Tyrosine Kinase Inhibitors Can Differentially Inhibit Integrin-dependent and CAM-stimulated Neurite Outgrowth

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Abstract. We have used monolayers of parental 3T3 cells and 3T3 cells expressing one of three transfected cell adhesion molecules (CAMs) (NCAM, N-cadherin, and L1) as a culture substrate for rat cerebellar neurons. A number of tyrosine kinase inhibitors have been tested for their ability to inhibit neurite outgrowth over parental 3T3 monolayers which we show to be partly dependent on neuronal integrin receptor function, as compared with neurite outgrowth stimulated by the above three CAMs. Whereas genistein (100 μM), lavendustin A (20 μM), and tyrphostins 34 and 47 (both at 150 μM) had no effect on integrin dependent or CAM stimulated neurite outgrowth, the erbstatin analogue (10–15 μg/ml) and tyrphostins 23 and 25 (both at 150 μM) specifically inhibited the response stimulated by all three CAMs. CAM stimulated neurite outgrowth can be accounted for by a G-protein-dependent activation of neuronal calcium channels; experiments with agents that directly activate this pathway localized the erbstatin analogue site of action upstream of the G-protein and calcium channels, whereas tyrphostins have sites of action downstream from calcium channel activation. These data suggest that activation of an erbstatin sensitive tyrosine kinase is an important step upstream of calcium channel activation in the second messenger pathway underlying the neurite outgrowth response stimulated by a variety of CAMs, and that this kinase is not required for integrin-dependent neurite outgrowth.

During development the majority of neurons migrate to their final destination where they establish appropriate patterns of synaptic connections and complete the elaboration of axonal and dendritic processes (Jacobson, 1992). The molecular basis of pathway selection and target recognition is obviously complex and is likely to involve the interaction of a panoply of factors with receptor molecules present on the neuronal growth cone. In this context, contact of the growth cone with other cells or with the extracellular matrix can directly stimulate axonal growth with integrin receptors and cell adhesion molecules (CAMs)[1] mediating such interactions (for review see Bixby and Harris, 1991; Reichardt and Tomaselli, 1991; Doherty and Walsh, 1992). Recent studies on CAMs suggest that their ability to activate a neuronal second messenger pathway might be the basis whereby they stimulate axonal growth (Doherty et al., 1991*a*; Saffell et al., 1992; Williams et al., 1992).

Whereas the transmembrane signaling pathways that mediate the action of soluble trophic factors such as the nerve growth factor (NGF) are relatively well characterized (for example see Hempstead et al., 1992), those that mediate the actions of integrins and CAMs remain poorly understood. The binding of antibodies or soluble ligands to integrins and CAMs can trigger the activation of a number of intracellular messenger pathways. For example a prominent response that follows the clustering of integrin receptors is increased tyrosine phosphorylation of a 125-kD protein tyrosine kinase called FAK (for review see Zachary and Rozengurt, 1992; Juliano and Haskill, 1993). Also a link between CAMs and tyrosine phosphorylation is suggested by the observation that the binding of antibodies to NCAM or L1 (but not N-cadherin), or indeed the addition of soluble NCAM or L1 to a growth cone–enriched fraction is associated with a substantial reduction in phosphotyrosine on tubulin, possibly consequential to an inhibition of the *c-src* tyrosine kinase (Atashi et al., 1992). However the same antibodies can modulate a wide variety of other second messenger pathways in neuronal cells including protein kinase A, protein kinase C, heterotrimeric G-protein activity and levels of intracellular calcium and internal pH (Schuch et al., 1989; von Bohlen und Halbach et al., 1992). As the triggering mechanisms are non-physiological, and as there is considerable cross-talk between second messenger pathways, more direct assays are required to identify the key messengers that transduce functionally relevant information.

Antibody perturbation experiments have established the importance of NCAM, N-cadherin and L1 for cell-contact dependent axonal growth both in vitro (for example see Bixby et al., 1987; Neugebauer et al., 1988; Seilheimer and

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1. *Abbreviation used in this paper*: CAM, cell adhesion molecule.

Schachner, 1988) and in the case of NCAM also in vivo (Landmesser et al., 1990). The neurite outgrowth promoting activity of individual CAMs can be directly studied by culturing neurons on confluent monolayers of 3T3 fibroblasts that stably express (after transfection) physiological levels of human NCAM (Doherty et al., 1990), chick N-cadherin (Doherty et al., 1991*b*), or human L1 (Williams et al., 1992). Recent studies have shown that the activation of a pertussis toxin sensitive pathway that culminates in calcium influx into neurons is both necessary and sufficient to account for the ability of all three CAMs to stimulate neurite outgrowth from primary neurons and the PC12 neuronal cell line (for review see Doherty and Walsh, 1992). This pathway is specific for CAMs in that integrin, NGF and cyclic AMP (cAMP) dependent neurite outgrowth is not inhibited by pertussis toxin or calcium channel antagonists.

A number of observations suggest that receptor and non-receptor tyrosine kinases might play an important role in axonal growth. Soluble trophic factors such as NGF and FGF function by binding to receptors that have tyrosine kinase domains (Chao et al., 1992). Also non-receptor tyrosine kinases such as *c-src* can be localized to the growth cone and are expressed at high levels during periods of axonal growth and regeneration (Maness et al., 1988; Le Beau et al., 1991). In fact the transforming oncogene *v-src* can directly induce neurite outgrowth from PC12 cells (Cox and Maness, 1991). A possible involvement of non-receptor tyrosine kinases in the CAM signaling pathway is suggested by interaction of the CAM fasciclin 1 and the abelson (abl) tyrosine kinase in pathway finding in *Drosophila* as revealed by genetic analysis (Elkins et al., 1990).

In the present study we have tested the effects of a large number of tyrosine kinase inhibitors on neurite outgrowth stimulated by NCAM, N-cadherin and L1. Our results show that the erbstatin analogue (Umezawa et al., 1990) can inhibit neurite outgrowth stimulated by all three CAMs at concentrations that do not affect neurite outgrowth stimulated by extracellular matrix components. This agent did not inhibit neurite outgrowth induced by direct activation of the CAM pathway at the level of G-protein activation or calcium influx into neurons. Overall the results suggest that a variety of cell adhesion molecules stimulate neurite outgrowth by activating an erbstatin sensitive tyrosine kinase that acts upstream of a pertussis toxin sensitive G-protein and calcium channel activation.

