Purified N-Cadherin Is a Potent Substrate for The Rapid Induction of Neurite Outgrowth

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Abstract. N-cadherin is the predominant mediator of calcium-dependent adhesion in the nervous system (Takeichi, M. 1988. *Development (Camb.).* 102: 639–655). Investigations using antibodies to block N-cadherin function (Bixby, J. L., R. L. Pratt, J. Lilien, and L. F. Reichardt. 1987. *Proc. Natl. Acad. Sci. USA.* 84:2555–2569; Bixby, J. L., J. Lilien, and L. F. Reichardt. 1988. *J. Cell Biol.* 107:353–362; Tomaselli, K. J., K. N. Neugebauer, J. L. Bixby, J. Lilien, and L. F. Reichardt. 1988. *Neuron.* 1:33–43) or transfection of the *N*-cadherin gene into heterologous cell lines (Matsunaga, M., K. Hatta, A. Nagafuchi, and M. Takeichi. 1988. *Nature (Lond.).* 334:62–64) have provided evidence that N-cadherin, alone or in combination with other molecules, can participate in the induction of neurite extension. We have developed an affinity purification procedure for the isolation of whole N-cadherin from chick brain and have used the isolated protein as a substrate for neurite outgrowth. N-cadherin promotes the rapid extension of neurites from chick ciliary ganglion neurons, which extend few or no neurites on adhesive but noninducing substrates such as polylysine, tissue culture plastic, and collagens. N-cadherin is extremely potent, more so than the L1 adhesion molecule, and comparable to the extracellular matrix protein laminin. Compared to laminin, however, N-cadherin promotes outgrowth from ciliary ganglion neurons extremely rapidly and with a distinct morphology. These results provide a direct demonstration that N-cadherin is sufficient to induce neurite outgrowth when substrate bound and suggest that the mechanism(s) involved may differ from that induced by laminin.

C ELL–CELL interactions are fundamental to all aspects of animal development, as well as to the functioning of the mature organism. Since the pioneering experiments of Steinberg et al. (1973) and Takeichi (1977), it has become clear that a major role in these interactions is played by calcium-dependent adhesion systems. Calcium-dependent adhesion is accomplished principally through the actions of the cadherins, a family of cell surface glycoproteins (reviewed by Takeichi, 1988). At least five cadherins have been identified, and the discovery of others may be anticipated (Takeichi, 1988; Crittenden et al., 1988).

In the nervous system, calcium-dependent adhesion appears to be regulated predominantly by N-cadherin, a 136,000-M_r cell surface glycoprotein (reviewed by Takeichi, 1988). N-cadherin is probably identical to molecules initially identified by other laboratories as N-calCAM (Grunwald et al., 1982; Crittenden et al., 1988) and A-CAM (Volk and Geiger, 1984, 1986). N-cadherin has been implicated in a wide spectrum of patterning events, including the formation and maintenance of adherens junctions (Volk and Geiger, 1986), the formation of somites (Duband et al., 1987), the histogenesis of neural retina (Matsunaga et al., 1988b), the morphogenetic movements during initial formation of the neural tube (Kintner, C., personal communication), and the regulation of axon growth (Bixby et al., 1987, 1988; Tomaselli et al., 1988; Matsunaga et al., 1988a; Neugebauer et al., 1988).

Axon growth must be regulated by a complex combination of (a) intrinsic, genetically programmed abilities; (b) soluble and chemotactic factors (e.g., nerve growth factor); and (c) substrate-bound cues. It has been appreciated for a number of years that macromolecules of the extracellular matrix (ECM)[1] are important as substrate-bound inducers of neurite outgrowth, at least in vitro. In particular, laminin (LN) is the most potent known ECM protein in this regard (e.g., Manthorpe et al., 1983; Lander et al., 1985). More recently, examination of process outgrowth induced by the surfaces of other cells has strongly suggested a role for cell adhesion molecules in this process (Bixby et al., 1987, 1988; Chang et al., 1987; Tomaselli et al., 1988; Seilheimer and Schachner, 1988). The particular cell adhesion molecules that are involved include L1 (Chang et al., 1987; Lagenauer and Lemmon, 1987; Bixby et al., 1988); the L1-related molecules, F11 (contactin) and neurofascin (Chang et al., 1987; Rathjen et al., 1987; Brummendorf et al., 1989; Ranscht,

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1. *Abbreviations used in this paper:* CG, ciliary ganglion; DOC, deoxycholate; ECM, extracellular matrix; LN, laminin; N-CAM, neural cell adhesion molecule.

1988); neural cell adhesion molecule (N-CAM) (Bixby et al., 1987; Neugebauer et al., 1988; Doherty et al., 1989); and N-cadherin (references cited above). Some evidence suggests that the myelin-associated glycoprotein may also be involved in neurite extension (Johnson et al., 1989).

There are two lines of evidence that indicate a role for N-cadherin in neuronal process growth. First, antibodies recognizing N-cadherin inhibit neurite growth on the surfaces of several types of nonneuronal cells (e.g., Bixby et al., 1987, 1988; Tomaselli et al., 1988). Second, immortal cells transfected with a cDNA clone of the gene encoding N-cadherin are a much better substrate for neurite growth from retinal explants than the control, untransfected cells (Matsunaga et al., 1988a).

The calcium-independent cell adhesion molecule, L1, has been shown to mediate neurite growth from chicken and murine central neurons when purified and used as a substrate, although similar experiments with purified N-CAM were less definitive (Lagenauer and Lemmon, 1987; Lemmon et al., 1989). In this paper, we report analogous experiments using purified chicken N-cadherin. Our results demonstrate that N-cadherin alone is sufficient to induce neurite growth and that this molecule is similar in potency and effectiveness to the most potent known ECM molecule, LN. In addition, neurite outgrowth induced by N-cadherin has characteristics that distinguish it from LN-induced growth, suggesting that different underlying mechanisms may be involved.

