Extracellular Matrix Molecules and Cell Adhesion Molecules Induce Neurites through Different Mechanisms

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Abstract. It has recently become clear that both extracellular matrix (ECM) glycoproteins and various cell adhesion molecules (CAMs) can promote neurite outgrowth from primary neurons, though little is known of the intracellular mechanisms through which these signals are transduced. We have previously obtained evidence that protein kinase C function is an important part of the neuronal response to laminin (Bixby, J. L. 1989. *Neuron.* 3:287–297). Because such CAMs as L1 (Lagenauer, C., and V. Lemmon. 1987. *Proc. Natl. Acad. Sci. USA.* 84:7753–7757) and N-cadherin (Bixby, J. L. and R. Zhang. 1990. *J. Cell Biol.* 110:1253–1260) can be purified and used as substrates to promote neurite growth, we have now tested whether the response to CAMs is similarly dependent on protein kinase C. We find that inhibition of protein kinase C inhibits growth on fibronectin or collagen as well as on laminin. In contrast, C kinase inhibition actually potentiates the initial growth response to L1 or N-cadherin. The later "phase" of outgrowth on both of these CAMs is inhibited, however. Additionally, phorbol esters, which have no effect on neurite growth when optimal laminin concentrations are used, potentiate growth even on optimal concentrations of L1 or N-cadherin. The results indicate that different intracellular mechanisms operate during initial process outgrowth on ECM substrates as compared to CAM substrates, and suggest that protein kinase C function is required for continued neurite growth on each of these glycoproteins.

In recent years, substantial progress has been made in the identification both of the molecules in the neuron's environment that regulate the formation of axons, and of the neuronal cell surface receptors that recognize these molecules. Particularly in the case of substrate-attached glycoproteins, however, our understanding of the intracellular events that result in the transduction of these growth signals has not kept pace. An understanding of these transduction events would clearly give important clues to the mechanisms underlying axon growth.

The substrate factors that are known to induce axonal growth include macromolecules of the extracellular matrix (ECM),[1] such as laminin (LN) and fibronectin (Akers et al., 1981; Manthorpe et al., 1983; Lander et al., 1985) and membrane-bound cell adhesion molecules (CAMs) (Jessel, 1988; Reichardt et al., 1989). The list of CAMs implicated in the induction of axon growth includes members of the immunoglobulin superfamily (NCAM, L1/8D9, F11/contactin, neurofascin, TAG-1, and MAG) and the calcium-dependent CAM, N-cadherin (Bixby et al., 1987; Doherty et al., 1989; Moos et al., 1988; Chang et al., 1987; Lagenauer and Lemmon; 1987; Brummendorf et al., 1989; Ranscht, 1988; Rathjen et al., 1987; Furley et al., 1990; Johnson et al., 1989; Matsunaga et al., 1988). Some of these proteins have been directly demonstrated to promote neurite growth as purified substrates (Lagenauer and Lemmon, 1987; Bixby and Zhang, 1990; Furley et al., 1990). Neuronal receptors for each of these three classes of molecule have been identified. Although numerous putative receptors for ECM macromolecules have been described, those that have been shown to be involved in axon growth are members of the β1 family of integrin receptor heterodimers (e.g., Tomaselli et al., 1986; Bozyczko and Horwitz, 1986). The relevant receptors for the cadherins and immunoglobulin superfamily members are likely to be the proteins themselves, in a "homophilic" interaction (Edelman, 1985; Lemmon et al., 1989; Bixby et al., 1988; Takeichi, 1988; Doherty et al., 1990*a*, *b*).

The transduction of neuronal growth signals by integrins and CAMs is poorly understood. For soluble neurite outgrowth-promoting agents, such as nerve growth factor and fibroblast growth factor, pathways involving tyrosine kinases, cAMP, and protein kinase C have all been implicated in the cellular response (e.g., Cremins et al., 1986; Maher, 1988; Damon et al., 1990). The issue is further complicated by the evidence that different effectors of neurite growth may act through different pathways (Koizumi et al., 1988; Damon et al., 1990). Both the integrin receptors and the CAMs cited above can have extensive intracellular domains, and are likely to be directly involved in the process of signal transduction. In the case of integrins and those immunoglobulin superfamily members that have been studied, this transduc-

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; ECM, extracellular matrix; LN, laminin.

tion may involve activation of protein kinase C, among other putative signals (Banga et al., 1986; Burn et al., 1988; Cambier and Ransom, 1987; Weiss and Imboden, 1987). However, the relationship of these findings to the induction of axon growth remains to be determined. Recent evidence indicates that protein kinase C function is necessary for the neuronal response to LN, and suggests that induction of neurite outgrowth by LN involves activation of protein kinase C (Bixby, 1989). Because ECM proteins and CAMs act in concert to induce process growth from neurons cultured on non-neuronal cells, we have speculated that these different classes of signal operate through common intracellular pathways (Bixby et al., 1988). In the present paper we test this hypothesis by asking whether the induction of neurite outgrowth by L1 and N-cadherin, like that of LN, depends on the function of protein kinase C.

