

The Effects of cAMP on Differentiation of Cultured Schwann Cells: Progression from an Early Phenotype (04⁺) to a Myelin Phenotype (P₀⁺, GFAP⁻, N-CAM⁻, NGF-Receptor⁻) Depends on Growth Inhibition

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Abstract. The present experiments were designed to clarify the relationship between cAMP elevation, proliferation and differentiation in Schwann cells. They were carried out on short-term cultures of cells obtained from neonatal rat sciatic nerves.

It was found that the myelin-related phenotype was expressed in response to agents that elevate or mimic intracellular cAMP (forskolin, cholera toxin, cAMP analogues), provided cell division was absent. This phenotype included upregulation of the major myelin protein P₀ and downregulation of GFAP, N-CAM, A5E3, and NGF receptor. In contrast, when cells were cultured in conditions where cell division occurred, elevation of intracellular cAMP produced an alternative response, characterized by DNA synthesis

and absence of myelin-related differentiation. The cAMP mediated induction of an early Schwann cell antigen, 04, followed a different pattern since it was induced equally in dividing and nondividing cells.

These observations are consistent with the proposal that during development of the rat sciatic nerve: (a) cAMP elevation, possibly induced by axon-associated factors, is a primary signal responsible for the induction of 04 expression in proliferating Schwann cells during the premyelination period; (b) subsequent withdrawal of cells associated with the larger axons from the cell cycle acts as a permissive secondary signal for induction of myelination, since in quiescent cells the ongoing cAMP elevation will trigger myelination.

THE adenylyl cyclase-cAMP second messenger pathway has been implicated in the regulation of growth and development in several cell types, including Schwann cells, the major glial element in peripheral nerves. Thus, cAMP elevation stimulates DNA synthesis in Schwann cells cultured in the presence of serum or growth factors (9, 10, 14, 40, 42, 43, 51). In cultured Schwann cells it also triggers the surface expression of two lipids, galactocerebroside and 04 (sulfatide), both of which are found on myelin-forming and non-myelin-forming Schwann cells in adult nerves (20, 32, 50, 51). During development of the rat sciatic nerve, galactocerebroside appears at embryonic day (E) 18–19 in cells destined to form myelin, and during the second to fifth postnatal week in non-myelin-forming cells. 04 on the other hand is an early Schwann cell differentiation marker appearing at E16. The effect of cAMP on induction of the major myelin glycoprotein P₀ is more controversial. Although it has been reported that elevation of cAMP does not induce P₀ in short-term cultured Schwann cells (49, 51), other studies show that cAMP induces expression of P₀ and also induces P₀ mRNA and myelin basic protein mRNA in long-term Schwann cell cultures that have previously been exposed to mitogenic stimulation (24).

These *in vitro* studies suggest that cAMP might be an intracellular signal during several different stages of Schwann cell development. In synergy with other factors, it might drive Schwann cell proliferation in developing nerves; it could act as a signal triggering early Schwann cell differentiation, *i.e.*, 04 appearance; or it could be a signal for the emergence of the myelin phenotype or for the final maturation of nonmyelin-forming cells.

The proposal that cAMP elevation is involved in regulation of these disparate events during the *in vivo* development of the rat sciatic nerve raises at least three questions. First, does cAMP elevation unambiguously mimic myelin-related differentiation in rat Schwann cells? Second, if so, what is the timing mechanism that *in vivo* would allow cAMP elevation to induce 04 at E16 (32) but myelin-related differentiation, as exemplified by P₀ expression, 5–6 d later (around birth) (29,55). Third, is induction of the myelin phenotype by cAMP in cultured cells incompatible with stimulation of DNA synthesis, as predicted by the observation that *in vivo* myelin-related differentiation does not occur in dividing cells (56).

To answer these questions we have investigated the relationship between induction of the myelin phenotype, induction of 04 expression and stimulation of DNA synthesis. We

show for the first time that in short-term Schwann cell cultures elevation of intracellular cAMP not only induces expression of P_0 but, in the same cells, triggers downregulation of several proteins normally downregulated during myelination *in vivo*, including GFAP, N-CAM, and A5E3, and also nerve growth factor (NGF) receptors (NGFR) detected by the 217c antibody (8, 15, 18, 21, 23, 26, 27, 31, 36). Significantly, we find that in short-term Schwann cell cultures, these changes only take place if the proliferative response to cAMP is prevented. In contrast, O4 expression is readily induced by cAMP in dividing cells.