Materials and Methods

Reagents

Fertile white leghorn eggs were obtained from SPAFAS, Inc. (Norwich, CT). Nitrocellulose (BA83) was obtained from Schleicher & Schuell, Inc. (Keene, NH). Cell culture media and sera were from the University of Miami Cancer Center Media Facility (Miami, FL). Protein G-Sepharose was from Pharmacia Fine Chemicals (Piscataway, NJ), and dimethylpimelimidate was from Pierce Chemical Co. (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-mouse IgG and anti-rabbit IgG coupled to alkaline phosphatase were obtained from Promega Biotec (Madison, WI). The NCD-2 rat/mouse hybridoma cells were the generous gift of Masatoshi Takeichi (Kyoto University, Japan), and ascites fluid from this hybridoma was developed in nude mice. The rabbit anti-N-cadherin antiserum (RR2) was the kind gift of Jack Lilien (University of Wisconsin, Madison, WI). IgG was purified from this serum as described (Bixby et al., 1987). LN was purified from the Engelbreth-Holm-Swarm sarcoma as described (Bixby and Reichardt, 1985) or was the generous gift of Deborah Hall (Athena Neurosciences, S. San Francisco, CA). Rat tail collagen was purified as described (Bornstein, 1958). Purified chick muscle actin was the kind gift of David Burgess. Chick brain tubulin was purified from E17 chick brain as described, using two rounds of polymerization and depolymerization (Vallee, 1986).

Methods

NCD-2 Affinity Column. A high-efficiency antibody affinity column was prepared using a modification of the method of Schneider et al. (1982). 4 ml of NCD-2 ascites fluid (approximate IgG concentration, 3.5 mg/ml) was run continuously over a column of protein G-Sepharose (1.7 ml) at room temperature for 3 h. The column was then washed with 15 ml of PBS followed by 10 ml of 0.2 M triethanolamine, pH 8.2. Bound antibody was cross-linked to the column by washing with 25 ml of freshly-prepared 20 mM dimethylpimelimidate in the triethanolamine buffer. The column was washed with 20 mM ethanolamine, pH 8.2, and stored in 50 mM Hepes, 0.1 M NaCl, pH 7.5, with 0.02% NaN₃. About 6 mg of antibody was coupled to the column in this procedure.

Purification of N-Cadherin. A crude membrane fraction was prepared from embryonic day-18 chick brain as described previously (Bixby and Reichardt, 1985), except that the buffers contained 2 mM calcium chloride and 1 μg/ml antipain. This preparation was extracted with 0.5% Na deoxy-

cholate (DOC) for 45 min at 4°C and centrifuged at 35,000 *g* for 40 min. The supernatant fraction was run over the NCD-2 affinity column overnight at 4°C. The column was washed with 25 ml of high salt buffer (0.1 M Tris, pH 8, 0.5 M NaCl, 0.1% DOC, 1 mM CaCl₂) followed by low salt buffer (0.1 M Tris, pH 8, 0.1 M NaCl, 0.25% DOC, 0.05% SDS, 1 mM CaCl₂) until the OD₂₈₀ of the wash reached a plateau. Bound antigen was eluted with 50 mM diethylamine, pH 11.5, 0.2% DOC, 1 mM CaCl₂, neutralized with 0.14 vol of 1 M Hepes, pH 7.4, and lyophilized in a Speed-Vac concentrator. Gel electrophoresis and Western blotting were performed as described (Bixby and Reichardt, 1985) except that the second antibody used was coupled to alkaline phosphatase rather than peroxidase. Protein was determined by electrophoresis and staining with Coomassie blue, comparing the unknowns with a set of albumin standards.

Preparation of Substrates. Nitrocellulose-coated culture dishes (Falcon Labware, Oxnard, CA) were prepared essentially as described by Lagenauer and Lemmon (1987). In early experiments, 1–4 μl of test protein solutions were spotted onto nitrocellulose-coated 35- or 60-mm tissue culture dishes, but diffusion of the protein sometimes led to ambiguities in interpretation. In subsequent experiments, we coated 96-well plates with nitrocellulose (3 μl nitrocellulose solution/well) and spotted 1 μl of protein solution in the center of each well. N-cadherin was diluted in Hepes-buffered HBSS containing Ca⁺⁺ and Mg⁺⁺, to which 0.1% DOC was added. LN was diluted in divalent cation-free PBS. Proteins were allowed to adhere to the substrate for 15 min, after which the plates were washed four times with HBSS, and blocked by incubation at 37°C with serum-containing culture medium.

Cell Culture. Preparation of ciliary ganglion (CG) neuron cultures has been described (Bixby and Reichardt, 1985; Tomaselli et al., 1986). Antibodies were added to the wells at 2× final concentration, and the neurons were added in an equal volume of medium. In the antibody "preincubation" experiments, a 1:6 dilution of NCD-2 ascites in culture medium (50 μl) was used to block the substrate for 1 h at 37°C. The antibody solution was removed and the wells were rinsed with medium before the neurons were added. For the "recovery" experiments, CG neurons were prepared as usual and incubated in a sterile 15-ml tube at 37°C and 5% CO₂ until the time of plating. Neurons were triturated with a pasteur pipette before plating, but this did not prevent extensive clumping of the cells. Cultures were fixed and analyzed as described (Bixby, 1989).

