Characterisation of the second messenger pathway underlying neurite outgrowth stimulated by FGF

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SUMMARY

Cerebellar neurons, cultured on monolayers of 3T3 fibroblasts or on a polylysine/laminin-coated substratum, responded to recombinant basic FGF by extending longer neurites. The response was biphasic reaching a maximum at 5 ng/ml FGF, but desensitising at 100-200 ng/ml FGF. The response to FGF could be inhibited by a tyrosine kinase inhibitor (the erbstatin analogue), by a diacylglycerol lipase inhibitor (RHC-80267) and by a combination of N- and L-type calcium channel antagonists or other agents that negate the effects of calcium influx into neurons. The response to FGF could be fully mimicked by arachidonic acid added directly to the cultures, or generated via activation of phospholipase A_2 with melittin. The response to melittin, but not to FGF or arachidonic acid, was inhibited by 4-bromophenacyl bromide, a phospholipase A_2 inhibitor. The response to arachidonic acid was also biphasic and high concentrations of this agent could cross-desensitise the FGF response and vice versa. The response to arachidonic acid could be fully inhibited by the agents that block or negate the effects of calcium influx into neurons, but was not inhibited by the tyrosine kinase or diacylglycerol lipase inhibitors. These data suggest that FGF stimulates neurite outgrowth by activating a cascade that involves activation of phospholipase $C\gamma$ to produce diacylglycerol, conversion of diacylglycerol to arachidonic acid by diacylglycerol lipase and the activation of voltage-gated calcium channels by arachidonic acid.

Key words: FGF, axonal growth, second messengers

INTRODUCTION

During development, neuronal survival and differentiation can be influenced by a variety of local signals or signals derived from intermediate or final target tissues (reviewed in Dodd and Jessell, 1988; Oppenheim, 1991). The neurotrophins are a family of well-characterised molecules, the prototypic member being nerve growth factor (NGF), that control neuronal survival during development and may also play a role in maintaining neuronal viability in the mature nervous system, particularly after injury (reviewed in Hefti et al., 1989). Fibroblast growth factor (FGF) was originally identified as a potent mitogen for murine and human fibroblasts. More recently basic and acidic FGF have been recognised as being prototypic members of a much larger family of soluble growth factors that have mitogenic effects on a wide range of cell types (reviewed in Gospodarowicz, 1990; Slack, 1990). An important role for FGFs in the development of the nervous system is supported by a number of observations. For example, basic FGF supports the survival and/or morphological differentiation of a number of different neuronal types placed in tissue culture (Hatten et al., 1988; Walicke, 1989; Morrison et al., 1986, Hughes et al., 1993). Also, when supplied intraventricularly, basic FGF supports the survival of axotamised forebrain cholinergic neurons (Anderson et al., 1988; Araujo et al., 1993).

FGFs exert their effects by activating cell surface receptor tyrosine kinases (reviewed in Schlessinger and Ullrich, 1992; Jaye et al., 1992). The FGF receptor (FGFR) gene family contains four well-characterised members encoded by distinct genes (reviewed in Jaye et al., 1992). In the developing nervous system, neurons predominantly express the FGFR1 (also called *Flg*) whereas glial cells express the FGFR2 (Wanaka et al., 1990; Asai et al., 1993). The binding of FGF to its receptor is thought to lead to receptor dimerisation which allows for autophosphorylation on defined tyrosine residues. This in turn creates binding sites for the SH2 domains of a number of transduction molecules including *src* and PLCγ. In this way a number of independent signalling cascades are activated and these may act independently or synergistically to stimulate survival, mitogenesis or differentiation (for review see Schlessinger and Ullrich, 1992).

Phosphorylation of tyrosine 766 in the carboxyterminal tail of the FGFR allows the binding and activation (by tyrosine phosphorylation) of PLCγ. It has been reported that an FGFR with a single point mutation at tyrosine 766 fails to associate with or activate PLCγ in response to FGF, even though the receptor is still autophosphorylated on other sites. Mitogenic responses to FGF are not compromised by dominant negative expression of this mutated receptor demonstrating that activation of PLCγ is not required for this response (Mohammidi et al., 1992; Peters et al., 1992).

The signalling steps involved in mediating the effects of

FGFs on axonal growth have not been elucidated. Calcium is recognised as a key second messenger that can modulate growth cone motility (reviewed in Kater and Mills, 1991 and Doherty and Walsh, 1994). There is an 'optimal' or 'set-point' level of calcium at which maximal rates of axonal growth are observed and this allows for agents that can modulate calcium to promote and/or inhibit axonal growth. Agents that might modulate axonal growth by changing calcium levels or fluxes in growth cones include neurotransmitters (Kater and Mills, 1991), three distinct cell adhesion molecules (CAMs) that promote cell-contact-dependent neurite outgrowth (NCAM, N-cadherin and L1; Doherty and Walsh, 1994) and a myelin-derived factor that can induce growth cone collapse (Bandtlow et al., 1990). The direct turning of neuronal growth cones in an applied electric field (Bedlack et al., 1992) and directed migration of granule cells in the developing cerebellum (Komuro and Rakic, 1992) can also be inhibited by agents that prevent calcium influx into neurons via voltage gated calcium channels.

