Ca^{2+} Influx and Neurite Growth in Response To Purified N-Cadherin and Laminin

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Abstract. The signaling mechanisms underlying neurite growth induced by cadherins and integrins are incompletely understood. In our experiments, we have examined these mechanisms using purified N-cadherin and laminin (LN). We find that unlike the neurite growth induced by fibroblastic cells expressing transfected N-cadherin (Doherty, P., and F. S. Walsh. 1992. *Curr. Opin. Neurobiol.* 2:595–601), growth induced by purified N-cadherin in chick ciliary ganglion (CG), sensory, or forebrain neurons is not sensitive to inhibition by pertussis toxin. Using fura-2 imaging of single cells, we show that soluble N-cadherin induces Ca^{2+} increases in CG neuron cell bodies, and, importantly, in growth cones. In contrast, N-cadherin can induce Ca^{2+} decreases in glial cells. N-cadherin-induced neuronal Ca^{2+} responses are sensitive to Ni^{2+}, but are relatively insensitive to diltiazem and ω-conotoxin. Similarly, neurite growth induced by purified N-cadherin is inhibited by Ni^{2+}, but is unaffected by diltiazem and conotoxin. Soluble LN also induced small Ca^{2+} responses in CG neurons. LN-induced neurite growth, like that induced by N-cadherin, is insensitive to diltiazem and conotoxin, but is highly sensitive to Ni^{2+} inhibition. K^+ depolarization experiments suggest that voltage-dependent Ca^{2+} influx pathways in CG neurons (cell bodies and growth cones) are largely blocked by the combination of diltiazem and Ni^{2+}. Our results demonstrate that cadherin signaling involves cell type-specific Ca^{2+} changes in responding cells, and in particular, that N-cadherin can cause Ca^{2+} increases in neuronal growth cones. Our findings are consistent with the current idea that distinct neuronal transduction pathways exist for cell adhesion molecules compared with integrins, but suggest that the involvement of Ca^{2+} signals in both of these pathways is more complex than previously appreciated.

In recent years there has been rapid progress in our understanding of the extrinsic molecules regulating axon growth in vivo and neurite growth in vitro. It is clear that axon growth can be regulated both by soluble "neurotrophic" factors, and by substrate-acting factors (for review see Bixby and Harris, 1991). There appear to be three major classes of substrate-acting neuronal growth factors: glycoproteins of the extracellular matrix (ECM),[1] Ca^{2+}-independent cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily, and Ca^{2+}-dependent CAMs of the cadherin family (Bixby and Harris, 1991; Reichardt et al., 1990). The known number of members of these families and our understanding of the complexity of their interactions have increased steadily.

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1. *Abbreviations used in this paper*: CAM, cell adhesion molecules; CG, ciliary ganglion; DRG, dorsal root ganglion; ECM, extracellular matrix, F, fluorescence intensity; LN, laminin; PDL, poly-D-lysine; ROI, region of interest.

In general, three in vitro approaches have proven successful in attempts to define the roles of axonal growth-promoting molecules. In the first approach, neurons are grown on physiologically relevant cellular substrates, and specific antibodies are added to block the function of proteins suspected to be involved in neurite growth (Tomaselli et al., 1986; Bixby et al., 1987; Chang et al., 1987). A second method is to coat the surface of a tissue culture dish with a purified growth-promoting molecule. This approach had been in widespread use for secreted proteins (such as those of the ECM), but was made considerably more versatile with the introduction of methods for preparing substrates from membrane proteins (Lagenauer and Lemmon, 1987). Finally, it is possible to transfect genes encoding growth-promoting molecules into heterologous cell types, such that the transfected cells express the molecules on their surfaces in a relatively isolated context (Matsunaga et al., 1988; Doherty et al., 1990).

Each of these approaches has advantages and disadvantages. The most physiologically relevant situation involves growth on cell types normally expressing the growth-promoting molecules and normally encountered by the neurons,

but the use of antibody blockade alone can cause some problems of interpretation. The use of purified proteins offers the advantage of studying known quantities of a single growth-promoting molecule in isolation from other proteins. However, purified membrane proteins may be altered by the isolation procedure and are presented in the absence of a biological membrane. The use of transfected cells is clearly advantageous in that proteins can be studied in a relatively simple cellular context without the use of antibody blockade, but the proteins are then expressed on an abnormal cell type, and the cells used for expression contribute to neurite growth in ways that are only partly understood. With these considerations, it is noteworthy, and perhaps even surprising, that the three methods have yielded almost completely complementary data, such that a growth-promoting molecule defined one way can be confirmed as such in another (e.g., Bixby et al., 1987; Matsunaga et al., 1988; Bixby and Zhang, 1990, for N-cadherin).

Most of the substrate-associated neuronal growth-promoting molecules were originally defined as adhesion molecules, and early models for the induction of neurite growth emphasized adhesive functions (Letourneau 1975; Hammarback et al., 1988). When a greater variety of physiologically relevant substrates was employed, however, it became clear that simple adhesion models could not be correct, and that the ability to promote neurite growth was a separate function of so-called "adhesion molecules" (Tomaselli et al., 1986; Gunderson, 1987; Hall et al., 1987; Doherty et al., 1990; Frei et al., 1992; Lemmon et al., 1992; Appel et al., 1993). For this reason, it was postulated that CAMs and ECM receptors interact with cytoskeletal elements and modulators of intracellular "second messengers" to transduce growth signals (Bixby and Reichardt, 1988), and this hypothesis was subsequently lent experimental support (Bixby, 1989; Schuch et al., 1989; Bixby and Jhabvala, 1990; Atashi et al., 1992; von Bohlen und Halbach et al., 1992).

In a landmark series of papers, F. Walsh, P. Doherty, and their colleagues have recently provided strong evidence for the involvement of specific intracellular signals in neurite growth induced by CAMs, including L1, NCAM, and N-cadherin (Doherty et al., 1991*a*,*b*, 1992; Saffel et al., 1992; Williams et al., 1992, 1994*a*). These authors have formulated a "unifying" hypothesis that the sole mechanism through which N-cadherin and other CAMs induce neurite growth is through a G protein-dependent activation of voltage-dependent (N- and L-type) calcium channels, while integrin-dependent neurite growth involves neither PTX-sensitive G proteins nor intracellular Ca^{2+} changes. This model has recently been extended to include the activation of a receptor-type tyrosine kinase (Williams et al., 1994*a*).

The model described above requires that CAMs induce Ca^{2+} signals in responding neurons. Although NCAM and L1 have been shown to induce Ca^{2+} increases in some neurons (von Bohlen und Halbach et al., 1992), this has not been demonstrated for N-cadherin. In addition, responses in single cells or in growth cones, the presumed locus of signal transduction, have not been measured for any CAM. It would be important to demonstrate CAM-induced Ca^{2+} signals in growth cones, since a large body of evidence suggests that Ca^{2+} levels in growth cones play a significant role in neurite growth (Kater and Mills, 1991). We have therefore examined changes in $[Ca^{2+}]_i$ in response to N-cadherin in single chick ciliary ganglion (CG) neurons. Because cadherins and CAMs interact with the cytoskeleton in ways that are not obviously dependent on Ca^{2+} influx but could influence axon growth, we also examined the effects of PTX and Ca^{2+} channel antagonists on neurite growth using purified N-cadherin. Finally, because activated integrins can cause changes in $[Ca^{2+}]_i$ in a variety of cell types (Leavesley et al., 1993; Schwartz, 1993; Shankar et al., 1993), Ca^{2+} and neurite growth measurements were also carried out with laminin (LN). Our results suggest that PTX-sensitive G proteins are not required for N-cadherin–induced neurite growth, but that signals involving changes in Ca^{2+} are likely to be important not only for growth induced by cadherins, but also for growth induced by integrins.

Materials and Methods

Materials

Fertilized eggs were purchased from SPAFAS (Preston, CT), and were incubated at 38°C until use. ^{32}P-NAD (30 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). PTX was purchased from List Biological Laboratories (Campbell, CA), and in one case was a gift from Dr. I. Litosch (University of Miami, FL). Cholera toxin and GTP-γ-S were purchased from Sigma Chem. Co. (St. Louis, MO). Diltiazem and ω-conotoxin GVIA were gifts from Dr. D. Adams (University of Miami, FL). Mouse NGF was a gift from Dr. E. Johnson (Washington University, St. Louis, MO). All other chemicals were reagent grade. Tryptic fragments of N-cadherin from chick brain and the soluble form of N-cadherin from chick retina (NCAD90) were purified as described (Shirayoshi et al., 1986; Paradies and Grunwald, 1993).

Methods

Cell Culture. Cell culture substrates (nitrocellulose coated with LN, N-cadherin, rat tail collagen, poly-D-lysine, or serum proteins) were prepared as described previously (Bixby and Jhabvala, 1990, 1992). Embryonic day 8 (E8) CG neurons and E7 forebrain neurons were cultured as described (Bixby and Jhabvala, 1992). E9 dorsal root ganglion neurons were cultured as in Holz et al. (1989), except that the NGF was used at a concentration of 30 ng/ml. For the GTP-γ-S experiment, GTP-γ-S at a nominal concentration of 100 μM was loaded into cells by the trituration method of Borasio et al. (1989). That the procedure was capable of permeabilizing neurons was confirmed by loading rhodamine-labeled antibodies into the cells with the same technique.

Analysis of Neurite Growth. Cultures were fixed and washed as described (Bixby and Jhabvala, 1992), and the percentage of cells with neurites (greater than two cell diameters) was determined for 150–300 neurons in each condition. Analysis of neurite lengths was performed with the JAVA video analysis system. The length of the longest neurite was determined for 20–45 neurons in each condition, or for 10–20 clusters of neurons in the case of the CG neurons preincubated for 24 h with PTX (single cells were not observed after 24 h in suspension), and the mean ± SEM was determined for each condition. Neurons without neurites were excluded from the length measurements.

ADP-ribosylation of G Proteins. Forebrains from E7 chicks were dissected and dissociated into single cells as in the culture procedure. Cells were spun down, resuspended in 50 mM Tris, 5 mM $MgCl_2$, pH 7.5, and homogenized with a ground glass homogenizer. The homogenate was centrifuged 5 min at 800 *g*, and the supernatant (S/N) from the first spin was centrifuged at 15,000 *g* for 25 min. The S/N was removed and the pellet (crude membranes) was frozen at −80°C for later use in assays. Assays were performed with 20 μg of crude membranes in a volume of 25 μl, with 20 mM thymidine, 0.5 mM GTP, 0.5 mM ATP, 2 mM $MgCl_2$, 6 mM DTT, 30 μM ^{32}P-NAD, and 50 mM $NaPO_4$, pH 7.5, to which 1 μg/ml PTX was added (or omitted for controls). The tubes were incubated for 30 min at 37°C, and the reaction was stopped by addition of TCA to 10%. After precipitation for 5 min on ice, the proteins were spun down, washed 2× with 500 μl cold acetone, and resuspended in SDS sample buffer for loading onto polyacrylamide gels.