Results

Purification of N-Cadherin

Previous reports have described procedures for the purification of tryptic fragments of N-cadherin (Shirayoshi et al., 1986; Crittenden et al., 1987). One of these procedures involved the use of the monoclonal antibody NCD-2 to purify microgram quantities of a soluble fragment of N-cadherin from enzymatically digested brain membranes (Shirayoshi et al., 1986). To purify the intact native molecule, we used the same antibody in combination with a detergent extract of chick brain membranes. Preliminary experiments demonstrated that N-cadherin could be quantitatively solubilized by 0.5% DOC and that solubilization in CHAPS or Triton X-100 was significantly less efficient (not shown). We therefore bound DOC-solubilized material to an affinity column of NCD-2 and eluted the column under denaturing conditions. The eluted material consisted of a major band of 136,000 M_r (the size of intact N-cadherin), a series of bands (which varied in density among different preparations) between 90 and 120 kD, and minor bands near 200 and 43 kD (Fig. 1). Immunoblots revealed that all of the high molecular mass bands comigrated with species recognized by the NCD-2 antibody, while the 43-kD band was unlabeled (Fig. 1). Fig. 1 also shows that the antibody recognized the 136-kD band (and sometimes a second band at 80 kD) in intact membrane fractions but did-not recognize bands at 90–120 kD. This suggests that the other NCD-2–positive bands were generated during the purification, despite the presence of protease inhibitors. Our conclusion is that the vast majority
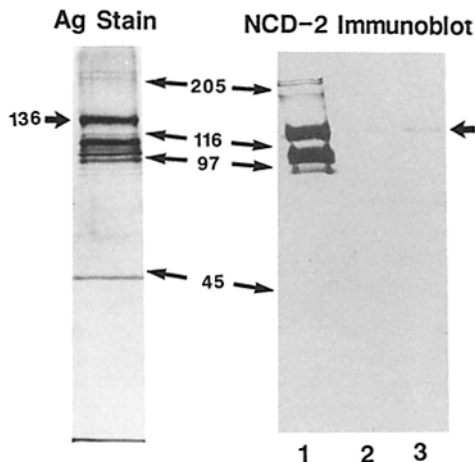
Ag Stain   NCD-2 Immunoblot

Figure 1. Analysis of purified N-cadherin. The left-hand panel shows a silver-stained 7% polyacrylamide gel of the eluate from the NCD-2 affinity column. There is a major band at 136 kD plus a series of prominent bands between 95 and 115 kD. Minor bands are seen at 43 and 210 kD. Similar gels stained with Coomassie blue demonstrated that the relative intensity of these bands is a reliable indication of their relative abundance (not shown). The right-hand panel shows an immunoblot using the NCD-2 antibody (1:600 dilution of ascites). (Lane *1*) 600 ng NCD-2 column eluate; (lane *2*) 30 μg DOC extract of brain membranes after running over NCD-2 column; (lane *3*) 30 μg DOC extract before running over NCD-2 column. Note that all of the silver-stained bands correspond to proteins recognized by the NCD-2 antibody, with the exception of the 43-kD band. Note also that the major band visible in the DOC extract is the 136-kD band, suggesting that the other NCD-2 positive bands were generated during purification and handling. The positions of molecular mass standards (run separately for the two conditions) are indicated by the arrows between the two panels.

of the high relative molecular mass material is authentic N-cadherin and products of its partial proteolytic degradation. Both the unlabeled 43-kD band and a second minor species (52,000 M_r) seen in some other preparations are likely to be cytoskeletal proteins nonspecifically retained on

the column (data not shown). Although our N-cadherin is not pure, it is clearly highly enriched for NCD-2–immunoreactive material.

Purified N-Cadherin Is an Inducer of Process Outgrowth

Since N-cadherin has been shown to be involved in neurite outgrowth by both central and peripheral neurons, we asked whether purified N-cadherin was sufficient to induce outgrowth when used as a substrate. Because our N-cadherin preparation was solubilized in detergent, we used a modification of the method of Lagenauer and Lemmon (1987) to immobilize the protein on a thin layer of nitrocellulose in tissue culture plates. Using their results with L1 as a guide, our initial experiments used N-cadherin at 50–100 μg/ml.