Materials and Methods

Materials

Fertile White Leghorn chicken eggs were obtained from SPAFAS, Inc. (Norwich, CT), and incubated at 38°C. LN, fibronectin, and type IV collagen were purified as described (Bixby, 1989), and some of the LN was the gift of Deborah Hall (Athena Neurosciences, S. San Francisco, CA). The 1A6 (anti-NCAM), 8D9 (anti-L1), and NCD-2 (anti-N-cadherin) antibodies were the kind gifts of David Gottlieb (Washington University, St. Louis), Vance Lemmon (Case Western Reserve, Cleveland), and Masatoshi Takeichi (Kyoto University, Japan), respectively. Ascites fluid containing these antibodies was generated either in BALB/c mice (1A6, 8D9) or in nude mice (NCD-2). Nitrocellulose (BA83) was from Schleicher & Schuell, Inc. (Keene, NH), protein A-Sepharose and protein G-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ), and H7 and HA1004 were from Seikagaku America (St. Petersburg, FL). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Methods

Purification of CAMs. High-efficiency antibody affinity columns of 1A6 and 8D9 antibodies were prepared essentially as described by Schneider (1982) and Bixby and Zhang (1990). About 1–2 mg of antibody per ml of protein A- or protein G-Sepharose were coupled. A crude membrane fraction was prepared from embryonic day 17 chick embryos as described (Bixby and Reichardt, 1985), and was extracted with 0.5% CHAPS for 45 min at 4°C. The extract was centrifuged at 35,000 *g* for 40 min., and the supernatant run over the appropriate affinity column overnight at 4°C. Washing and elution were as described (Bixby and Zhang, 1990), except that the buffers contained 0.5% CHAPS instead of deoxycholate. Eluted, neutralized antigens were lyophilized and stored at −80°C. N-cadherin was isolated as described (Bixby and Zhang, 1990), and SDS-PAGE protocols have been described (Bixby and Reichardt, 1985).

Cell Culture. Substrates of various glycoproteins on nitrocellulose-coated 96-well plates were prepared as in Bixby and Zhang (1990), with NCAM and L1 being treated in the same way as N-cadherin, and FN and collagen being treated as for LN. Preparation of ciliary ganglion cultures and treatment with H7 have been described (Bixby and Reichardt, 1985; Bixby, 1989). Cultures were fixed after 6–18 h in vitro and analyzed as discussed previously (Bixby, 1989), except that neurite length measurements were made with the aid of a digital micrometer (VIA 100; Boeckeler Instruments, Tucson, AZ), after calibration with a stage micrometer. For percentage measurements, at least 150 and usually 200 neurons per condition were counted. Statistical tests used include Student's *t* test, and the "d" test for comparison of two percentages (Bailey, 1981).

Results

Purification of CAMs

In previous work, we described an affinity purification procedure for isolating N-cadherin from brain membranes (Bixby and Zhang, 1990). We modified this procedure to isolate two other CAMs from chick brain (L1 and NCAM) using the anti-L1 mAb 8D9 (Lemmon and McCloon, 1986), and the anti-NCAM mAb 1A6 (Lemmon et al., 1982). The 8D9 antigen, which we call L1, is a member of a group of related molecules that includes NILE, L1, NgCAM, 8D9, and G4 (see Lemmon et al., 1989). The antibodies we used have been shown to be useful for affinity purification of their respective antigens (Lemmon and McCloon, 1986). Fig. 1 is a silver-stained gel of the purified CAMs. The 1A6 antibody isolated the characteristic 180-, 140-, and 120-kD NCAM species, as well as a smear of proteins from 180–250 kD, representing the highly polysialated species present in embryonic brain (Rothbard et al., 1982). The 8D9 antibody bound the 190-, 135-, and 80-kD species characteristic of L1 (Lemmon and McCloon, 1986). Both antigens were contaminated with proteins of lower molecular weight, but these were a low percentage of total protein, based on staining both with silver and with Coomassie blue (not shown). These results demonstrate that our procedure allows the isolation of highly enriched preparations of L1 and NCAM.