Incubation Protocol for Fura-2-loading. 25-mm glass coverslips were rinsed several times with HBSS buffered with 10 mM Hepes, pH 7.4, and then loaded at room temperature with a sonicated combination of 5 μM fura-2/AM (Molecular Probes, Inc., Eugene, OR), 2.5% FCS, in 2.0 ml of HBSS for 45–60 min. After several washes with HBSS, the coverslips were mounted into an open Dvorak-Stotler chamber and placed on the stage of a Nikon Diaphot microscope.

Ratio Imaging. Epifluorescence illumination with a 100 W mercury lamp filtered through 10-nm-wide band-pass filters centered on 340 or 380 nm (Chroma Technology Corp., Brattleboro, VT) excited the Ca^{2+} indicator dye. The fluorescence image of the emitted light (600 nm > x > 510 nm) was visualized with a 40×, 1.3 NA oil immersion objective (CF Fluor DL series, Nikon, Inc.), directed out the side port of the microscope to a KS1381 micro-channel plate image intensifier (Opelco Inc., Washington, D.C.), coupled to a Hamamatsu 2400 CCD camera. The camera output was digitized and analyzed with an IC300 Imaging Workstation (Inovision Corp., Durham, NC). Individual cell bodies, processes, or growth cones were identified as regions of interest (ROIs) within the digitized image. 8–32 video frames were averaged at each of the two excitation wavelengths and the ratio within each ROI was then computed on a pixel by pixel basis, excluding pixels that failed to reach a threshold value. Ratios were computed every 10 s, or more frequently as needed. Average ratio values for each ROI (or cell) were written to disk for later analysis and graphing. Results were obtained from neurons 4–20 h in culture; no consistent differences in Ca^{2+} responses at different culture times were apparent.

Calibration and Computation of $[Ca^{2+}]$. Fluorescence intensity (F) at each wavelength (340 and 380nm) was measured with aliquots of calcium-free and calcium-saturated solutions containing 10 μM fura-2 (K^+ salt, Molecular Probes), 120 mM KCl, 10 mM Hepes, pH 7.4. These values were used to compute three terms: R_{min}, R_{max}, and β. The average value of the calibration constants for the experiments reported here are: R_{min} = 0.17, R_{max} = 3.56, β = 4.8. The ratio data can then be transformed into Ca^{2+}-concentrations using the equation of Grynkiewicz et al. (1985) and assuming a K_d for Ca^{2+}/fura-2 of 250 nM. Given remaining uncertainties about the K_d of the dye in the cytoplasm of these cells, such transformed data are only an approximation and may be in error by a factor of approximately two. We therefore chose to plot the ratio values in our figures and not the transformed values. Approximate values of $[Ca^{2+}]_i$ are given in the figure legends.

Pressure Application of NCAD90. Soluble fragments of N-cadherin from brain, or NCAD90 from retina (10 μg/ml in HBSS) were loaded into pulled glass microelectrodes (tip diameter ~0.5 μm), and pressure-applied to all the cells in a microscopic field. Application was normally for 1 min at 5–15 psi.

Results

Role of G Proteins in LN- or N-Cadherin-induced Growth

Because of our interest in the transduction of substrate-associated axonal growth signals, we performed preliminary experiments to test the possible role of heterotrimeric G proteins in this process. We used PTX preincubation to inactivate sensitive G proteins, and tested growth of cultured neurons on LN substrates. PTX had no consistent effect on the percentages of neurons with neurites in cultures of CG neurons or neurons from the E7 forebrain (Table I), and there was no obvious effect of PTX on the lengths of individual neurites (several hundred cells examined). The lack of effect of PTX on LN-stimulated neurite growth is consistent with conclusions reached by Doherty et al. (1991*a*). In addition, when we attempted to activate G proteins artificially, we found no clear effect on the growth of CG neurons (on either LN or poly-D-lysine [PDL] substrates). Experiments included cholera toxin (80–400 ng/ml; n = 2), aluminum fluoride (5 mM fluoride, 50 μM aluminum; n = 1), and GTP-γ-S (100 μM; n = 1). These results suggest that activation of G proteins is not sufficient to promote neurite growth from these neurons, though further experiments would be necessary to substantiate this conclusion.

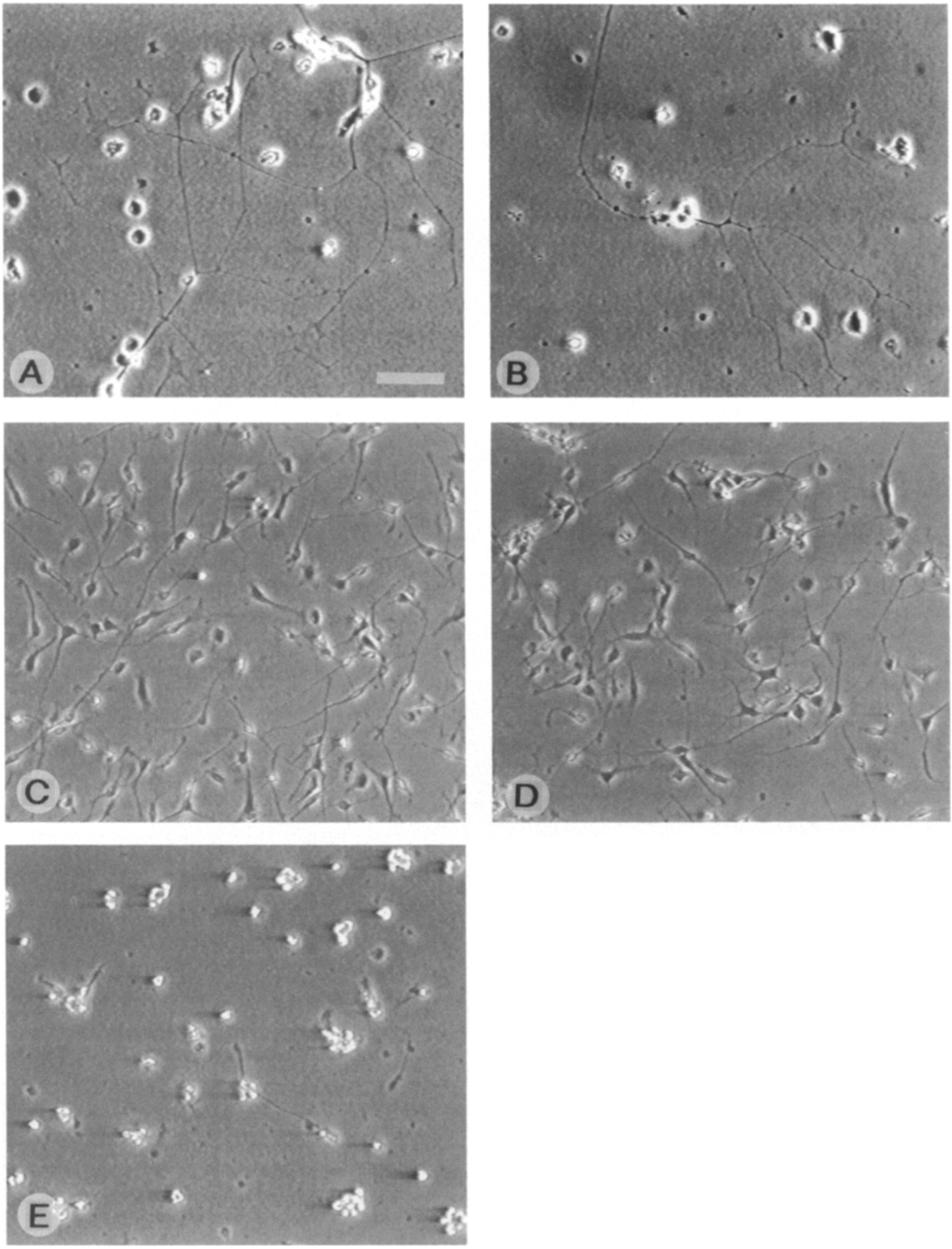
Table I. Effect of PTX on Laminin-induced Neurite Growth

Neurons	H in vitro	Condition	% with neurites
CG	19	Control	79%
		PTX	82%
		Control	82%
		PTX	71%
	4	Control	46%
		PTX	38%
FB	16	Control	94%
		PTX	94%

CG neurons or E7 forebrain (*FB*) neurons were grown for the indicated times on nitrocellulose treated with 20 μg/ml laminin, after pretreatment for 3–4 h with 0.5–1 μg/ml PTX. The percentage of neurons with neurites longer than two cell diameters is given. Although not quantified, the length of neurites also did not appear affected by PTX treatment.

Prompted by the reports from Doherty, Walsh, and colleagues demonstrating PTX-sensitive neurite growth of some neurons on 3T3 cells expressing N-cadherin, we were led to reexamine the effect of PTX using purified N-cadherin as a substrate. The results of our studies can be summarized briefly. For three different neuronal populations, using a variety of experimental protocols, we found no effect of PTX on neurite growth induced by purified N-cadherin. This was true whether we examined the percentage of neurons with neurites in a culture or the length of the longest neurite from a neuron or cluster of neurons (Fig. 1; Table II). The experimental protocols will be described briefly. In a first series of experiments, the CG neurons were preincubated for 4 h with PTX (1 μg/ml), then plated for 4–5 hours (n = 2) or 18–23 h (n = 2) in the continued presence of PTX before fixation. In another set of cultures the CG neurons were preincubated in suspension with PTX (1 μg/ml) for 24 h, then plated in the presence of PTX for 4–5 h (n = 2). The forebrain neurons were preincubated for 3 h in PTX, then grown for 16–19 h in the presence of PTX before fixation (n = 3). The dorsal root ganglion (DRG) neurons were directly plated in PTX-containing medium and grown for 18–23 h before fixation. The DRG neurons were grown in sub-optimal concentrations of nerve growth factor to prevent a high "background" growth of neurites in the absence of N-cadherin. "Control" substrates included serum proteins and PDL for the CG and forebrain neurons, and collagen IV and PDL for the DRG neurons. Although neurons attached and sometimes grew neurites on these substrates, the growth induced by N-cadherin was always much more robust (e.g., Fig. 1, Table II; see also Bixby and Zhang, 1990; Bixby and Jhabvala, 1990, 1992).