In the present study, we have tested whether calcium influx into neurons is required for the neurite outgrowth response from primary neurons that is stimulated by basic FGF. Our results suggest that FGF stimulates neurite outgrowth by activating a second messenger pathway that requires calcium influx into neurons via N- and L-type calcium channels. The response to FGF can be mimicked by direct application of arachidonic acid (AA) to neurons or by the treatment of neurons with melittin which generates endogenous AA by activating phospholipase A$_2$ (PLA$_2$). The response to FGF can be specifically inhibited by an inhibitor of diacylglycerol (DAG) lipase, an enzyme that normally generates AA from DAG. This and other evidence suggests that FGF stimulates neurite outgrowth from primary neurons by stimulating a cascade that involves activation of PLCγ to produce DAG, conversion of DAG to AA by DAG lipase and the stimulation of calcium influx into neurons as a consequence of the action of AA on voltage-gated calcium channels. Recent evidence suggests that cell-contact-dependent neurite outgrowth stimulated by NCAM, N-cadherin and L1 involves activation of a similar, if not identical, cascade (Doherty et al., 1991a; Williams et al., 1994a,b; Doherty and Walsh, 1994).

MATERIALS AND METHODS

Cell culture

3T3 fibroblast cells were routinely maintained on confluent dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and kept at 37°C in 8% CO$_2$. Monolayers for co-cultures were established by seeding 80,000 cells per chamber well of 8-chamber tissue culture slides (Lab-Tek) that had been coated with polylysine (PL) (70,000-150,000 M_r from Sigma at 17 µg/ml in distilled H$_2$O for 20-30 min at room temperature) followed by fibronectin (from SIGMA at 10 µg/ml in DMEM for 2 hours at 37°C). Single cell suspensions of postnatal day 4 (PND4) rat cerebellar neurons were obtained as previously described (Doherty et al., 1990, 1992) and co-cultures were established by seeding 3,000 cerebellar neurons onto confluent monolayers of 3T3 cells established over a 24 hour period. In some experiments, 5,000 cerebellar neurons were seeded into wells coated with PL followed by laminin (PL/LN; laminin was obtained from SIGMA and was coated for 2 hours at 37°C at 10 µg/ml in DMEM). Neuronal cultures were generally main-

tained for ~16 hours in DMEM/SATO medium (for details see Doherty et al., 1990) supplemented with 2% FCS and various reagents as indicated. The cultures were then fixed with paraformaldehyde, methanol permeabilised and the cerebellar neurons stained for GAP-43 as previously described (Doherty et al., 1991b, 1992; Williams et al., 1992). Greater than 90% of the phase-bright cells expressed GAP-43. The average length of the longest neurite on each neuron was determined using a Sight Systems Image Manager (Sight Systems, Newbury, England). All GAP-43-stained neurons were included in the analysis irrespective of the length of their neurites.

Protein extraction and western blotting for phosphotyrosine modified proteins

Cultures of cerebellar neurons were established by seeding 5×10^6 cells into PL-coated 35 mm tissue culture plates. After ~20 hours, control and test cultures were washed twice with ice-cold PBS and harvested on ice into 20 mM Tris/HCl (pH 7.4) containing 1 mM EDTA, 1% NP40, 1% Aprotinin, 1 mM PMSF and 1 mM vanadate. Protein concentrations were determined and 50 µg of extracts were resolved by electrophoresis on 7.5% polyacrylamide minigels and transferred to nitrocellulose membranes as previously described (Williams et al., 1992). The nitrocellulose membranes were incubated for 4 hours with phosphate-buffered saline (PBS) containing 2% soya milk and 0.2% Tween 20. After washing, the membranes were incubated with an anti-phosphotyrosine mAb PY72 (obtained from Dr Bart Sefton, Salk Institute and used at a 1:300 dilution). After washing, bound PY72 was detected with a peroxidase-conjugated goat anti-mouse serum (from Amersham and used at 1:3000 dilution). Both antibodies were diluted into PBS/Tween 20 and incubated for 1 hour at room temperature. Immunoreactive bands were visualised using Amersham's ECL detection kit according to manufacturers instructions.

Other reagents

Recombinant basic FGF was obtained from Amersham. Verapimil and 4-bromophenacyl bromide (BPB) were purchased from SIGMA; ω-conotoxin, BAPTA/AM, the erbstatin analogue and melittin were purchased from Calbiochem and arachidonic acid and RHC-80267 were from Biomol. Pertussis toxin was a gift from Dr J. Kenimer. All of the reagents were used at concentrations that have no non-specific effects on the ability of 3T3 monolayers to support neurite outgrowth (e.g. see Doherty et al., 1991; Williams et al., 1992; results from the present study). Some experiments were conducted in media containing 0.25 mM CaCl$_2$ (rather than 1.8 mM CaCl$_2$) with, in both instances, the MgCl$_2$ concentration being kept at 1.8 mM. In these experiments varying concentrations of divalent cations were added to calcium- and magnesium-free DMEM.

RESULTS

FGF stimulates neurite outgrowth from cerebellar granule cells cultured on a cellular monolayer or on PL/LN