These observations, and earlier work on the regulation of galactocerebroside expression are consistent with the proposal that cAMP acts as a second messenger of an early and general Schwann cell differentiation signal. They also suggest that factors regulating Schwann cell proliferation control the transition from the premyelin to the myelin phenotype.

Materials and Methods

Schwann Cell Culture

Sciatic nerves were dissected from early postnatal rats (5–7 d) and sheathed. 5-d rats were used in most experiments. The tissue was dissociated essentially by the method of Brockes et al. (4), and maintained in DMEM with added glutamine, insulin, and 10% calf serum. Cytosine arabinoside, 10^{-5} M, was added after 24 h for 48 h to give cultures that were 95% pure (4). On the fourth day cells were plated on to coverslips coated with polylysine, polylysine plus laminin, or extracellular matrix as required, at a density of between 2.5–20,000 cells/coverslip. Cells were left for 24 h before further treatment. In most experiments, Schwann cells were cultured on laminin but in some experiments, polylysine or coverslips coated with extracellular matrix from bovine corneal endothelial cells were used. Cells were cultured in defined medium consisting of a 1:1 mixture of DMEM and Ham's F12 medium supplemented with selenium ($100 \mu\text{g}\cdot\text{ml}^{-1}$), triiodothyronine ($0.1 \mu\text{g}\cdot\text{ml}^{-1}$), transferrin ($100 \mu\text{g}\cdot\text{ml}^{-1}$), putrescine ($16 \mu\text{g}\cdot\text{ml}^{-1}$), thyroxine ($0.4 \mu\text{g}\cdot\text{ml}^{-1}$), progesterone ($60 \mu\text{g}\cdot\text{ml}^{-1}$), 30% BSA (Path-o-cyte, ICN Immunologicals, Lisle, IL) ($0.3 \text{ mg}\cdot\text{ml}^{-1}$), dexamethasone ($38 \text{ ng}\cdot\text{ml}^{-1}$), insulin ($5 \mu\text{g}\cdot\text{ml}^{-1}$) with or without the addition of NGF ($10 \text{ U}\cdot\text{ml}^{-1}$), glucose ($2 \text{ mg}\cdot\text{ml}^{-1}$), 10% calf serum or glial growth factor (GGF) at a concentration of $1.8 \mu\text{g}\cdot\text{ml}^{-1}$. The GGF was a semipure fraction (GGF-CM) prepared by Dr. A. J. D. Goodearl (Ludwig Institute, London) as described elsewhere (47). Calf serum was added in concentrations ranging from zero to 10%.

Cell Culture in the Presence of Cholera Toxin

Cholera toxin (CTx) (Sigma Chemical Co., St. Louis, MO) ($150 \text{ ng}\cdot\text{ml}^{-1}$) was added to Schwann cell cultures for 1 h at the start of experiments, and 24 h later for an additional hour. In experiments lasting >4 d, CTx was added for 1 h at 96 and 168 h. As a control for the specificity of the CTx, the β subunit was used in some experiments at the same concentration and using a similar regime to that used for CTx.

Cell Culture in the Presence of Forskolin

In some experiments forskolin, a reversible activator of adenylyl cyclase was added to cells cultured in media as described above. A 10 mM stock in alcohol was diluted in medium and added at concentrations varying from 0.01 to 200 μM . At the higher concentrations a control containing alcohol alone was added to the experiment. The forskolin was replaced in new medium every 24 h.

Cell Culture in the Presence of cAMP Analogues

In these experiments Schwann cells were placed on coverslips coated with

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CTx, cholera toxin; GCF, glial growth factor; IBMX, 3-isobutyl-1-methylxanthine; NGFR, nerve growth factor receptor.

polylysine, laminin, or extracellular matrix. They were cultured in media as described above. cAMP analogues, $\text{N}^6,2'-O$ -dibutyryladenine 3':5'-cyclic monophosphate (db-cAMP) (5×10^{-4} M) plus 8-bromoadenosine 3':5'-cyclic monophosphate (8 bromo-cAMP) (5×10^{-4} M) were added at the start of the experiment. At 24 h and 48 h, fresh medium containing the two analogues, each at a concentration of 5×10^{-5} M, was added to the cultures. If experiments lasted >3 d, this dose was repeated every day until the end of the experiment. Similar concentrations of butyrate were used in control experiments.