To ensure that the PTX was active, we took two kinds of precautions. First, the experiments were done with six different lots of PTX, none of which was stored for longer than two months before use, and most of which were used within three weeks. Second, we tested directly the ability of our PTX preparations to ADP-ribosylate G proteins, after their use in neurite outgrowth experiments. A crude membrane fraction was prepared from E7 forebrain cells (the source of the forebrain neurons used for culture). Membranes were incubated with and without PTX, and ^{32}P-labeled NAD was used to visualize ADP-ribosylated proteins on SDS-polyacrylamide gels. A diffuse band in the molecular mass range of 41–43 kD was labeled by this procedure when PTX was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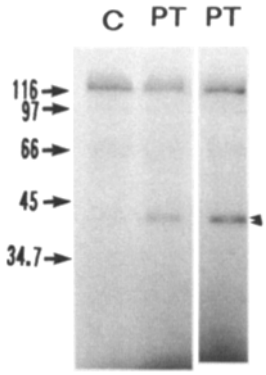

Figure 2. Autoradiographs of ADP-ribosylated G proteins. Crude membranes were ADP-ribosylated with 1 μg/ml PTX (*PT*) as described in the Materials and Methods, then the proteins were separated on SDS-PAGE and autoradiographed. A diffuse band at 41 kD is visible in the PTX-treated lanes, but not the control lane. The double arrowheads in the far lane point to the doublet at this apparent MW that was sometimes resolved. The first two lanes are from a single experiment, and the third lane is from a separate experiment. Arrows at left indicate M_r in kD.

used at 1 μg/ml (Fig. 2). In some gels this band could be resolved as a doublet (Fig. 2, *right lane*). These results demonstrate both that our PTX preparations were active and that E7 forebrain neurons accumulate at least two PTX-sensitive G proteins. In addition, previous reports have demonstrated that CG neurons and sensory neurons are affected by incubation with PTX (Holz et al., 1989; Dryer et al., 1991; Meriney et al., 1994).

Diltiazem and ω-conotoxin Do Not Affect CG Neurite Growth on Purified LN- or N-cadherin

When PC12 cells are grown on N-cadherin–transfected 3T3 cells, the neurite outgrowth attributable to the transfected N-cadherin is inhibited by a combination of diltiazem and ω-conotoxin, which are thought to block, respectively, L- and N-type calcium channels (Doherty et al., 1991*a*). The response of cerebellar neurons to L1 and hippocampal neurons to NCAM in transfected cells is also inhibited by these Ca^{2+} channel blockers (Doherty et al., 1992; Williams et al., 1992). Interestingly, while the rise in $[Ca^{2+}]_i$ induced by L1 in PC12 cells is blocked by diltiazem, the rise in $[Ca^{2+}]_i$ in cerebellar and sensory neurons is unaffected by the same treatment (Schuch et al., 1989; von Bohlen und Halbach et al., 1992). The results from transfected cells have been interpreted to imply that neurite outgrowth induced by CAMs is mediated solely through the G-protein–dependent opening of Ca^{2+} channels (Williams et al., 1992). To test this idea for the case of purified N-cadherin, we examined the effect of the combination of diltiazem and ω-conotoxin on neurite growth from CG neurons. The combination of 20 μM diltiazem and 0.4 μM ω-conotoxin had no apparent effect on the percentage of cells with neurites, or on the length of neurites elaborated by CG neurons in 18–23 h (Table III; $n = 2$; a third experiment with 40 μM diltiazem and 0.2 μM ω-conotoxin gave the same result). These results indicate that influx of Ca^{2+} through channels blocked by these drugs is not required for the neurite growth induced by purified N-cadherin. In agreement with previous suggestions, we also found that neurite growth on LN was unaffected by diltiazem and conotoxin (Table III). We did not measure the effect of long-term application of these drugs on intracellular Ca^{2+} levels. The diltiazem used in these experiments was pharmacologically active, since it could inhibit K^+-stimulated Ca^{2+} influx into CG neurons (see below), and could completely block beating of cultured heart cells (see Lampidis et al., 1992) at concentrations as low as 2 μM (data not shown).

Table II. Effect of PTX on N-Cadherin-induced Neurite Growth

Neurons	H in vitro	Condition	% with neurites	Avg. length
				μm
CG	3–4	Control	38%	ND
		PTX	40%	ND
		Control	75%	ND
		PTX	83%	ND
	4–5	Control	ND	92 ± 6
		PTX	ND	104 ± 5
		Control	ND	127 ± 16
		PTX	ND	124 ± 3
	18–23	Control	ND	293 ± 17
		PTX	ND	316 ± 16
		Control	ND	306 ± 27
		PTX	ND	316 ± 30
FB	16	Control	95%	61 ± 5
		PTX	95%	60 ± 4
		Control	94%	ND
		PTX	95%	ND
DRG	18–23	Control	26%	178 ± 21
		PTX	24%	211 ± 15
		Control	28%	183 ± 16
		PTX	25%	182 ± 18
		Control	ND	137 ± 13
		PTX	ND	165 ± 12

Ciliary ganglion (*CG*), E7 forebrain (*FB*), or E8 sensory (*FRG*) neurons were incubated in control conditions or with 1 μg/ml PTX, on nitrocellulose substrates treated with 10 μg/ml N-cadherin for the indicated times. The average length of the longest neurite from a cell or cell cluster (cells without neurites are not considered) is given in μm (mean ± SEM), and the percentage of neurons with neurites longer than two cell diameters is given. Each line represents a single experiment. ND, not determined. In all but the DRG experiments, neurons were preincubated with PTX for 3–4 h (FB; CG 18–23, 4–5) or 24 h (CG 4–5) before plating.

Pharmacology of K^+-stimulated Ca^{2+} Influx

Because our results with Ca^{2+} channel blockers differ from those of Doherty and colleagues using other neurons, we wished to characterize pharmacologically the types of Ca^{2+} influx pathways present in the CG neurons. In these experiments, we induced Ca^{2+} influx by depolarizing the neurons with 80 mM K^+, and monitored changes in intracellular Ca^{2+} with fura-2 ratio imaging. Depolarization led to large increases in Ca^{2+} in the cell body, with a characteristic transient followed by a plateau (Fig. 3 *a*). 1 mM $NiCl_2$, which should block both L- and T-type channels (Narahashi and Herman, 1992), reversibly reduced both the transient and the sustained component of the K^+ response. 20 μM

Figure 1. Phase contrast video micrographs of E9 sensory neurons (*A* and *B*) and E7 forebrain neurons (*C–E*) incubated in the presence (*B* and *D*) and absence (*A*, *C*, and *E*) of PTX (1 μg/ml). Neurons were grown on substrates of 10 μg/ml N-cadherin (*A–D*) or serum proteins (*E*). Neurite growth is unaffected by PTX for either neuronal type. The neurite growth is induced by N-cadherin and not simply by adhesion, as is shown by *E*, in which growth is meager despite neuronal adhesion. Bar, 50 μm.

Table III. Effect of Diltiazem and ω-Conotoxin on Neurite Growth

Substrate	H in vitro	Condition	% with neurites	Avg. Length
				μm
Ncad	6	Control	68%	56 ± 4
		Diltiazem/conotoxin	69%	61 ± 5
	18	Control	ND	293 ± 17
		Diltiazem/conotoxin	ND	314 ± 36
	23	Control	ND	306 ± 27
		Diltiazem/conotoxin	ND	316 ± 26
Laminin	6	Control	81%	98 ± 5
		Diltiazem/conotoxin	82%	102 ± 7

CG neurons were grown for the indicated times on either 10 μg/ml N-cadherin (*Ncad*) or 20 μg/ml laminin, in the presence or absence of 20 μg/ml diltiazem plus 400 nM ω-conotoxin. The percentage of neurons with neurites longer than two cell diameters, and the length of the longest neurite (for those neurons with neurites) were quantified. Lengths are expressed as mean ± SEM. In the long-term cultures, the % with neurites could not be quantified, because of extensive clumping of cells. No differences between control and treated were apparent by visual inspection.

diltiazem, an inhibitor of L-type Ca^{2+} channels, also blocked a substantial portion of the K^+-induced Ca^{2+} response, but was somewhat less effective than Ni^{2+} at blocking the transient component. The combination of diltiazem and Ni^{2+} reversibly inhibited most of the K^+-evoked Ca^{2+} influx (Fig. 3 *a*; $n = 6$). These results suggest that the Ca^{2+} influx pathways in CG neuronal cell bodies are sensitive to Ni^{2+} and to diltiazem, and that the contribution of voltage-dependent Ca^{2+} channels insensitive to both of these drugs is minor. However, the important Ca^{2+} changes during neurite growth may conceivably be confined to the growth cones and distal neurites. Therefore, we also examined Ca^{2+} influx pathways in two growth cones. The shape of the Ca^{2+} response to depolarization could be somewhat different in the growth cones from that seen in the cell bodies, suggesting the possibility that the populations of Ca^{2+} channels differ in the two regions. Nevertheless, the majority of the Ca^{2+} increase could be blocked by the combination of diltiazem and Ni^{2+} (Fig. 3 *b*). We conclude that, for CG neurons, most of the voltage-dependent Ca^{2+} influx can be blocked by the combination of diltiazem and Ni^{2+}, in both growth cones and cell bodies.

N-Cadherin-induced Changes in $[Ca^{2+}]_i$

In PC12 cells and at least some neuronal populations, L1 and NCAM have been shown to cause $[Ca^{2+}]_i$ increases, when measured from a population of cells (Schuch et al., 1989; von Bohlen and Halbach et al., 1992). To examine whether N-cadherin can cause Ca^{2+} changes in single CG neurons, we prepared soluble fragments of N-cadherin and pressure-applied these to neurons growing on LN-coated coverslips. In initial experiments, we used fragments prepared by trypsinization of brain membranes and isolated by affinity chromatography (Shirayoshi et al., 1986; data not shown). For most of our experiments, however, we used a naturally occurring soluble fragment of N-cadherin (NCAD90) isolated from chick retina (Paradies and Grunwald, 1993). NCAD90 has previously been shown to retain adhesive and neurite-promoting functions (Paradies and Grunwald, 1993). No qualitative differences in the effects of the two preparations were seen; we therefore used NCAD90 because of its higher purity.