Materials and Methods

Cell Culture and Neurite Outgrowth

Rat cerebellar neurons isolated at PND4 were cultured for ~20 h on confluent monolayers of parental 3T3 cells or clones of 3T3 cells expressing the 140-kD isoform of NCAM, N-cadherin or L1 (Williams et al., 1992). Co-cultures were established by seeding 3,000 cerebellar neurons onto confluent monolayers of parental or transfected 3T3 cells established in individual chambers of eight chamber Lab-tek slides (Lab-tek, Naperville, IL). The co-culture media was SATO supplemented with 2% FCS with Ca^{2+} and Mg^{2+} at 4 mM and 0.25 mM, respectively. Cerebellar neurons were allowed to attach to 3T3 monolayers for 4 h before drugs were added to discount effects on initial adhesion. Co-cultures were fixed with paraformaldehyde, methanol permeabilized and the cerebellar neurons stained for GAP-43. The average length of the longest neurite on each neuron was determined using a sight system image manager (Sight Systems, Newbury, England) as previously described (Doherty et al., 1991*a*). The PC12 neuronal cell line was maintained as previously described (Doherty et al., 1991*a*) and co-cultures established by seeding 1,500 cells onto monolayers of parental 3T3 cells or clones of 3T3 cells expressing NCAM, N-cadherin or L1 (see above). After ~40 h these co-cultures were fixed and the average length of the longest neurite determined following staining for the Thy-1 antigen on PC12 cells (Doherty et al., 1991*a*). PC12 cells that had been primed with NGF for 4–6 d were subcultured into individual wells of eight-chamber slides coated with poly-lysine and laminin (Doherty et al., 1988). The primed cells were cultured for 16–24 h in the presence and absence of a number of tyrosine kinase inhibitors.

Tyrosine Kinase Inhibitors

A wide range of commercially available tyrosine kinase inhibitors were tested in this study. Genistein, a competitive inhibitor of the ATP binding site of tyrosine kinases, known to inhibit tyrosine phosphorylation in whole cells (Mustelin et al., 1990) and lavendustin A (Onoda et al., 1989) were both obtained from Calbiochem Novabiochem, Ltd. (Nottingham, U.K.). The tyrphostins (47, 23, 25, and 34), a group of low molecular weight compounds based on erbstatin inhibit a number of tyrosine kinases in intact cells (Lyall, 1989; Levitsky, 1990) and were a kind gift from Dr. A. Levitsky. The stable methyl 2,5-dihydroxycinnamate analogue of erbstatin, (hereafter referred to as the erbstatin analogue) inhibits EGF receptor autophosphorylation in cultured cells (Umezawa et al., 1990) was obtained from Calbiochem Novabiochem Ltd. All these agents were diluted in medium from DMSO stock solutions and added to the cultures 4 h after plating the neurons. Controls for these experiments included DMSO diluted in medium to the same extent as the drug stocks.

ELISA Assays for CAM Expression

The level of human NCAM expression in transfected 3T3 cells and of rat NCAM in cerebellar neurons was determined by ELISA as previously described (Doherty et al., 1990). In all assays the detecting antibody was either a bivalent $F(ab)'_2$ fraction of horseradish peroxidase–conjugated anti–mouse Ig or anti–rabbit Ig obtained from Sigma Chemical Co. (St. Louis, MO), and used at 1:1,000 dilution. The binding of this antibody in the absence of primary antibodies was determined and subtracted from the positive values.

Other Reagents

Cholera toxin and mastoparan were purchased from the Sigma Chemical Co. A rabbit antiserum raised against a rat liver glycoprotein that specifically recognizes and blocks the function of β_1 integrins was obtained as a purified immunoglobulin fraction and added to cultures at up to 50 μg/ml (Gullberg et al., 1989). Pertussis toxin and calcium channel antagonists were used as previously described (e.g., see Williams et al., 1992).

Results

Effects of Tyrosine Kinase Inhibitors on Neurite Outgrowth over 3T3 Fibroblasts

Agents can inhibit tyrosine kinases by competitively binding to either the ATP binding site or alternatively the substrate binding site. Genistein and lavendustin A bind to the ATP binding site (for review see Levitsky, 1990) and can inhibit tyrosine kinases in intact cells (for examples see Bixby and Jhabvala, 1992; Devary et al., 1992). In the present study we found no effect of these agents on the ability of cerebellar granule cells to extend neurites over confluent monolayers of 3T3 fibroblasts (Table I). Erbstatin and tyrphostins inhibit tyrosine kinases by binding to the substrate binding site (Levitsky, 1990). The methyl 2,5 dihydroxycinnamate analogue of erbstatin (hereafter referred to as the erbstatin analogue) is a potent inhibitor of the EGF receptor-associated tyrosine kinase (Umezawa et al., 1990). This agent had no effect on neurite outgrowth over 3T3 fibroblasts when used at 10–15 μg/ml (Table I), however at higher concentrations it was extremely toxic to the cerebellar neurons. The tyr-

Table I. The Effects of a Variety of Agents on Neurite Outgrowth over 3T3 Fibroblasts

Agent	Neurite outgrowth, percent control
(*a*) Genistein (100 μM)	119.7 ± 9.4
Lavendustin A (20 μM)	98.4 ± 2.6
Erbstatin analogue (10–15 μg/ml)	100.0 ± 5.3
Tyrphostin 23 (200 μM)	107.2 ± 14
Tyrphostin 25 (200 μM)	105.3 ± 15
Tyrphostin 34 (150 μM)	105 ± 12
Tyrphostin 47 (150 μM)	112 ± 10
(*b*) Anti-β_1 integrin (25 μg/ml)	55.8 ± 5.4*
(50 μg/ml)	49.2 ± 3.9*

Cerebellar neurons were cultured on confluent monolayers of 3T3 fibroblasts in control media, media supplemented with characterized tyrosine kinase inhibitors as shown (*a*), or media supplemented with antibodies that block integrin receptor function at the given concentrations (*b*). After ~16 h the co-cultures were fixed and the mean length of the longest neurite per cell determined. In each experiment measurements were made on 120–180 neurons sampled from the replicate cultures, and the mean neurite length in treated cultures expressed as a percentage of that measured in the control culture. In *a* the results show the mean ± SEM from three independent experiments. In *b* the mean ± SEM determined in one representative experiment is shown. Over this period of culture the 100% control value was usually of the order of 30 μm (for example see Figs. 1 and 3). The drugs were also tested for their effects on neuronal cell number and the only drug that had a significant effect was Genistein which reduced neuronal cell number by up to 80%. However, the remaining neurons were healthy and extended neurites as normal (see above).

* $P = 0.005$, statistical analysis was performed using *t*-test.