Previous experiments have shown that neurons of the chick CG do not grow neurites on noninducing substrates like tissue culture plastic or poly-D-lysine, at least during the first 16–20 h of culture (e.g., Tomaselli et al., 1986). This is true despite the ability of the CG neurons to adhere to these substrates and extend lamellipodia. In contrast, CG neurons rapidly extended processes on substrates coated with N-cadherin, and 80–90% of CG neurons extended processes within 16–20 h (Fig. 2). It is clear from Fig. 2 that N-cadherin can be an effective substrate for neuronal attachment as well. Although neurons were relatively dense in the N-cadherin–coated regions, one could see (after 20 h of culture) cell-sparse zones in the portions of the plate outside the region where protein was spotted. The line between protein-coated and control neurons could be quite sharp (Fig. 2). The ability of N-cadherin to promote neuronal adhesion was not studied systematically. In general, both the percentage of neurons with neurites and the length of individual neurites on N-cadherin substrates were similar to those seen with the most potent ECM protein, LN, in cultures examined 16–20 h after plating. N-cadherin can therefore be categorized as a substrate-acting protein capable of inducing rapid process outgrowth. The rapid attachment and spreading of ganglionic nonneuronal cells on N-cadherin also demonstrates
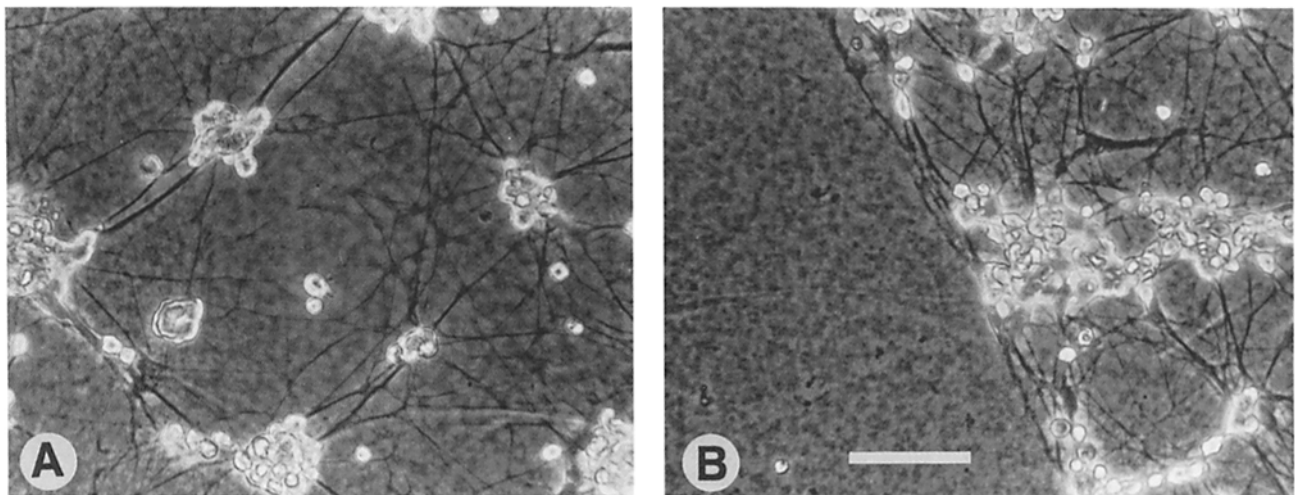

Figure 2. Phase–contrast photomicrographs of CG neurons cultured for 19 h on a nitrocellulose-coated 60-mm dish spotted with N-cadherin at 100 μg/ml. *A* shows the extensive process outgrowth in the center of the N-cadherin spot, while *B* shows the boundary between N-cadherin and blocked nitrocellulose. Neuronal attachment and growth is limited to the N-cadherin region. Bar, 50 μm.

Table I. CG Neuron Outgrowth on Various Substrates

Time of culture	N-Cadherin	LN	Nitrocell.	PDL	Collagen
h	%	%	%	%	%
4–8	56 ± 7.5 (5)	46 ± 8 (7)	0.1 ± 0.1 (4)	0.75 (2)	0.5 (2)
15–20	79 ± 5.5 (5)	66 ± 11 (5)	16 ± 1.5 (5)	21 (1)	24 (1)

CG neurons were grown for the indicated times on various substrates. The percentage of neurons with neurites (mean ± SEM; $n > 2$) is shown for each condition, with the number of experiments in parentheses. *N-Cadherin*, 50 μg/ml N-cadherin; *LN*, 50 μg/ml LN; *Nitrocell.*, nitrocellulose blocked with serum proteins; *PDL*, poly-D-lysine; *Collagen*, rat tail collagen. The number of neurons attached to the various substrates was not different.

that this protein is a potent adhesion molecule for other types of neural cell (see below; Fig. 5).

In these early experiments, in which proteins were spotted onto large (35- or 60-mm) culture dishes, there were two complications. First, as noted above, there could be significant differences among different substrates in the ability of cells to adhere, which complicates experiments designed to examine process outgrowth under different conditions. Second, it was not always clear where the boundary was between different "spots" of substrate. Both of these problems were obviated in subsequent experiments with the use of 96-well plates. In these plates, the plating efficiency of the cells (measured after fixation and washing) did not differ systematically for different substrates (data not shown).

In the 96-well plates, the ability of substrates to support attachment could be distinguished from the ability to promote process outgrowth. Although both N-cadherin and LN induced rapid and extensive neurite growth, substrates consisting of nitrocellulose alone (blocked by incubation with serum-containing medium), nitrocellulose coated with poly-D-lysine, or nitrocellulose coated with rat tail collagen gave quite different results. CG neurons generally did not grow neurites under these conditions for 6–8 h (Table I), although a significant percentage was able to form short lamellipodia (not shown). A small percentage of CG neurons grew neurites after 15–20 h (Table I), further demonstrating the ability of CG neurons to attach to these substrates.

Neurite outgrowth observed on substrates of highly purified N-cadherin was mediated by N-cadherin and not by a contaminant. This was demonstrated in two ways. First, substrates were prepared using purified preparations of actin (250 μg/ml) and tubulin (250 μg/ml), the major cytoskeletal components in the crude brain membranes and the likely sources of the contaminating bands (not shown). These substrates were no more effective than uncoated nitrocellulose blocked with serum-containing medium, whether examined after 8 or 21 h (Table II). In the same experiments, N-cadherin at 50 μg/ml induced extensive process outgrowth. Second, specific antibodies to N-cadherin inhibited process outgrowth on N-cadherin but did not affect growth on LN (Fig. 3). In these experiments, two different antibodies were used. The first is the NCD-2 monoclonal antibody described by Takeichi and colleagues, which we used for the purification of N-cadherin. NCD-2 ascites fluid at a dilution of 1:11 (approximate IgG concentration, 300 μg/ml) inhibited growth on N-cadherin by ~80% while having no significant effect on LN-induced growth. IgG purified from a polyclonal antiserum to N-cadherin, RR2 (Crittenden et al., 1987; Bixby et al., 1987), also reduced growth on N-cadherin by 80% without affecting growth on LN. In addition, neurites that

