

Calcitonin Gene-related Peptide Promotes Schwann Cell Proliferation

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Abstract. Schwann cells in culture divide in response to defined mitogens such as PDGF and glial growth factor (GGF), but proliferation is greatly enhanced if agents such as forskolin, which increases Schwann cell intracellular cAMP, are added at the same time as PDGF or GGF (Davis, J. B., and P. Stroobant. 1990. *J. Cell Biol.* 110:1353-1360). The effect of forskolin is probably due to an increase in numbers of PDGF receptors (Weinmaster, G., and G. Lemke. 1990. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:915-920).

Neuropeptides and β -adrenergic agonists have been reported to have no effect on potentiating the mitogenic response of either PDGF or GGF. We show that the neuropeptide calcitonin gene-related peptide (CGRP) increases Schwann cell cAMP levels, but the

cells rapidly desensitize. We therefore stimulated the cells in pulsatile fashion to partly overcome the effects of desensitization and show that CGRP can synergize with PDGF to stimulate Schwann cell proliferation, and that CGRP is as effective as forskolin in the pulsatile regime.

CGRP is a good substrate for the neutral endopeptidase 24.11. Schwann cells in vivo have this protease on their surface, so the action of CGRP could be terminated by this enzyme and desensitization prevented. We therefore suggest that CGRP may play an important role in stimulating Schwann cell proliferation by regulating the response of mitogenic factors such as PDGF.

THERE are two circumstances in which Schwann cells, the glial cells of peripheral nerves, proliferate: during development and after nerve injury. During development, they proliferate as they migrate out along growing axons (Eccleston, 1992). After nerve injury, they undergo two waves of proliferation: one occurs acutely, adjacent to the injury site, whereas the second occurs over a prolonged period as the nerve undergoes Wallerian degeneration and regeneration and extends distally from the injury site (Fawcett and Keynes, 1990). Although it is still not clear how Schwann cell division is controlled in vivo, there has been considerable effort to identify the mitogens that regulate the proliferation of rat sciatic nerve Schwann cells in culture. PDGF, FGF-1 and FGF-2, TGF- β , and glial growth factor (GGF)¹ have all been shown to promote the proliferation of these cells. In addition, Schwann cells in culture respond to

axons and axonal membrane fractions (Eccleston, 1992; Ratner et al., 1988). A striking feature of the response of cultured Schwann cells to each of the defined growth factors, however, is that the response is greatly potentiated if the intracellular level of cAMP is raised by treatment with dibutyryl cAMP, cholera toxin, or forskolin (Raff et al., 1978a,b; Ridley et al., 1989; Davis and Stroobant, 1990; Weinmaster and Lemke, 1990; Chen et al., 1991; Stewart et al., 1991; Goodearl et al., 1993; Marchionni et al., 1993). With the exception of GGF (Goodearl et al., 1993), there is significant expansion of Schwann cell numbers in response to each of these mitogens only if cAMP levels are also increased with agents such as forskolin. The growth factors themselves do not signal via cAMP, but increases in cAMP increase the levels of mRNAs encoding growth factor receptors in Schwann cells (Cohen et al., 1992; Weinmaster and Lemke, 1990), which may account, in part at least, for the synergy.

It is not known if cAMP signaling pathways play an important part in the control of Schwann cell proliferation in vivo and if they do, what endogenous signals are responsible for increasing cAMP in these cells. Potential candidates, such as catecholamines and neuropeptides, have previously been reported not to promote Schwann cell proliferation in culture (Raff et al., 1978a; Davis and Stroobant, 1990; Stewart et al., 1991). In this study, we have reinvestigated the action of

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1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; CGRP, calcitonin gene-related peptide; GGF, glial growth factor; IBMX, 3-isobutyl-1-methyl-xanthine; PHI, peptide histidine-isoleucine; VIP, vasoactive intestinal peptide.

several neuropeptides that are known to stimulate adenylate cyclase in various cell types, that are made by motor neurons, sensory neurons, or both, and whose synthesis has been shown to increase after peripheral nerve injury. These include calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and peptide histidine-isoleucine (PHI). CGRP is normally made by both sensory and motor neurons, and its synthesis is increased in motor neurons after nerve injury (Arvidsson et al., 1990; Dumoulin et al., 1990; Noguchi et al., 1990). VIP and PHI are normally expressed in only a small subset of sensory neurons in the dorsal root ganglion, but many sensory neurons express both VIP and PHI after injury (McGregor et al., 1984; Shehab and Atkinson, 1986; Nielsch and Keen, 1989). We also tested galanin and neuropeptide Y, which do not usually act via cAMP pathways but increase in sensory neurons after injury (Villar et al., 1989; Kashiba et al., 1992; Wakisaka et al., 1992; Hokfelt et al., 1994). The increased synthesis of these peptides after nerve injury suggests that they may play a special part in nerve regeneration, especially as the synthesis of other neuropeptides and transmitter-related enzymes decreases after nerve injury and during nerve regeneration (for review see Hokfelt et al., 1994). We also compared the effects of the neuropeptides with the β -adrenergic agonist isoproterenol, which classically stimulates adenylate cyclase, although the enzymes that synthesize adrenergic transmitters decrease after nerve injury (Cheah and Geffen, 1973).