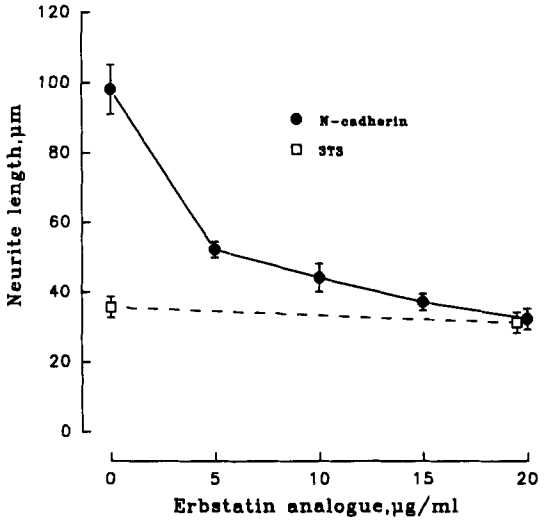

Figure 1. The erbstatin analogue inhibits N-cadherin dependent neurite outgrowth in a dose-dependent manner. Cerebellar neurons were cultured on monolayers of parental 3T3 cells or 3T3 cells expressing chick N-cadherin in control media or media supplemented with the erbstatin analogue (5–20 μg/ml). Co-cultures were maintained for 20 h before being fixed and stained for GAP-43. The mean length of the longest neurite per cell was measured and each value is the mean ± SEM of 120–180 neurons sampled from replicate cultures.

phostins are a relatively new family of tyrosine kinase inhibitors that are also derivatives of erbstatin (Lyall et al., 1989; Levitsky, 1990). They differ in their fine specificity but can inhibit a number of tyrosine kinases in intact cells (Levitsky, 1990). At concentrations in the range of 150–200 μM tyrphostins 23, 25, 47, and 34 failed to modulate neurite outgrowth over 3T3 monolayers (Table I).

Integrin receptors on neurons mediate neurite outgrowth over laminin and collagen (Bixby and Harris, 1991; Reichardt and Tomaselli, 1991). A wide range of tyrosine kinase inhibitors including genistein, lavendustin A, herbimycin A, and a number of tyrphostins do not inhibit (and in some cases actually potentiate) neurite outgrowth over laminin or collagen (Bixby and Jhabvala, 1992; Miller et al., 1993). We have also found that genistein, tyrphostin 47 and the erbstatin analogue do not inhibit basal or NGF stimulated neurite outgrowth from primed PC12 cells cultured on a laminin coated substratum (data not shown). Neurite outgrowth over fibroblasts also depends on neuronal integrins binding to extracellular matrix molecules (e.g., see Bixby and Harris, 1991). We have previously shown that antibodies that block β_1 integrins can partially inhibit neurite outgrowth over 3T3 monolayers (Doherty et al., 1991*b*). In the present study antibodies that bind to β_1 integrins inhibited basal neurite outgrowth over 3T3 monolayers by ~50% (Table I). As a control antibodies that bind to cell adhesion molecules on neurons have no effect on neurite outgrowth over 3T3 monolayers (for example see Williams et al., 1992). We do not know what receptors mediate the remaining component of neurite outgrowth over 3T3 cells, however these cells are capable of extending neurites over poly-lysine coated substrata (Frei et al., 1992). None of the tyrosine kinase inhibitors listed in Table I affected neurite outgrowth over the 3T3 cell monolayers. Thus evidence from a number of studies is that tyrosine kinase inhibitors do not inhibit integrin dependent neurite outgrowth.

Effects of the Erbstatin Analogue on Neurite Outgrowth Stimulated by CAMs

Neurons extend longer neurites when grown over genetically modified fibroblasts that express transfected N-cadherin, (Doherty et al., 1991*a*), NCAM (Doherty et al., 1990), or L1 (Williams et al., 1992). All of the tyrosine kinase inhibitors listed in Table I were tested for their ability to inhibit neurite outgrowth stimulated by the above CAMs. Of all of the agents tested the erbstatin analogue was the most potent in blocking CAM stimulated neurite outgrowth. The effects of a range of erbstatin analogue concentrations on neurite outgrowth over 3T3 monolayers and monolayers expressing N-cadherin is shown in Fig. 1. A substantial (~70%) inhibition of the N-cadherin response is apparent at 5 μg/ml. In this experiment a complete inhibition of the response was apparent at ~15 μg/ml of the erbstatin analogue. Similar concentrations of the erbstatin analogue are required to block tyrosine kinases in intact cells (Umezawa et al., 1990). The effect of the erbstatin analogue on neurite outgrowth over monolayers expressing the L1 glycoprotein and the 140-kD isoform of NCAM was also tested with the results of a representative experiment shown in Fig. 2. In this experiment all three CAMs stimulated neurite outgrowth over the basal level found on parental 3T3 monolayers in control media, but the response was absent in media supplemented with the erbstatin analogue. Overall in a total of nine experiments (three tests with each CAM) the CAM response was inhibited by 87 ± 8% (mean ± SEM, $n = 9$) whereas basal outgrowth over 3T3 monolayers, at least 50% of which can be attributed to integrin receptor function, was unaffected (Table I).

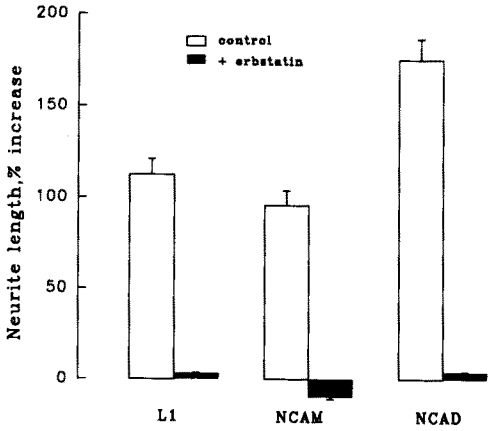

Figure 2. CAM-dependent neurite outgrowth is completely inhibited by the erbstatin analogue. Cerebellar neurons were cultured on monolayers of control 3T3 fibroblasts or 3T3 fibroblasts transfected with either human L1, NCAM, or chick N-cadherin, in control media or media supplemented with the erbstatin analogue at 10 μg/ml. Cultures were fixed and stained and the mean neurite length of the longest process per cell was measured for 120–180 neurons sampled from replicate cultures. The results show the percentage increase in mean length ± SEM over controls from representative experiments for each CAM. Basal neurite outgrowth over 3T3 monolayers in control media was of 35.6 ± 2.6 μm (mean ± SEM, n = 3 independent experiments).

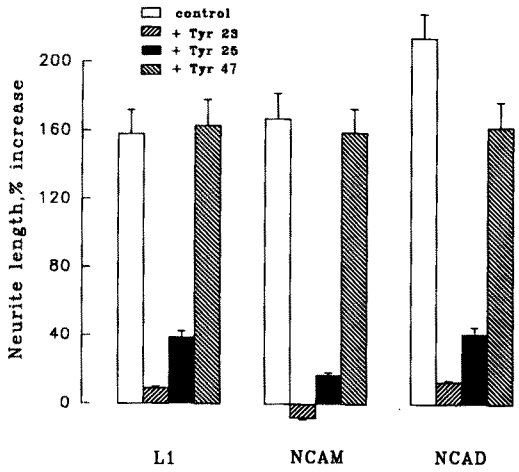

Figure 4. The tyrphostins show differential specificity as inhibitors of CAM dependent neurite outgrowth. Co-cultures were established in the presence or absence of tyrphostins 23, 25, or 47 (all at 150 μM) for cerebellar neurons grown on monolayers of control 3T3 fibroblasts or fibroblasts expressing one or other of the CAMs (NCAM, N-cadherin, or L1). The mean neurite length of the longest neurite per cell was measured for 120–180 neurons sampled from replicate cultures after 20 h of incubation. The results show the percentage increase in neurite length (mean ± SEM) for neurons on CAM expressing relative to parental 3T3 cells in the presence and absence of the tyrphostins for a representative experiment.