were able to grow on N-cadherin in the presence of NCD-2 or RR2 were much shorter than in the control. The concentration of RR2 IgG used in these experiments was the same as that previously shown to inhibit N-cadherin function on cells and was assumed to be functionally saturating (Crittenden et al., 1987; Bixby et al., 1987). Inhibition by NCD-2 antibodies did not require cross-linking of cell surface N-cadherin since preincubation of the N-cadherin substrate with NCD-2 also inhibited outgrowth by >80% (Fig. 3 B). In these assays, there were no effects of the antibodies on the number of neurons attached to the substrate, as assessed by the number of neurons remaining in a given field (data not shown). We conclude that the outgrowth-promoting activity we have demonstrated in our preparation resides in N-cadherin. While we cannot be certain of the explanation for the inability of these antibodies to inhibit growth completely, it is worth noting that nitrocellulose-coated plates blocked with serum proteins are better substrates (for CG neuron outgrowth) than tissue culture plastic or polylysine.

In dose–response experiments, N-cadherin proved extremely potent when compared with other substrate-bound ECM proteins or cell adhesion molecules. Concentrations of N-cadherin as low as 0.1 μg/ml were capable of inducing growth above control conditions, and the response was half-maximal at ~3 μg/ml (Fig. 4). LN, the most potent known ECM protein, gave a half-maximal response at ~5 μg/ml (Fig. 4). This is similar to what has been observed previously for LN on tissue culture plastic, using these same neurons (Tomaselli et al., 1986). The potency of N-cadherin in our experiments compares favorably with the ability of the calcium-independent adhesion molecules, L1 and N-CAM, to stimulate growth. Lagenauer and Lemmon (1987) reported that for chick tectal neurons concentrations of L1 <10 μg/ml were ineffective at producing adhesion or neurite out-

Table II. Neurite Growth on N-Cadherin and Cytoskeletal Substrates

Experiment	Time in vitro	N-Cadherin	Tubulin	Actin	Control
	h	%	%	%	%
1	21	85	19	18	20
2	21	72	ND	12	9
3	8	72	0	0.7	0

CG neurons were grown for the indicated times on substrates of 50 μg/ml N-cadherin, 250 μg/ml tubulin, 250 μg/ml actin, or nitrocellulose blocked with medium. The percentage of neurons with neurites in the culture is shown for each substrate. *Control*, blocked nitrocellulose. Qualitatively similar observations were made in two other cultures done in 60-mm dishes; uncertainty about boundaries of tubulin and actin spots precluded quantification.
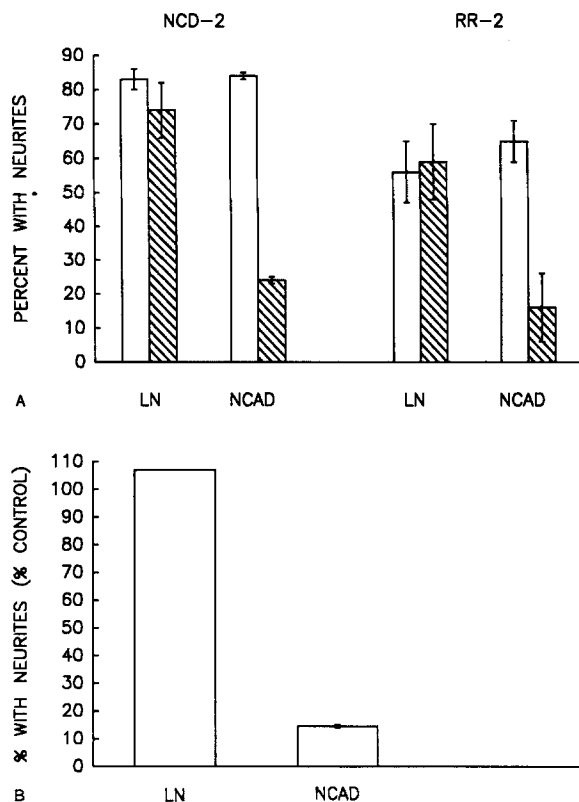
Figure 4. Dose–response curve for substrate-bound N-cadherin. Neurons were grown for 8 h on substrates of N-cadherin (*open circles*), LN (*closed squares*), or rat tail collagen (*closed circle*). The percentage of neurons with neurites is shown for the concentrations of protein listed on the abscissa. The LN response and the N-cadherin response are quite similar, with half-maximal responses at ~4 μg/ml. Neurites do not grow on collagen at these short times. Similar dose–response curves were obtained for N-cadherin in four other independent experiments, with half-maximal responses ranging from 0.75 to 5 μg/ml (mean = 2.5 μg/ml; n = 5). The lowest effective N-cadherin concentration tested was 0.1 μg/ml. The mean half-maximal response to LN was 5 μg/ml (n = 3).