We show that, although CGRP is a potent stimulator of adenylate cyclase activity in Schwann cells, the resulting increase in Schwann cell intracellular cAMP is transient, as the cells rapidly desensitize. Consequently, continuous treatment with CGRP does not promote Schwann cell proliferation in the presence of PDGF. By contrast, pulsatile treatment with CGRP is as potent at promoting Schwann cell proliferation in the presence of PDGF as is pulsatile treatment with forskolin and PDGF. These results suggest that CGRP may play an important role in stimulating Schwann cell proliferation in development, injury, or both. In addition, we show that sciatic nerve fibroblasts respond to CGRP, VIP and PHI, suggesting that these neuropeptides may also regulate fibroblast behavior in nerve regeneration.

Materials and Methods

Animals and Materials

Newborn rats were obtained from the breeding colony of the University College London Animal Facility. Recombinant human PDGF-BB was purchased from R&D Systems, Inc. (Minneapolis, MN). The neuropeptides CGRP, VIP, PHI, galanin, and neuropeptide Y, as well as forskolin, isoproterenol, 3-isobutyl-1-methyl-xanthine (IBMX), 5-bromo-2'-deoxyuridine (BrdU), poly-D-lysine, laminin (Sigma Chemical Co., Poole, UK; catalogue-L2020), trypsin (type III), collagenase (Sigma Chemical Co.; catalogue-C9263), BSA (fraction V and crystalline fraction V), and DNase were purchased from Sigma Chemical Co. (St. Louis, MO). FCS, Earl's balanced salt solution (EBSS), L-15, Ham's F12, and DME with glutamax-I media were purchased from GIBCO BRL (Paisley, Scotland). Tissue culture plastics were from Falcon Labware (Oxnard, CA). The monoclonal anti-Thy 1.1 (OX7) and anti-BrdU hybridomas were gifts from A. Williams (Oxford University, Oxford, UK), and D. Mason (Oxford University), respectively. Purified mAb OX42 was from Sera-Lab Ltd. (Sussex, UK), and anti-mouse Ig antibodies as well as conjugates were purchased from Dako-Patts (Glostrup, Denmark). The dual range 125 I-cAMP radioimmunoassay kit was from Amersham International (Amersham, UK).

Purification of Fibroblasts and Schwann Cells by Sequential Immunopanning

Cell Preparation. Sciatic nerves were dissected from 10–20 rats taken on postnatal days 5–7, finely chopped with a scalpel and forceps, then incubated at 37°C for 35 min in EBSS containing 0.025% trypsin, 0.1% collagenase, and 0.004% DNase; enzymes were inactivated by addition of 10% FCS and the suspension centrifuged. The cell pellet was resuspended in L-15 medium with 0.1% BSA, further dissociated by trituration with a 1-ml Pipetteman (Anachem, Luton, UK), and then filtered through nylon mesh (20- μ m pore size) to obtain a single-cell suspension.

Preparation of Panning Dishes. The method is a modification of that used by Barres et al. (1992). Panning dishes were prepared by incubating 100-mm Petri dishes overnight at 4°C with 10 ml of 0.2 M carbonate-bicarbonate buffer, pH 9.5, containing 50 μ g of rabbit anti-mouse Ig. Following aspiration of buffer, the dishes were washed three times with PBS before coating with cell type-specific mAb for at least 1 h in PBS with 0.2% BSA at room temperature; the plates were washed three times before the cell suspensions were added.

Immunopanning Procedure. Single-cell suspensions were applied sequentially to panning dishes coated with either OX42 (1 μ g/ml) to remove macrophages and other white blood cells or OX7 (hybridoma supernatant, diluted 1:20) to remove Thy-1⁺ fibroblasts. The cell suspension was incubated with each dish for 30 min at room temperature and agitated vigorously at 15 and 30 min to prevent nonspecific binding; the medium containing nonadherent cells was then transferred to the next dish. The adherence of cells to each type of panning dish was monitored microscopically until no further adhesion of cells was seen, and the depleted cell suspension was then transferred to the next type of panning dish.

To prepare pure nerve fibroblasts, the cell suspension was incubated sequentially on one or two OX42 dishes, then on one or two OX7 dishes. The fibroblasts that stuck to OX7 dishes were washed at least six times under microscopic control until all loosely attached cells were removed. They were then incubated overnight in a CO₂ incubator at 37°C in DME with glutamax-I and Ham's F12 media mixed 1:1 and supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml) (DME/F12) plus 10% FCS. The following day the fibroblasts were harvested by trypsinization (0.125% trypsin in EBSS for 10 min at 37°C); the enzyme was inactivated with 10% FCS, and the cells were squirted off the dish with a 1-ml Pipetteman. After centrifugation, the fibroblasts were resuspended in DME/F12 with 10% FCS and were plated into 24-well tissue culture plates, either with or without glass coverslips.

To prepare purified Schwann cells, the cells previously incubated with OX42 and OX7 dishes were incubated with further OX7 dishes to maximally remove Thy-1⁺ fibroblasts; in general, a cell suspension from 20 7-d-old rats required at least two OX42 dishes and four or five OX7 dishes to deplete the Schwann cell preparation of other cell types. After centrifugation, the cell pellet was washed several times with DME/F12 containing 0.5% crystalline BSA, resuspended in defined tissue culture medium, and plated into 24-well tissue culture plates, either with or without glass coverslips.