The Effects of Tyrphostins on Neurite Outgrowth Stimulated by CAMs

The effects of tyrphostins 23, 25, 34, and 47 were tested on neurite outgrowth stimulated by L1, NCAM, and N-cadherin. Two of these agents were able to inhibit CAM stimulated neurite outgrowth at concentrations that have no effect on basal neurite outgrowth over monolayers of parental 3T3 fibroblasts. Fig. 3 shows that tyrphostin 23 and 25 can inhibit N-cadherin dependent neurite outgrowth in a dose dependent manner, but full inhibition requires the presence of 150–200 μM of the test agent. The results also show tyrphostin 23 to have a greater potency than tyrphostin 25. The effect of these agents on neurite outgrowth stimulated by L1 and NCAM was also determined with results of a representative experiment shown in Fig. 4. Again at 150 μM tyrphostin 23 completely inhibited the response to all three CAMs, whereas tyrphostin 25 inhibited each response by ~80%. In a series of experiments we found no effect of tyrphostins 34 and 47 on neurite outgrowth stimulated by any of the above CAMs, and this is shown for tyrphostin 47 in Fig. 4. Again, similar concentrations of tyrphostins (~100 μM) are required to block tyrosine kinases in intact cells (Lyall et al., 1989).

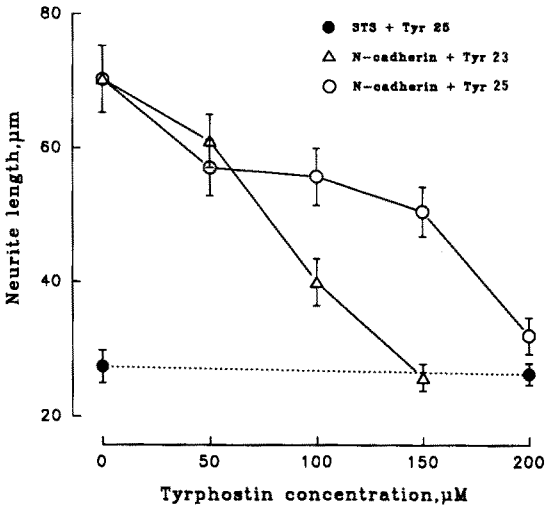

Figure 3. Tyrphostins 23 and 25 specifically inhibit N-cadherin dependent neurite outgrowth. Cerebellar neurons were cultured on monolayers of control 3T3 cells or 3T3 cells transfected with N-cadherin in the presence or absence of varied concentrations of tyrphostins 23 or 25 (50–200 μM). Co-cultures were maintained for 20 h before being fixed and stained for GAP 43. The results show the mean neurite length for the longest neurite per cell for 120–180 neurons sampled from replicate cultures, each value given ± SEM. Tyrphostin 23 had no effect on neurite outgrowth over monolayers of control 3T3 cells at up to 200 μM (see Table I).

Genistein and Lavendustin A Do Not Inhibit CAM-stimulated Neurite Outgrowth

Genistein can be used to inhibit tyrosine phosphorylation in live cells at 100–150 μM, whereas lavendustin A is effective in the very low micromolar range (for examples see Bixby and Jhabvala, 1992; Devary et al., 1992). In the present study we found no inhibition of NCAM-, N-cadherin-, and L1-dependent neurite outgrowth from cerebellar neurons or PC12 cells when these agents were added to cultures at up to 100 μM (genistein) or 20 μM (lavendustin A), alone or together. At the highest concentrations these agents were slightly toxic for cerebellar neurons but not for PC12 cells. The results for a representative experiment for N-cadherin

Table II. The Effects of Genistein and Lavendustin A on Neurite Outgrowth from Naive PC12 Cells

Monolayer	Control media	Genistein (100 μM)	Lavendustin A (10 μM)
3T3	18.0 ± 1.8 (114)	18.2 ± 1.4 (135)	17.9 ± 1.3 (148)
3T3 + NCAD	56.8 ± 3.0 (161)	53.6 ± 3.0 (146)	57.4 ± 3.8 (122)

PC12 cells were cultured on monolayers of control 3T3 cells or transfected 3T3 cells expressing N-cadherin in control medium or medium supplemented with 100 μM genistein or 10 μM lavendustin A. After 40 h the cultures were fixed and the mean length of the longst neurite per cell was measured (Doherty et al., 1991*a*). The results show the absolute values ± SEM in μm for the given number of PC12 cells sampled from replicate cultures. Similar results were obtained in two additional experiments and neither drug affected PC12 cell number (data not shown).

stimulated neurite outgrowth from the PC12 neuronal cell line are shown in Table II.

Effects of Agents That Block the CAM Responses on Neurite Outgrowth Stimulated by Cholera Toxin

The above results show that three tyrosine kinase inhibitors (the erbstatin analogue and tyrphostins 23 and 25) can block CAM stimulated, but not integrin-dependent neurite outgrowth. Neurite outgrowth from cerebellar neurons grown on 3T3 monolayers can also be stimulated by cholera toxin presumably via increased cAMP and activation of protein kinase A. This response differs from the CAM response in that it cannot be inhibited by pertussis toxin (which inactivates heterotrimeric G-proteins) or by agents that block or negate the effects of calcium influx into neurons (Williams et al., 1992). To test if the above agents are inhibiting a step relatively exclusive to the CAM pathway, or are affecting a more general step involved in neurite outgrowth stimulated by exogenous agents, cerebellar neurons were grown on monolayers of 3T3 cells in the presence of cholera toxin (1 ng/ml) in media further supplemented with the erbstatin analogue (10 μg/ml) or tyrphostins 23 and 25 (both at 200 μM). The results of a representative experiment show very clearly that whereas tyrphostin 25 can completely inhibit the cholera toxin response, the erbstatin analogue and tyrphostin 23 have no effect (Fig. 5). We can conclude that these agents do not inhibit protein kinase A.

