

Platelet-derived Growth Factors and Fibroblast Growth Factors Are Mitogens for Rat Schwann Cells

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Abstract. Rat sciatic nerve Schwann cells in culture respond to a limited range of mitogens, including glial growth factor, transforming growth factors beta-1 and beta-2 (TGF- β 1, TGF- β 2), some cell membrane-associated factors, and to agents such as cholera toxin and forskolin which raise intracellular levels of cAMP. These responses require the presence of FCS, which exhibits little or no mitogenic activity in the absence of other factors. However, we recently found that forskolin greatly potentiates the mitogenic signal from TGFs- β 1 and β 2, raising the possibility that cAMP might couple other factors to mitogenesis. We have therefore screened a range of candidate mitogens using DNA synthesis assays. Other than TGFs- β and glial growth factor, none of the factors tested were mitogenic in the presence of 10% serum alone. With

the addition of forskolin, however, porcine PDGF, human PDGF, acidic and basic FGF were potent mitogens for rat Schwann cells, stimulating DNA synthesis and increasing cell number. Cholera toxin and dibutyrylcyclicAMP, but not 1,9-dideoxyforskolin, can substitute for forskolin indicating that the mitogenic effect is mediated via adenylyl cyclase activation. Porcine PDGF gave half-maximal stimulation at 15 pM, and human PDGF an equivalent response at 1 nM. Basic FGF was half maximal at 5 pM, acidic FGF at 1 nM.

The recognition of PDGFs and FGFs as mitogens for Schwann cells has many implications for the study of Schwann cell proliferation in the development and regeneration of nerves, and in Schwann cell tumorigenesis.

SCHWANN cells, the glial cells of the peripheral nervous system, surround all peripheral nerve axons, and in the case of myelinated nerve fibers, elaborate the myelin sheaths necessary for fast impulse conduction. There are many important developmental and functional interactions between Schwann cells and axons. Of particular interest for this paper is the proliferation of Schwann cells in three contexts. During development, Schwann cells proliferate and migrate out along newly formed axons, surrounding them, and myelinating them in some cases (3, 32). If the nerve is injured, through mechanical trauma, neurotoxins or demyelinating diseases for example, Schwann cells again proliferate to restore the integrity of the Schwann cell sheath and aid regeneration of functional nerve fibers (1, 42). Thirdly, Schwann cell tumors may arise on any peripheral nerve, as in the cases of Von Recklinghausen's neurofibromatosis, bilateral acoustic neurofibromatosis, and spontaneous, non-hereditary acoustic neuromas (47). The origin and nature of the factors involved in these proliferative processes are poorly understood.

Attempts to define the factors involved in Schwann cell proliferation have revealed positive responses by various types of Schwann cell in culture to a limited range of mitogens (38, 51), including a few polypeptide growth factors (35), molecules derived from central and peripheral nervous system membranes (13, 28, 37, 39, 43, 44, 49, 53), and ana-

logues of cAMP and agents, such as cholera toxin, that raise intracellular levels of cAMP (36). Glia maturation factor, reported to be a rat Schwann cell mitogen, has recently been found to contain contaminants, including (FGF)¹ (7, 27). For the past ten years, glial growth factor (GGF) has been viewed as the only soluble polypeptide growth factor for rat sciatic nerve Schwann cells and was purified on the basis of this activity (8). GGF, a 31-kD basic polypeptide isolated from bovine pituitaries and present in the nervous system of higher vertebrates (26), has subsequently been implicated in the limb regeneration of Urodele amphibians (9). In addition, a GGF-like factor has been detected in extracts of human acoustic neuromas (12). Four striking features of the responses of rat sciatic nerve Schwann cells to mitogens were (a) that these cells grew extremely slowly in 10% FCS (doubling time \sim 8 d) and incorporated very little 5'-[¹²⁵I]-iodo-2'-deoxyuridine ([¹²⁵I]-UdR) or [³H]thymidine during 48-h assays (33, 36); (b) that GGF required serum for its mitogenic activity (26); (c) that a range of well-known soluble polypeptide growth factors (including PDGF, FGF, EGF, and nerve growth factor), pituitary hormones, neuropep-

1. *Abbreviations used in this paper:* aFGF, acidic FGF; bFGF, basic FGF; dbcAMP, dibutyrylcAMP; FCP, fetal calf plasma; GGF, glial growth factor; hPDGF, human PDGF; IL, interleukin; pPDGF, porcine PDGF; PDGF-AA, PDGF with AA chain composition; PDGF-BB, PDGF with BB chain composition; pPDGF, porcine PDGF; TGF, transforming growth factor.

tides, and neurotransmitters were inactive (38); and (d) that partially purified GGF ([GGF-CM] GGF-carboxymethyl cellulose fraction), in the presence of serum, would synergize with agents that stimulated intracellular cAMP (33, 35).