Schwann Cell Cultures

Purified Schwann cells were plated into 24-well plates either with or without coverslips. Each well was previously coated with poly-D-lysine (1 mg/ml in water) washed three times and air dried, and then incubated with laminin (10 μ g/ml in DME/F12). In the case of coverslips, the laminin was confined to a 50- μ l drop, whereas wells without coverslips were completely covered by the laminin. The cells were plated at 50,000–100,000 cells per well, either in 500- μ l (no coverslips) or 50- μ l drops (on coverslips); in the latter case the wells were topped up to 500 μ l after the cells attached to the coverslips. The growth medium for Schwann cells was DME/F12 (see above) with defined additives modified from Bottenstein and Sato (1979) as follows: 100 μ g/ml transferrin, 16 μ g/ml putrescine, 5 μ g/ml insulin, 50 ng/ml thyroxine, 50 ng/ml triiodothyronine, 39 ng/ml sodium selenite, 100 μ g/ml crystallized BSA. After 24 h the cultures were washed three times with media to remove any debris from the wells. Schwann cells were usually grown for 2–3 d and washed twice with defined medium before starting an experiment.

Fibroblast Cultures

Fibroblasts were plated at a density of 5,000–10,000 per well into Primaria grade 24-well plates without coverslips or into 24-well plates with poly-D-

lysine-coated coverslips; growth medium was DME/F12 plus 10% FCS. The cells were grown for 2–3 d and washed with DME/F12 twice before starting an experiment.

Purity and Yield of Schwann Cells and Fibroblasts

Freshly dissociated cells, as well as cells prepared by immunopanning, were allowed to settle onto coverslips for 1 h and were then fixed with 4% paraformaldehyde; cells were also fixed at 24 h or 4 d after plating. The cells were stained with either OX42 or OX7 mAbs followed by biotinylated anti-mouse Ig and then streptavidin-fluorescein. Some coverslips were labeled with a rabbit anti-S100 antiserum followed by biotinylated anti-rabbit Ig or OX7 followed by biotinylated anti-mouse Ig and streptavidin-horse radish peroxidase, and the antibody labeling was visualized by reaction with diaminobenzidine; the nuclei were then stained with hematoxylin.

1 or 24 h after dissociation, mixed P7 sciatic nerve cells contained ~1.5% OX42⁺ cells (macrophages), ~13% OX7⁺ cells (Thy-1⁺ fibroblasts), and ~85% S100⁺ cells (Schwann cells). After negative panning on OX42 and OX7 dishes, the proportion of S100⁺ Schwann cells was 98%, whereas the remaining cells were OX7⁺ and had the morphology of fibroblasts after 24 h in culture; there were no OX42⁺ cells after panning. After 4 d in serum-free medium without growth factors, almost all the Thy-1⁺ cells were pyknotic. Fibroblasts prepared by positive panning on OX7 plates were 99.9% Thy-1⁺ at the time of plating, and these cells survived well in serum-containing medium as well as in serum-free medium for several days at the density plated. After panning, the yield of S100⁺ Schwann cells and Thy-1⁺ fibroblasts from 20 P7 animals was ~15 × 10⁶ and 0.6 × 10⁶, respectively.

cAMP Assays

To ensure an accurate time of exposure to test materials in all wells, the experiment was terminated by inverting the culture plate on blotting paper and then lysing the cells in each well by inverting the test plate over a similar plate containing ice-cold 65% ethanol in each well and then inverting the two plates together. cAMP was then extracted for at least 2 h at -20°C, and the ethanol-containing cAMP was transferred to borosilicate glass tubes and evaporated by vacuum using a Speed Vac (model SC100; Savant, Farmingdale, NY). The cAMP-containing pellet was resuspended in assay buffer, and the cAMP content of each sample was measured in duplicate using the nonacetylation method of the cAMP RIA kit as directed, except that all components of the RIA were diluted 1:3 to increase the number of samples per kit (this did not affect the accuracy of the assay). Radioactivity in the antibody pellet was determined in a gamma counter (NE1600; Nuclear Enterprises, Edinburgh, UK).

BrdU Labeling and Immunofluorescence

DNA synthesis by purified Schwann cells was detected by addition of BrdU at a concentration of 10 μM for the final 24–30 h of culture. Living cells were incubated with OX7 (supernatant diluted 1:1 in L15 with 5% calf serum and 0.1% sodium azide) followed by Texas red goat anti-mouse IgG (1:100) to label the fibroblasts. They were then fixed in 4% paraformaldehyde for 1 min and treated with 2 N HCl for 10 min to denature the DNA and with 100 mM sodium borate (pH 8.5) for 10 min. The cells were then incubated sequentially with anti-BrdU antibody (supernatant diluted 1:1 in PBS containing 100 mM lysine, 5% calf serum, Triton X-100, and 0.1% sodium azide), biotinylated sheep anti-mouse Ig, and streptavidin fluorescein (Amersham International, Amersham, UK; both diluted 1:100 in the same solution). Coverslips were mounted in Citifluor (City University, London, UK), sealed with nail polish, and examined with a fluorescence microscope (Zeiss). The Thy-1 surface labeling (red and green) was easily distinguished from the nuclear BrdU labeling (green).

Results

Accumulation of cAMP by Schwann Cells and Fibroblasts

We tested the ability of various neuropeptides and isoproterenol to stimulate the accumulation of cAMP in purified Schwann cells or fibroblasts that were in culture for either 3 or 8 d. As shown in Fig. 1, both CGRP and isoproterenol increased intracellular cAMP in Schwann cells at both ages;

the response to isoproterenol was more pronounced in 8-d cells, whereas the basal levels of cAMP and the response to CGRP were similar in 3 and 8-d cells. The magnitude of the CGRP response depended on the culture conditions, as cells plated at high density gave a larger increase in cAMP (approximately eightfold) than the same number of cells plated over a larger area (approximately threefold), even though basal levels were similar. VIP, PHI, galanin, and neuropeptide Y had little effect.