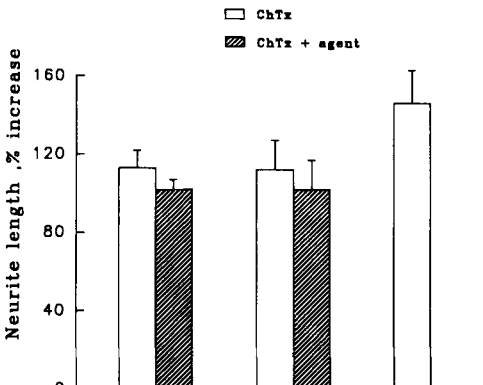

Figure 5. Cerebellar neurons were grown over monolayers of parental 3T3 cells in control media, media supplemented with cholera toxin (1 ng/ml) or media supplemented with cholera toxin and the given tyrosine kinase inhibitors (see above). After ~20 h the cultures were fixed and the mean length of the longest neurite determined as in Fig. 1. The results show the percentage increase in mean neurite length induced by cholera toxin in the presence and absence of inhibitors pooled from three independent experiments.

Effects of Agents That Block the CAM Responses on Neurite Outgrowth Stimulated by K^+ Depolarization

Neurite outgrowth stimulated by CAMs differs from that stimulated by integrins in that calcium influx into neurons via both N- and L-type calcium channels is both necessary and sufficient to account for the response (for review see Doherty and Walsh, 1992). As such, direct activation of calcium channels by a specific agonist or by K^+ depolarization can stimulate neurite outgrowth from neurons cultured on 3T3 monolayers. To determine if the erbstatin analogue and tyrphostins 23 and 25 inhibited a step upstream or downstream of calcium channels their effects on neurite outgrowth stimulated by 40 mM KCl was determined (Williams et al., 1992). The results pooled from six independent control experiments and three experiments with the erbstatin analogue (at 10–15 μg/ml) or tyrphostins 23 and 25 (both at 200 μM) are shown in Fig. 6. On its own KCl increased the length of

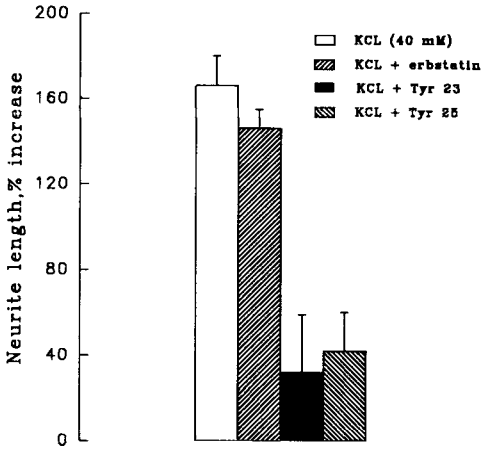

Figure 6. K^+-induced neurite outgrowth is inhibited by tyrphostin 23 and 25 but not the erbstatin analogue. Cerebellar neurons were cultured on monolayers of parental 3T3 cells in the presence or absence of KCl at 40 mM, in control media or media further supplemented with either the erbstatin analogue (10–15 μg/ml) tyrphostin 23 or 25 (both at 200 μM). After 20 h the mean length of the longest neurite per cell was determined for 120–180 neurons from replicate cultures, and the percentage increase in neurite length over controls was determined. The results are pooled to show the mean ± SEM from six independent experiments with K^+ alone and three independent experiments with K^+ and the given tyrosine kinase inhibitor.

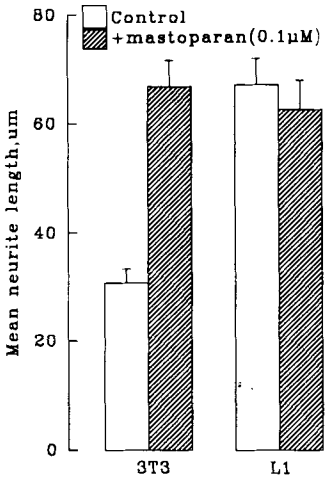

Figure 7. Cerebellar neurons were cultured on monolayers of parental 3T3 cells or 3T3 cells expressing L1 in control media or media supplemented with mastoparan (0.1 μM). After 20 h the cultures were fixed and the mean length of the longest neurite determined as in Fig. 1. Each value is the mean ± SEM of 120–180 neurons sampled from replicate cultures in a representative experiment.

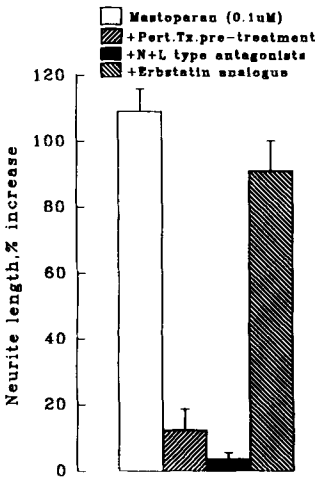

Figure 8. Cerebellar neurons were grown on monolayers of parental 3T3 cells in control media, media supplemented with mastoparan (0.1 μM) or mastoparan plus calcium channel antagonists (verapamil at 10 μM, ω-conotoxin at 0.25 μM) or the erbstatin analogue (10 μg/ml). In one set of experiments neurons that had been pre-treated with 800 ng/ml pertussis toxin for 3 h before establishment of co-cultures were tested for their responsiveness to mastoparan. After ~20 h the cultures were fixed and the mean length of the longest neurite determined as in Fig. 1. The results show the percentage increase in mean neurite length induced by mastoparan as indicated. The control mastoparan response was pooled from 10 independent experiments, with the other results pooled from three independent experiments. Bars show 1 SEM. With the exception of mastoparan none of the agents had any effect on basal neurite outgrowth (for example, see Williams et al., 1992).

the longest neurite to, on average, 260% of the control value. This response can be inhibited by reducing extracellular calcium to 0.25 mM, by blocking N- and L-type calcium channels or by pre-loading neurons with a calcium chelator (Williams et al., 1992). Tyrphostins 23 and 25 inhibited the KCl response by 75–80% whereas the erbstatin analogue had no significant effect (Fig. 6). These data suggest that the erbstatin analogue blocks the neurite outgrowth stimulated by CAMs at a site upstream of calcium channel activation, whereas the tyrphostins act at other or additional sites downstream of this step.

The Erbstatin Analogue Does Not Inhibit Neurite Outgrowth Stimulated by Direct Activation of G-Proteins

Pertussis toxin can inhibit calcium influx into PC12 cells triggered by the binding of antibodies to NCAM and N-cadherin (Schuch et al., 1989). Pre-treatment of neurons with pertussis toxin inhibits neurite outgrowth stimulated by NCAM, N-cadherin, and L1 at a site upstream of calcium channel activation (Williams et al., 1992). Mastoparan activates pertussis toxin sensitive G-proteins (Higashijima et al., 1990) and we have found that this agent can stimulate neurite outgrowth from cerebellar neurons cultured on 3T3 monolayers and that this response is not additive with a CAM response (Fig. 7). Mastoparan also stimulated neurite outgrowth on a poly-lysine/laminin-coated substratum (data not shown). In three independent experiments the response to mastoparan was essentially completely inhibited by pretreating neurons with pertussis toxin (Fig. 8). The response was also completely blocked by calcium channel antagonists (Fig. 7), or by pre-loading neurons with BAPTA/AM or reducing extracellular calcium to 0.25 mM (data not shown). In contrast the erbstatin analogue only had a small and non-significant inhibitory effect. These data demonstrate that mastoparan stimulated neurite outgrowth requires activation of a neuronal pertussis toxin sensitive G-protein, and calcium influx into neurons. However it does not require activation of an erbstatin analogue sensitive tyrosine kinase.