Purified L1 Promotes Outgrowth from CG Neurons

Lagenauer and Lemmon devised an innovative technique for preparing substrates of membrane proteins. They have used this technique to demonstrate that purified L1, at a concentration of 100 μg/ml, promotes extensive neurite growth from mouse cerebellar and chick tectal neurons (Lagenauer and Lemmon, 1987; Lemmon et al., 1989; see also Kadmon et al., 1990). We found that L1 also induced outgrowth from chick CG neurons (Fig. 2 *A*), though not as effectively as for the central neurons. When CG neurons were cultured for 6–8 h on L1 (100 μg/ml), the percentage with neurites averaged 16 ± 4% (n = 10). No neurites grew on nitrocellulose blocked with serum proteins or BSA over this time period. A concentration of 50 μg/ml L1 induced neurites slightly but significantly better than "blocked" nitrocellulose, and no growth was seen when concentrations of 20 μg/ml or less

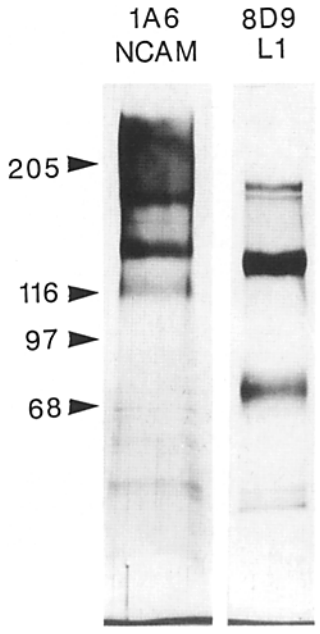

Figure 1. SDS gel of purified NCAM and L1. Approximately 1 μg of NCAM (from 1A6 column, left lane) or L1 (from 8D9 column, right lane) was loaded. The gel was stained with silver. Arrowheads to the left of the NCAM lane indicate the positions of molecular mass standards. The 180-, 140-, and 120-kD NCAM bands are clearly visible, as well as a smear from 180–240 kD that represents the embryonic form. In the L1 lane, the 190-, 135-, and 80-kD forms are all evident. Lower molecular weight contaminants can be seen in both preparations.