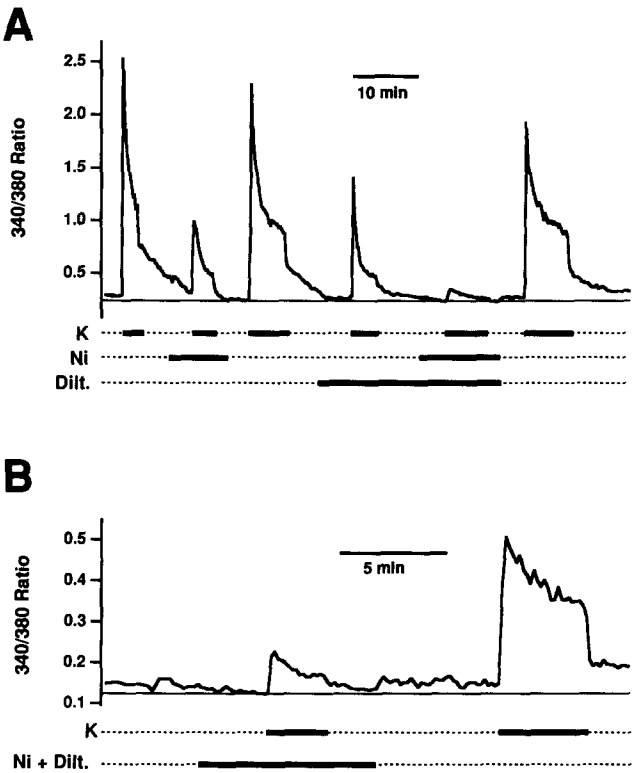

Figure 3. K^+-stimulated Ca^{2+} influx in CG neurons. Plots of 340/380 ratios for a cell body (*A*) and a growth cone (*B*) exposed to 80 mM K^+ (*K*) in the presence and absence of the indicated drugs. Diltiazem (*Dilt*) was used at 20 μM, and $NiCl_2$ (Ni) was used at 1 mM. Solid bars indicate the presence of various additives. (*A*) Both Ni^{2+} and diltiazem reversibly reduce the Ca^{2+} response in a cell body, and the combination virtually abolishes the response. Note that the initial peak is somewhat more sensitive to Ni^{2+} than to diltiazem. A final application of the 80 mM K^+ solution shows that the cell body still gave a robust Ca^{2+} response to depolarization. (*B*) In a growth cone, the response to elevated K^+ is virtually abolished by the combination of $NiCl_2$ and diltiazem, but is readily apparent after washout of the drugs. Approximate values of $[Ca^{2+}]_i$ are: 0.2 = 30 nM; 0.5 = 150 nM; 2.0 = 1.5 μM.

Pressure application of NCAD90 (10 μg/ml; 60 s) to CG neurons led to increases in Ca^{2+} in both cell bodies and growth cones (Figs. 4 and 5). A summary of our results is presented in Table IV. Clear Ca^{2+} responses were seen in most of the cell bodies examined (58%; $n = 45$). These responses were variable with respect to latency, magnitude, and overall shape (Fig. 4 *B*). The average latency after NCAD90 application was 7.8 ± 1.4 min. Although we do not know the origin of this delay, it likely reflects the time required for a number of transduction steps following binding of NCAD90 to its cell surface receptor (see Schuch et al., 1989; Doherty and Walsh, 1992, for suggested models). In any case, this delay seems to rule out a direct mechanism such as the opening of Ca^{2+} channels by the NCAD90 receptor. Most of the cell body responses consisted of long-lasting plateaus (Fig. 4 *B*, *top*), while others were transient, "spike-like" elevations (Fig. 4 *B*, *bottom*). In the vast majority (20/21) of cases, internal Ca^{2+} remained elevated above

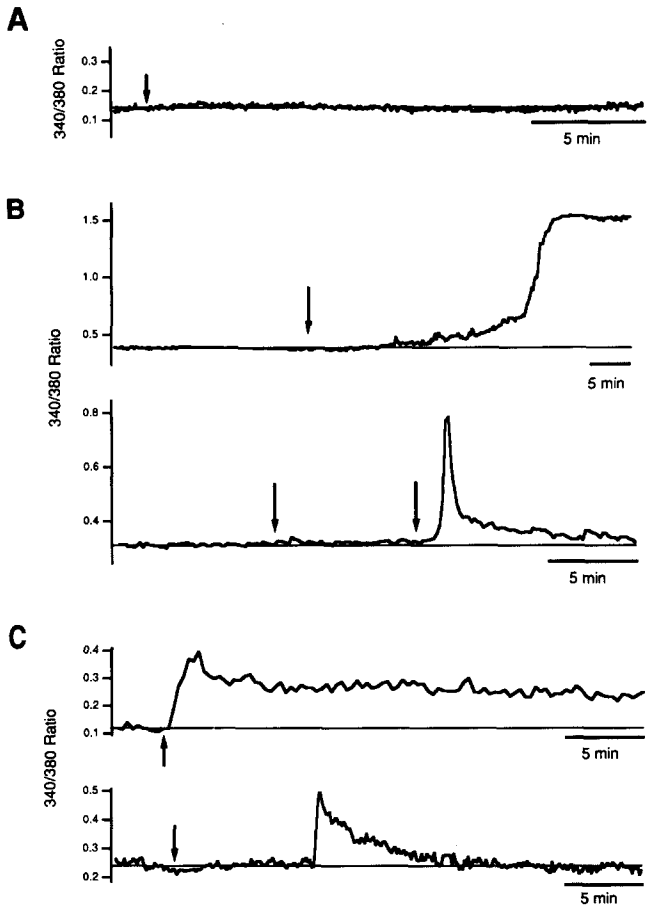

Figure 4. Ca^{2+} responses to NCAD90 in CG neurons. NCAD90 (10 μg/ml) was pressure-applied for 1 min at the times indicated by the arrows. All five traces are from separate experiments; traces in A and B are from cell bodies, traces in *C* are from growth cones. (*A*) Pressure application of anti-NCAM IgG (500 μg/ml) does not lead to a Ca^{2+} change for at least 25 min. (*B*) Neuronal cell bodies responded both with long, plateau-like elevations in Ca^{2+} (*top*), and with more transient elevations (*bottom*). (*C*). Growth cones also responded with both sustained (*top*) and transient (*bottom*) elevations in Ca^{2+}; latencies in growth cones tended to be shorter (*upper trace*). Approximate values of $[Ca^{2+}]_i$ are: 0.2 = 30 nM; 0.5 = 150 nM; 1.5 = 800 nM.

baseline for at least 15 min after the initial rise. The average peak value of the Ca^{2+} response was 947 nM, about five times the baseline level in this group of cells.

Two types of result indicate the specificity of these responses. First, pressure application of anti-NCAM IgG (Bixby et al., 1987), monoclonal anti-L1 (8D9, Lemmon and McLoon, 1986), or monoclonal anti-N-cadherin (NCD-2; Shirayoshi et al., 1986) did not lead to similar Ca^{2+} increases in CG neurons (see Fig. 4; 1/24 cells showed a small increase). Second, baseline Ca^{2+} levels were very stable with ongoing UV excitation in the absence of applied NCAD90; in 10 cells we examined continuously over 75 min, baseline levels went from an average of 119 ± 5 nM during the first 5 min of the recording to 120 ± 5 nM during the last 5 min.

Comparison of the NCAD90 responses to those seen with other CAMs is difficult, since these have been used only on populations of neurons (von Bohlen und Halbach et al., 1992). In these previous experiments, however, similar concentrations of L1 led to responses that began after ~5 min, and reached a peak at 20 min. The magnitude of the L1 response was ~3.6 times the baseline value (von Bohlen und Halbach et al., 1992). The similarity of the two response types is consistent with the notion that these two classes of CAMs induce Ca^{2+} signals through similar mechanisms as has been suggested (Doherty and Walsh, 1992).

NCAD90 application also led to Ca^{2+} responses in growth cones (Figs. 4 c and 5; Table IV). We did not investigate growth cone responses systematically, but have seen clear responses in 12 growth cones in four different preparations. Consistent with previous observations in invertebrates (Connor et al., 1990), baseline values of Ca^{2+} were lower in the growth cones than in cell bodies. The absolute magnitudes of the Ca^{2+} changes were also lower in the growth cones, but the average increase compared to the baseline value was quite similar (Table IV). Responses occurred in the growth cones with a latency of only 3.5 ± 1.2 min, significantly faster than in the cell bodies. As was seen with cell bodies, growth cones exhibited both sustained (Fig. 4 *c*, *top*) and transient (Fig. 4 *c*, *bottom*) responses, but were more likely than cell bodies to give transient responses. To our knowledge, this is the first evidence that CAMs can cause Ca^{2+} responses in neuronal growth cones.

Although growth cones and cell bodies both showed Ca^{2+} increases in response to NCAD90, this was not uniform throughout the nerve cell. We have examined 16 neurites (not immediately adjacent to growth cones or cell bodies) and have never seen a Ca^{2+} response (e.g., Fig. 5). This could reflect either a lack of binding sites for N-cadherin or a lack of the appropriate signaling machinery in neurites.

In some cases we have seen Ca^{2+} responses to NCAD90 in non-neuronal (presumed glial) cells. Interestingly, several of these responses consisted of Ca^{2+} decreases upon NCAD90 application (Fig. 6). We have not concentrated on non-neuronal cell responses, and therefore do not know the relative frequency of Ca^{2+} increases vs. Ca^{2+} decreases. However, we have never seen this kind of response in a neuron. These results therefore suggest that the kind of Ca^{2+} response elicited by N-cadherin can be cell type specific. Glial responses to NCAD90 might be expected since these cells are known to express N-cadherin (Hatta et al, 1987; Bixby et al., 1988).

We also examined the response of CG neuronal cell bodies to pressure-applied LN (20 μg/ml; 60-s application). Of 23 cell bodies examined, 11 (48%) exhibited Ca^{2+} elevations within the first 5 min after pressure application which could be transient or sustained (Fig. 7). These aspects are similar to the NCAD90 responses. However, the magnitudes of the Ca^{2+} increases were very small. From an average baseline value of 53 ± 6 nM, peak levels of Ca^{2+} during the response were only 69 ± 6 nM. Although small (16 nM), these elevations were significant ($p < 0.05$). It is possible that the small average size of the LN responses reflects an inadequate presentation of this ECM protein (in solution vs. as an insoluble matrix component).