The erbstatin analogue does not inhibit neurite outgrowth stimulated by agents that activate protein kinase A (see above). Likewise the erbstatin analogue does not inhibit neurite outgrowth stimulated by low concentrations of phorbol esters which work via activation of protein kinase C (Williams, E. J., unpublished observation). Direct measurements of protein kinase C activity in cultures of cerebellar neurons also showed this to be unaffected by 20 h of treatment with 10 μg/ml of the erbstatin analogue (data not shown). The erbstatin analogue does however inhibit tyrosine phosphorylation in cultures of cerebellar granule cells (Doherty et al., 1994). Thus the effects of the erbstatin analogue on neurite outgrowth can most likely be attributed to its ability to inhibit tyrosine kinases.

The possibility that the erbstatin analogue inhibits the response by affecting expression of the CAMs in the monolayer or neuron was tested by quantitating this in control and treated cultures by ELISA (see Materials and Methods). Cultures of cerebellar neurons or NCAM expressing 3T3 cells were maintained for 16 h in the presence and absence of the erbstatin analogue (10 μg/ml) before being assayed for NCAM expression. The level of NCAM in the neurons (105 ± 2%) and transfected fibroblasts (101 ± 4%) was not affected by the erbstatin analogue (both values are expressed as a percentage of the control and for each value individual determinations were made on seven replicate cultures). Although we cannot exclude the possibility that the erbstatin analogue might directly affect the structure of all three CAMs this would be extremely unlikely and there is no precedent for such an action of a tyrosine kinase inhibitor. Thus the erbstatin analogue sensitive step, presumably inhibition of a tyrosine kinase, most probably resides downstream of the CAM binding step.

Discussion

The results of the present study show that three tyrosine kinase inhibitors (the erbstatin analogue and tyrphostins 23

and 25) can completely inhibit neurite outgrowth over 3T3 monolayers stimulated by NCAM, N-cadherin, or L1 at concentrations that do not inhibit integrin dependent neurite outgrowth over the same monolayers. Thus the possibility that the agents have non-specific effects on the neuron's ability to extend neurites, or the monolayer's ability to support this can be discounted. The data provides substantive evidence for the postulate that CAMs and integrins stimulate neurite outgrowth via fundamentally different mechanisms (Bixby and Jhabvala, 1990; Doherty et al., 1991*a*) and provide the first evidence for activation of a tyrosine kinase being required for the CAM response.

We have previously established that the above CAMs stimulate cell contact-dependent neurite outgrowth by activating a second messenger pathway that culminates in calcium influx into neurons (Doherty and Walsh, 1992). Although we have shown that the activity of a pertussis toxin sensitive G-protein is required upstream of calcium channel activation, the initial steps in the pathway were not identified. In the present study we have shown that the erbstatin analogue is the most restrictive of a number of tyrosine kinase inhibitors in that it does not inhibit neurite outgrowth that results from direct stimulation (by K^+ depolarization) of the CAM second messenger pathway at the level of calcium channel activation. This observation firmly places the erbstatin analogue sensitive step upstream of calcium channel activation. The possibility that the analogue inhibits a kinase other than a tyrosine kinase is extremely unlikely based on the known pharmacological profile of erbstatin and also the fact that more general kinase inhibitors such as staurosporine and H7 do not inhibit CAM stimulated neurite outgrowth (Doherty et al., 1991*a*). In this context we have also shown that the erbstatin analogue does not inhibit neurite outgrowth stimulated by agents that activate protein kinase A or C, or by K^+ depolarization. We have also previously shown that activation of protein kinase A or C does not lead to calcium dependent neurite outgrowth (Doherty et al., 1991*a*; Williams et al., 1992).

The possibility that the target for the erbstatin analogue resides in the monolayer rather than the neuron seems unlikely for a number of reasons. For example, K^+ and cholera toxin-stimulated neurite outgrowth over 3T3 monolayers is not affected by the erbstatin analogue. Also the erbstatin analogue does not modulate the expression of CAMs in the transfected 3T3 cells. A direct link between NCAM and L1 and tyrosine phosphorylation in neuronal growth cones has also been established by the observation that antibodies to these CAMs or the soluble ligands themselves can trigger the loss of phosphate groups from tyrosine on tubulin (Atashi et al., 1992).

Neurite outgrowth stimulated by NCAM, N-cadherin, or L1 in the substratum requires the function of the same CAM in the neuron suggesting that all three molecules operate via a homophilic binding mechanism (for example see Lemmon et al., 1989; Doherty et al., 1991*b*; Williams et al., 1992). The possibility that the erbstatin analogue works by setting into motion a train of events leading to loss of the receptor, (i.e., the CAM), from the neurons can be discounted as we found no difference in the level of NCAM expression by cerebellar neurons after treatment with this tyrosine kinase inhibitor. Thus we can conclude that the erbstatin analogue blocks CAM-dependent neurite outgrowth at a step downstream from receptor binding and upstream from calcium channel activation most likely by inhibiting a tyrosine kinase. It is also apparent that this kinase is required for neurite outgrowth stimulated by three quite distinct CAMs but not neurite outgrowth that depends on integrins or neurite outgrowth stimulated by cholera toxin.

The tyrphostins are derivatives of erbstatin that differ in their fine specificity as inhibitors of a number of tyrosine kinases (Levitsky, 1990). Two of these (23 and 25) inhibited neurite outgrowth stimulated by CAMs but not integrins but only at an order of magnitude greater concentration than the erbstatin analogue. These agents also inhibited neurite outgrowth stimulated by K^+ depolarization and tyrphostin 25 also blocked neurite outgrowth stimulated by cholera toxin. As a control tyrphostins 34 and 47 did not block any of the above responses. We can conclude that these agents are not acting at a single specific site in the CAM pathway upstream from calcium channel activation.