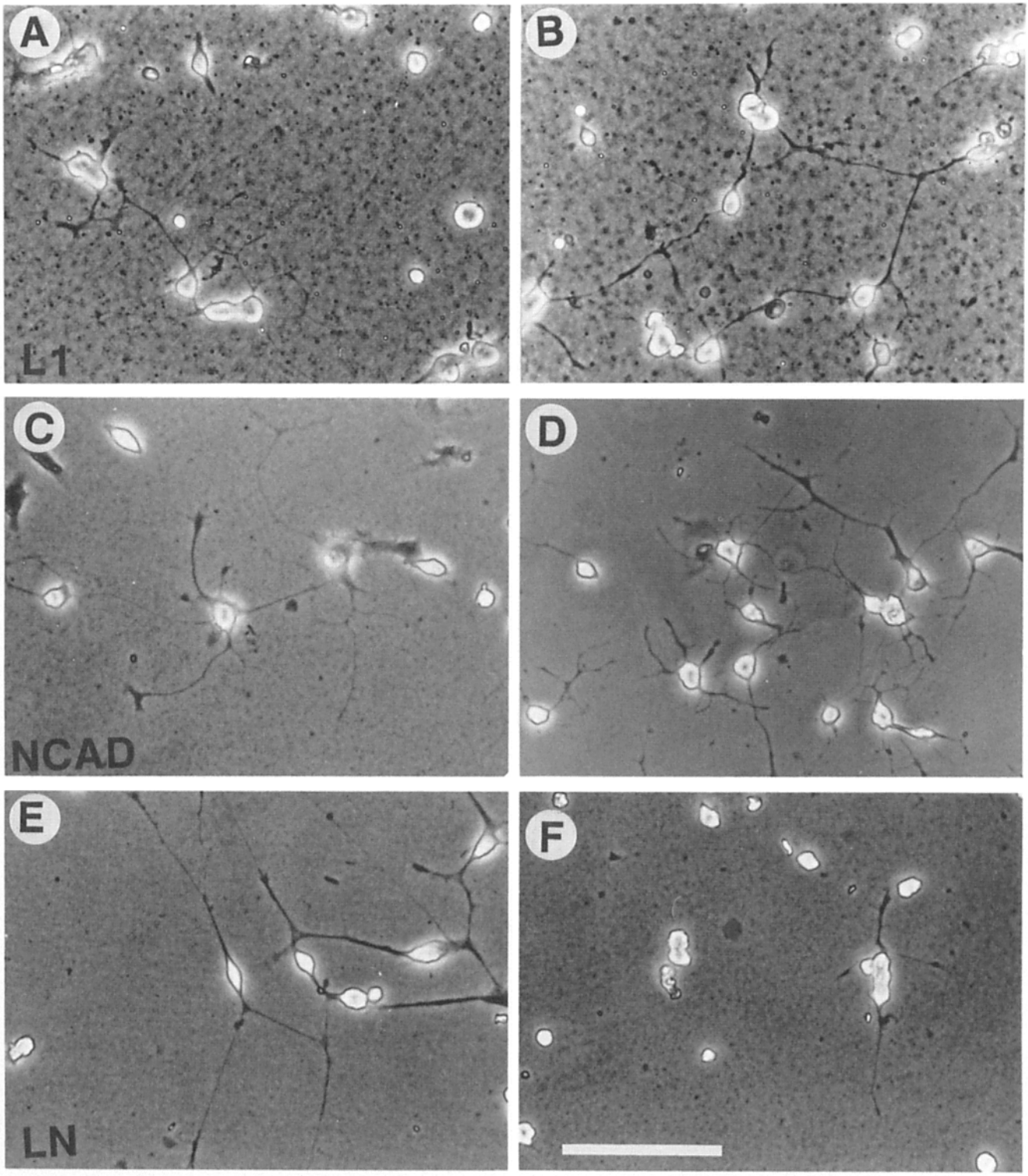

Figure 2. Phase-contrast photomicrographs of CG neurons grown on various substrates in the presence and absence of H7. Neurons were cultured for 8 h on 100 μg/ml L1 (*A* and *B*), 25 μg/ml N-cadherin (*C* and *D*), or 50 μg/ml LN (*E* and *F*), in control medium (*A*, *C*, and *E*) or in medium containing 100 μM H7 (*B*, *D*, and *F*). Process outgrowth is potentiated by H7 on L1 and N-cadherin, but is strongly inhibited on LN. The LN/H7 field is not representative, because most fields had no neurons with neurites, but is shown to illustrate that neurite lengths are also decreased. Bar, 50 μM.

were used. This dose-response relationship is similar to that obtained for L1 by Lagenauer and Lemmon (1987). In contrast to these results, purified NCAM (80–100 μg/ml), when used as a substrate for CG neurons, produced growth above control levels in only three of eight experiments. These results are consistent with those of Lagenauer and Lemmon (1987), who found NCAM-induced growth in two of four experiments with chick central neurons. Because NCAM is

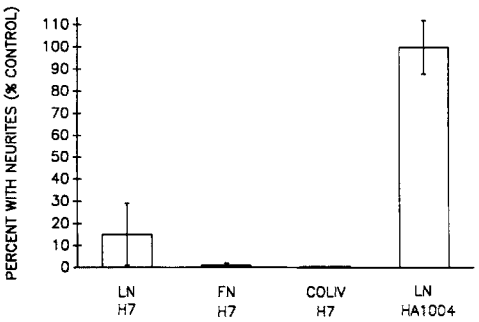

Figure 3. Effects of H7 and HA1004 on neurite outgrowth by CG neurons on ECM substrates. The percentage of neurons with neurites (as a percentage of the control value; mean ± SEM) is given for neurons cultured on 10–50 μg/ml LN, 40 μg/ml fibronectin (*FN*), or 10 μg/ml collagen type IV (*COLIV*) in the presence of 100 μg/ml H7, or HA1004 (*extreme right-hand bar*). For FN and COLIV, neurons were grown for 16 h. For LN, neurons were grown for 5–18 h. The mean percentages of neurons with neurites for controls were LN/H7, 58 ± 6% (n = 18); FN, 62 ± 12% (n = 4); COLIV, 24 ± 10% (n = 4); LN/HA1004, 65 ± 6% (n = 7). H7 strongly inhibits process outgrowth on all three ECM substrates, while HA1004 has no significant effect on LN-induced growth.

clearly capable of participating in the induction of neurite growth when present in intact cell membranes (Bixby et al., 1987; Doherty et al., 1989), these results suggest that a specific conformation and/or association with other molecules is important for NCAM function.

Inhibition of C Kinase Inhibits Growth on ECM Proteins, but Stimulates Growth on CAMs

Inhibitors of protein kinase C (H7, sphingosine) inhibit CG neuron process outgrowth on LN substrates, and phorbol esters potentiate growth on suboptimal, but not optimal, LN substrates (Bixby, 1989). These results suggest that LN normally activates C kinase in CG neurons, and that this activation is required for induction of neurite growth. Other ECM proteins (fibronectin, collagen IV), like LN, act through integrin receptors (Hynes, 1987), and can promote neurite growth, though not very effectively in the case of CG neurons (e.g., Tomaselli et al., 1986). To test whether C kinase is also required for these responses, we plated CG neurons on optimal concentrations of collagen IV and fibronectin, in the presence and absence of H7. H7 completely blocked growth on these two ECM substrates (Fig. 3). Our results suggest that all three ECM proteins require the function of neuronal C kinase to promote process outgrowth.