Recently it has been found that transforming growth factors (TGFs) $\beta 1$ and $\beta 2$ are mitogenic for rat sciatic nerve Schwann cells (40), suggesting that these cells may be less restricted in their responses to growth factors than previously suggested. During attempts to establish the responses of rat sciatic nerve Schwann cells to purified soluble factors, we found that forskolin, a diterpene drug that reversibly activates adenylyl cyclase with similar effects to cholera toxin (45), greatly potentiates the mitogenic signal from TGFs- β (40), raising the possibility that adenylyl cyclase agonists might couple other growth factors to mitogenesis and reinforcing the idea that Schwann cells might respond to a wide range of soluble growth factors. As a result, we have screened, using incorporation of [125 I]-UdR, a variety of candidate mitogens in serum-containing medium, in the presence and absence of forskolin. This screening, coupled with measurements of cell number and morphological observations, has revealed two additional classes of growth factors to be mitogenic for rat sciatic nerve Schwann cells.

Materials and Methods

Materials

Insulin, NGF-7S, NGF-2.5S, prolactin, follicle-stimulating hormone, ACTH, vasopressin, oxytocin, met-enkephalin, leu-enkephalin, substance P, neurotensin, angiotensin-I, angiotensin-II, vasoactive intestinal peptide, somatostatin, bombesin, gastrin releasing peptide, bradykinin, histamine, dopamine, norepinephrine, epinephrine, serotonin, gamma-amino-*n*-butyric acid, glutamate, glycine, acetylcholine, ATP, cholera toxin, isobutylmethylxanthine, dibutyryl cAMP (dbcAMP), fatty acid-free BSA, 1- β -D-arabinofuranosylcytosine, poly-D-lysine hydrobromide (*M*, 30–70 kD), and rabbit complement were obtained from Sigma Chemical Co. (St. Louis, MO); porcine PDGF (pPDGF), human PDGF (hPDGF), acidic and basic FGF (aFGF and bFGF), TGFs- $\beta 1$, - $\beta 2$, interleukin-1 α (IL-1 α), IL-1 β , IL-2, IL-3, IL-4, IL-6, tumor necrosis factor- α , tumor necrosis factor- β (British Biotechnology Ltd., Oxford, England); insulin-like growth factor-I, insulin-like growth factor-II (KabiGen AB, Stockholm, Sweden); batch tested FCS, fetal calf plasma (FCP) (Imperial Laboratories Ltd., Andover, England); tissue culture plastics (Costar, Data Packaging Corp., Cambridge, MA); DME, 2.5% trypsin in PBS, and 0.05% trypsin/0.02% EDTA in modified Puck's saline (Gibco Laboratories, Scotland); collagenase (type I) (Worthington Biochemical Corp., Freehold, NJ); forskolin, 1,9-dideoxyforskolin, cholera toxin (Calbiochem-Behring Corp., San Diego, CA); anti-Thy-1.1 (Serotec Ltd., Oxford, England); [125 I]-UdR (Amersham International, Amersham, England); rabbit anti-rat S-100 antibody (gift of Dr. R. Mirsky, University College, London); recombinant human PDGF with BB chain composition (PDGF-BB) and PDGF with AA chain composition (PDGF-AA) (gift of Drs. S. M. Gale and C. George-Nascimento, Chiron Corp., Emeryville, CA); EGF (gift of Dr. G. Panayotou, Ludwig Institute for Cancer Research, London); GGF-CM (0.13 mg/ml protein by Bio-Rad assay with gamma-globulin standard), prepared according to the method of Brookes (8) (gift of Dr. A. D. J. Goodearl, Ludwig Institute for Cancer Research).

Preparation of Schwann Cells

Schwann cells from rat sciatic nerves were isolated essentially using the method of Brookes et al. (8, 11). Briefly, sciatic nerves were dissected from 2–5-d-old Sprague-Dawley rat pups, dissociated with 0.1% collagenase and 2.5% trypsin in DME, and plated into polylysine-coated tissue culture flasks in DME, 10% FCS. After ~24 h the medium was replaced with fresh medium containing 10 mM 1- β -D-arabinofuranosylcytosine. 3–4 d later the cells were washed and fed with DME, 10% FCS, 5 μ M forskolin, 0.5 μ g/ml GGF-CM. Once confluent, 2–3 d later, the cells were detached from the flask with 0.05% trypsin, 0.02% EDTA in modified Puck's saline, and

washed and treated with anti-Thy-1.1 and rabbit complement for 30 min to remove contaminating fibroblasts. Schwann cells were grown in DME, 10% FCS, 5 μ M forskolin, 0.5 μ g/ml GGF-CM, fed every second day, and passaged every 4–7 d as described above. The Schwann cell cultures were >99% pure and contained <1% fibroblasts, as determined by morphology and S-100 staining.