Figure 3. Inhibition of N-cadherin–induced neurite outgrowth by antibodies to N-cadherin. (*A*) The percentage of neurons with neurites is given for cultures grown on 50 μg/ml LN (*LN*) or on 50 μg/ml N-cadherin (*NCAD*) in the presence (*hatched bars*) or absence (*open bars*) of antibody. The left side shows the results for NCD-2 (ascites diluted 1:11), and the right side shows the results for RR-2 IgG (1 mg/ml). Bars show the mean and standard error of three experiments for NCD-2 (18–21 h in vitro) and the mean and range of two experiments with RR2 (8 h in vitro). The antibodies inhibit growth on N-cadherin by 80% without affecting growth on LN. (*B*) The percentage of neurons with neurites is given (as a percent of control) for neurons cultured 6 h on substrates preincubated with NCD-2 (ascites diluted 1:6). Error bar for N-cadherin gives the range of two experiments. For LN, both experiments gave the same result. LN, 10 μg/ml LN; NCAD, 10 μg/ml N-cadherin.

growth and that purified N-CAM was a very weak inducer of process growth. In preliminary experiments, we have also found this to be true for CG neurons. In the three experiments we have done so far, using 7-h culture periods, L1 at 100 μg/ml (mean 35% with neurites) is clearly less effective than N-cadherin at 25 μg/ml (mean 64% with neurites), and the L1 response is clearly diminished at concentrations of 50 μg/ml (mean 8% with neurites). Therefore, of the limited known examples, N-cadherin is at least as potent an inducer of process outgrowth as other cell adhesion molecules. N-cadherin proved comparable to LN in its effectiveness as well. As is the rule in neurite outgrowth assays, there was variability from experiment to experiment in the number and length of neurites on a given substrate. However, the similar effectiveness of LN and N-cadherin could be demonstrated by calculating the ratio (the percent of neurons with neurites on N-cadherin to the percent of neurons with neurites on LN) for a series of experiments. In seven independent experiments, this ratio averaged 1.07 ± 0.08 (mean ± SEM).
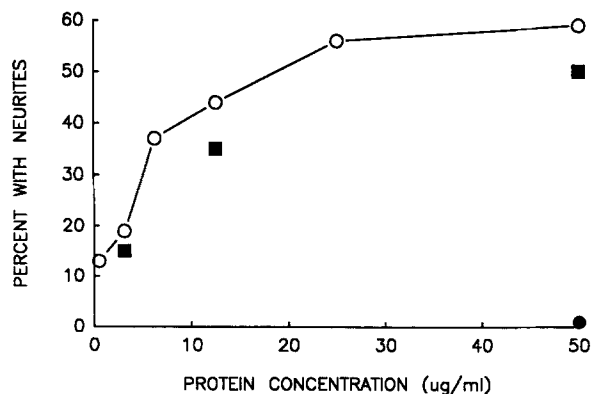
N-Cadherin–induced Growth Is Characteristic

While N-cadherin is similar to LN in potency and effectiveness, the response of neurons to N-cadherin can readily be distinguished from that seen on LN. This first became clear in examination of cultures fixed after relatively short (6–8 h) times in vitro. In these cultures, there were obvious morphological differences between neurons grown on N-cadherin and those grown on LN. N-cadherin–induced neurites were relatively short, highly branched, and curved. In contrast, neuronal processes on LN were longer, straighter, and less branched (Fig. 5). Fig. 5 also shows a phenomenon we observed consistently in these short-term cultures. Ganglionic nonneuronal cells attached and spread rapidly on N-cadherin so that "spread" nonneuronal cells were more numerous on N-cadherin than on LN.

When we examined cultures at earlier time points, we found that CG neurons also responded more rapidly to N-cadherin than to LN. Some neurites could be seen on N-cadherin substrates within 45 min of plating the cells (Fig. 6; earlier times were not examined). CG neurons do not respond to LN in <1–2 h (not shown). By 2 h of culture, 40% of neurons on N-cadherin had short neurites, while only 6% of those on LN had initiated growth (Fig. 6).