The effects of FGF on neurite outgrowth from PND4 cerebellar granule cells was determined for neurons cultured at low density over confluent monolayers of 3T3 fibroblasts or over a PL/LN-coated substratum. The first set of experiments allows for a comparison between the results of the present study with a number of studies that have tested the effects of pharmacological reagents on neurite outgrowth over control and transfected 3T3 fibroblasts that have been modified to express a number of CAMs (see discussion). In the second set of experiments, we can confirm that the effects of FGF and other agents are not mediated by the monolayer cells.
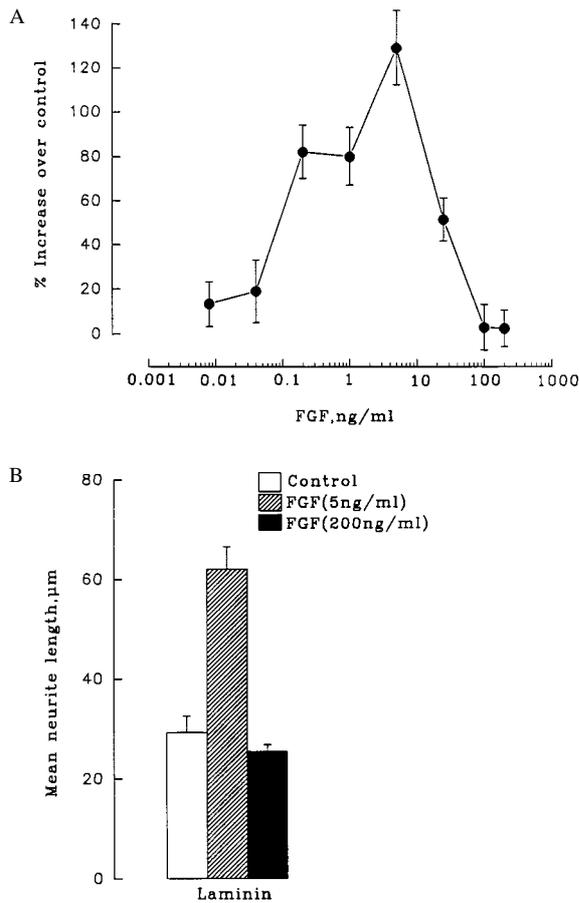
A

B

Fig. 1. FGF stimulates neurite outgrowth from cerebellar granule cells. Cerebellar granule cells were cultured in control media or media supplemented with FGF on confluent monolayers of 3T3 fibroblasts (A) or on a PL/LN-coated substratum (B). After 16 hours, the cultures were fixed and stained for GAP-43 and the length of the longest neurite on each cell was then determined. (A) The mean neurite length measured in a single representative experiment with each value derived from 100-150 sampled neurons. (B) Pooled results from three independent experiments. Bars show ± s.e.m.

A wide range of postmitotic neurons, including cerebellar granule cells, express the FGFR1 (e.g. see Wanaka et al., 1990; Heuer et al., 1990; Asai et al., 1993) and FGF can promote survival and/or neurite outgrowth from a variety of dissociated neurons placed in tissue culture (see introduction). Fig. 1A shows that FGF stimulates neurite outgrowth from cerebellar neurons cultured on 3T3 cell monolayers in a dose-dependent manner; however, the response is clearly complex. In a total of 8 independent experiments, the mean length of the longest neurite increased from a basal value of 31.6±1.8 μm in control cultures to 63.9±1.9 μm in cultures treated with 5 ng/ml FGF (both values mean ± s.e.m.). In contrast, in a total of four independent experiments, high concentrations of FGF (100-200 ng/ml) had no effect on neurite outgrowth (the value in treated cultures was 99.0±2.1% of the control value, mean ± s.e.m.). Biphasic effects of FGF on neuronal survival and neurite outgrowth have been noted in other studies (e.g. see Hatten et al., 1988).

An analysis of the number of GAP-43-positive neurons, and

Table 1. Effects of 5 ng ml FGF on neuronal survival and neurite outgrowth

	Neurons/well	% Neurons with a neurite	% Neurons with a neurite >40 μm
3T3	1538±64 (3)	83.7±2.3 (3)	22.4±2.2 (12)
3T3 + FGF	1594±111 (3)	80.6±2.5 (3)	61.9±2.9 (12)
PL/LN	1996±98 (3)	58.9±2.3 (3)	27.4±4.8 (3)
PL/LN + FGF	2022±76 (3)	61.2±2.7 (3)	63.3±2.4 (3)

Cultures of cerebellar granule cells used for the analysis of mean neurite length (for details see Fig. 1) were also analysed for total number of GAP-43-positive cells and the percentage of these cells with a clearly discernable neurite (of approximately 1 cell diameter) or a neurite greater than 40 μm in length. Measurements were made from replicate cultures for the given number of independent experiments. Neuronal counts were determined by sampling approximately 10% of the total area of the culture well.

the percentage of these cells with a clearly discernible neurite of approximately one cell diameter or more, shows that at 5 ng/ml FGF does not promote survival or stimulate neuritogenesis per se in our cultures (Table 1). Furthermore the percentage of cells extending a neurite of greater than 40 μm was substantially increased on treatment with 5 ng/ml FGF for neurons cultured on both 3T3 monolayers and laminin (Table 1). Thus we can conclude that the effects of FGF on neurite outgrowth are not related to changes in neuronal survival or to a stimulation of neuritogenesis per se.

FGF had an essentially identical effect on cerebellar neurons cultured on a PL/LN-coated substratum (Fig. 1B). Results pooled from three independent experiments showed the mean length of the longest neurite increased from 29.3±3.2 μm in control media to 62±4.5 μm in media supplemented with 5 ng/ml FGF (mean ± s.e.m.). Again higher concentrations of FGF failed to stimulate neurite outgrowth (see Fig. 1B). We can conclude that FGF stimulates neurite outgrowth by directly acting on cerebellar granule cells and that high concentrations of FGF can desensitise this response.