DNA Synthesis Assay

DNA synthesis in Schwann cells was assessed by measuring incorporation of [125 I]-UdR, according to the method of Brookes (8). Cells were cultured in the absence of GGF-CM or forskolin for 2–3 d, then seeded into 96-well plates at 10,000 cells/well in 100 μ l/well DME, 10% FCS or FCP. After overnight incubation, samples were added to the assay plates. Factors were prepared in 4 mM HCl or PBS, containing 1 mg/ml BSA (fatty acid free). Forskolin stocks were prepared in dimethyl sulphoxide. All factors were serially diluted in DME and added in a constant volume to give a total volume of 120 μ l/well and the required final concentration. Dose responses of solvents alone had no effect (data not shown). The cells were then incubated with samples for 48 h, 0.2 μ Ci/well [125 I]-UdR being added for the last 24 h. Medium was then aspirated, cells detached using 0.05% trypsin, 0.02% EDTA in modified Puck's saline, and harvested (Multimash 2000; Dynatech Laboratories Inc., Chantilly, VA) onto glass-fiber filter mats for counting (Gamma 5500; Beckman Instruments Inc., Palo Alto, CA).

Measurement of Cell Proliferation

24-well plates were prepared by coating with poly-D-lysine, at 100 μ g/ml water using 2.5 μ g/cm² polylysine for 5 min, followed by one wash with water and air drying. Schwann cells were cultured for 3 d in the absence of forskolin or GGF-CM and seeded into the wells at a density of 60,000 cells/well. After overnight incubation, samples were added at a concentration equivalent to that which gave maximal stimulation in the DNA synthesis assays (2.6 μ g/ml GGF-CM, 10 nM aFGF, 100 pM bFGF, 3 nM pPDGF, 3 nM hPDGF). The cells were incubated for 4 d, photographed, trypsinized, and counted in an hemocytometer.

Results

Screening of Candidate Mitogens

A range of soluble polypeptide growth factors, pituitary hormones, neuropeptides, and neurotransmitters to which rat Schwann cells might respond were assayed for their ability to stimulate DNA synthesis in the presence of 10% FCS with and without forskolin. In general, a wide range of concentrations was tested, and most factors were highly pure preparations (Table I). GGF-CM (Fig. 1 A) and TGFs- β (Table I) were mitogenic in the presence of serum with and without forskolin, as previously reported (26, 33, 40). Most of the factors tested in the presence or absence of forskolin gave no mitogenic response, in general agreement with previous reports showing that Schwann cells respond to few mitogens (35, 38, 51).

Forskolin Couples PDGFs and FGFs to Mitogenesis

The addition of forskolin to the assay, however, revealed the potent mitogenic effects of particular PDGF isoforms, and also aFGF and bFGF. The mitogenic response in the presence of forskolin alone was generally in the range of 200–1,000 cpm, these low values being due to the use of FCS pre-screened for a low forskolin background. pPDGF (BB chain composition) gave half-maximal stimulation at ~15 pM and maximal activity at ~10 nM (Fig. 1 B). Recombinant human PDGF-BB, in which there is no possibility of contaminating A-chains, behaved similarly to pPDGF, reaching the same maximum stimulation as pPDGF (data not shown). In contrast hPDGF (mainly AB chain composition) had not yet

Table I. Screen of Candidate Mitogens for Rat Schwann Cells

| Factor | Concentration tested | Activity* |
|---------------------------------|----------------------|-----------|
| TGF- β 1 | 100 fM-1 nM | a, af |
| TGF- β 2 | 100 fM-1 nM | a, af |
| pPDGF | 300 fM-30 nM | af |
| hPDGF | 10 fM-3 nM | af |
| PGDF-BB | 3 pM-30 nM | af |
| PDGF-AA | 3 pM-30 nM | - |
| aFGF | 10 fM-100 nM | af |
| bFGF | 10 fM-1 nM | af |
| EGF | 40 fM-100 nM | - |
| Insulin | 10 fM-100 nM | - |
| Insulin-like growth factor I | 1 pM-10 nM | - |
| Insulin-like growth factor II | 1 pM-10 pM | - |
| Nerve growth factor (2.5S) | 400 fM-400 pM | - |
| Nerve growth factor (7S) | 700 fM-7 nM | - |
| IL-1 α | 1 fM-10 nM | - |
| IL-1 β | 2 fM-20 nM | - |
| IL-2 | 700 fM-7 nM | - |
| IL-3 | 700 fM-7 nM | - |
| IL-4 | 700 fM-7 nM | - |
| IL-6 | 500 fM-5 nM | - |
| Tumor necrosis factor- α | 10 pM-100 nM | - |
| Tumor necrosis factor- β | 2 pM-20 nM | - |
| Luteotrophic hormone | 1 pM-1 μ M | - |
| Follicle stimulating hormone | 1 pM-1 μ M | - |
| Adrenocorticotrophic hormone | 1 pM-1 nM | - |
| Vasopressin | 1 pM-1 μ M | - |
| Oxytocin | 1 pM-1 μ M | - |
| Met-enkephalin | 1 pM-1 μ M | - |
| Leu-enkephalin | 1 pM-1 μ M | - |
| Substance P | 670 fM-670 nM | - |
| Neurotensin | 1 pM-1 μ M | - |
| Angiotensin-I | 900 fM-900 nM | - |
| Angiotensin-II | 900 fM-900 nM | - |
| Vasoactive intestinal peptide | 10 pM-10 nM | - |
| Somatostatin | 10 pM-100 nM | - |
| Gastrin releasing peptide | 30 fM-100 nM | - |
| Bradykinin | 10 pM-1 μ M | - |
| Histamine | 1 pM-1 μ M | - |
| Dopamine | 5 pM-50 nM | - |
| Norepinephrine | 5 pM-5 μ M | - |
| Epinephrine | 5 pM-5 μ M | - |
| Serotonin | 1 pM-1 μ M | - |
| Gamma amino butyric acid | 1 pM-1 μ M | - |
| Glutamic acid | 1 pM-1 μ M | - |
| Glycine | 1 pM-1 μ M | - |
| Acetylcholine | 1 pM-1 μ M | - |
| ATP | 2 pM-2 μ M | - |