Because the ability of N-cadherin to promote neurite growth has been reported to depend on the activation of N- and L-type voltage-dependent Ca^{2+} channels, we examined the sensitivity of the Ca^{2+} response to NCAD90 using various Ca^{2+} channel blockers. CG neurons were incubated

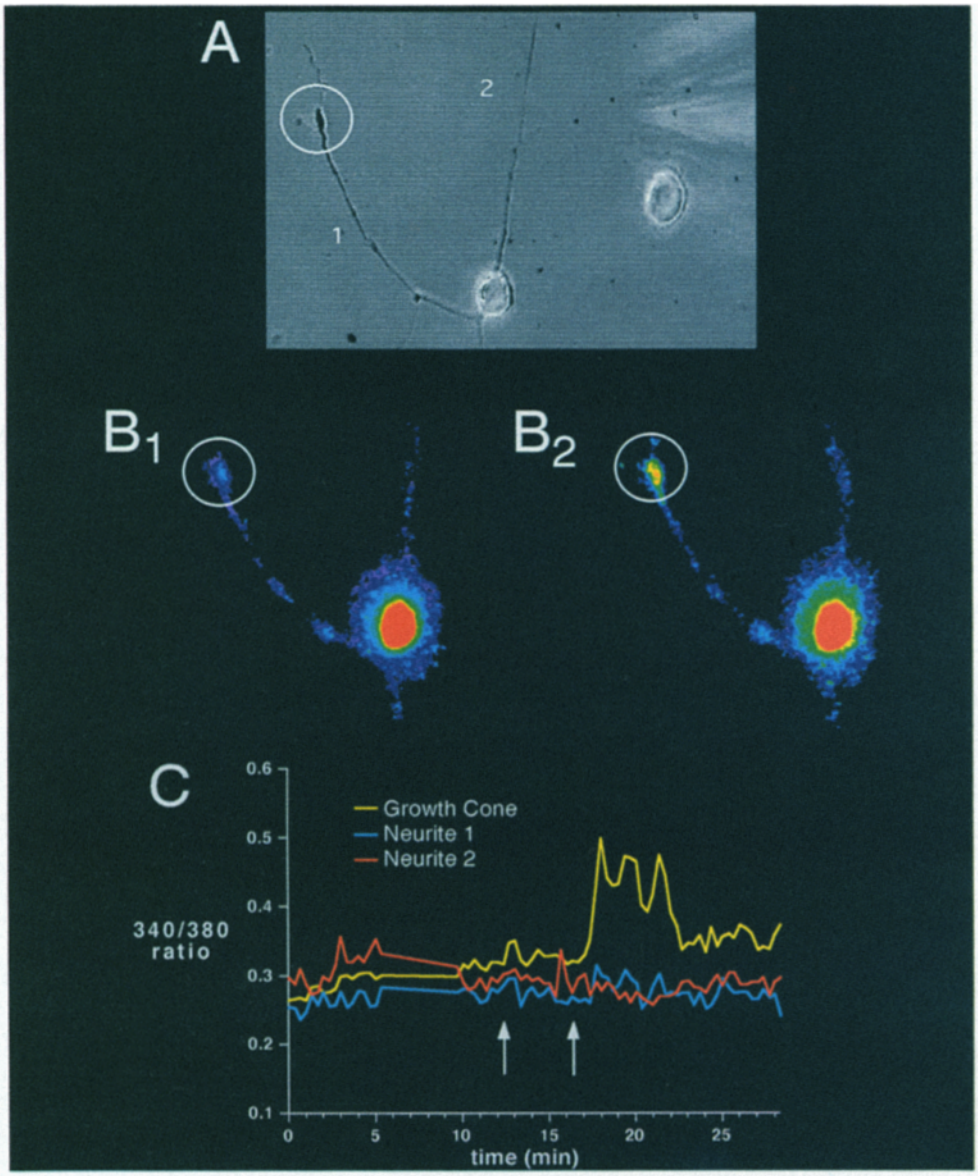

Figure 5. A Ca^{2+} response is induced by NCAD90 in a growth cone, but not in adjacent neurites. (*A*) Phase-contrast image of a CG neuron cultured on LN; the pressure pipette containing NCAD90 can be seen at right. The growth cone is circled, and two neurites are numbered (1 and 2). (*B*) Pseudocolor images of fura-2-fluorescence ratios before (B1) and after (B2) application of NCAD90 through the pipette. The Ca^{2+} increase in the growth cone is clear, and the tips of two filopodia appear in B2. (*C*) 340/380 ratio data plotted against time for the growth cone (yellow), neurite 1 (green) and neurite 2 (red). NCAD90 was applied for 1 min at the times indicated by the arrows. There is a response in the growth cone, but not in the two neurites. It should be noted that there was also a Ca^{2+} increase in the cell body, but this could not be reliably measured because the high gain necessary to see the growth cone signal saturated the cell body image. White circles in *A* and *B* are 25 μm in diameter. Approximate values of $[Ca^{2+}]_i$ are: 0.2 = 30 nM; 0.5 = 150 nM.

Table IV. Summary of Calcium Responses to NCAD90

Population	% Responding	Baseline value	Peak value	Response latency	Ca^{2+} increase	Normalized peak	% w/ Plateau	% w/ Maintained elevation	N
		nM	*nM*	*min*	*nM*				
Growth cones	NA	21 ± 7	125 ± 54	3.5 ± 1.2	108 ± 52	5.2	50%	58%	12
Cell bodies	58% (45)	202 ± 34	947 ± 150	7.8 ± 1.4	736 ± 133	3.6	71%	94%	21
Cell bodies (dilt./cono.)	43% (32)	170 ± 26	1108 ± 189	7.0 ± 1.6	953 ± 203	5.6	70%	93%	13
Cb neurons (L1)*	NA	70 ± 10	250 ± 30	5	180	2.6	NA	NA	NA

* Data from von Bohlen und Halbach et al., 1992.

Average values (mean ± SEM) are given separately for growth cones, cell bodies, and cell bodies in the presence of diltiazem/ω-conotoxin (*dilt./cono.*); data for responses of cerebellar neuronal populations to L1 or anti-L1 have been extracted from von Bohlen und Halbach et al. (1992) for comparison. The "% responding" is based on all cells tested; the N's are given in parentheses. The N's for the other eight measurements are given separately because some responses were truncated artificially and could not be analyzed in full. The "response latency" was defined as the time between the first application of NCAD90 and the beginning of the calcium increase. The "calcium increase" was defined as the peak value minus the baseline value. The "normalized peak" measurement was the calcium increase divided by the baseline value, and allows a comparison of the magnitude of the response when baseline values are different. A "plateau" was defined as an elevation that reached a peak and did not decline for at least 10 min. "Maintained elevation" was defined as a response that reached a maintained level above baseline following the peak resposne. The baselines, peaks, and response latencies were significantly different ($p < 0.02$) for growth cones vs. cell bodies; none of our three normalized peaks were different from each other. The baselines, peak values, and response latencies for cell bodies were not different from cell bodies in the presence of diltiazem/ω-conotoxin. Note that the response latencies for the cell bodies were similar to those reported for a population of cerebellar neurons responding to L1 and L1 antibodies (von Bohlen und Halbach et al., 1992).

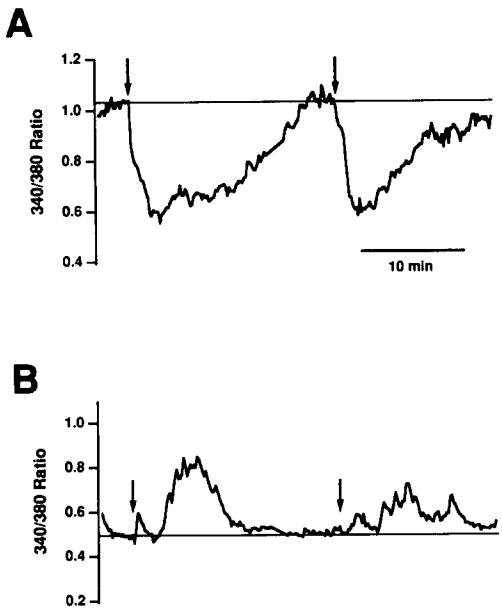

Figure 6. Ca^{2+} responses induced in a non-neuronal cell (*A*) and a neuron (*B*) in the same field. NCAD90 was applied to the field at the times indicated by the arrows. In the non-neuronal cell, a decrease in Ca^{2+} is evident that lasts for 15 min and can be induced again by a second application of NCAD90. In the neuron, the same application induces a Ca^{2+} increase; the response to the second application is less robust. Approximate values of $[Ca^{2+}]_i$ are: 0.5 = 150 nM; 1.0 = 400 nM.

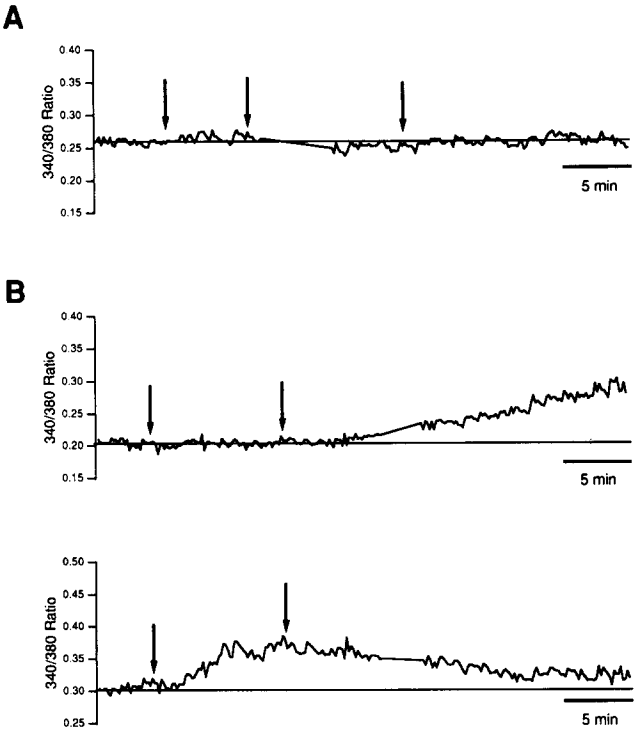

Figure 7. Ca^{2+} responses to pressure application of soluble laminin. LN (20 μg/ml) was pressure-applied for 1 min at the times indicated by the arrows. (*A*) Three applications of LN failed to elicit any Ca^{2+} response in this CG neuron. (*B*) Ca^{2+} responses from two other CG neurons in response to repeated application of LN. The upper record shows a sustained response while the lower one shows a transient response that started with a relatively short latency. Approximate values of $[Ca^{2+}]_i$ are: 0.2 = 30 nM; 0.3 = 70 nM.

in 20 μM diltiazem and 0.2 μM ω-conotoxin, and exposed to pressure application of 10 μg/ml NCAD90. NCAD90 led to Ca^{2+} increases in neurons that were similar to those seen in control conditions (Fig. 8; Table IV). Of 32 cells tested, Ca^{2+} responses were seen in 13 (41%), with an average latency after onset of application of 7 ± 1.6 minutes. Neither of these is significantly different from the corresponding values obtained in control saline. The magnitudes and the time courses of the Ca^{2+} increases were also similar to controls (Table IV). We conclude that the Ca^{2+} responses to NCAD90 in CG neurons are not strongly affected by these blockers of N- and L-type Ca^{2+} channels. This result is consistent with our observation that these agents do not inhibit N-cadherin–induced neurite growth from the same neurons. One must bear in mind, however, that our Ca^{2+} measurements were made with soluble N-cadherin and were short-term, while neurite outgrowth experiments were long-term and used insoluble N-cadherin.