FGF increases the level of phosphotyrosine-modified proteins

In primary neurons, FGF is likely to mediate its effects through binding to FGFRs and stimulating tyrosine phosphorylation of cellular substrates. We have used western blotting with an anti-phosphotyrosine mAb(PY72) to assess the effects of FGF on steady state levels of tyrosine phosphorylation in cerebellar neurons cultured on a PL/LN-coated substratum. Fig. 2 shows an example of a representative experiment on the effects of differing concentrations of FGF on the level of phosphotyrosine proteins at 60 minutes after the addition of FGF. Specific increases in immunoreactivity are clearly seen in a number of proteins including some with apparent relative molecular masses of 120, 106 and 100 (a doublet) $\times 10^3$. The response was dose-dependent and reached a maximal value at 200 ng/ml FGF. The response to high levels of FGF (200 ng/ml) was not obviously diminished with respect to low levels of FGF (5 ng/ml) even after 20 hours of treatment (data not shown). This result suggests that the failure of high concentrations of FGF to stimulate neurite outgrowth cannot be attributed to desensitisation of the FGFR. An alternative possibility, supported by the results of Fig. 2, is that FGF can stimulate the production of a second messenger within the neurons and that it is this second messenger that has biphasic effects on neurite outgrowth.
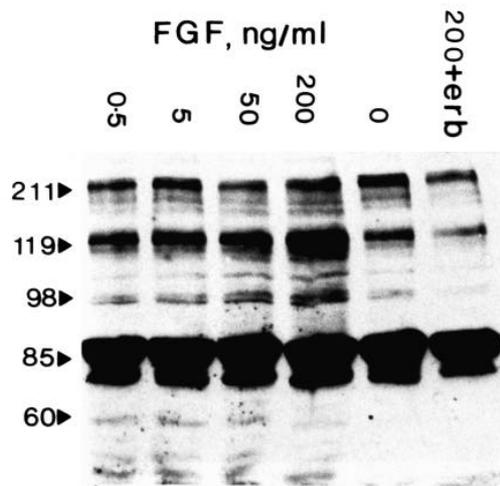
Fig. 2. FGF increases the level of phosphotyrosine modified proteins. Cultures of cerebellar neurons were treated for 60 minutes with varying levels of FGF as indicated, before being extracted and analysed for phosphotyrosine modified proteins using the PY72 mAb (see methods). Results also show the effect of the addition of the erbstatin analogue at 10 μg/ml on the response to FGF at 200 ng/ml (lane marked 200+erb).

We tested a number of tyrosine kinase inhibitors for their ability to inhibit the increase in tyrosine phosphorylation induced by FGF. Of particular interest was the methyl 2,4-dihydroxycinnamate derivative of erbstatin (commonly referred to as the erbstatin analogue), which can inhibit the EGF receptor tyrosine kinase (Umezawa et al., 1990) and which acts as a specific inhibitor of neurite outgrowth stimulated by CAMs (Williams et al., 1994a). Pretreatment of neurons for 30 minutes with the erbstatin analogue inhibited FGF-induced increases in tyrosine phosphorylation of all of the substrates apparent in Fig. 2.

FGF stimulated neurite outgrowth is inhibited by the erbstatin analogue and pertussis toxin

The erbstatin analogue inhibits neurite outgrowth stimulated by transfected CAMs at a site upstream from calcium channel activation, but has no effect on neurite outgrowth stimulated by a wide variety of other agents including K^{+} depolarisation, cholera toxin, which activates protein kinase A, and phorbol esters, which activate protein kinase C (Williams et al., 1994a). In the present study, we tested the effects of the erbstatin analogue on FGF-stimulated neurite outgrowth from cerebellar neurons grown on 3T3 monolayers or on a PL/LN-coated substratum with the results from three independent experiments pooled to generate Fig. 3. The erbstatin analogue had no effect on basal neurite outgrowth over the 3T3 monolayers or over PL/LN (see Fig. 3 legend) confirming that it does not inhibit integrin-dependent neurite outgrowth (Williams et al., 1994a). In contrast the erbstatin analogue completely inhibited the FGF response for neurons cultured on both substrata. Pertussis toxin (1 μg/ml), which inactivates heterotrimeric G-proteins, also inhibited the neurite outgrowth response stimulated by FGF (Fig. 3). Pertussis toxin has no effects on basal neurite outgrowth over 3T3 monolayers or neurite outgrowth stimulated by K^{+} depolarisation (Williams et al., 1992).
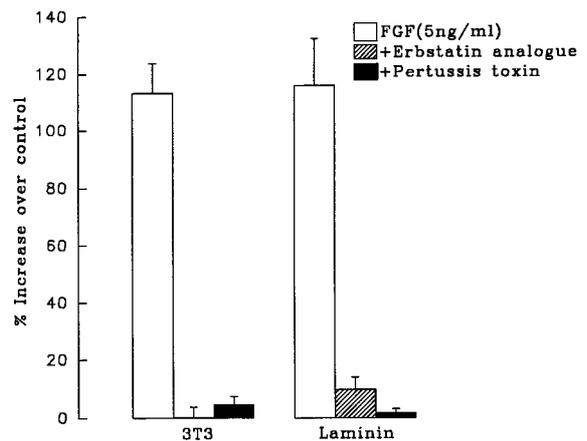

Fig. 3. The erbstatin analogue fully inhibits FGF-dependent neurite outgrowth. Cerebellar neurons were cultured in the presence and absence of FGF (5ng/ml) on confluent monolayers of 3T3 cells or on a PL/LN-coated substratum (see text for details). In each experiment, the FGF-induced increase in mean neurite length was measured in the absence and presence of the erbstatin analogue (10 μg/ml) or pertussis toxin (1 μg/ml). The results show the mean percentage (±1 s.e.m.) increase in mean neurite length over control calculated in three independent experiments. In control media, basal neurite outgrowth over 3T3 cells and PL/LN was 28.2±1.9 μm and 30.8±3.4 μm, respectively. In the presence of the erbstatin analogue, the respective values were 108±3.4% (3T3 monolayers) and 106±4.7% (PL/LN) of the controls. In the presence of pertussis toxin, the respective values were 110±3.5% (3T3 monolayers) and 98.6±4.5% (PL/LN). All values are the mean ± s.e.m., $n=3$ independent experiments.