* -, no effect; a, active; af, active with forskolin.

reached a maximum at 3 nM and was \sim 70-fold less active than pPDGF (Fig. 1 B), suggesting that the reduction in potency was due to the A-chain component. Since highly purified human PDGF contains variable and undetermined quantities of BB and AA chains (17), we also tested recombinant human PDGF-AA, which, although it is as highly active as pPDGF in mitogenic assays with Swiss 3T3 cells (our unpublished results), gave no response with rat Schwann cells in the presence of forskolin (Table I).

aFGF and bFGF were also strongly mitogenic for rat

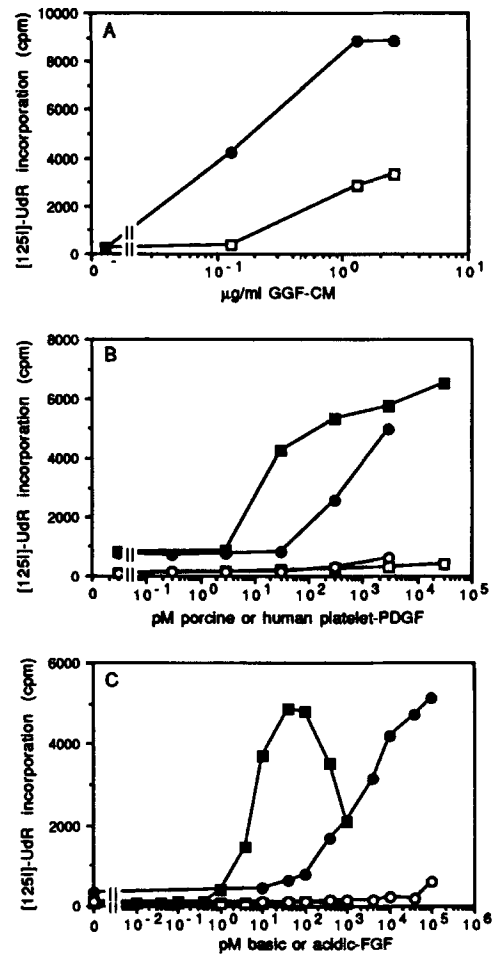


Figure 1. DNA synthesis in rat sciatic nerve Schwann cells in the presence of GGF-CM, PDGFs, and FGFs. Factors were added, with (closed symbols) or without (open symbols) 5 μ M forskolin, to cells in DME, 10% FCS, in 96-well plates for 48 h. DNA synthesis was measured by incorporation of [¹²⁵I]-UdR over the last 24 h. (A) GGF-CM; (B) pPDGF (■) or hPDGF (●); (C) bFGF (■) or aFGF (●). Each figure is a typical example of at least three separate experiments, where each value represents the mean of duplicate observations not differing from the mean by >10%.

Schwann cells in the presence of serum and forskolin (Fig. 1 C). bFGF gave a half-maximal stimulation at \sim 5 pM and reached a maximum at 40-100 pM, whereas aFGF had not reached a clear maximum at 50 nM, and only gave a stimulation approaching the half maximal of bFGF at 1 nM (Fig. 1 C). Interestingly, bFGF gave strong inhibition at concentrations over 300 pM, emphasizing the need to carry out mitogenic assays over a wide range of concentrations. aFGF was \sim 200-fold less active than bFGF, as has been found in other systems.

The Response to PDGFs or FGFs Is Dependent upon Raised Intracellular cAMP

The observation that forskolin is required to effect the mitogenicity of PDGFs or FGFs suggests that cAMP may be the mediator of the required cooperation. However, forskolin is known to have effects other than the activation of adenylyl cyclase leading to a rise in intracellular cAMP (45), such as membrane perturbation and activation of ion channels (19),

inhibition of glucose transport, and desensitization of acetylcholine receptors (21, 52). For these reasons we investigated whether the forskolin effect required for the mitogenicity of PDGFs or FGFs is due to the activation of adenylyl cyclase by using the forskolin analogue 1,9-dideoxyforskolin. This analogue, which fails to activate adenylyl cyclase but shows similar biochemical properties to forskolin in other respects (45, 19, 21, 52), is unable to support the stimulation of DNA synthesis by PDGFs or FGFs (Fig. 2). Similarly, other agents that are known to cause a rise in intracellular cAMP, such as cholera toxin or dbcAMP also couple FGFs or PDGFs to mitogenesis (Fig. 2).