A large fraction of the depolarization-induced Ca^{2+} influx of CG neurons can be blocked by 1 mM $NiCl_2$. We therefore investigated whether Ni^{2+} affected the NCAD90-induced Ca^{2+} influx in these neurons. Application of 1 mM $NiCl_2$ decreased the "resting" Ca^{2+} levels in CG neurons (Fig. 9), suggesting that CG neurons grown on LN have a steady-state Ca^{2+} influx that is sensitive to Ni^{2+}. This might represent a LN-induced Ca^{2+} influx, though further experiments would be necessary to test such an idea. The effect of Ni^{2+} on the NCAD90 response varied from cell to cell. In most neurons (5/8), the NCAD90-induced Ca^{2+} response was almost completely blocked by Ni^{2+} (Fig. 9 *a*). In others, however, Ni^{2+} was relatively ineffective (2/8; Fig. 9 *b*), and in one case the Ca^{2+} response was partially sensitive to block by Ni^{2+} (data not shown). In contrast to diltiazem and ω-conotoxin, therefore, Ni^{2+} was found to be an effective blocker of the N-cadherin–induced Ca^{2+} response in CG neurons.

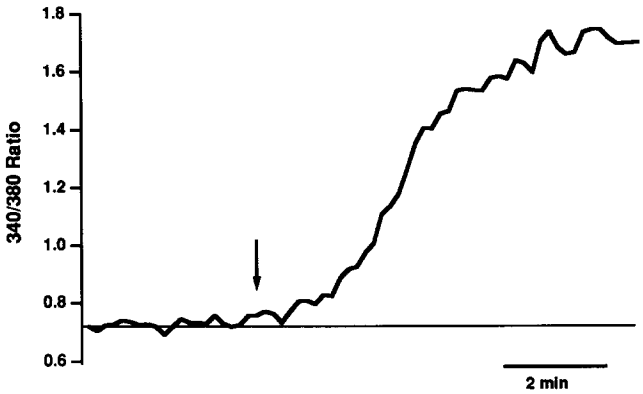

Figure 8. NCAD90 can elicit Ca^{2+} responses in the presence of diltiazem and conotoxin. A 1-min application of NCAD90 (*arrow*) to a CG neuron in the presence of 20 μM diltiazem and 0.2 μM ω-conotoxin results in a long-lasting Ca^{2+} response, similar to those seen in control saline (see Fig. 4). The response lasted for the duration of the experiment (20 min, not shown). Approximate values of $[Ca^{2+}]_i$ are: 0.7 = 250 nM; 1.5 = 800 nM.

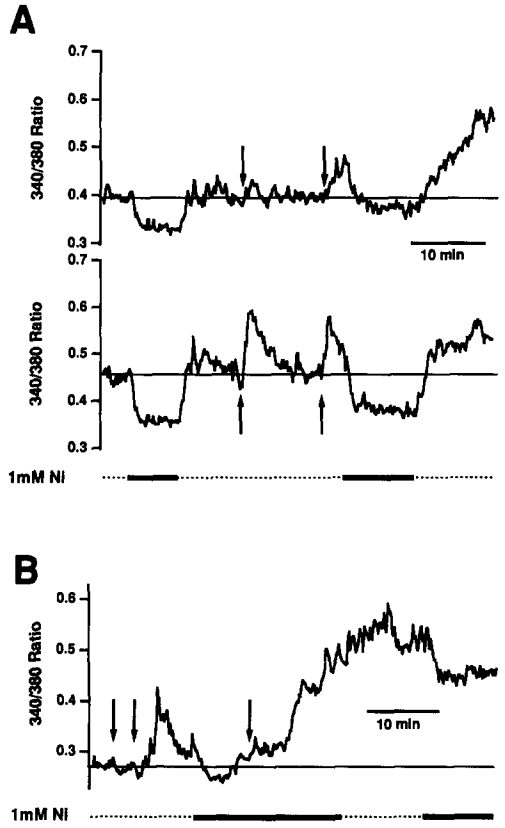

Figure 9. Sensitivity of cell body NCAD90 responses to Ni^{2+}. The presence of $NiCl_2$ (1 mM Ni) is indicated by the solid bars; NCAD90 was applied for 1 minute at the times indicated by the arrows. (*A*) Ni^{2+}-sensitive responses in two neurons. Ni^{2+} application results in decreases in "resting" Ca^{2+} levels. Application of NCAD90 results in brief Ca^{2+} responses. Responses to the second NCAD90 application are truncated by the addition of Ni^{2+}; washout of Ni^{2+} after 10 min leads to recovery of the Ca^{2+} responses. These latter responses are sustained. (*B*) Ni^{2+}-intensitive response. As in the first cell, Ca^{2+} levels are depressed by Ni^{2+}. However, NCAD90 leads to a Ca^{2+} response in the presence of Ni^{2+}, and subsequent washout of Ni^{2+} has relatively little effect. Readdition of Ni^{2+} fails to significantly inhibit the response. Approximate values of $[Ca^{2+}]_i$ are: 0.3 = 70 nM; 0.5 = 150 nM; 0.6 = 200 nM.

Effect of Ni^{2+} on Neurite Growth

Because Ni^{2+} is capable of inhibiting N-cadherin-induced Ca^{2+} responses, we tested whether it also affected N-cadherin-induced neurite growth. CG neurons were grown on LN, N-cadherin, or PDL, in the presence or absence of 1 mM $NiCl_2$, and neurite outgrowth was assessed at 7 h after plating. Neurite growth on N-cadherin was essentially unaffected, with no change in the number of neurons with neurites, and a small (but not statistically significant) reduction in the mean length of neurites (Figs. 10, *a* and *c* and 11). In contrast, growth on LN was severely reduced, with a 60% reduction in the number of neurons with neurites, and a 50% reduction in the length of those neurites (Figs. 10, *b* and *d* and 11). Surprisingly, there was an increase in the percentage of neurons growing neurites on PDL in the presence of Ni^{2+}. Whereas only 2.1 ± 0.6% (mean ± SEM) of CG neurons grew neurites in PDL in control cultures, this increased to 11.4 ± 3.5% in the presence of Ni^{2+} (n = 3 experiments; Fig. 11).

To investigate the effect further, we allowed CG neurons to grow for 17 h in the presence of 1 mM $NiCl_2$. This resulted in severe disruption of neurite growth both on

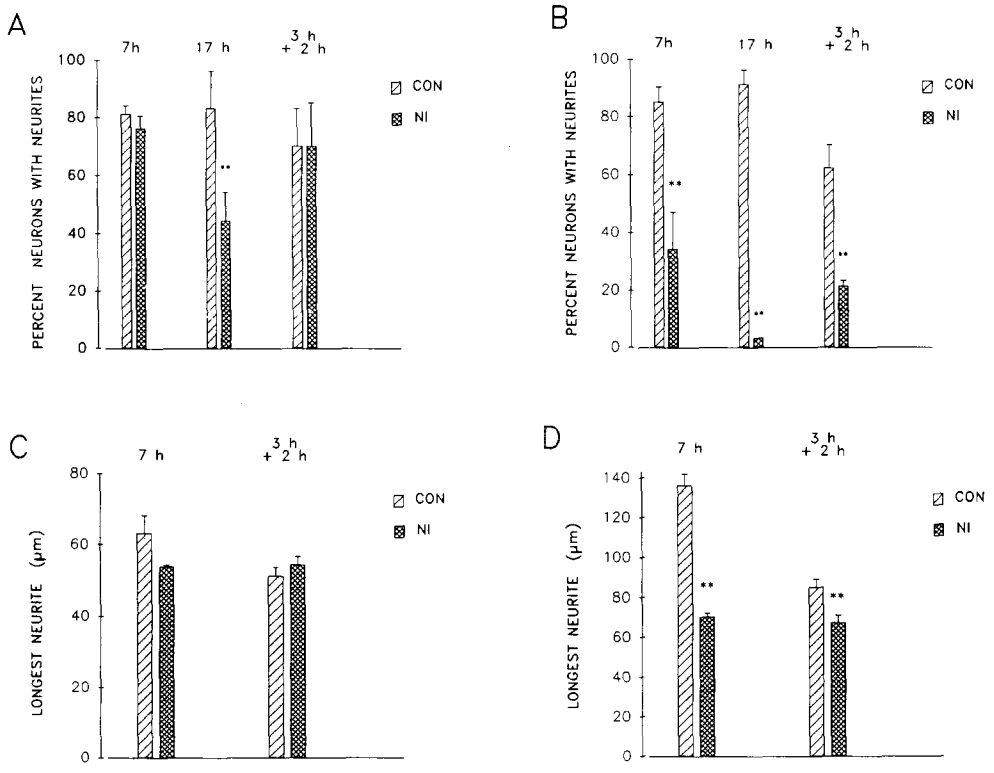

Figure 10. Effect of Ni^{2+} on neurite outgrowth from CG neurons grown on LN and N-cadherin. Neurons were grown for 7 or 17 h on 10 μg/ml N-cadherin (*A* and *C*) or 20 μg/ml LN (*B* and *D*) in the absence (*hatched bars*) or presence (*crosshatched bars*) of 1 mM $NiCl_2$, or were grown for 3 h in the absence of Ni^{2+}, then for 2 h in the absence (*hatched*) or presence (*crosshatched*) of Ni^{2+}. Both the percentage of neurons with neurites (*A* and *B*) and the mean length of the longest neurite (*C* and *D*) were quantified. The 7 h data are from three independent experiments; the other two experiments were each performed twice. All experiments were done in duplicate. Statistical differences from control values are indicated, where appropriate, by double asterisks ($p < 0.001$).