FGF-stimulated neurite outgrowth requires calcium influx into neurons

Cerebellar neurons were cultured in the presence and absence of a maximally active concentration of FGF (5 ng/ml) in control media or media containing L- and N-type calcium channel antagonists (verapimil at 10 μM and ω-conotoxin at 0.25 μM). The combination of calcium channel antagonists had no effect on basal neurite outgrowth over 3T3 monolayers or the PL/LN-coated substratum (Williams et al., 1992; see also Table 3), but completely inhibited FGF-stimulated increases in neurite outgrowth on both substrata (the pooled results from three independent experiments on each substratum are shown in Fig. 4). In a single experiment on 3T3 monolayers, antagonising the L-type channel inhibited the FGF response by 73%, whereas antagonising the N-type channel inhibited the response by 64%.

BAPTA/AM is a membrane permeable calcium chelator that can be trapped in cells following hydrolysis of its acetoxymethyl ester group. Following preloading of neurons, BAPTA/AM can prevent neurite outgrowth stimulated by CAMs or by K^{+} depolarisation by chelating and thereby attenuating changes in intracellular calcium. BAPTA/AM has no non-specific effects on the ability of neurons to extend neurites per se, nor the ability of 3T3 cells to support neurite outgrowth (for details see Williams et al., 1992). In the present study, pretreatment of neurons with BAPTA/AM (20 μM for 3 hours), or the presence of this agent throughout the culture period, completely inhibits neurite outgrowth stimulated by FGF with
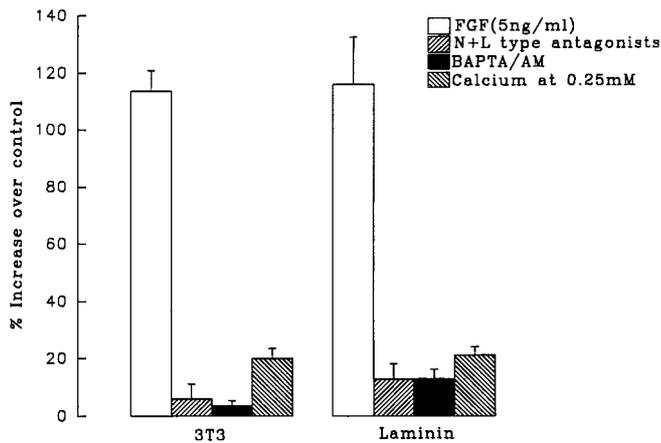
Fig. 4. Agents that perturb calcium flux across the neuronal membrane inhibit the FGF response. Cerebellar neurons were cultured in control media supplemented with FGF (5 ng/ml) on monolayers of 3T3 fibroblasts or on a PL/LN-coated substratum. For each experiment, the FGF-induced increase in mean neurite length was measured in the absence or presence of verapimil (10 μm) and ω-conotoxin (0.25 μM), BAPTA/AM (20 μM) or extracellular calcium reduced to 0.25 mM. Cultures were maintained for ~16 hours prior to fixing and staining. The above results show the percentage increase in mean neurite length over controls pooled from three independent experiments, each value is the mean ± s.e.m. In control media, basal neurite outgrowth over 3T3 cells and PL/LN was 28.2±1.9 μm and 30.8±3.4 μm respectively. In the absence of FGF, N- and L-type antagonists had no effect on neurite outgrowth over 3T3 cells (107±3.7 % control) or PL/LN (93.4±4.5 % control). The respective values in BAPTA/AM-treated cultures were 108±2.5 % (3T3 monolayers) and 104±3.7% (PL/LN) and 90±4.6 % (3T3 monolayers) and 90±5.4 % (PL/LN) in low Ca^{2+} media. All values are the mean ± s.e.m, n=3.

Fig. 5. Arachidonic acid (AA) stimulates neurite outgrowth from cerebellar neurons in a dose-dependent manner. Cerebellar granule cells were cultured in control media or media supplemented with AA (0.1-200 μM) on confluent monolayers of 3T3 fibroblasts for 16 hours before being fixed and stained for GAP-43. The length of the longest neurite on each cell was then determined. The results show the mean neurite length and each value is derived from measurements made from 100-150 neurons in a representative experiment.

the pooled results from three independent experiments shown in Fig. 4.

Reduction of extracellular calcium to 0.25 mM, which would substantially reduce calcium influx into neurons, also inhibited FGF stimulation of neurite outgrowth from neurons cultured on 3T3 monolayers (three independent experiments) or neurons cultured on a PL/LN-coated substratum (one experiment, both results shown in Fig. 4). Again, as with the calcium channel antagonists and BAPTA/AM, reducing extracellular calcium to 0.25 mM has no non-specific effects on neurite outgrowth. For example agents that block or negate the effects of calcium influx into neurons have no effect on basal neurite outgrowth, which depends on integrin receptor function or neurite outgrowth stimulated by agents that activate protein kinase A or C (for details see Williams et al., 1992).