Since H7 also inhibits cyclic nucleotide-dependent kinases (though not as well as C kinase; Hidaka et al., 1984), this conclusion would be strengthened if the inhibitory effect on neurite growth could be linked specifically to inhibition of C kinase. This can be done using the related drug HA1004, which is a more potent inhibitor of the cyclic nucleotide-dependent kinases than H7, but blocks C kinase relatively poorly (Hidaka et al., 1984). Fig. 3 shows that HA1004, at concentrations equal to those used previously (and in this study) for H7, has no effect on CG neuron outgrowth on LN. Fig. 3 also demonstrates that H7 strongly inhibits process growth on LN, even when concentrations of 10–50 μg/ml LN are used to coat nitrocellulose substrates (see also Fig. 2, *E* and *F*).

The availability of purified CAMs that induce neurite growth allows a test of the generality of this C kinase result. H7 does not prevent the growth of neurites induced by Con A in CG neurons, suggesting that C kinase function is not required for the cytoskeletal rearrangements accompanying axon growth (Bixby, 1989). Because the physiological relevance of concanavalin A is not clear, however, it is important to test other "natural" growth inducers. Fig. 4 illustrates the results of H7 treatment when CG neurons are grown on N-cadherin or L1 substrates (see also Fig. 2, *B* and *D*). The effects of H7 on these substrates were dramatically different from those seen with ECM proteins. H7 increased the percentage of neurons with neurites, either on N-cadherin or on L1. This effect was more pronounced with L1 substrates than with N-cadherin. Fig. 4 also demonstrates the effects of HA1004 on CG neurons grown on N-cadherin or L1 substrates. For both CAMs, HA1004 tended to increase the percentage of neurons with neurites, but not to the same extent as H7. These results suggest that inhibition, both of protein kinase C and of cyclic nucleotide-dependent kinases, can augment process outgrowth on CAMs. Both the larger effect of the drugs on L1 substrates and the larger scatter in the data (compared to N-cadherin) are likely to reflect the relatively poor and variable response seen with L1 in control conditions.

As noted above, the response of CG neurons to affinity-purified NCAM was extremely weak and inconsistent. However, the trend in these data was similar to that seen for the other two CAMs. In three experiments, the mean percentage of neurons with neurites on NCAM was 1.7 ± 1.1% for controls, and 12 ± 3% for H7-treated cultures. This can be compared with BSA-coated wells in the same experiments, for which growth was minimal and not significantly different in the two conditions (mean 1 ± 0.6%, controls; 2.7 ± 1.8%, H7-treated). Therefore, although these were among the experiments in which NCAM did not promote neurite growth

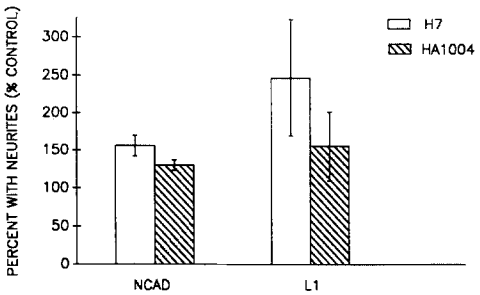

Figure 4. Effects of H7 and HA1004 on neurite outgrowth by CG neurons on L1 and N-cadherin substrates. The percentage of neurons with neurites (as a percentage of the control value; mean ± SEM) is given for neurons cultured on 5–10 μg/ml N-cadherin (*NCAD*) or 80–100 μg/ml L1, in the presence of 100 μg/ml H7 (*open bars*) or 100 μg/ml HA1004 (*hatched bars*). The mean percentages of neurons with neurites in the controls were NCAD, 51 ± 7% (n = 10, H7; n = 4, HA1004); L1, 30 ± 8% (n = 9, H7; n = 4, HA1004). Both H7 and HA1004 produced a potentiation of neurite growth ($p < 0.001$ for all except L1/HA1004, for which $p < 0.05$). Cultures were grown for 6–16 h.

Table I. H7 Effects at Early and Late Times on Various Substrates

Substrate	% with neurites (% of control)	
	6–8 h	15–18 h
Ncadherin	174 ± 14 (7)	114 ± 9 (3)
L1	317 ± 109 (6)	106 ± 20 (3)
LN	22 ± 13 (11)	5 ± 6 (7)

For each condition, the percentage of neurons with neurites in the H7-treated cultures were expressed as a percentage of the control result, then averaged (mean ± SEM). Data were grouped according to time in culture. The number of experiments for each condition is given in parentheses following the averages. The percentages of neurons with neurites for controls were LN 6–8, 58%; LN 15–18, 58%; N-cadherin 6–8, 48%; N-cadherin 15–18, 66%; L1 6–8, 19%; L1 15–18, 46%.

significantly better than BSA in control conditions, a neurite-promoting effect of NCAM was "unmasked" in the presence of H7.