Cell Culture in Isobutylmethylxanthine

A 50 mM stock solution of 3-isobutyl-1-methyl-xanthine (IBMX), an inhibitor of cAMP hydrolysis, (Sigma Chemical Co., St. Louis, MO) in 50% ethanol was diluted 1:5 in PBS. This solution (10 mM in 10% ethanol) was used to give a final dilution of 100 μM in Schwann cell cultures at the start of the experiment. At 24 and 48 h, fresh medium containing 100 μM IBMX was added to the cultures. Control cultures contained 0.1% ethanol.

Substratum Coating

13-mm-diam round glass coverslips were incubated with polylysine ($1 \text{ mg}\cdot\text{ml}^{-1}$) at room temperature for 24 h, washed with six changes of sterile distilled water over a period of 4 d, and allowed to dry. Laminin-coated coverslips were prepared by incubating polylysine-coated coverslips with 50 μl of laminin solution (Gibco Laboratories) ($20 \text{ ng}\cdot\text{ml}^{-1}$ in DME) for 2 h before plating with Schwann cells. Extracellular matrix coated coverslips were prepared from bovine endothelial cells as described previously (10) and stored in sterile PBS at 4°C until required for use.

Antibodies

Rabbit anti- P_0 was produced and characterized by Brockes et al. (5) and used at a dilution of 1:200. Rabbit anti-N-CAM was produced and characterized by Gennarini et al. (16) and used at a dilution of 1:500. Rabbit anti-GFAP and rabbit anti-S100 (Dako Immunoglobulins A/s; Dakopatts, Copenhagen, Denmark) were used at a dilution of 1:100 and 1:1,000, respectively. Ascites fluid containing mouse mAb to galactocerebroside (IgG3), produced and characterized by Ranscht et al. (44) was used at a dilution of 1:200. Supernatant containing mouse mAb O4 (IgM) (52) was used at a dilution of 1:1. Ascites fluid containing mouse mAb A5E3 (IgG2a) (30) was used at a dilution of 1:500. Ascites fluid to mouse mAb to P_0 (0.4) produced and characterized by Dr. C. Linington (Max Planck Institut für Psychiatrie, Martinsreid, FRG) was used at a dilution of 1:500. Mouse monoclonal anti-GFAP (clone G-A-5; Boehringer, Mannheim, FRG) was used at a dilution of 1:4. Mouse mAb 217c supernatant (37), which has recently been shown to recognize NGFR (15), was used at a dilution of 1:500. Purified mouse mAb 192-IgG (1.3 mg/ml) (53) which also recognizes NGFR was used at a dilution of 1:1,000. Mouse monoclonal anti-BrdU (Bu20a) (IgG1) supernatant was used at a dilution of 1:20 (17). Fluorescein or tetramethyl rhodamine-conjugated to goat anti-rabbit Ig (G-anti-RIg-F1 or G-anti-RIg-Rd) (Cappel Laboratories, Ltd., Malvern, PA) absorbed with mouse Ig, were used at a dilution of 1:300. Fluorescein or tetramethyl rhodamine conjugated to goat anti-rabbit Ig (G-anti-MIg-F1 or G-anti-MIg-Rd) (Cappel Laboratories, Ltd.) absorbed with rabbit Ig, were used at a dilution of 1:150. Biotinylated sheep anti-mouse Ig and streptavidin-Texas Red (Amersham International, Amersham, UK) were both used at a dilution of 1:100.

Immunofluorescence

Antibodies were diluted in MEM-H with 10% calf serum when used on living cells, or in PBS containing 0.1 M lysine, 0.02% sodium azide, and 10% calf serum when used on fixed cells unless otherwise stated. All incubations of cultured cells grown on glass coverslips were carried out at room temperature for 30 min. Living cells were incubated with O4, galactocerebroside, A5E3, 217c, or 192-IgG antibodies followed by G-anti-MIg-Rd or G-anti-MIg-F1, or with N-CAM antibodies followed by G-anti-RIg-Rd or G-anti-RIg-F1, then permeabilized in 95% ethanol/5% acetic acid for 10 min at -20°C . The cells were then incubated in P_0 antiserum followed by G-anti-RIg-F1. In some experiments cells were fixed in 95% ethanol/5% acetic acid and then incubated in mAbs to P_0 or GFAP followed by biotinylated sheep anti-mouse Ig then streptavidin-Texas Red. Cells to be labeled with S100 antibodies were fixed in 4% paraformaldehyde for 20 min before permeabilization in 95% ethanol/5% acetic acid for 10 min at -20°C .