Arachidonic acid can mimic the FGF response

The above results suggest that calcium influx into neurons is required for the FGF response. A second messenger is likely to be involved in the activation of calcium channels and candidates can be identified by determining if they can mimic the neurite outgrowth response stimulated by FGF. Activation of protein kinase A or C stimulates neurite outgrowth from cerebellar neurons (eg see Williams et al., 1992); however, these responses do not require calcium influx into neurons. AA has
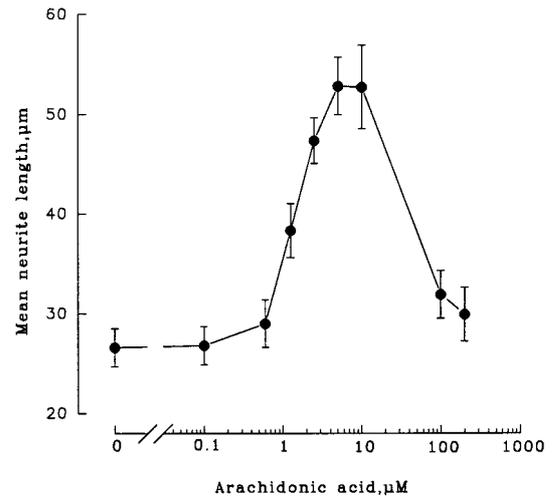
recently been shown to modulate the activation of voltage-sensitive calcium channels in myocytes with a maximal response apparent at 10-20 μM with a reduced response at >100 μM (Huang et al., 1992). AA can stimulate a dose-dependent neurite outgrowth response from PND4 cerebellar neurons cultured on monolayers of 3T3 fibroblasts with the results from a representative experiment shown in Fig. 5 (see also Williams et al., 1994b). The response to AA was of a similar magnitude to the FGF response and, like the FGF response, was also clearly biphasic. AA was also able to stimulate neurite outgrowth from neurons cultured on a PL/LN-coated substratum with the dose-response curve shifted slightly to the left. For example, at 2.5 μM, AA increased the mean length of the longest neurite from 34.2±2.6 μm to 66.4±3.5 μm for neurons grown on the PL/LN substratum (values mean ± s.e.m pooled from three independent experiments). We can conclude that AA can directly stimulate neurite outgrowth from cerebellar neurons and that high concentrations can desensitise the response.

AA stimulated neurite outgrowth requires calcium influx into neurons

We tested the effects of a variety of agents on the neurite outgrowth response to AA. Similar results were obtained for neurons cultured on 3T3 monolayers and on PL/LN with the results from the co-culture experiments shown in Table 2. In a total of three independent experiments, AA (10 μM) increased the mean length of the longest neurite by 139±7% in control media, but had no significant effect when verapamil (10 μM) and ω-conotoxin (0.25 μM) or BAPTA/AM (20 μM) were included in the media (see Table 1) and this confirms previous results (Williams et al., 1994b). In contrast, the response to AA

Table 2. Effect of various agents on AA-induced neurite outgrowth

Agent	% Inhibition of AA response
N- and L-type antagonists	93.7±2.3 (3)
BAPTA/AM (20 μM)	94.4±6.2 (3)
Erbstatin analogue (10 μg/ml)	9.5±2 (3)
BPB (50 μM)	5.8±8.2 (3)
RHC-80267 (50 μM)	−0.8±6.4 (3)
Pertussis toxin (1 μg/ml)	2.7±1.7 (3)
FGF (200 ng/ml)	95.6±9.6 (1)

Cerebellar neurons were cultured in the presence and absence of AA (10 μM) on confluent monolayers of 3T3 cells. In a typical experiment AA increased the mean neurite length per neuron from a basal value of 25.6±1.9 μm to 60.9±4.2 μm (mean ± s.e.m. for details see text). The effects of N- and L-type calcium channel antagonists (ω-conotoxin at 0.25 μM and verapimil at 10 μM), the calcium chelator BAPTA/AM, the erbstatin analogue, the PLA_2 and DAG lipase inhibitors (BPB and RHC-80267 respectively) pertussis toxin (1 μg/ml) or high FGF (200 ng/ml) on the response to AA was measured in a number of experiments. The above results show the percentage inhibition of the AA response and each value is the mean ± s.e.m. determined from the given number of experiments. These agents had no effect on basal neurite outgrowth (e.g. see Table 3 and text for details).

Table 3. Activation of PLA_2 stimulates calcium-dependent neurite outgrowth

	Control media	Melittin (0.1 μM)
control	25.6±2.8 μm(135)	64.7±4.8 μm(136)
N- and L-type antagonists	26.9±2.8 μm(124)	30.2±3.4 μm(120)
BAPTA/AM(20 μM)	25.2±2.9 μm(156)	33.4±2.4 μm(121)
BPB(50 μM)	23.6±3.9 μm(142)	30.6±2.7 μm(140)
RHC-80267(50 μM)	26.4±3.2 μm(126)	59.9±3.9 μm(130)

