from the media or by blockage of the L-type calcium channels. The authors concluded that, as in this study, the stimulating effects may be due to ganglioside potentiation of a Ca^{2+}-dependent signaling pathway. However, in this context NGF induced morphological differentiation of PC12 cells can be distinguished from that induced by CAMs in that the former is not inhibited by either pertussis toxin or calcium channel antagonists (Doherty et al., 1991*a*).

Neither this study nor that by Hilbush and Levine can distinguish between a ganglioside modulatory role directly at the level of calcium channel function as opposed to an intracellular site of action at perhaps the level of a calcium-dependent kinase. The clear association between increased β-cholera toxin binding and biological responses would be consistent with a cell membrane site of action. Further evidence for gangliosides acting at this level has recently been obtained. Hilbush and Levine (1991) have found that exogenous GM1 can enhance the increase in intracellular calcium that accompanies K^+-depolarization of PC12 cells (J. M. Levine, personal communication).

Evidence that endogenous gangliosides may modulate the function of calcium channels has been obtained by the binding of ligands to GM1 in the membrane of intact cells. Frieder and Rapport (1987) have reported that antibodies to GM1 can enhance the release of γ-aminobutyric acid induced by K^+ depolarization, but not that induced by the calcium ionophore A23187. In addition, they excluded a role for Na^+ channels in the ganglioside response. Dixon et al. (1987) have shown that the β subunit of cholera toxin, which binds to GM1 but has no other cellular effects, can trigger sustained rises in cytoplasmic free calcium in rat lymphocytes and that this is likely to arise from a net influx of extracellular calcium. These data support the possibility that exogenous gangliosides may function in a similar manner to their endogenous counterparts. In this context some gangliosides show highly restricted patterns of expression during development. For example a ganglioside antigen recognized by the Jones mAb first appears in central rat retina at E12–13 when retinal ganglion cells start to differentiate and extend their axons, and by E17–18 it is expressed in a dorsoventral gradient over the retina (Constantine-Paton et al., 1986). Interpretation of the positional information encoded by such a distribution could conceivably be at the level of modulation of the signals arising from CAMs.

In summary, many studies have suggested that calcium levels in the neuronal growth cone may control growth cone mo-

tility and thereby affect changes in axonal growth (for review see Kater and Mills, 1991). We have previously provided evidence that CAMs, which undoubtedly play a role in axonal growth and guidance in vivo (e.g., see Greeningloh et al., 1991) operate via a calcium signaling pathway. In the present study, gangliosides have been shown to modulate this physiologically relevant pathway. Based on these observations, we would postulate that endogenous gangliosides, and also, exogenous gangliosides that associate with neurons in vivo, may modulate axonal growth during development and after injury primarily by modulating CAM-induced calcium influx into neurons.

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