As shown in Fig. 2, isoproterenol also increased cAMP in purified sciatic nerve fibroblasts, and in the presence of the phosphodiesterase inhibitor IBMX, CGRP, VIP, and PHI did as well, but the effects of VIP and PHI were only seen in the older cultures. Galanin and neuropeptide Y had no effect. As shown in Fig. 3, *a* and *b*, the CGRP-induced response was maximal at 5 × 10⁻⁸ M in both Schwann cells and fibroblasts, whereas the isoproterenol response was maximal at 10⁻⁶ M (not shown). The VIP-induced response in fibroblasts was maximal at 5 × 10⁻⁷ M (Fig. 3 *b*).

Desensitization of Schwann Cell Response to CGRP and Isoproterenol

As shown in Fig. 4 *a*, the increase in cAMP in Schwann cells

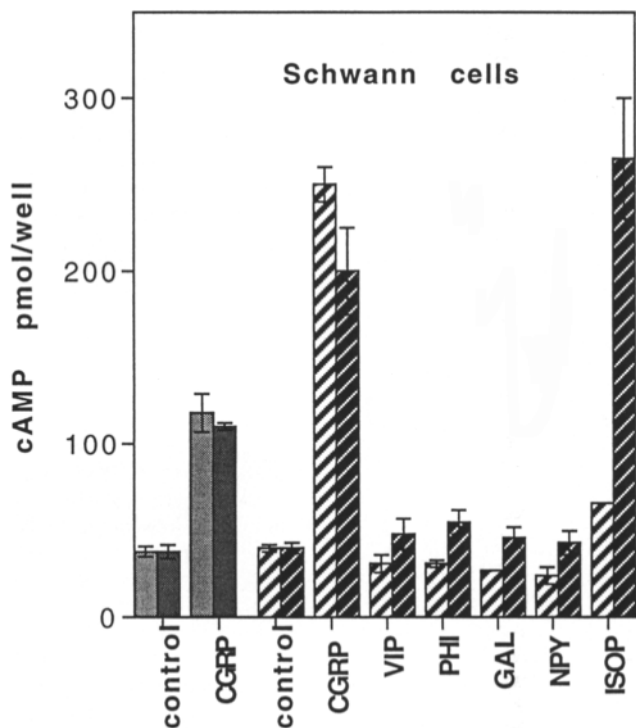


Figure 1. Intracellular levels of cAMP in Schwann cells in response to 10⁻⁶ M isoproterenol (ISOP) or 5 × 10⁻⁸ M neuropeptides. After immunopanning, 50,000 cells were plated in defined medium either over the entire well (sparse, stippled bars) or confined to the area of a 50-μl drop (dense, hatched bars). After either 3 d (light hatched or stippled bars) or 8 d (dark hatched or stippled bars) in culture, drugs were added for 5 min; cells were extracted and cAMP measured as described. In this and the following experiments, 0.1% crystalline BSA was included in all drug solutions to prevent neuropeptides from sticking to the tissue culture plastic. The data presented in this and Figs. 2–6 are means ±SD for triplicate wells. All experiments are typical of results obtained in at least three different platings. ▨ 3d SC, sparse; ▨ 3d SC, dense; ▩ 8d SC, sparse; ▩ 8d SC, dense.

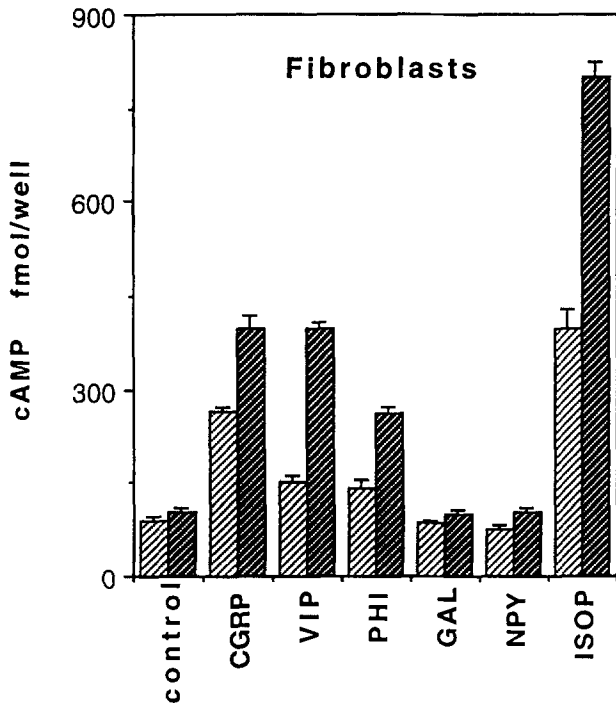


Figure 2. Intracellular levels of cAMP in sciatic nerve fibroblasts in response to either 10^{-6} M isoproterenol (ISOP) or 5×10^{-8} M neuropeptides. After immunopanning, 5,000 cells were plated in 10% FCS and allowed to divide in culture for either 2 d (light hatched bars) or 7 d (dark hatched bars). The cells were washed and incubated in defined DME/F12 for 4 h with 200 μ M IBMX added for 2 h. The cells were then stimulated for 2 min with the drugs in the presence of IBMX; cells were extracted, and cAMP was measured as described. ▨ 2d, +IBMX; ▩ 7d, +IBMX.