Cerebellar neurons were cultured on confluent monolayers of 3T3 fibroblasts in control media and media supplemented with the PLA_2 activator melittin as shown. To discount possible effects of this agent on G-protein activity, neurons were pretreated with pertussis toxin for ~2 hours prior to co-culturing. The media was then further supplemented with either N- and L-type calcium channel antagonists (verapamil at 10 μM and ω-conotoxin at 0.25 μM) calcium chelating agent BAPTA/AM the PLA_2 inhibitor (BPB) or the DAG lipase inhibitor (RHC-80267) as indicated. After ~16 hours, the co-cultures were fixed and the mean length of the longest neurite per cell determined. The results show the mean ± s.e.m. for the given number of neurons sampled in a representative experiment. Similar results were obtained in three independent experiments.
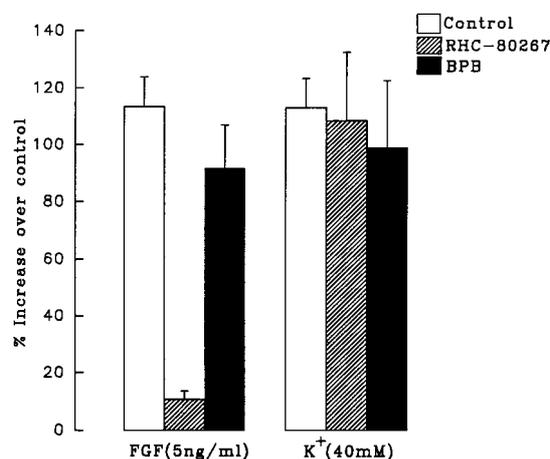
was not inhibited by the erbstatin analogue or by pertussis toxin (Table 1). High concentrations of AA might fail to stimulate neurite outgrowth as a consequence of desensitisation of calcium channels and it follows that the biphasic response to FGF (see Fig. 1A) might also be attributable to an AA-induced desensitisation of calcium channels. If this is the case high concentrations of FGF should inhibit neurite outgrowth stimulated by AA and vice versa (see above). We have found that at 200 ng/ml FGF can also inhibit the neurite outgrowth response to AA (Table 2). High concentrations of FGF had no effect on neurite outgrowth stimulated by K^+ depolarisation or agents that activate protein kinase A or C (data not shown).

A similar response to AA can also be induced by melittin which stimulates the production of AA by activating PLA_2. The response was again inhibited by agents that block or negate the effects of calcium influx into neurons (Table 3). The response could be substantially inhibited by a specific inhibitor of PLA_2 (BPB e.g. see Snyder et al., 1992). This drug had no effect on neurite outgrowth stimulated by AA (results pooled from three independent experiments shown in Table 2) or neurite outgrowth stimulated by K^+ depolarisation, or agents that activate protein kinase A or C (data not shown). These data clearly show that this drug has no non-specific effects on neurite outgrowth. We can conclude that whether exogenously supplied or endogenously produced, AA can fully mimic the FGF response via a mechanism that requires calcium influx into neurons.

FGF-stimulated neurite outgrowth requires DAG lipase activity

AA can be generated in cells either by PLA_2 or by the sequential activities of a PLC to generate DAG and conversion of this by DAG lipase to AA (reviewed in Piomelli, 1993). It follows that, if AA is required for the FGF response, then the response should be specifically blocked by inhibition of PLA_2 and/or DAG lipase. Results pooled from three independent experiments and summarised in Fig. 6 show that the FGF response can be completely abolished by a DAG lipase inhibitor (RHC-

Fig. 6. The FGF response is blocked by an inhibitor of DAG lipase. Cerebellar granule cells were cultured on confluent monolayers of 3T3 fibroblasts in the presence or absence of FGF (5 ng/ml) or K^+ (40 mM). In each experiment, the FGF- and K^+-induced increase in mean neurite length was measured in control media or media containing RHC-80267 (50 μM) or BPB (50 μM). These agents had no effect on basal neurite outgrowth (e.g. see Table 3) but the DAG lipase inhibitor RHC-80267 specifically inhibited the FGF response. The results show the percentage (+s.e.m.) increase in mean neurite length pooled from three independent experiments.

80267 at 50 μM) but is relatively unaffected by the PLA_2 inhibitor (BPB at 50 μM). As a control, we found no effect of the DAG lipase inhibitor on basal neurite outgrowth (Table 3) or neurite outgrowth stimulated by AA (Table 2) melittin (Table 3) or agents that activate protein kinase A or C (data not shown).

DISCUSSION

Fibroblast growth factors constitute a family of polypeptides that exhibit mitogenic activity towards a wide variety of cells of mesenchymal, neuronal and epithelial origin (Burgess and

Maciag, 1989; Gospodarowicz, 1990). In addition to their mitogenic activities, FGFs stimulate survival and/or neurite outgrowth from hippocampal neurons, cerebral cortical neurons and spinal motor neurons (Walicke, 1989; Morrison et al., 1986; Hughes et al., 1993). The ability of FGFs to influence cellular differentiation, as well as the demonstration that these factors are expressed during embryogenesis suggests that they may play other important roles during development. For example, experiments using *Xenopus* embryos have shown that FGF can act as an inducer of mesoderm and might serve in this capacity during normal embryogenesis (Slack, 1990; Amaya et al., 1991).

Our results clearly show that recombinant basic FGF can stimulate neurite outgrowth from cerebellar granule cells cultured either on monolayers of 3T3 fibroblasts or on a PL/LN-coated substratum. It has been previously reported that FGF can stimulate the survival of mouse cerebellar granule cells (Hatten et al., 1988). In our cultures, neuronal survival was not influenced by exogenous FGF and this may be related to the relatively rich medium that we use and/or the presence of a small number of astroglial cells. The lack of effect of FGF on neuronal survival and the percentage of cells with a discernable neurite confirms that FGF is a neurite growth promoting factor for cerebellar neurons (Hatten et al., 1988). The FGF response is completely blocked by a combination of N- and L-type calcium channel antagonists or by preloading neurons with BAPTA/AM or by reducing extracellular Ca^{2+} to 0.25 mM. This is the first evidence that the ability of FGF to induce axonal growth critically relies on the influx of calcium through N- and L-type calcium channels. The effects of FGF are mediated by activation of a cell surface receptor tyrosine kinase (FGFR) and we have shown that the FGF-induced increases in tyrosine phosphorylation and neurite outgrowth can be abolished by a tyrosine kinase inhibitor, the erbstatin analogue, in a highly specific manner.