The Effect of H7 on CAM-induced Outgrowth Differs at Early and Late Times

If experiments performed with H7 are segregated according to time in vitro, it becomes apparent that the potentiation of process growth by H7 was a transient phenomenon. The percentage of neurons with neurites was nearly doubled on N-cadherin substrates, and tripled on L1 substrates, when early (6–8 h) cultures were considered (Table I, see Fig. 2). In contrast, H7-treated cultures were essentially identical to controls in this regard by 15–18 h in vitro. The inhibition of outgrowth by H7 on LN substrates was consistent, and seen both at early and at late times in culture (Table I). There was a tendency for the inhibition to be more marked at later times, but this tendency did not reach statistical significance.

Examination of cultures grown for 6–8 h in the presence of H7 suggested that the stimulatory effect seen on CAM substrates was solely on the number of neurons with neurites (neurite initiation), and not on the length of individual neurites. To verify this quantitatively, we measured the length of neurites for representative cultures. There were no differences between control and H7-treated cultures in the number or length of neurites per cell, when assayed at these early times in vitro (Table II). This was true both for L1 and for N-cadherin. In contrast, the inhibitory effect of H7 on LN-induced neurite growth was manifest both in the percentage of cells with neurites and in the length and number of neurites that did grow (Table II). That is, the small percentage of neurons that grew neurites on LN in the presence of H7 had shorter and fewer neurites than in the control cells.

As noted above, the potentiation of neurite growth by H7 (an increase in the number of neurite-bearing cells) was no longer evident at late times in culture. Although the number of neurons with neurites was not different from controls in these cultures, the length of neurites was actually reduced by H7 over this time period. This was consistently observed, but normally not quantified. In one experiment neurite lengths were measured for the case of N-cadherin substrates. H7 decreased the lengths of individual neurites and the total amount of process growth per cell by ~50% (Table II). The number of neurites per cell did not differ from controls. The decrease in neurite lengths relative to controls may reflect a retraction of neurites caused by H7, or a cessation of neurite growth. In this regard it may be significant that the lengths of neuronal processes in the 16-h H7-treated culture were similar to that found for the 8-h control (or treated) cultures. Although we did not measure neurites in long-term L1 cultures, the qualitative impression was similar to that seen with N-cadherin.

In summary, the effect of C kinase inhibition on CAM-induced process outgrowth is complex. At early times (6–8 h in vitro), the number of neurons with neurites is increased, but the length of individual neurites is unaffected. Later (15–18 h in vitro), the same percentage of cells have neurites as in the controls, but these neurites are significantly shorter, as though growth had been inhibited beyond a certain point. This contrasts with the results seen with ECM substrates, where H7 inhibits all aspects of process outgrowth at all times examined.

Table II. Neurite Length Measurements

Substrate	Condition	h	Average neurite length	Average neurites/neuron	Average total neurite length	Neurons with neurites
						%
Ncadherin	Control	8*	52 ± 3.3	2.6 ± 0.28	133 ± 15	53
(5 μg/ml)	H7		47 ± 3.9	2.8 ± 0.31	131 ± 15	89‖
	Control	16	95 ± 7	3.2 ± 0.25	310 ± 31	52
	H7		55 ± 6‖	2.9 ± 0.22	158 ± 14‖	65§
Laminin	Control	8*	74 ± 5	2.9 ± 0.2	215 ± 19	63
(10 μg/ml)	H7		63 ± 5‡	1.4 ± 0.13‖	90 ± 11‖	10‖
L1	Control	8	44 ± 3	2.9 ± 0.31	127 ± 22	50
(100 μg/ml)	H7		46 ± 3	2.5 ± 0.25	115 ± 15	67§

Neurites were measured for a minimum of 16 neurons per condition, taken from duplicate wells in a single experiment except where noted (8 h LN, N-cadherin), when two such experiments were examined. For each condition, the average length of individual neurites, the average number of neurites per neuron, and the average total neuritic length, for those neurons that have neurites, are shown (mean ± SEM). The final column gives the percentage of neurons with neurites in the cultures measured. Figures that are significantly different from control values are marked.

* Data combined from two experiments.

‡ $p < 0.05$.

§ $p < 0.01$.

‖ $p < 0.001$.