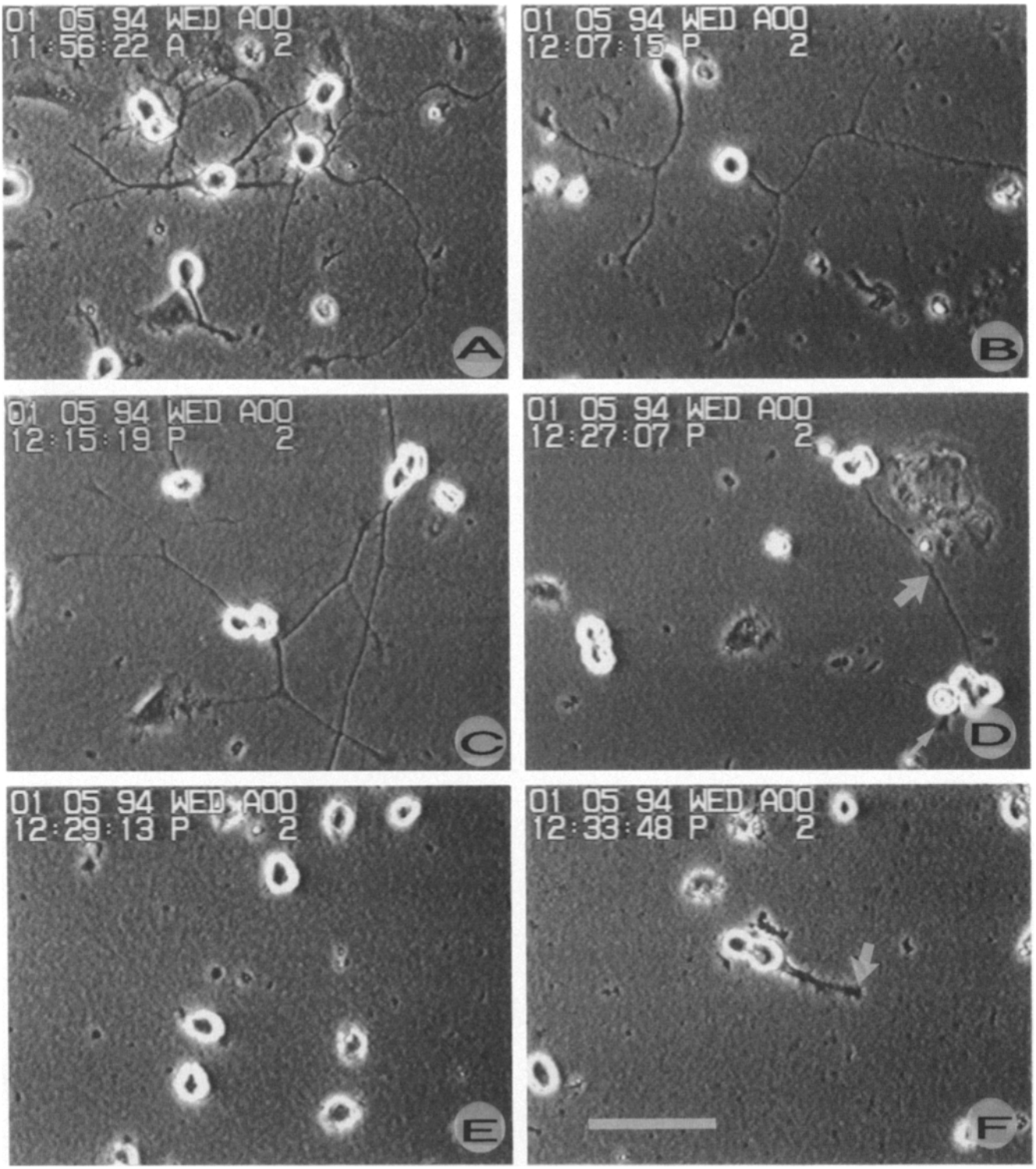

Figure 11. Phase contrast photomicrographs of CG neurons grown on 10 μg/ml N-cadherin (*A* and *B*), 20 μg/ml LN (*C* and *D*), or PDL (*E* and *F*) for 7 h in the absence (*A*, *C*, and *E*) or presence (*B*, *D*, and *F*) of 1 mM $NiCl_2$. Large arrow in *D* points to neurite, small arrow to small process less than 1 cell diameter in length. Arrow in *F* points to a neurite that grew on PDL in the presence of Ni^{2+}. Bar, 50 μm.

N-cadherin and on LN. On N-cadherin, the percentage of neurons with neurites was decreased almost 50% (Fig. 10 *a*), and those neurites that were present were both shorter and thinner than control neurites (Fig. 12, *a* and *b*). On LN, almost all neurite growth was abolished (Figs. 10 *b* and 12, *c* and *d*). The sparse neurite growth seen on PDL, however, was still somewhat increased by Ni^{2+} (from 1% with neurites to 9%; Fig. 12, *e* and *f*). Because neurite growth at 17 h

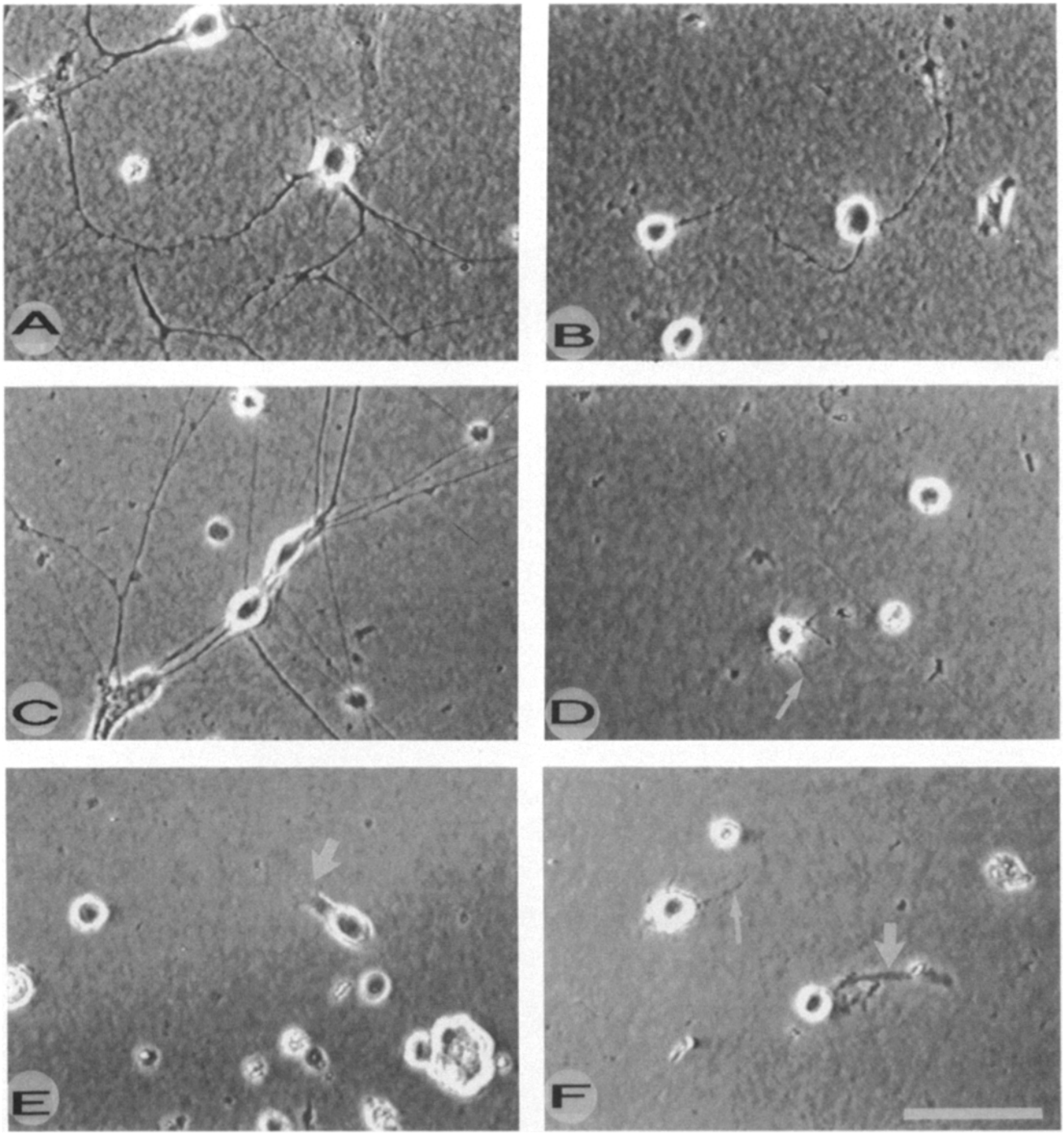

Figure 12. Phase contrast photomicrographs of CG neurons grown on 10 μg/ml N-cadherin (*A* and *B*), 20 μg/ml LN (*C* and *D*), or PDL (*E* and *F*) for 17 h in the absence (*A*, *C*, and *E*) or presence (*B*, *D*, and *F*) of 1 mM $NiCl_2$. Only a few tiny processes can be seen on LN in the presence of Ni^{2+} (*D*, *arrow*), and neurites are plentiful but short and spindly on N-cadherin in the presence of Ni^{2+} (*B*). In contrast, neurites are evident on PDL, even after 17 h in Ni^{2+} (compare *arrows* in *E* and *F*); these are not any shorter than in control conditions. Bar, 50 μm.

in the presence of Ni^{2+} was less than at 7 h, on both N-cadherin and LN, Ni^{2+} must result in the retraction of preexisting neurites. To see whether there is also an effect on initial outgrowth, we grew CG neurons for 3 h on LN or N-cadherin in normal medium, then switched to Ni^{2+}-containing medium for just two additional hours. With this short incubation in Ni^{2+}, no effect on neurite growth was seen with *N*-cadherin (Fig. 10, *a* and *c*). However, both the percentage of neurons with neurites and the length of neurites was decreased in the LN cultures (Fig. 10, *b* and *d*). Because the percentage of neurons with neurites at 5 h in Ni^{2+} (on LN) was comparable to that expected at 3 h (see Bixby and Zhang, 1990), it appears that neurite initiation was strongly inhibited. In summary, Ni^{2+} inhibited neurite growth on

both LN and N-cadherin, but not in an equivalent manner. More than 7 h of growth in Ni^{2+} was required to see an effect with N-cadherin, and half of the neurites were still present after 17 h. On LN, however, less than 2 h of exposure was sufficient to inhibit neurite growth, and the effect was severe by 7 h. In contrast, growth on PDL was actually potentiated by Ni^{2+}. Although the mechanism of neurite growth on PDL is not clear, this result demonstrates that the effect of Ni^{2+} is not simply to make the neurons "sick," and is in accord with the healthy appearance of the neuronal cell bodies on all substrates, even at 17 h (Fig. 12).

Discussion

We have shown that NCAD90 is capable of producing Ca^{2+} increases both in neuronal cell bodies and in growth cones, and that LN can induce small Ca^{2+} increases in cell bodies. Furthermore, Ni^{2+}, which blocks a large fraction of K^+-stimulated Ca^{2+} influx in these cells, inhibits neurite growth both on N-cadherin and on LN. Our results have implications for the mechanisms through which CAMs and integrins signal neurite growth.