The kinetic differences noted above between N-cadherin– and LN-induced neurite growth could be due to differences in the response time of the neurons or to differences in the recovery of the relevant neuronal receptors from inactivation by trypsin during the cell preparation. Although the susceptibility of LN receptors to trypsinization is not known, digestion of trypsin in the absence of Ca++ leads to loss of cell surface cadherins (Takeichi, 1988). We attempted to address this issue by allowing a recovery period of 2–2.5 h between trypsinization and plating of the cells. This manipulation clearly allowed some form of recovery because "recovered" CG neurons could extend processes on LN by 30 min after
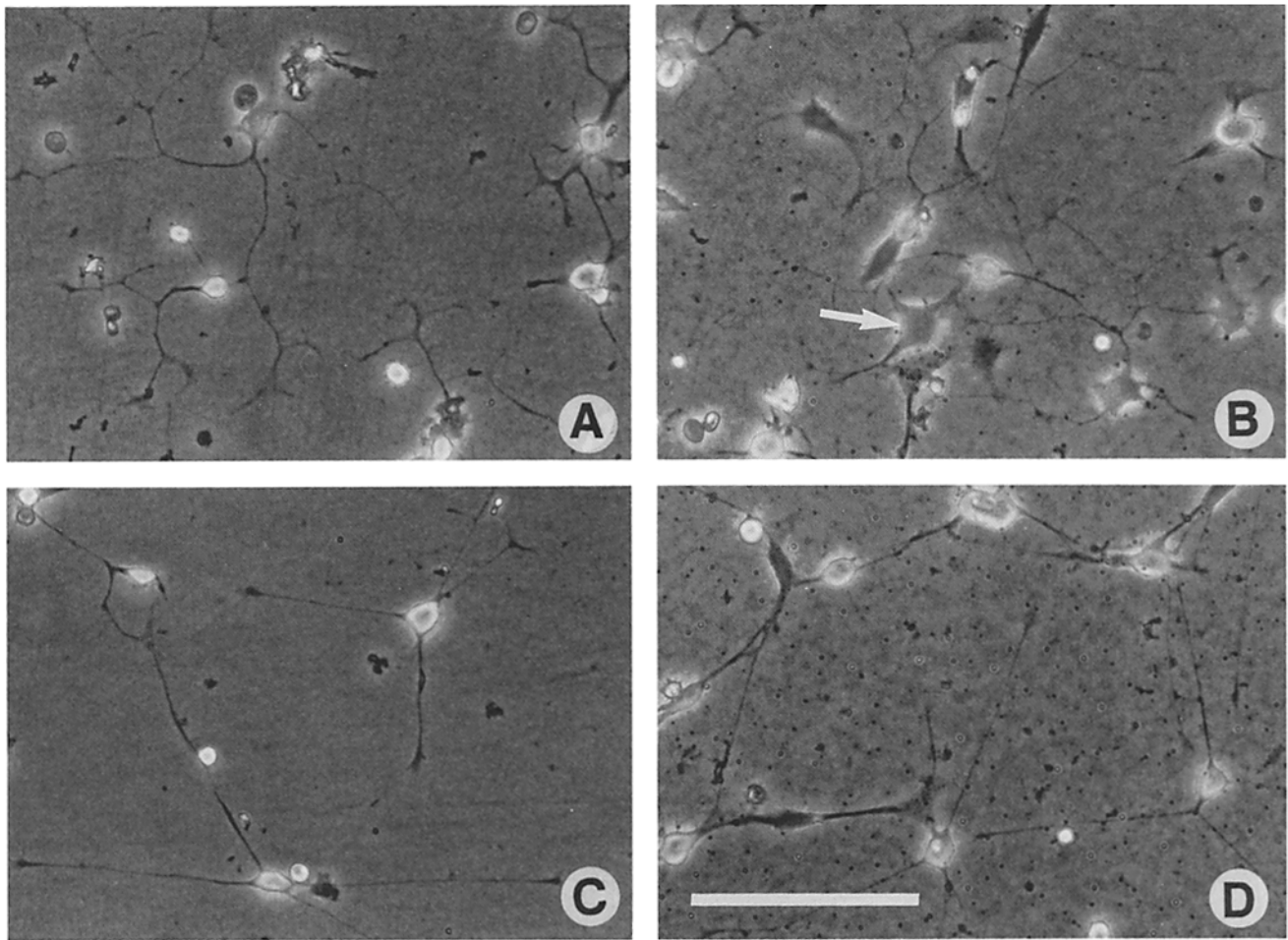
Figure 5. Phase–contrast photomicrographs of CG neurons grown for 8 h on 30 μg/ml N-cadherin (*A* and *B*) or 50 μg/ml LN (*C* and *D*). *A* and *C* were taken from one experiment, *B* and *D* were from a second experiment. Neurites formed on LN are relatively straight and unbranched. Note the obviously adherent and flattened nonneuronal cells in *B* (compare with *D*), one of which is marked with an arrow. Bar, 50 μm.
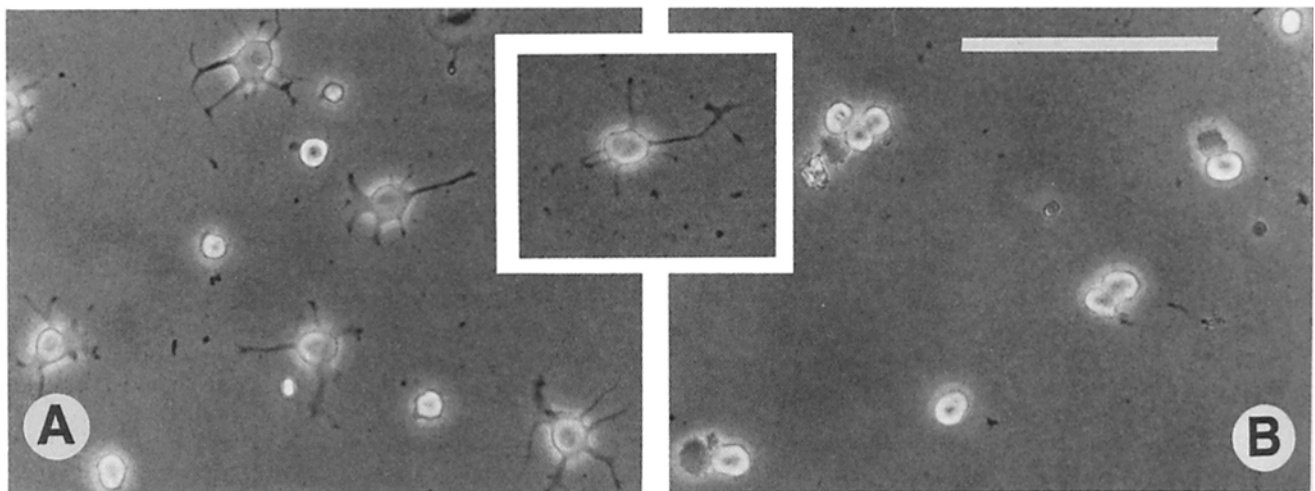

Figure 6. Phase–contrast photomicrographs of CG neurons grown for 2 h on 15 μg/ml N-cadherin (*A*) or 50 μg/ml LN (*B*). Numerous processes have formed on the N-cadherin substrate but not on the LN. (*Inset*) Example of a neuron with processes after 45 min on N-cadherin. This is not a "typical" example but is one of the longest to form during that time. No neurites were observed to form on LN in <1.5 h. Bar, 50 μm.

plating. The differences between the two substrates (with respect to time of neurite initiation) were lessened by using this recovery period but were not abolished. In four different experiments, when cells were fixed between 0.5 and 2 h after plating, the ratio (the percent with neurites on N-cadherin to the percent with neurites on LN) averaged 1.73 ± 0.32 (mean ± SEM). These results were complicated by the fact that recovered cells tended to settle in clumps, rather than individually, and that clumps were more likely than single cells to extend processes on any kind of substrate. The contribution of cell–cell interactions to our results could not be independently assessed. Therefore, while the quantitative contribution of recovery from trypsinization cannot be determined, our results suggest that, at least for CG neurons, N-cadherin receptors are able to produce neurites more rapidly than those responding to LN.