Plasma Can Replace Serum for the Mitogenic Response

Since these results indicate that PDGFs in the presence of elevated cAMP are mitogenic, and since TGFs- β have previously been shown to be mitogenic for rat Schwann cells (40), the use of serum in mitogenic assays raises two questions. Firstly, although FCS prescreened for low background activity was used in the screen, could levels of platelet-derived factors have influenced the results? Secondly, although cAMP has previously been considered to be mitogenic (36), could this response be due to coupling with the PDGF and TGF- β present in the FCS? Schwann cells exposed to FGFs or PDGFs in the presence of FCP showed the same mitogenic responses as seen with FCS; giving no response in the presence of FCP alone and responses of the same shape and magnitude as in Fig. 1 when incubated with FCP plus forskolin; and demonstrating that a platelet factor(s) is not the serum constituent required for response to the soluble growth factors, and that FCP equals FCS in providing factors required for maintenance and the responses to growth factors and forskolin (data not shown). The background DNA synthesis of the cells with forskolin was higher with FCS than FCP, suggesting that the serum-derived platelet factors may be required for the mitogenic effect of forskolin. Fig. 3 shows the mitogenic response to cholera toxin (Fig. 3 A) or forskolin (Fig. 3 B) in the presence of FCP and two lots of FCS. Both lots of FCS give a higher response than FCP but one is particularly mitogenic in the presence of 0.5 μ M forskolin, which is characteristic of TGF- β (40), a major component in bovine sera. A small response still remains in the presence of FCP, which may be due to a failure to remove all platelets from the plasma or before some released their contents, but also possibly due to the secretion of some growth factors by the Schwann cells themselves (34). However, the data show that cAMP elevating agents are largely dependent upon platelet factors from serum for their mitogenic activity, sug-

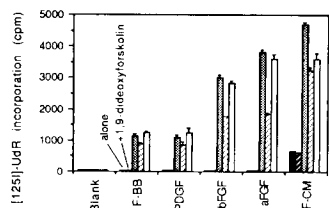


Figure 2. DNA synthesis in rat sciatic nerve Schwann cells exposed to PDGF-BB (3 nM), hPDGF (3 nM), bFGF (50 pM), aFGF (10 nM), or GGF-CM (2.6 μ g/ml) in the presence of DME, 10% FCP, and either blank (\blacksquare), 5 μ M 1,9-dideoxyforskolin (\square), 5 μ M forskolin (\boxtimes), or 100 pM cholera toxin (\square) for 48 h. Errors = SEM, $n = 4$. One of two experiments.

gesting that cAMP may be mitogenic for rat Schwann cells only in the presence of platelet factors such as PDGFs and TGFs- β , or with FGFs.

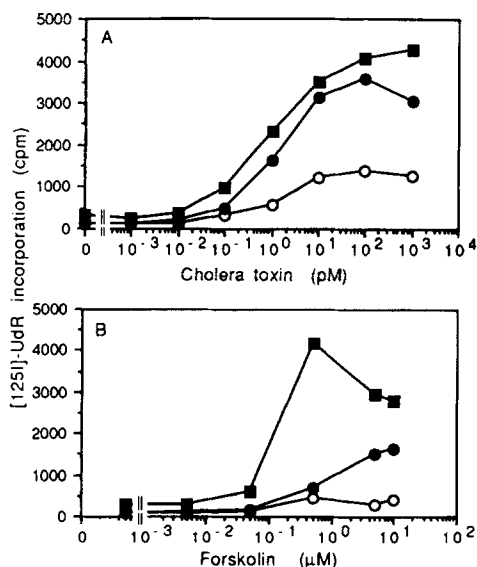


Figure 3. DNA synthesis in rat sciatic nerve Schwann cells after incubation for 48 h in DME and, (○) 10% FCP, (●) 10% FCS (screened for low background growth), or (■) 10% FCS (high background), in the presence of dose responses to either (A) cholera toxin or (B) forskolin. Each value represents the mean of duplicate observations not differing from the mean by >10%, and are typical of three separate experiments.

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Effects of PDGFs and FGFs on Cell Proliferation

To establish whether these highly suggestive changes in [125 I]-UdR incorporation reflect a true mitogenic response by the cells, it was necessary to observe the effects of PDGFs and FGFs in the presence of forskolin on cell proliferation. In agreement with previous studies of rat sciatic nerve Schwann cell growth in the presence of forskolin we found it necessary to coat tissue culture plastic surfaces with polylysine to maintain the adherence of dividing cells over periods of more than a few days (33). Furthermore, because our studies with FCP led to lower background growth than in serum, 10% FCP was used in place of 10% FCS. Table II presents changes in cell number after exposure of Schwann cells to factors, at concentrations estimated to be optimal in