Ca^{2+} Responses to N-cadherin

Previous experiments have shown that populations of PC12 cells or mouse cerebellar neurons can respond to CAMs of the Ig superfamily (NCAM and L1) with Ca^{2+} increases (Schuch et al., 1989; von Bohlen und Halbach et al., 1992). We have now extended these results to N-cadherin, a CAM with a very different structure. Further, imaging of single cells has allowed us to demonstrate that neuronal growth cones can respond to CAMs with Ca^{2+} increases, an important result for interpreting the relationship of Ca^{2+} changes to neurite growth. The analysis of individual cells revealed a diversity of responses to the NCAD90 stimulus. Neurons either were unresponsive, produced brief "spiky" Ca^{2+} increases, or produced large and prolonged Ca^{2+} increases. In non-neuronal cells, N-cadherin could produce Ca^{2+} decreases, indicating that not only the magnitude but also the direction of the Ca^{2+} response can vary. These results raise the possibility that cadherins in general use changes in $[Ca^{2+}]_i$ in their signaling pathways.

Studies with other neurons and cell lines have suggested that N-cadherin–dependent neurite growth is completely sensitive to blockade by the combination of diltiazem and ω-conotoxin, linking N-cadherin signaling to the activation of N- and L-type Ca^{2+} channels (Doherty and Walsh, 1992). In CG neurons, diltiazem and ω-conotoxin are not strong inhibitors either of N-cadherin–induced Ca^{2+} increases or of N-cadherin–dependent growth. In contrast, Ni^{2+}, which is not selective for N- and L-channels, inhibited both processes. Our results therefore support the prediction that N-cadherin binding can result in the opening of voltage-sensitive Ca^{2+} channels, but suggest that exact pathways may differ from neuron to neuron. This view is consistent with the findings of von Bohlen und Halbach et al. (1992). They found that Ca^{2+} signals induced in PC12 cells by a different CAM, L1, can be blocked by diltiazem, but that those induced by L1 in cerebellar neurons are insensitive to the same drug.

The mechanisms through which N-cadherin induces Ca^{2+} increases in CG neurons are not completely clear. Some role of Ca^{2+} influx through voltage-sensitive channels is suggested, but not proven by the Ni^{2+} blockade. The insensitivity of some NCAD90 responses to Ni^{2+} suggests that either more than one Ca^{2+} entry pathway and/or a release of intracellular Ca^{2+} is involved. CG neurons are known to have caffeine-sensitive Ca^{2+} stores (Sorimachi, 1993), and there is evidence in other neurons that release from such stores may be important in neurite growth (Kocsis et al., 1994).

Ca^{2+} Signals and Neurite Growth on N-cadherin and LN

The relationship of N-cadherin–induced Ca^{2+} changes to the induction of neurite growth remains to be fully elucidated. Although Ni^{2+} is an effective inhibitor of the Ca^{2+} responses induced by soluble N-cadherin, it has little effect on N-cadherin–induced neurite growth over 7 h in vitro. Diltiazem, which together with Ni^{2+} can inhibit the vast majority of K^+-induced Ca^{2+} influx (in CG neurons), had no effect on neurite growth induced by purified N-cadherin. Our studies therefore do not support the idea that Ca^{2+} influx through voltage-sensitive channels is the sole means through which N-cadherin promotes growth, as has been proposed (Doherty and Walsh, 1992). Because Ni^{2+} can eventually inhibit N-cadherin–induced neurite formation and/or stability, however, our results are consistent with the view that elevation of $[Ca^{2+}]_i$ is important in such growth.

The neurite growth induced by LN from CG neurons is highly sensitive to Ni^{2+}, a substance demonstrated to inhibit K^+-dependent Ca^{2+} influx in the same cells. Addition of Ni^{2+} to neurons growing on LN results in a reduction in intracellular Ca^{2+}, consistent with the possibility that LN induces a tonic Ca^{2+} influx. Application of soluble LN to CG neurons induced a small but measurable Ca^{2+} increase. Finally, we have seen two cases of "spontaneous" Ca^{2+} transients in CG neuronal growth cones migrating on LN (data not shown). Although not conclusive, these results, taken together, are consistent with a role for Ca^{2+} influx in LN-induced neurite growth. CG neuron growth on LN is dependent on the function of β1 integrins (Tomaselli et al., 1986). In endothelial cells, osteoclasts, and neutrophils, integrins, including those of the β1 family, have been shown to mediate Ca^{2+} signals (Ng-Sikorski et al., 1991; Schwartz, 1993; Shankar et al., 1993). Although previous studies suggest that there is no role for Ca^{2+} changes in integrin-dependent neurite growth (Doherty and Walsh, 1992), these studies are not conclusive. It therefore seems worthwhile to investigate the possibility that integrin-dependent Ca^{2+} signals are involved in neurite growth on LN.

PTX-sensitive G Proteins and Neurite Growth

Our results demonstrate that neurite growth induced by purified N-cadherin is insensitive to PTX, in three different neuronal populations. Therefore, the hypothesis that PTX-sensitive G proteins are a component of N-cadherin signaling required for neurite growth (Williams et al., 1994*a*) is unlikely to be correct in its simplest form. Recently, the formulators of the G protein hypothesis have suggested that, though PTX-sensitive G proteins can influence CAM-mediated neurite growth, they are not a "linear component" of the CAM signaling pathway (Williams et al., 1994*b*). This conclusion is not inconsistent with our results, since G proteins

could certainly modulate neurite growth signals in ways that were not apparent in our experiments.

Relationship of Our Results to Previous Studies

Our conclusions about N-cadherin–induced neurite growth contrast somewhat with those drawn on the basis of experiments using fibroblasts transfected with L1, NCAM, and N-cadherin as the growth substrate for either PC12 cells, hippocampal neurons, or cerebellar neurons (Doherty et al., 1991*a*,*b*; Williams et al., 1992, 1994*a*). These authors state that all three CAMs stimulate neurite growth entirely through a G protein–dependent activation of N- and L-type Ca^{2+} channels (mediated by a membrane-proximal tyrosine kinase). Because the status of the G protein hypothesis is uncertain (see above), we will not consider this issue in detail. Instead, we shall concentrate on explanations for the apparent discrepancy in the Ca^{2+} results.

A major difference in our experiments from those cited is the use of a purified protein, rather than transfected fibroblasts. It has been suggested that, when purified CAMs are used as a substrate, "CAM-dependent adhesion per se may be sufficiently permissive to allow for neurite outgrowth" (Williams et al., 1992). The idea that "simple" adhesion is sufficient to promote outgrowth is not new, but it is at odds with available data. When actually measured, the ability of purified "adhesion" proteins to promote adhesion is poorly correlated with their ability to promote neurite growth (Gunderson, 1987; Lemmon et al., 1992). Second, some substrates of immobilized antibodies to adhesion proteins can promote growth, but others promote strong attachment and spreading of neurons without triggering neurites (Hall et al., 1987). Third, a wide variety of "natural" and artificial substrates can be shown to promote adhesion but not neurite growth of a diverse array of neurons (e.g., Tomaselli et al., 1986; Bixby et al., 1988; Neugebauer et al., 1991). Fourth, neurite growth induced by purified CAMs responds differently to activators and inhibitors of protein kinase C compared with ECM-induced growth (Bixby and Jhabvala, 1990). Fifth, the domains of NCAM or L1 optimal for cell adhesion are not the same as those that are optimal for neurite growth (Frei et al., 1992; Appel et al., 1993). Finally, in the specific case of N-cadherin, the neurite growth promoted by the purified molecule has kinetic and morphological features not duplicated by any other substrates tested so far (Bixby and Zhang, 1990). This is difficult to explain by "adhesion per se."

A related possibility that must be considered is that neurite growth induced by purified N-cadherin is specific (not due to simple adhesion), but unrelated mechanistically to the growth induced by N-cadherin in membranes. This possibility is rendered unlikely by the findings discussed in the Introduction that the proteins found to promote growth on cells and those that promote growth as purified molecules are the same, and that in both cases CAMs seem mechanistically different from ECM proteins (Bixby and Jhabvala, 1990; Doherty and Walsh, 1992). An additional consideration is quantitative. Growth on purified N-cadherin by CG neurons results in neurites of 60 μm in 6 h, or 300 μm in 18–23 h (Table III). In studies in which blocking antibodies were used to assess the relative contributions to neurite growth of various CAMs, the neurite growth from CG neurons attributable to N-cadherin on Schwann cells (average 50 μm per neurite in 6 h, 200 μm per neurite in 18 h) or on astrocytes (200 μm per neurite in 16–20 h) was quantitatively similar (Bixby et al., 1988; Tomaselli et al., 1988). This quantitative similarity suggests that purified N-cadherin is a reasonable model for the study of CAM-induced neurite growth. Growth induced by N-cadherin–transfected fibroblasts (and those transfected with NCAM or L1) was considerably less (25–50 μm in 16–24 h; Williams et al., 1992, 1994*a*,*b*). This could be due to differences in the responsiveness of different neuronal populations to CAMs, difference in available CAM density, or to the lack of trophic factors required for optimal growth (e.g., Hatten et al., 1988). It is therefore not clear whether there is a fundamental difference between the ability of N-cadherin to promote growth as a purified substrate and its ability to promote growth as a molecule expressed on the surface of a heterologous cell.

If substrate-associated CAMs do trigger neurite growth through different mechanisms than the same proteins in membranes, this would have implications for CAM function in vivo. Several CAMs, including NCAM, L1, and N-cadherin, have secreted forms, especially in the embryo (Martini and Schachner, 1986; Gower et al., 1988; Roark et al., 1992). It may be that the effects of these secreted forms are distinct from those of the forms found on cell surfaces.

In summary, our results suggest a modification of the current view of N-cadherin signal transduction leading to neurite growth. Robust neurite growth induced by N-cadherin can be insensitive to PTX; PTX-sensitive G proteins therefore are not required for such growth. However, as would be predicted from previous results, Ca^{2+} signals are in fact induced by N-cadherin, both in cell bodies and in growth cones. These signals can be insensitive to diltiazem and ω-conotoxin, but are at least partially sensitive to other blockers of voltage-dependent Ca^{2+} channels. Neurite growth can be induced by N-cadherin in conditions in which Ca^{2+} responses should be greatly diminished, but growth is inhibited in the long term. Finally, neurite growth on LN may also be dependent on Ca^{2+} signals, though the nature of such signals remains to be elucidated.

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