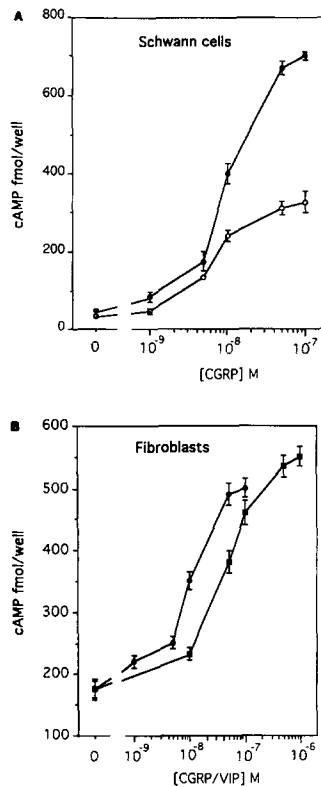


Figure 3. (a) CGRP dose-response curve for cAMP accumulation in Schwann cells. Cells were plated at high density (50,000 cells per 50- μ l drop) and grown in defined medium for 4 d. Cells were washed, and 200 μ M IBMX was added to some wells; after 2 h various doses of CGRP were added for 5 min either with (solid circles) or without (open circles) IBMX. (b) CGRP and VIP dose-response curves for cAMP accumulation in sciatic nerve fibroblasts. Cells were plated at 5,000 cells per well and allowed to divide in culture with 10% FCS for 3 d. After washing with DME/F12, 200 μ M IBMX was added for 2 h; various doses of either CGRP (circles) or VIP (squares) were then added for 2 min and the cells extracted.

induced by a maximal dose of CGRP was eightfold higher than basal levels after 5 min without IBMX (11-fold higher with IBMX), but declined rapidly, even in the presence of IBMX, so that it was only twofold higher than basal levels after 1 h. The attenuation of the response to CGRP in fibroblasts was even more rapid (Fig. 4 b). The attenuation of the cAMP response was not due to degradation or inactivation of the peptide because, when culture medium from Schwann cells incubated with CGRP for 3 h was transferred to fresh cultures, a comparable stimulation of cAMP was seen after a further 5 min, just as with fresh CGRP. Moreover, adding fresh CGRP to the Schwann cells treated with CGRP for 3 h failed to increase cAMP in the cells after 5 min (Fig. 5). The response to isoproterenol also declined with time in similar fashion to the CGRP response.

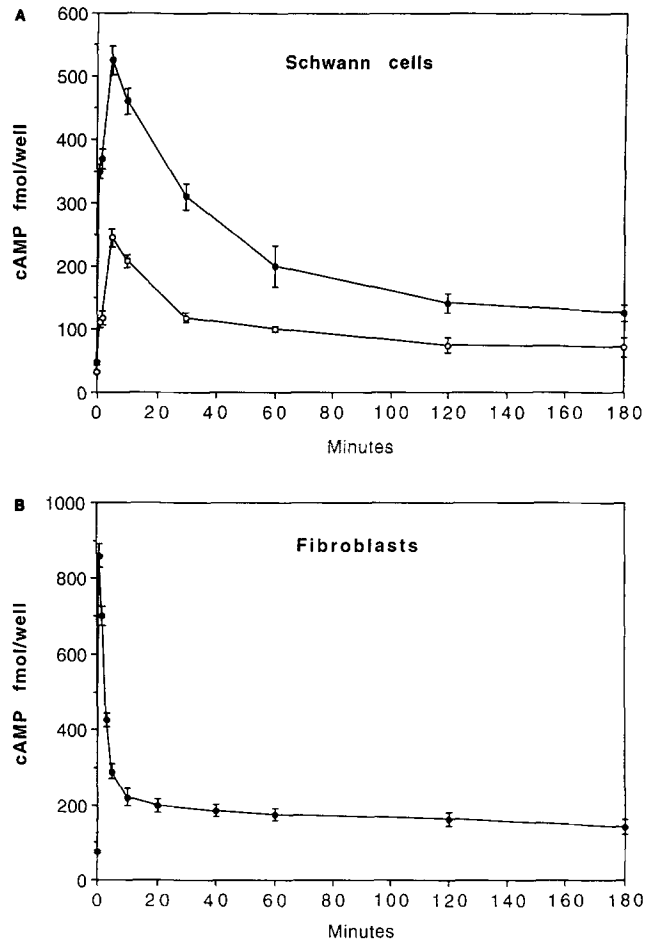


Figure 4. (a) Time course for cAMP accumulation in response to CGRP in Schwann cells plated at high density and grown for 4 d in defined medium. CGRP (5×10^{-8} M), either with (solid circles) or without (open circles) IBMX, was then added for various times in decreasing order and the incubation terminated at zero time. For each time point with IBMX, the drug (200 μ M) was added for a total of 2 h. (b) Time course for cAMP accumulation in response to CGRP in sciatic nerve fibroblasts plated at 5,000 cells per well and grown for 3 d in 10% FCS. After washing, 200 μ M IBMX was added to all wells for 2 h; CGRP (5×10^{-8} M) was then added for various times in decreasing order and the incubation terminated at zero time.

Potential of the Mitogenic Response to PDGF by CGRP and Isoproterenol

To study the mitogenic effect of CGRP and isoproterenol on Schwann cells, we needed to overcome the attenuation of the CGRP and isoproterenol-induced responses. We did this by adding CGRP- and isoproterenol-induced responses. We did this by adding CGRP and isoproterenol, with and without PDGF, in a pulsatile fashion, interrupted by washing steps, and compared the results with those when the agents were added only twice over 48 h. We also compared the effects of CGRP and isoproterenol with that of forskolin. Mitogenesis was assessed by the incorporation of BrdU into Thy-1 cells in immunofluorescence assays as described in Materials and Methods. When viewed under an inverted phase microscope, there was no obvious morphological difference between cultures that were repeatedly washed in the pulsing regime and those that were not; moreover, there was no difference in the unstimulated levels of cAMP in cultures with and without washing (not shown).