Table II. Cell Growth with Forskolin plus FGFs or PDGFs

| Addition | Cells/Well*† |
|-------------|----------------------|
| No addition | 83,500 \pm 6,500 |
| GGF | 249,000 \pm 1,750 |
| pPDGF | 207,500 \pm 15,500 |
| hPDGF | 107,500 \pm 3,000 |
| aFGF | 213,000 \pm 6,000 |
| bFGF | 143,500 \pm 8,500 |

* $p < 0.05$, † \pm SEM ($n = 4$) one of two experiments. Schwann cells were seeded into 24-well plates, coated with polylysine in DME, 10% FCP at a density of 60,000 cells/well. After 24 h incubation, growth factors were added to a final concentration of 2.6 μ g/ml GGF-CM, 10 nM aFGF, 100 pM bFGF, 3 nM pPDGF, 3 nM hPDGF, 5 μ M forskolin, and incubated for a further 4 d at 37°C, 10% CO $_2$. The cells were then photographed and detached by trypsinization for counting in a hemocytometer.

the DNA synthesis assays, over a period of 4 d. Porcine PDGF and aFGF stimulated increases in cell number which were, respectively, 149 and 155% above the background forskolin value, while bFGF and hPDGF gave increases of 72 and 29%, respectively. The lower responses may be due to differences in the requirements of the cells for each individual growth factor over the longer incubation time of 4 d compared to the DNA synthesis assays (Fig. 1) of only 2 d. The lower response to bFGF, however, may also be partly due to the more narrow effective concentration range of this factor and the use of a concentration close to the upper limit of this range. Although the presence of the polylysine substratum introduces an additional variable, which possibly influences the responses to soluble growth factors, the data nevertheless confirm the DNA synthesis results above and

that pPDGF, hPDGF, aFGF, and bFGF are capable of stimulating Schwann cell proliferation.

The Morphology of Cells Growing in PDGFs or FGFs

Schwann cells in culture express a range of different morphologies, especially in response to mitogens. In addition to the characteristic bipolar, aligned cells, a second morphology may be seen that is flatter, darker, fibroblast-like, and more frequent in the presence of forskolin or cholera toxin. The cells may also take on a very spindly appearance in serum-free medium (10, 51). Fig. 4 shows the appearance of the cells when stimulated with GGF-CM, aFGF, bFGF, pPDGF, or hPDGF in the presence of FCP and forskolin for 4 d. Serum and forskolin alone resulted in the Schwann cells