The response to FGF is biphasic with neurons treated with 5 ng/ml FGF responding by an approximate doubling in neurite length as compared to control cultures whereas neurons treated with 100-200 ng/ml FGF show no response. The biphasic nature of the response is not an uncommon phenomenon (e.g. see Hatten et al., 1988) and could result from desensitisation of the FGFR. However, this seems unlikely in this case since these high concentrations of FGF still lead to increased tyrosine phosphorylation of neuronal proteins. A more plausible explanation could be that the accumulation of a second messenger, downstream of receptor activation, can desensitise the pathway.

It has been suggested that AA is capable of modulating voltage gated calcium channels in a dose-dependent manner with high concentrations (100-200 μM) showing a reduced effect (Huang et al., 1992). A comparable neurite outgrowth response to that induced by FGF can be elicited by AA when added directly to the culture media. Melittin can also stimulate a similar response that can be inhibited by a PLA_2 blocker suggesting that it generates AA within the neurons via activation of this enzyme. The results clearly show that AA-induced neurite outgrowth mimics the FGF response both in the biphasic nature of the response, and the requirement of calcium influx via voltage gated N- and L-type calcium channels. It is known that activated FGFRs can bind and activate PLCγ, and that the sequential activity of PLCγ which produces DAG,

followed by DAG lipase can generate AA. FGF-induced neurite outgrowth is fully inhibited by the DAG lipase inhibitor RHC-80267 at concentrations that do not affect neurite outgrowth stimulated by arachidonic acid or use of depolarising concentrations of KCl. Conversely the FGF response is not blocked by the PLA_2 inhibitor BPB at concentrations that abolish neurite outgrowth induced by melittin, which activates PLA_2. This suggests that the generation of AA by the activity of DAG lipase and not PLA_2 forms a crucial step in the FGF signalling pathway upstream of calcium influx. The biphasic nature of the dose response to FGF could be explained by the accumulation of AA which might operate by desensitising the activation of voltage gated calcium channels. Thus we would postulate that FGF-dependent induction of neurite outgrowth requires the activation of its receptor tyrosine kinase followed by the sequential activities of PLCγ and DAG lipase to generate AA which in turn might directly activate calcium channels. Further metabolism of AA is unlikely to be required for the response as the structurally related oleic acid can also modulate calcium currents (Huang et al., 1992) and stimulate neurite outgrowth (E. J. W., unpublished observation), but cannot be metabolised to prostaglandins or leukotrienes.

Biological responses that require calcium influx into cells are not always accompanied by a change in the apparent resting level of calcium within the cell (for example see Rasmussen, 1989; Doherty et al., 1993). This can be accounted for by the failure of the current technologies to measure highly localised changes in calcium within cells or by the fact that the rate of calcium exchange across the membrane, rather than the level of free calcium, might serve as a signal in a transduction process. For example, the filopodia of the growth cone can serve as a sensor and transducer of extracellular signals and as few as 60 calcium ions moving into a filopodia can double the calcium concentration (Davenport et al., 1993). In this context, the observation by Mattson and collaborators (1989) that FGF does not elevate calcium in hippocampal neurons is not at odds with the present study. However, it remains possible that FGF responses in some neurons might involve activation of a different pathway. For example, the induction of a neuronal phenotype on treatment of naive PC12 cells with FGF (Togari et al., 1985) is a transcription-dependent response that requires a sequence of *src* and *ras* activities (Kremer et al., 1991). We also found no effect of calcium channel antagonists on this response (P.D. unpublished observation). In contrast, the FGF stimulation of neurite outgrowth from NGF-primed PC12 cells, which is a more appropriate model of neurite outgrowth from a postmitotic neuron (Greene, 1984) can be inhibited by calcium channel antagonists (P.D. unpublished observation).

There is evidence that pertussis toxin-sensitive heterotrimeric G-proteins can modulate the function of FGFRs (Jarvis et al., 1992) yet pertussis toxin does not inhibit the mitogenic activity of FGF (Chambard et al., 1987; however see Logan and Logan, 1991). In the present study, we have found that pertussis toxin can completely inhibit the neurite outgrowth response stimulated by FGF upstream from the site of action of AA. In some cells, activation of PLCγ by the EGF receptor tyrosine kinase can be inhibited by pertussis toxin (Yang et al., 1991). A similar phenomenon for FGFR-induced activation of this enzyme could account for the sensitivity of the neurite outgrowth response, and not the mitogenic response (see Introduction), to pertussis toxin. However, it remains

possible that pertussis toxin has a more general effect on signalling through the FGFR (Jarvis et al., 1992; Logan and Logan, 1991).

Cell-contact-dependent neurite outgrowth stimulated by NCAM, N-cadherin and L1 can be inhibited by the erbstatin analogue, pertussis toxin, RHC-80267 and agents that block or negate the effects of calcium influx into neurons. We have postulated that the above CAMs might bind to and activate the FGFR in neurons (Williams et al., 1994a; Saffell et al., 1994). The results of the present study lend support to this postulate by demonstrating that neurite outgrowth stimulated by activation of the FGFR with FGF involves activation of a second messenger pathway that is pharmacologically indistinguishable from that activated by the above CAMs.

In summary, whereas activation of PLCγ by the FGFR tyrosine kinase has been shown to lead to calcium mobilisation from intracellular stores and calcium influx across the cell membrane (e.g. see Peters et al., 1992), it has been established that this pathway is not required for mitogenic responses. The results of the present study suggest that FGF stimulation of neurite outgrowth from cerebellar neurons involves the sequential activities of PLCγ and DAG lipase to generate AA, which in turn can stimulate neurite outgrowth via a pathway that requires calcium influx into neurons through N- and L-type calcium channels.

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