Fig. 6 *a* shows the percentage of Schwann cells that incorporated BrdU into their nucleus during the last 24 h of the 48-h experiment when the reagents were added daily without washing. Forskolin and PDGF both increased BrdU incorporation about threefold, while CGRP and isoproterenol had no effect. As reported by others (Davis and Stroobant, 1990; Weinmaster and Lemke, 1990; Chen et al., 1991), forskolin and PDGF together acted synergistically, inducing a 14-fold increase. In contrast, when the reagents were added every 3 h, separated by 3-h wash-out periods, both CGRP and isoproterenol increased BrdU incorporation about threefold, comparable to the effect of forskolin; moreover, CGRP and PDGF together were as effective as forskolin and PDGF together (eightfold stimulation), and both combinations were synergistic, whereas isoproterenol and PDGF were simply additive (Fig. 6 *b*). Whereas the combination of forskolin and PDGF gave a 14-fold stimulation with the unpulsed regime (Fig. 6 *a*), it gave only an eightfold stimulation in the pulsed regime (Fig. 6 *b*), indicating that the synergy is greater if adenylate cyclase is continually stimulated.

Discussion

Schwann cells generally grow very slowly in culture, with a

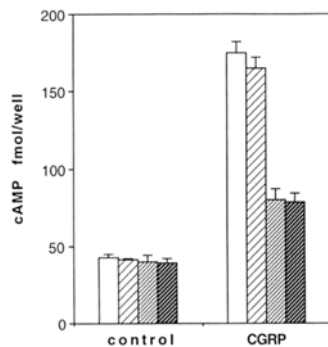


Figure 5. Activity of CGRP after incubation with Schwann cells was determined by transferring culture medium from a set of Schwann cells incubated with 10^{-8} M CGRP for 3 h to a fresh set of Schwann cells for 5 min. The levels of cAMP in fresh cells stimulated with the transferred medium (*first bar*) was compared with the levels of cAMP in fresh cells stimulated with fresh CGRP (*second bar*) and the level of

cAMP in the cells after 3 h of incubation with CGRP (*third bar*); in addition fresh CGRP was added for 5 min to another set of Schwann cell previously incubated with CGRP for 3 h (*fourth bar*). The controls are sets of cultures treated in the same way but without the addition of CGRP.

doubling time of 8–10 d even in the presence of 10% serum (Raff et al., 1978a; Davis and Stroobant, 1990). Similarly, low levels of DNA synthesis are seen in Schwann cells grown in defined media, with only a small percentage of cells incorporating BrdU during a 24-h pulse in this study and that of Stewart et al. (1991). The mitogens PDGF-BB, FGF-1, FGF-2, and TGF- β have little effect on cell proliferation when added either with serum (Ridley et al., 1989; Davis and Stroobant, 1990; Eccleston et al., 1990; Weinmaster and Lemke, 1990) or without (Chen et al., 1991; Stewart et al., 1991; Schubert, 1992). In the presence of 5–10 μ M forskolin, however, each of these mitogens causes Schwann cells to double in number in 1–2 d (Ridley et al., 1989; Davis and Stroobant, 1990; Weinmaster and Lemke, 1990). Although GGF is a mitogen without forskolin, forskolin also enhances the effect of GGF on Schwann cell proliferation (Davis and Stroobant, 1990; Goodearl et al., 1993; Marchionni et al., 1993). Indeed, a common way of obtaining large numbers of Schwann cells for biochemical studies is to “expand” the cultures with either GGF or PDGF-BB in the presence of forskolin. In this article, we show that 1 μ M of forskolin increases the incorporation of BrdU into DNA in

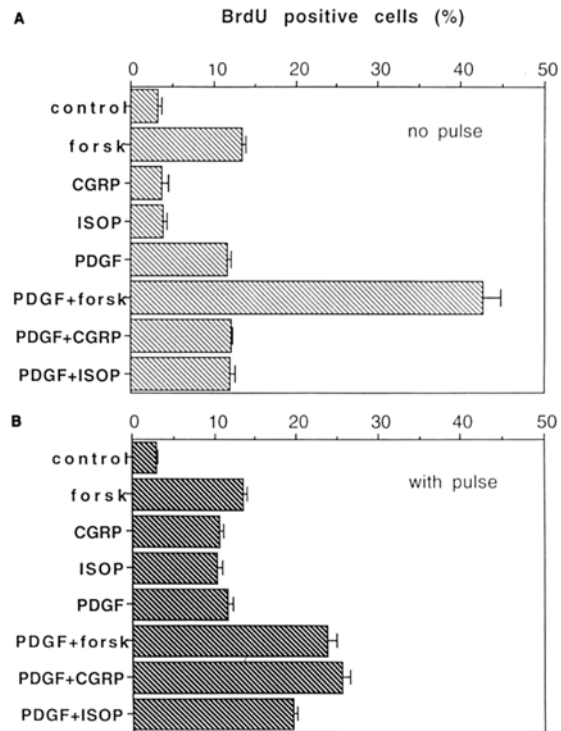


Figure 6. Schwann cells were plated at high density and grown in defined medium for 3 d. (*a*) Drugs were added on the second and third day for a total of 48 h. (*b*) Drugs were added every 6 h for 3 h starting on the second day and continuing for a total of 37 h; 3 h after each addition of drugs, wells were carefully washed with 2×1 ml of medium and left to incubate before adding fresh drugs 3 h later. For the final 11 h of incubation, the drugs were left in continuously. BrdU was added for the last 24 h of incubation for both *a* and *b*. Concentrations of drugs were as follows: 1 mM forskolin; 10^{-8} M CGRP; 10^{-6} M isoproterenol; 10 ng/ml PDGF-BB. The number of Schwann cells that had synthesized DNA in the last 24 h of culture was determined by counting the number of BrdU⁺ cells in random fields as a percentage of the total Schwann cells; at least 300 cells were counted on each coverslip.