The results of the present study show that a wide range of tyrosine kinase inhibitors have no effect on neurite outgrowth over 3T3 fibroblasts that is mediated by integrins or CAMs. Genistein, lavendustin A, and tryphostins 34 and 47 have been shown to inhibit a wide range of tyrosine kinases in vitro and in some instances also in intact cells and these included *c-src*, *v-src*, *v-abl*, *yes*, *lck*, the PDGF receptor, and the EGF receptor (Akiyama et al., 1987; Trevillyan et al., 1990; O'Del et al., 1991; Lyall et al., 1989; Devary et al., 1992; Umezawa et al., 1990; Miller et al., 1993). The results suggest that none of these kinases are required for neurite outgrowth stimulated by a variety of agents and this is perhaps most interesting for *c-src* which has been postulated to play such a role (see introduction). In fact genistein and lavendustin have actually been reported to stimulate neurite outgrowth over short periods of culture (4–5 h) from a variety of neurons cultured on laminin or on purified L1 (Bixby and Jhabvala, 1992; also see Miller et al., 1993), leading the authors to suggest that tyrosine kinases might actually suppress neurite outgrowth. We did not see a similar response to genistein and lavendustin with cerebellar neurons or naive PC12 cells. However, we have found that genistein but not erbstatin or tyrphostin 47 stimulates neurite outgrowth from primed PC12 cells cultured on laminin in both the absence and presence of NGF and a similar result has recently been reported by others (Miller et al., 1993). Thus some tyrosine kinases most likely suppress neurite outgrowth. We have also shown that activation of pertussis toxin sensitive G-proteins with mastoparan can fully mimic the neurite outgrowth responses to CAMs and that this is not inhibited by the erbstatin analogue. We would therefore postulate that homophilic binding of CAMs can lead to the activation of a tyrosine kinase and that downstream events require the activity of a pertussis toxin sensitive G-protein. In this context the oncogenic *v-fps* tyrosine kinase can stimulate GTP binding to a ~50-kD protein that might be an alpha-subunit of a heterotrimeric G-protein (Alexandropoulos et al., 1991). Also the EGF-receptor tyrosine kinase can be co-precipitated with a Gi-α subunit and EGF induced tyrosine phosphorylation of phospholipase C can be inhibited by pertussis toxin (Yang et al., 1991). Furthermore a number of G-protein alpha subunits have been identified as substrates

for tyrosine kinases with phosphorylation leading to an increase in the rate of GTP-γ-s binding (Hausdorf et al., 1992). Experiments are currently underway to determine the identification of the G-protein involved in the CAM pathway with a longer term view of determining if tyrosine phosphorylation of its α subunit is required for CAM dependent neurite outgrowth.

Although a role for non-receptor tyrosine kinases such as *src*, *abl*, *yes*, and *fyn*, which are all expressed at high levels in the nervous system (Maness and Cox, 1992) might seem unlikely in view of the fact that the tyrosine kinase inhibitors genistein and lavendustin A and tyrphostin 34 and 47 do not block the CAM response (see below), it is not possible to be certain that these kinases are fully inhibited by the drugs. However, other candidates should also be considered and these include receptor tyrosine kinases, particularly those that have immunoglobulin like extracellular domains. As CAMs can bind to other CAMs via *cis*-interactions in the same membrane (Kadmon et al., 1990) an intriguing possibility is that NCAM, N-cadherin and L1 might be capable of co-clustering and thereby activating a receptor tyrosine kinase that contains immunoglobulin like domains as part of its extracellular structure.

One possible candidate is the FGF receptor tyrosine kinase. In support we have recently found that CAMs and FGF stimulate neurite outgrowth by activating a common second messenger pathway in primary neurons, and that the erbstatin analogue can inhibit the tyrosine kinase activity of the FGF receptor. A direct link between CAMs and the FGF receptor has been established by showing that an antibody to the FGF receptor can inhibit CAM stimulated neurite outgrowth, and that peptides derived from a putative CAM binding domain on the FGF receptor can specifically and differentially inhibit neurite outgrowth stimulated by NCAM, N-cadherin, and L1 (Williams, E. J., F. S. Walsh, and P. Doherty, manuscript submitted for publication).

In summary, the results of the present study provide the first evidence that activation of a tyrosine kinase is an important step upstream of G-protein activation in the CAM pathway leading to neurite outgrowth. This kinase appears to be exclusive to the CAM pathway. The results provide a firm basis for further studies of the nature of the relationship between two important classes of morphoregulatory molecules, namely CAMs and tyrosine kinases.

We would like to thank Alex Levitsky for the tyrphostins and Bart Sefton for the PY72 antibody. We also thank Hendrika Rickard for typing this manuscript.

This work was supported by the Dunhill Trust and the Medical Research Council.

Received for publication 27 April 1993 and in revised form 22 November 1993.

References

Akiyama, T., J. Ishada, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fokami. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 262:5592–5595.

Alexandropoulos K., C. K. Joseph, R. Spangler, and D. A. foster. 1991. Evidence that a G-protein transduces signals initiated by the protein tyrosine kinase *v-fps*. *J. Biol. Chem.* 266:15583–15586.

Atashi, S. R., S. G. Klintz, C. A. Ingraham, W. T. Matten, M. Schachner, and P. F. Maness. 1992. Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. *Neuron.* 8:831–842.

Bixby, J. L., and W. A. Harris. 1991. Molecular mechanisms of axon growth and guidance. *Annu. Rev. Cell. Biol.* 7:117–159.

Bixby, J. L., and P. Jhabvala. 1990. Extracellular matrix molecules and cell adhesion molecules induce neurites through different mechanisms. *J. Cell Biol.* 111:2725–2732.

Bixby, J. L., and P. Jhabvala. 1992. Inhibition of tyrosine phosphorylation potentiates substrate-induced neurite growth. *J. Neurobiol.* 23:468–480.

Bixby, J. L., R. S. Pratt, J. Lilien, and L. F. Reichardt. 1987. Neurite outgrowth on muscle cell surfaces involves extracellular matrix receptors as well as Ca^{2+}-dependent and independent cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 84:2555–2559.

Chao, M. V. 1992. Neurotrophin receptors: a window into neuronal differentiation. *Neuron.* 9:583–593.

Cox, M. E., and P. F. Maness. 1991. Neurite extension and protein tyrosine phosphorylation elicited by inducible expression of the v-src oncogene in a PC12 cell line. *Exp. Cell. Res.* 195:423–431.

Devary, Y., R. A. Gottlieb, T. Smeal, and M. Rasin. 1992. The mammalian ultraviolet response is triggered by activation of *src* tyrosine kinases. *Cell.* 71:1081–1091.

Doherty, P., and F. S. Walsh. 1992. Cell adhesion molecules, second messengers and axonal growth. *Curr. Opin. Neurobiol.* 2:595–601.

Doherty, P., E. Robinson, and F. S. Walsh. 1988. Analysis of PC12 cell adhesion to muscle and non-muscle cells and components of the extracellular matrix. *Exp. Cell Res.* 179:233–242.

Doherty, P., M. Fruns, P. Seaton, G. Dickson, C. H. Barton, T. A. Sears, and F. S. Walsh. 1990. A threshold effect of the major isoforms of NCAM on neurite outgrowth. *Nature (Lond.).* 343:464–466.

Doherty, P., S. V. Ashton, S. E. Moore, and F. S. Walsh. 1991*a*. Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G-protein dependent activation of L- and N-type neuronal calcium channels. *Cell.* 67:21–33.