Discussion

We have designed a simple and effective scheme for obtaining highly enriched preparations of active N-cadherin. Although the preparations are not pure, we estimate that >95% of the protein (in our best preparations) is N-cadherin or its breakdown products, using the criterion of NCD-2 immunoreactivity. It seems likely that the relative lack of contaminating proteins is due to our use of a detergent with a low micellar size, a rigorous washing protocol during the affinity step, and a high-efficiency, protein A–based antibody affinity column.

N-cadherin can function as a substrate-attached neurite outgrowth-promoting molecule. Previous experiments demonstrated that N-cadherin participates in the regulation of axon growth, but these studies were done with N-cadherin expressed in the complex environment of the nonneuronal cell plasma membrane, in which interactions between N-cadherin and other membrane components could not be controlled. Our results show directly that substrate-bound N-cadherin can induce neurite outgrowth in the absence of "*cis*" interactions with other plasma membrane molecules. The specificity of these results was demonstrated by inhibition with two different specific antibodies. Because the neurons were able to attach to "blocked" nitrocellulose in the absence of N-cadherin, the effect of antibodies on neurite growth could be independently assessed. Further, N-cadherin is not merely a "permissive" substrate in the sense that it induces rapid process growth in a population of neurons (CG neurons) that extend few or no neurites on a variety of substrates to which the neurons quantitatively adhere, including blocked nitrocellulose, collagen, tissue culture plastic, and poly-D-lysine (Tomaselli et al., 1986; Bixby, 1989; present study). Therefore, N-cadherin joins a short list of proteins (LN, L1, and fibronectin) capable of inducing rapid and extensive outgrowth in vitro when used as purified substrates.

N-cadherin can be removed from the plasma membrane while maintaining this adhesion and growth-promoting activity and, like proteins of the ECM, can support adhesion and rapid spreading of nonneuronal cells. Together with the recent identification of a novel, secreted cadherin (Ranscht, B., personal communication), this suggests the possibility that cadherins, like L1 and N-CAM (Martini and Schachner, 1986; Daniloff et al., 1986), may normally be expressed both on cell surfaces and in the ECM. Ultrastructural studies

that would resolve the localization issue have not yet been reported. We presume that the response of CG neurons to substrate-bound N-cadherin is mediated by N-cadherin on the neuronal cell surface, both because N-cadherin is known to function through "homophilic" binding (Takeichi, 1988) and because antibodies recognizing chicken N-cadherin inhibit neurite growth from chick neurons on rat nonneuronal cells, probably by recognizing the neuronal "receptor" (Bixby et al., 1988; Tomaselli et al., 1988).

Until now, LN has been the most potent known substrate-bound inducer of neurite growth. Within the limits of the assay, our results demonstrate that N-cadherin is comparable with LN in potency, and is at least as effective, in the sense that >85% of plated neurons produce neurites on N-cadherin in 15–16 h. Of other tested cell adhesion molecules, L1 appears to be significantly less potent than LN, and N-CAM exhibits only weak outgrowth-promoting activity when used as a purified substrate (Lagenauer and Lemmon, 1986; see Results). Whether the results obtained in vitro accurately reflect the importance of these molecules in vivo is not known. Recent results suggest, for example, that both N-CAM and myelin-associated glycoprotein can be demonstrated to potentiate neurite formation when expressed in heterologous cells (Johnson et al., 1989; Doherty et al., 1989). In addition, results obtained with cell surface proteins removed from the membrane must be viewed with caution. Nevertheless, the high potency and consistent effect of N-cadherin in our experiments seem to indicate an important role for this protein in axonal outgrowth.

The morphology of the neurite outgrowth produced by N-cadherin is distinct from that produced in response to LN. One striking manifestation of this difference is the curvature of the N-cadherin neurites, compared with the straight-line processes seen on substrates of LN, fibronectin, and collagens (e.g., Tomaselli et al., 1986; Bixby, 1989). Obviously, we do not know the mechanisms underlying these differences. We would speculate, however, that both the high degree of branching and the curving nature of N-cadherin neurites could be due to an unusually strong adhesion between neurite and substrate. This could be the result of differences in the number and/or localization of the relevant receptors. Alternatively, there may be differences in the intracellular responses produced by binding of these two adhesive molecules, as opposed to a purely mechanical effect. If this is the case, the ability of cadherins to interact specifically with cytoskeletal elements may be involved (e.g., Nagafuchi and Takeichi, 1988). These suggestions are testable, at least in principle.

CG neurons extend highly branched processes almost without delay when confronted with N-cadherin substrates. This behavior contrasts with that seen on LN and is in fact reminiscent of the response of these neurons to substrate-attached Con A. Our previous results showed that Con A can produce rapid neurite growth from CG neurons, but that this growth is limited in extent, has a different morphology, and seems to reflect a different underlying mechanism from that induced by LN (Bixby, 1989). For example, growth on LN is inhibited by protein kinase C inhibitors, while growth induced by Con A is not. Whether the early component of the N-cadherin response is mechanistically related to the Con A response is unknown, but clearly interesting to test, in view of N-cadherin's apparent physiological relevance (Bixby et

al., 1987, 1988; Tomaselli et al., 1988). One possibility is that N-cadherin induces a complex series of intracellular events sharing components of the LN response and of the Con A response. The availability of an assay using substrate-bound N-cadherin offers the possibility to test these speculations by investigation of receptor-mediated events inside the neuron (Bixby, 1989).

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