defined medium with PDGF from 10% to ~42% (40–60% in at least 10 different experiments), an increase that would decrease the doubling time from 10 to ~2 d. Thus significant proliferation of Schwann cells in culture in the presence of PDGF seems only to occur if the cells are treated at the same time with agents that raise intracellular cAMP levels.

Which of the potential ligands that might increase Schwann cell cAMP levels are present in the nerve and which might be available after nerve injury? Norepinephrine, the agonist for the β -adrenergic receptor, accumulates for 2 d after a nerve crush, but enzymes that synthesize this transmitter decrease in injured nerve (Cheah and Geffen, 1973). The neuropeptides VIP, PHI, and CGRP are all known to stimulate adenylate cyclase in many cell types. They are made and transported along peripheral axons and accumulate at the site of nerve injury (Lundberg et al., 1981; Kashihara et al., 1989). Interestingly, although CGRP in sensory neurons decreases after injury, it increases in motor neurons and continues to accumulate at the leading edge of regrowing axons. Both VIP and PHI, which are encoded by the same mRNA, are present in sympathetic neurons and a few sensory neurons; after injury, the majority of sensory neurons now express both VIP and PHI (for a review see Hokfelt et al., 1994). Thus CGRP, VIP, PHI, and norepinephrine are possible candidates for signals that increase Schwann cell cAMP. VIP and β -adrenergic agonists were shown previously to increase cAMP levels in cultured Schwann cells when added at high doses (Yasuda et al., 1988), but when they and CGRP were tested in a 48-h mitogen assay with PDGF (Davis and Stroobant, 1990) or GGF (Stewart et al., 1991), they could not mimic the effects of forskolin.

In this article, we show that CGRP and isoproterenol increase intracellular cAMP in cultured Schwann cells. The magnitude of the response of Schwann cells to CGRP (8–10-fold in 5 min with 5×10^{-8} M CGRP without IBMX) is among the largest reported for any cell type. VIP also increased cAMP in some experiments but the effect was small. The response of Schwann cells to both CGRP or isoproterenol rapidly desensitized, even in the presence of the phosphodiesterase inhibitor IBMX, suggesting that CGRP receptors desensitize in similar fashion to the well-documented desensitization of β -adrenergic receptors.

We thought it likely that attenuation of the cAMP response when CGRP and isoproterenol were added continuously for 48 h could explain the lack of effect of these agents on Schwann cell proliferation reported by Davis and Stroobant (1990) and Stewart et al. (1991). We therefore added them in pulsatile fashion with periods without agents to allow recovery from desensitization. We also used 10^{-8} M CGRP because we found that desensitization was reduced with this dose and compensated for the below-maximal initial response. In this way we were able to show that both CGRP and isoproterenol substantially increased the number of cells synthesizing DNA in 24 h from 10 to ~25% in the presence of PDGF, which would give a doubling time of ~4 d. Moreover, both CGRP and isoproterenol were as effective as 1 μ M of forskolin delivered in the same fashion.

Because the effect of forskolin on proliferation was less when it was given in a pulsatile fashion than when it was given continuously, it is clear that the time period over which adenylate cyclase is activated is crucial for the effectiveness of this signal in potentiating the action of PDGF. Weinmaster

and Lemke (1990) showed that increasing Schwann cell cAMP with forskolin led to increased synthesis of the PDGF β receptor and suggested that regulation of the number of mitogen receptors provides a general mechanism whereby cAMP potentiates the action of mitogens: only cells that respond in this fashion to forskolin show the increase in synthesis of mitogen receptor; cells that do not show potentiation with forskolin do not increase synthesis of mitogen receptors. In their study, Weinmaster and Lemke (1990) showed that the cells required 12–24 h of forskolin treatment to increase the amount of PDGF β receptor mRNA; a single 3-h pulse of forskolin was ineffective. As discussed by Weinmaster and Lemke (1990), other permissive effects of the cAMP signal are not excluded, but it is interesting that the erbB2 receptor is also upregulated in Schwann cells by forskolin, although it is not clear if this or a related receptor is the real receptor for GGF on Schwann cells (for a review see Mudge, 1993).