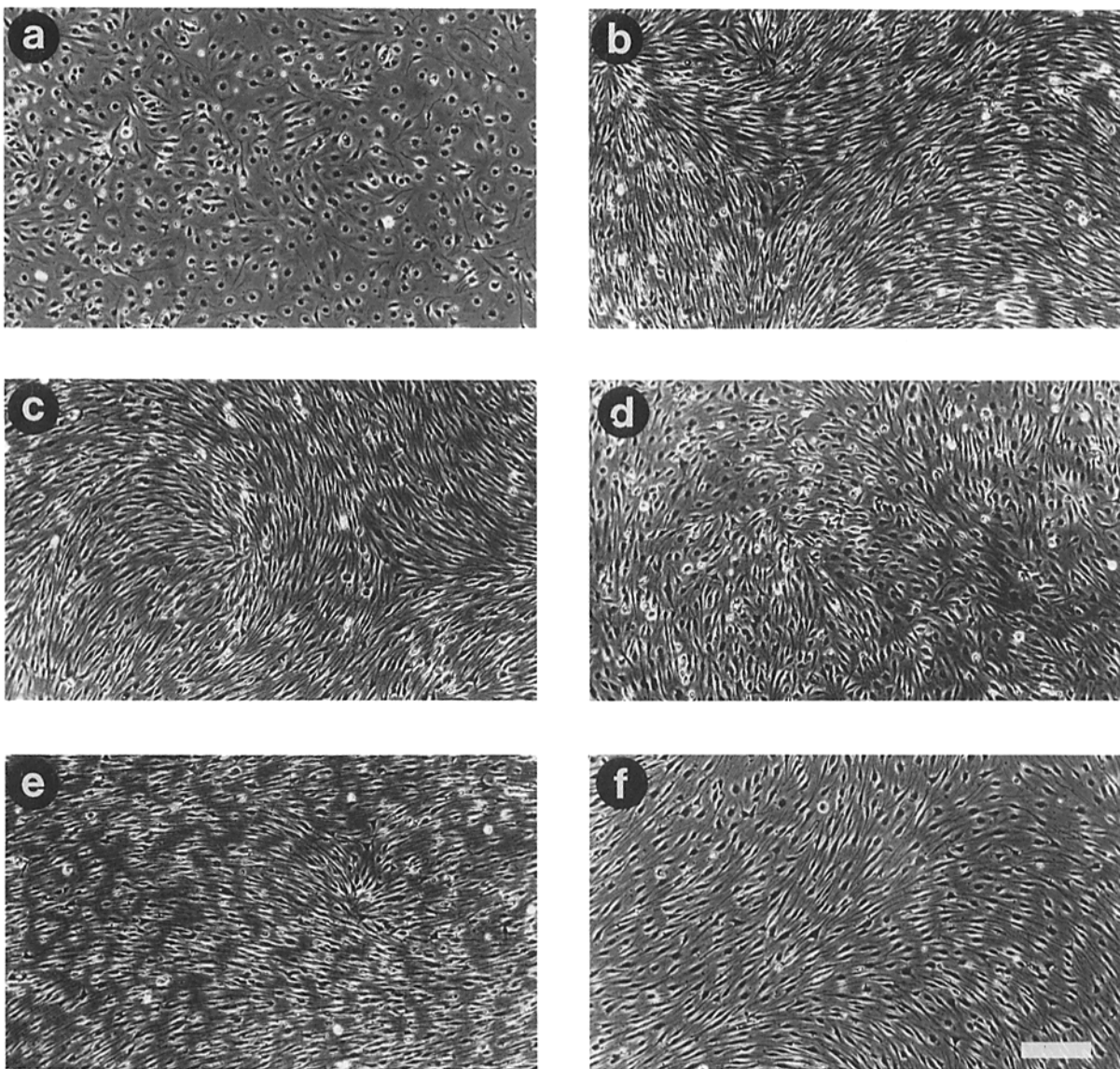


Figure 4. Phase-contrast microscopy of rat sciatic nerve Schwann cells after growing on polylysine-coated 24-well plates for 4 d in the presence of DME, 10% FCP, 5 μ M forskolin, and (a) no addition, (b) 2.6 μ g/ml GGF-CM, (c) 10 nM aFGF, (d) 100 pM bFGF, (e) 3 nM pPDGF, and (f) 3 nM hPDGF. Bar, 100 μ m.

exhibiting a pronounced flattening and fibroblast-like morphology (Fig. 4 *a*), whereas the addition of FGFs (Fig. 4, *c* and *d*) or PDGFs (Fig. 4, *e* and *f*) caused increases in cell density and a morphology similar to that seen upon stimulation with GGF-CM and forskolin (Fig. 4 *b*) (33), showing the characteristic alignment and packing of Schwann cells, with long bipolar processes.

Discussion

These results show that pPDGF, hPDGF, bFGF, and aFGF, which are inactive in the presence of serum alone and therefore have not previously been recognized as mitogens for rat sciatic nerve Schwann cells, are highly mitogenic for these cells when administered with forskolin, cholera toxin or dbcAMP.

It is clear that rat sciatic nerve Schwann cells are not mitogenically responsive to all three PDGF isoforms, BB, AB, and AA, as are some other cell types (18). The potent Schwann cell response to both pPDGF and recombinant PDGF-BB, the weaker response to hPDGF, and the nonmitogenicity of recombinant PDGF-AA, suggest that these cells only respond mitogenically to dimers containing at least one PDGF B-chain. Despite an apparent consensus that there are at least two classes of PDGF receptor, a B-chain-specific class and an A-chain- and B-chain-specific class, there are substantial differences within the field concerning the subunit composition of receptors (18, 46), which prevent further conclusions about the nature of PDGF receptors on the Schwann cells used in these experiments.

bFGF was 200-fold more active than aFGF in our assays, similar to observations with other cell types (6). The significance, however, of any differences in activity is unclear at present, as the two species have very different tissue distributions (16), and because aFGF appears to be equally active in some circumstances when stabilized with heparin, although the physiological relevance of this effect is unclear (15). In the past there have been conflicting reports as to whether FGF is a mitogen for Schwann cells. bFGF has been reported to be weakly mitogenic for rat Schwann cells (39), however, we and others (35, 42) have failed to observe any effect at concentrations of up to 1 nM bFGF and 40 nM aFGF in the absence of forskolin. Such apparent discrepancies may be due to differences in assay conditions, and in growth factor or cell preparations. In contrast, the response of mouse dorsal root ganglion Schwann cells to FGF and serum mitogens is undisputed (24), and PDGF stimulates cell proliferation in human fetal dorsal root ganglion Schwann cells (55). Some of these responses are apparently due to differences between species, or in tissue origin. Intracellular levels of cAMP, or part of the pathway initiated by cAMP, may control the responsiveness of the cells to PDGFs and FGFs, and higher endogenous levels in mouse and human Schwann cells may explain why they respond to these factors without the need for an additional increase in cAMP concentration.

The effects of forskolin detected in our screen suggested that cAMP has an important role to play in Schwann cell responses to growth factors, as it does for example in Swiss 3T3 cells (39). The results showing that the forskolin analogue 1,9-dideoxyforskolin, which does not activate adenylyl cyclase, is unable to couple PDGFs or FGFs to mitogenesis,

strongly suggest that the role of forskolin is to raise intracellular cAMP. This is further strengthened by the observation that another agent acting via this pathway, cholera toxin, is able to mimic forskolin, and dbcAMP is also effective. In view of these results it might be expected that physiological agents capable of raising intracellular cAMP might couple PDGFs and FGFs to mitogenesis. Vasoactive intestinal peptide, secretin, and β 1-adrenergic agonists (norepinephrine, epinephrine) have been shown to raise cAMP levels in Schwann cells (54). None of these, however, was able to potentiate the activity of GGF-CM in our assays, even in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (50 μ M) (our unpublished results). It is not clear whether this reflects an absence of receptors for the neuropeptides and adrenergic catecholamines in our Schwann cell preparations, or if a particular time of onset, duration, or level of intracellular cAMP is necessary for synergy with GGF-CM. However, clearly this is a promising experimental system in which to study the interactions between the cAMP generating pathways and those of the polypeptide growth factors.