Doherty, P., L. H. Rowett, S. E. Moore, D. A. Mann, and F. S. Walsh. 1991*b*. Neurite outgrowth in response to transfected NCAM and N-cadherin reveals fundamental differences in neuronal responsiveness to CAMs. *Neuron.* 6:247–258.

Doherty, P., J. Furness, E. Williams, and F. S. Walsh. 1994. Neurite outgrowth stimulated by the tyrosine kinase inhibitor Herbimycin A requires activation of tyrosine kinases and protein kinase C. *J. Neurochem.* In press.

Elkins, T., K. Zinn, L. McAllister, F. M. Hoffman, and C. S. Goodman. 1990. Genetic analysis of a drosophila neural cell adhesion molecule: interaction of fasciclin I and Abelson tyrosine kinase mutations. *Cell.* 60:565–575.

Frei, T., F. von Bohlen und Halbach, W. Willie, and M. Schachner. 1992. Different Extracellular domains of the Neural Cell Adhesion Molecule (N-CAM) are involved in different functions. *J. Cell Biol.* 118:177–194.

Gullberg, D., L. Terracio, T. K. Borg, and R. Rubin. 1989. Identification of integrin like matrix receptors with affinity for interstitial collagens. *J. Biol. Chem.* 264:12686–12694.

Hausdorff, W. P., J. A. Pitcher, D. K. Luttrell, M. E. Linder, H. Kurose, S. J. Parsons, M. G. Caron, and R. S. Lefkowitz. 1992. Tyrosine phosphorylation of G protein α-subunits by pp60 *c-src*. *Proc. Natl. Acad. Sci. USA.* 89: 5720–5724.

Hempstead, B. L., S. J. Rabin, L. Kaplan, S. Reid, L. F. Parada, and D. R. Kaplan. 1992. Overexpression of the *trk* tyrosine kinase rapidly accelerates nerve growth factor induced differentiation. *Neuron.* 9:883–896.

Higashijima, T., J. Burnier, and E. M. Ross. 1990. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. *J. Biol. Chem.* 265:14176–14186.

Jacobson, M. 1992. Developmental Neurobiology. Third Edition. Plenum Publishing Corp., New York.

Juliano, R. L., and S. Haskill. 1993. Signal transduction from extracellular matrix. *J. Cell Biol.* 120:577–585.

Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990. The neural cell adhesion molecule NCAM enhances L1-dependent cell–cell interactions. *J. Cell Biol.* 110:193–208.

Landmesser, L., L. Dahm, J. Tang, and U. Rutishauser. 1990. Polysialic acid as a regulator of intramuscular nerve branching during embryonic development. *Neuron.* 4:655–667.

Le beau, J. M., B. Tedeschi, and G. Walter. 1991. Increased expression of pp60 *c-src* protein tyrosine kinase during peripheral nerve regeneration. *J. Neurosci. Res.* 28:299–309.

Lemmon, V., K. L. Farr, and C. Lagenauer. 1989. L1 mediated axon growth occurs via a homophilic binding mechanism. *Neuron.* 2:1597–1603.

Levitzki, A. 1990. Tyrphostins–potential antiproliferative agents and novel molecular tools. *Biochem. Pharmacol.* 40:913–918.

Lyall, R. M., A. Zilberstein, A. Gazit, C. Gilon, A. Cavitak, and J. Schlesinger. 1989. Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF stimulated cell proliferation. *J. Biol. Chem.* 264:14503–14509.

Maness, P. F., M. Aubry, C. G. Shores, L. Frames, and K. H. Pfenninger. 1988. *c-src* Gene product in developing rat brain is enriched in nerve growth cone membranes. *Proc. Natl. Acad. Sci. USA.* 85:5001–5005.

Maness, P. F., and M. E. Cox. 1992. Protein tyrosine kinases in nervous system development. *Semin. Cell. Biol.* 3:117–126.

Miller, D. R., G. M. Lee, and P. F. Maness. 1993. Increased neurite outgrowth

induced by inhibition of protein tyrosine kinase activity in PC12 pheochromocytoma cells. *J. Neurochem.* 60:2134–2144.

Mustelin, T., K. M. Coggeshall, and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase pp56-lck by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 86:6302–6306.

Neugebauer, K. M., K. J. Tomaselli, J. Lilien, and L. F. Reichardt. 1988. N-cadherin, N-CAM and integrins promote retinal neurite outgrowth on astrocytes in vitro. *J. Cell Biol.* 107:1177–1187.

O'Dell, T. J., E. R. Kandel, and S. G. N. Grant. 1991. Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature (Lond.).* 353:558–560.

Onoda, T., H. Iinuma, Y. Sasaki, M. Hamada, K. Isshiki, H. Naganawa, T. Takeuchi, R. Tatsuta, and K. Umezana. 1989. Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from Streptomyces griseolavendus. *J. Nat. Prod. (Lloydia).* 52:1252–1257.

Reichardt, L. F., and K. J. Tomaselli. 1991. Extracellular matrix molecules and their receptors: functions in neural development. *Annu. Rev. Neurosci.* 14:531–570.

Saffell, J. L., F. S. Walsh, and P. Doherty. 1992. Direct activation of second messenger pathways mimics cell adhesion molecule dependent neurite outgrowth. *J. Cell Biol.* 118:663–670.

Schuch, U., M. J. Lohse, and M. Schachner. 1989. Neural cell adhesion molecules influence second messenger systems. *Neuron.* 3:13–20.

Seilheimer, B., and M. Schachner. 1988. Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neurons' growth on Schwann cells in culture. *J. Cell Biol.* 107:341–351.

Trevillyan, J. M., Y. Lu, D. Atluru, C. A. Phillips, and J. M. Bjorndhal. 1990. Differential inhibition of T cell receptor signal transduction and early activation events by a selective inhibitor of protein tyrosine kinases. *J. Immunol.* 145:3223–3230.

Umezawa, K., T. Hori, H. Tajima, M. Imoto, K. Ishiki, and T. Takeuchi. 1990. Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS (Feb. Eur. Biochem. Soc.) Lett.* 260:198–200.

Von Bohlen und Halbach, F., J. Taylor, and M. Schachner. 1992. Cell type specific effects of the neural adhesion molecules L1 and N-CAM on diverse second messenger systems. *Eur. J. Neurosci.* 4:896–909.

Williams, E. J., P. Doherty, G. Turner, R. A. Reid, J. J. Hemperly, and F. S. Walsh. 1992. Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. *J. Cell Biol.* 119: 883–892.

Yang, L., G. Baffy, S. G. Rhee, D. Mannings, C. A. Hansen, and J. R. Williamson. 1991. Pertussis toxin sensitive Gi protein involvement in epidermal growth factor induced activation of phospholipase C-y in rat hepatocytes. *J. Biol. Chem.* 266:22451–22458.

Zachary, I., and E. Rozengurt. 1992. Focal adhesion kinase ($p125^{FAK}$). A point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell.* 71:891–894.