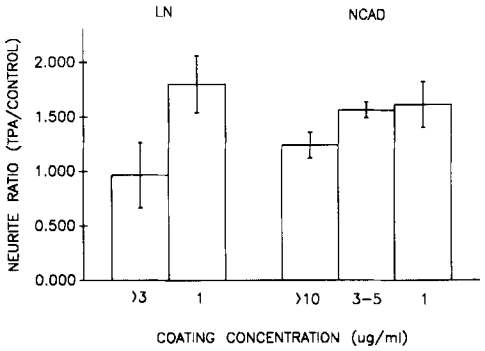

Figure 5. Effects of TPA (100 ng/ml) on neurite growth by CG neurons on LN and N-cadherin substrates. The neurite ratio (percentage with neurites in TPA/percentage with neurites in control) is given for various concentrations of LN (1 μg/ml or 5–50 μg/ml) or N-cadherin (*NCAD*, 1 μg/ml, 3–5 μg/ml, or 10–30 μg/ml). The mean percentages of neurons with neurites in the controls were LN >3, 72 ± 6% (n = 11); LN 1, 28 ± 11% (n = 6); NCAD >10, 54 ± 4% (n = 4); NCAD 3–5, 44 ± 4% (n = 3); NCAD 1, 39 ± 7% (n = 4). Neurons were grown on LN for 8 h and on N-cadherin for 6–7 h. Some of the LN data are from Bixby (1989). Growth is enhanced at 1 μg/ml LN ($p < 0.001$), and at all concentrations of N-cadherin ($p < 0.01$, 1 μg/ml, 3–5 μg/ml; $p = 0.05$, >10 μg/ml).

Phorbol Esters Potentiate Growth on Both Low and High CAM Concentrations

In previous experiments we noted that the phorbol ester TPA potentiated neurite growth on suboptimal concentrations of LN, but not on optimal concentrations (Bixby, 1989). These data are replotted (with some additional data) on the left-hand side of Fig. 5, and show that growth on 1 μg/ml LN was increased by 50% with TPA treatment, while no effect was seen when LN concentrations of >3 μg/ml were used (range 5–50 μg/ml). One interpretation of these results is that optimal LN concentrations activate C kinase to the level appropriate for rapid process outgrowth, so that further activation cannot increase growth. In contrast, the lack of inhibition of N-cadherin- or L1-induced process growth by H7 (at 6–8 h in vitro) suggests that activation of C kinase is not part of the initial response to these CAMs. If this is so, TPA might be expected to potentiate outgrowth on even high concentrations of CAMs, by activating a "parallel" pathway. Fig. 5 shows that this prediction is borne out. When neurons were grown for 6–7 h on N-cadherin, TPA (100 ng/ml) led to a significant increase in the number of neurons with neurites, for all N-cadherin concentrations from 1 to 30 μg/ml. To compare the LN and N-cadherin results, it should be noted that the dose-response curves for the two proteins are quite similar in our hands (Bixby and Zhang, 1990). Therefore, while TPA does not potentiate growth on optimal LN concentrations, it does so on optimal concentrations of N-cadherin.

TPA also potentiates growth on optimal concentrations of L1. When L1 was used at 100 μg/ml, TPA increased neurite growth to an average of 616 ± 270% of the control percentages (n = 3). It is difficult to compare these data directly to those obtained with LN and N-cadherin, because the control response to L1 was meager and variable (average percentage with neurites in controls, 7 ± 4%). Nevertheless, this concentration of L1 is likely to be optimal for growth (Lagenauer and Lemmon, 1987), so these data are consistent with the N-cadherin results. In two experiments, TPA did not lead to process growth on NCAM substrates (controls in these experiments were not different from BSA-blocked nitrocellulose). This agrees with previous results, which showed that activation of C kinase with TPA is not in itself sufficient to induce neurite growth in CG neurons (Bixby, 1989). In summary, TPA potentiates neurite growth on optimal CAM concentrations, in contrast to the results with LN substrates, and consistent with the H7 results.

Discussion

The major conclusion from these experiments is that ECM glycoproteins and some CAMs appear to differ in the mechanisms through which they induce process outgrowth, at least in the case of CG neurons. This, in turn, suggests that the receptors involved do not transduce signals in the same way. There is indirect evidence that the relevant receptors for ECM proteins are integrins (Hynes, 1987; Tomaselli et al., 1986) and those recognizing exogenous L1, NCAM, and N-cadherin are the cognate molecules in the neuronal membrane (Grumet and Edelman, 1988; Lemmon et al., 1989; Bixby et al., 1989; Takeichi, 1988; Doherty et al., 1990*a*). The study of signal transduction through these receptors is in its infancy. Integrins appear to interact both with protein kinase C and with particular elements of the cytoskeleton (Horwitz et al., 1986; Banga et al., 1986; Burn et al., 1988; Bixby, 1989). Little is known about signaling through CAMs, though one study using antibodies to trigger receptors is consistent with the involvement of protein kinase C and intracellular calcium (Schuch et al., 1989). Our results suggest that, though the pathways used by these various classes of receptor must converge at some level, the pathways themselves are distinct.