Recognition that the platelet factors PDGF and TGFs- β are mitogens for rat Schwann cells draws attention to a problem in the common use of serum in mitogenic assays. Our results show that although these cells do not require platelet factors for their responses to PDGFs, FGFs, and GGF-CM, the mitogenicity of adenylyl cyclase activators is highly dependent upon the presence of platelet factors, and thus it is likely that forskolin, cholera toxin, or dbcAMP are not mitogens in the absence of growth factors. It remains for the mode of control exerted by cAMP over the Schwann cell response to the FGFs and PDGFs, and secondly, for the nature of the factor(s) still remaining undefined in FCP, which are required by the Schwann cells in order for them to respond to these factors (35), to be determined.

Our observations using cultured cells suggest that PDGFs and FGFs may stimulate Schwann cell proliferation *in vivo* in situations where the cells are exposed to a second factor, or set of environmental conditions, that raises the intracellular levels of cAMP. In turn, these factors should now be considered in the processes of nerve development, myelination, regeneration, and Schwann cell tumorigenesis. The ensheathment and myelination of new axons during development begins with the proliferation of Schwann cells upon contact with the axon. *In vitro* experiments have established that membrane preparations from both the central and peripheral nervous system are mitogenic for Schwann cells, but that axolemmal membranes and PC12 membranes require the presence of serum. Although the evidence is not conclusive, a rise in cAMP does occur after stimulation with PC12 or dorsal root ganglion neuron-derived membranes (37), and cholera toxin does not synergize with axolemmal membranes in Schwann cell mitogenesis assays, suggesting that some of the membrane mitogens may act via cAMP (38, 49). In view of our results, the mitogenic signal from those membranes requiring the presence of serum may be due to a combination of serum platelet-derived factors and an increase in cAMP induced by the membrane mitogen. Interestingly, the expression of antigens associated with myelination by Schwann cells also appears to be dependent upon cAMP, since their level of expression diminishes upon removal of axonal contact but is restored by agents which raise cAMP (20, 29, 50).

PDGFs, FGFs, and TGFs- β have all been implicated in tissue repair after injury, being released by platelets, endothelial cells, mesenchymal cells, and macrophages at sites of injury and inflammation. The absence of leader sequences characteristic of secreted proteins from the FGFs makes their involvement uncertain, although it is possible they play a role after cell damage or unknown secretory mechanisms. Macrophages are known to infiltrate the sites of nerve injury and their experimental exclusion from injured nerves prevents the degradation of myelin and proliferation of Schwann cells that occurs after injury (5, 31). This suggests that their presence is essential for the generation of the mitogenic signal and an increase in cAMP does occur within 6–24 h of a crushing injury, concurrent with the appearance of macrophages. Although it is not clear what causes the rise in cAMP, it is known that macrophages secrete a mitogen for Schwann cells upon ingestion of myelin fragments (2, 4). Furthermore, activated macrophages are capable of producing PDGFs, FGFs, and agents such as prostaglandins that may raise cAMP levels in cells (14, 48). It is possible then, that macrophage-derived PDGFs and FGFs contribute to the stimulation of Schwann cell proliferation after nerve injury. Schwann cells in vitro may promote their growth through the autocrine production of growth factors (34). Similarly, Schwann cells in vivo may promote growth in schwannomas through the autocrine production of PDGFs or FGFs, particularly in association with conditions raising intracellular cAMP. In support of this possibility, mRNA for FGF has been detected in acoustic neuromas (30), and a group of pituitary tumors that has a constitutively active adenyl cyclase has recently been identified (25).

Our observations concerning the ability of forskolin to couple PDGFs and FGFs to stimulate Schwann cell proliferation, together with the previously documented stimulation by forskolin of GGF and TGFs- β directed mitogenesis (40), could be of relevance to the clinical treatment of demyelinating diseases or nerve injury, since physiological repair mechanisms in these diseases may involve the local production of one or more of these factors. The possibility of forskolin having therapeutic applications in these areas is further strengthened by positive results in stimulating amphibian and mammalian peripheral nerve regeneration (22, 23).

In conclusion we have shown that PDGFs and FGFs are mitogens for rat Schwann cells in vitro, and that their interactions with cAMP are potentially important for Schwann cell proliferation in a number of contexts. A variety of further studies will be of interest, including the determination of how cAMP controls responses to growth factors, comparisons of the responses of rat Schwann cells with other peripheral and central nervous system glial cell types, and the relevance of our observations to human Schwann cell biology and pathology in vivo.

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