CGRP is a very good substrate for the neutral endopeptidase 24.11 (Davies et al., 1992; Turner, A. J., personal communication). Both myelinating and nonmyelinating Schwann cells express endopeptidase 24.11 on their surface during development and postnatally, although it is suppressed in adult myelinating Schwann cells (Kioussi et al., 1992). Moreover, endopeptidase 24.11 is upregulated on both Schwann cells and endoneurial fibroblasts after injury to peripheral nerves (Kenny and Bourne, 1991). It is likely, therefore, that the activity of released CGRP could be terminated in much the same way that the action of acetylcholine is terminated by acetylcholinesterase at the neuromuscular junction, thus preventing desensitization of the receptors. Our attempts to minimize desensitization of Schwann cells in culture by washing out the drugs and allowing time for recovery was necessarily crude because of the limits imposed by changing the culture medium every few hours; more frequent changes were impractical. If endogenous endopeptidase 24.11 efficiently degrades CGRP in the small extracellular space within the endoneurium, prevention of desensitization in vivo would be much more effective than was the pulsatile treatment in our culture experiments. It is unclear whether inactivation and/or reuptake of norepinephrine occurs at the site of nerve injury. It seems likely that neuropeptides and norepinephrine are released from the tips of regenerating axons for the following reasons: (a) axons are electrically active during regeneration, and (b) large dense-core vesicles, which contain these molecules, can release their contents at nonsynaptic sites (Zhu et al., 1986).

The immunopanning method described here, using anti-Thy-1 antibodies to deplete fibroblasts from the dissociated sciatic nerve cell suspension, offers several advantages over previously described methods, such as complement-mediated cell lysis and/or pulsing with mitotic inhibitors such as cytosine arabinoside, which are used frequently to enrich for Schwann cells (Raff et al., 1978a; Ridley et al., 1989; Davis and Stroobant, 1990; Eccleston et al., 1990; Weinmaster and Lemke, 1990; Stewart et al., 1991; Schubert, 1992; Goodearl et al., 1993): cells are ready to plate for experiments on the same day as dissociation, the yield from the initial cell suspension is high so that "expansion" in mitogen and forskolin is not necessary, and the purity is >97%. Growing the Schwann cells in defined medium without serum increases the purity to >99% because the contaminating

fibroblasts, which are present at very low density at the time of plating, die within 2 d in culture. The Schwann cells, however, survive for >1 mo in the defined medium on laminin provided they are plated at high density. We found that plating the Schwann cells at high density also increased their adhesion to the coverslips, allowing extensive washing during the division experiments.

Our results with PDGF and forskolin are different from some other studies, even when the drugs were added continuously. We attribute this difference to our different plating procedure. We saw substantial DNA synthesis in response to PDGF and 1 μ M of forskolin when the cells were in serum-free defined medium: 40–60% of the Schwann cells incorporated BrdU in 24 h in contrast to the study by Stewart et al. (1991), in which only 6% of the cells incorporated BrdU in 24 h when exposed to PDGF and 10 μ M of forskolin (there was no DNA synthesis in 1 μ M and PDGF). Our results with forskolin and PDGF added continuously in defined medium are, however, similar to those reported for Schwann cells grown in serum (Chen et al., 1991; Davis and Stroobant, 1990; Weinmaster and Lemke, 1990). The relevant differences in culture methods could be that our cells were never exposed to forskolin, serum, or cytosine arabinoside before the experiment, and they were plated at high density on laminin. In addition, we found that high-density Schwann cells survive indefinitely in serum-free medium at this density, whereas lower density cultures die at \sim 10 d. Perhaps as a consequence of being healthier at high density, the magnitude of the increase in cAMP in response to CGRP was greater when compared with plating the same number of cells over a larger surface area. In the study by Yasuda et al. (1988) with VIP and isoproterenol, increases in cAMP required the presence of IBMX and concentrations of both drugs $>10^{-6}$ M; moreover, these effects did not plateau even at 10^{-4} M. In contrast, in our experiments, the effects with CGRP and isoproterenol on Schwann cells saturated at 5×10^{-8} M and 10^{-6} , respectively.

Fibroblasts also responded to CGRP and isoproterenol, as well as to both VIP and PHI, particularly in the older fibroblast cultures; the fibroblast response to CGRP was terminated more rapidly than was the Schwann cell response to CGRP, even in the presence of IBMX, suggesting that the CGRP receptors in the two cell types desensitize to CGRP at different rates. In contrast to the effects on proliferation of Schwann cells, forskolin inhibits the proliferation of sciatic nerve fibroblasts induced by PDGF (Kahn, M., and A. W. Mudge, unpublished results), which might favor regeneration by preventing formation of a fibroblast scar. Because sciatic nerve fibroblasts express endopeptidase 24.11 on their surface, a role for CGRP in regulating fibroblast cAMP-dependent effects is also possible. Both CGRP and VIP are also known to increase cAMP levels in macrophages (Wiik, 1989; Vignery et al., 1991) and we have also shown that these peptides increase cAMP in macrophages isolated from injured peripheral nerves (Bindemann, N. W., and A. W. Mudge, unpublished results); increasing levels of cAMP in macrophages is known to inhibit macrophage activation (Nong et al., 1989; Ohmori et al., 1990), and it may be important to inactivate macrophages as the axons regenerate.

In summary, our studies suggest that CGRP may be an endogenous ligand that activates Schwann cell adenylate cy-

clase and potentiates the mitogenic action of PDGF. Sciatic nerve fibroblasts and macrophages also increase their levels of cAMP after exposure to both CGRP and VIP, so that a number of cAMP-dependent nonneural cell functions such as fibroblast proliferation and macrophage activation may be regulated during nerve regeneration by the release of these neuropeptides as the nerve advances. Neuronal CGRP and VIP, which are upregulated after injury and uniquely activate adenylate cyclase, may thus serve as heralds of the axons approaching the uninervated nonneural cell territory eliciting an orchestrated cellular response conducive to nerve regeneration.

We thank Martin Raff and John Scholes for helpful comments on the manuscript and Barbara Barres for much advice on the immunopanning procedure.

M. Khan was supported by a Medical Research Council Training Grant Fellowship.

Received for publication 20 December 1994.

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