Together with the results of a previous study (Bixby, 1989), the present results indicate that protein kinase C function is necessary for neurite induction by ECM proteins, and suggest that these proteins normally activate C kinase as part of their signaling pathway. The earlier study demonstrated inhibition of the LN response by both H7 and sphingosine, a lipid inhibitor of C kinase. We extended these results to show inhibition of growth induced by FN and collagen IV. The specificity of the H7 result was confirmed in the present experiments using HA1004. This is important because sphingosine may not be a specific inhibitor of C kinase (e.g., Winicove and Gershengorn, 1988; J. L. Bixby, unpublished results). In contrast to the results with LN and other ECM proteins, the lack of inhibition by H7 of neurite growth on L1 and N-cadherin suggests that C kinase is not required for the initial response to these CAMs. However, growth does not continue at control levels for periods of 16–18 h. This could be explained by a biphasic growth signal, in which an initial phase not dependent on C kinase gives way to a later, C kinase–dependent phase. A somewhat similar suggestion has been made for the case of neurite growth induced by nerve growth factor in PC12 cells (Damon et al., 1990). Definitive evidence that any of these substrate molecules can activate C kinase can be obtained only with direct measurements of enzyme activity.

When CG neurons were grown on L1 or N-cadherin sub-

strates, H7 not only failed to inhibit growth, but actually increased the percentage of neurons with processes. We cannot fully explain this paradoxical effect. Because potentiation of neurite growth also occurred on these substrates with HA1004, the effect may be at least partially explained by inhibition of cAMP-dependent protein kinase (A kinase). Increasing intracellular cAMP (which activates A kinase) has been shown to be inhibitory to neurite growth in a number of neuronal types (Mattson et al., 1988; Lankford et al., 1988), including chick CG neurons (Bixby, 1989), and inhibiting this kinase (with H7 or HA1004) could have the opposite effect. However, the greater effect seen with H7 compared with HA1004 suggests that C kinase inhibition can also potentiate growth on CAMs. C kinase is clearly a control point for a variety of intracellular signalling events, and it may simply be that complete inhibition of this enzyme is too crude a manipulation to enable a complete dissection of its action.

Activation of C kinase with the phorbol ester TPA potentiates the CG neuron growth response on both high and low concentrations of L1 or N-cadherin. When the same neurons are grown on LN, TPA has an effect only on suboptimal concentrations of the substrate. Our interpretation of these results is that activation of C kinase is a part of the initial growth response to LN, but not to the CAMs. Therefore, N-cadherin (for example) plus TPA could be functionally similar to a combination of N-cadherin and LN. The possibility of synergism among these different substrate-bound growth factors may be physiologically relevant, since they appear to operate together on non-neuronal cell surfaces (Bixby et al., 1987, 1988; Tomaselli et al., 1988). In any case, the potentiation by TPA of neurite growth on CAMs would seem to rule out the possibility that the effect of TPA is solely to recruit or "unmask" the LN receptor, as has been suggested for the LFA1 integrin receptor on lymphocytes (Dustin and Springer, 1989).

Our findings with CG neurons grown on L1 extend the results of Lagenauer and Lemmon (Lagenauer and Lemmon, 1987; Lemmon et al., 1989), who showed that purified L1 can promote neurite growth from chick tectal neurons, and from chick and mouse cerebellar neurons. Although the potency of our purified L1 was similar, the efficacy of L1 for CG neurons was much lower than for the central neurons tested previously. In preliminary experiments, we have found that chick forebrain neurons (Kligman, 1982) also respond much better to L1 than do the CG neurons (our unpublished results). The general conclusion is that different populations of neurons will respond differentially to substrate-bound growth factors; and more specifically, central neurons may be more responsive to L1 than peripheral neurons.

Purified NCAM did not support consistent neurite growth from CG neurons. This negative result is in accord with those obtained by other workers using different neurons (Lagenauer and Lemmon, 1987; Kadmon et al., 1990). The particular form of NCAM used may not be critical since both embryonic and adult forms, of all three major types, have proven relatively ineffective when purified (Kadmon et al., 1990; present study). Nevertheless, various forms of NCAM can be shown to be involved in the induction of neurite growth, when present on cell surfaces, and CG neurons are among those that respond (Bixby et al., 1987; Neugebauer et al., 1988; Doherty et al., 1989, 1990*a*, *b*). Although it is possible that NCAM, unlike L1 or N-cadherin, irreversibly loses its activity when purified, we favor the explanation that NCAM acts in concert with other cell surface glycoproteins, rather than as an isolated molecule. For example, it could be that NCAM is brought into a functional conformation by interaction with other surface molecules. Work from Schachner's laboratory on adhesion to combinations of L1 and NCAM is consistent with this idea (Kadmon et al., 1990). Although isolated NCAM can clearly be effective in simple binding assays (e.g., Edelman, 1985), other molecules may be important for the full expression of its function.

In summary, our results suggest that ECM proteins and CAMs, through their distinct neuronal receptors, achieve a similar result (neurite growth) through different pathways. Although L1 and N-cadherin are representative of quite different CAM families, their mechanisms of action were not distinguished by our assay. Very little is known about the signaling mechanisms employed by these proteins, however, and future experiments may well uncover important differences in this regard.

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