DNA Replication

DNA synthesis by Schwann cells was detected by addition of bromodeoxyuridine (BrdU) at a concentration of 10^{-5} M for the final 24 h of culture (17). In double-labeling experiments, with P_0 antibodies cells were washed and fixed with 95% ethanol/5% acetic acid at -20°C for 10 min. They were then treated with 2 N HCl for about 10 min to denature DNA and 10 min with 0.1 M sodium borate (pH 8.5). Coverslips were incubated sequentially with anti-BrdU in PBS containing 0.1% Triton X-100 for 40 min, with G-anti-MiG-Rd, P_0 antibodies and G-anti-RiG-Fl, all for 30 min. In double-labeling experiments with 04 antibodies cells were labeled with 04 antibodies followed by G-anti-MiG-Rd, fixed in 2% paraformaldehyde for 10 min, 95% ethanol/5% acetic acid, 2 N HCl and sodium borate as above, followed by anti-BrdU and G-anti-MiG-Rd. There was no difficulty in distinguishing 04 surface labeling from the nuclear BrdU labeling despite the fact that both antibodies were monoclonal since the fixations used after use of the first two antibody layers reduced possible cross-reactivity. In some experiments, the number of cells per field (using $\times 63$ objective) was counted in cultures that had been subjected to different experimental treatments resulting in various BrdU percentages. It was found that the number of cells per field varied proportionally with the number of BrdU positive cells. This indicates that the BrdU percentage in these experiments is a measure of proliferation. All coverslips were mounted on microscope slides in Citifluor anti-fade mounting medium (Chemistry Department, City University, London, UK). Immunofluorescence was viewed in a Zeiss microscope using $\times 40$ dry or $\times 63$ oil-immersion phase contrast lenses, epi-illumination and rhodamine or fluorescein optics.

Immunoblotting

This was carried out using Schwann cells cultured in 75-mm tissue culture flasks coated with polylysine and laminin. Initially, Schwann cells were seeded at 5×10^5 – 10^6 Schwann cells per dish and treated with doses of cAMP analogues, forskolin or CTx for 3 d, as described earlier. Cells were cultured in defined medium. Control cultures were maintained without addition of analogues. Proteins were extracted from the cells using 0.1 ml of 2% SDS in 5 mM Tris-Cl, pH 6.8, containing 2 mM EGTA, 2 mM EDTA, and 2 mM PMSF per two dishes. The sample was boiled for 5 min, spun at 14,000 g for 5 min and 10 μl of supernatant withdrawn for protein determination using a protein estimation kit (Bio-Rad Laboratories, Richmond, CA). 2% mercaptoethanol was then added to the supernatant and the extracts subjected to SDS-PAGE using a 10–15% gradient acrylamide slab gel. The separated proteins were then transferred to nitrocellulose and immunoblotted essentially as described previously (19) using the P_0 antiserum at a concentration of 1:5,000, and ^{125}I -labeled donkey anti-rabbit Ig (ICN Biomedicals Ltd., Irvine, CA), 15×10^6 cpm/blot diluted in 20 ml of PBS containing 3% hemoglobin.

Results

cAMP Elevation Induces Expression of the Myelin Protein P_0 in Short-Term Cultures in Defined Medium

To avoid the complicating effects of serum, which has been included in almost all previous studies on cAMP and Schwann cell differentiation, we initially studied the effect of cAMP elevation on cultured short-term Schwann cells from 5-d rat sciatic nerve in serum-free, defined medium. Under these conditions cAMP does not promote Schwann cell DNA synthesis and the proliferation rate is extremely low (see below). Drug treatment was started after 5 d in vitro. By this time the cells had ceased to express immunohistochemically detectable levels of myelin proteins and glycolipids, such as P_0 , galactocerebroside, or 04 (22, 29, 32). We found that 3 d after elevation of cAMP by use of CTx, forskolin or cAMP analogues, expression of the major myelin protein P_0 was clearly seen in the majority of Schwann cells when they were examined by immunohistochemical methods using P_0 antibodies. The P_0 was also induced, albeit to a lesser extent, by IBMX, an inhibitor of phosphodiesterase, as ex-

Table I. Induction of P_0 in Defined Medium

Treatment	% P_0 -positive Schwann cells (\pm SEM)
Agents that elevate or mimic cAMP	
CTx ($150 \text{ ng}\cdot\text{ml}^{-1}$)	62 ± 6.5 ($n = 14$)
Forskolin ($2\text{--}4 \mu\text{M}$)	37 ± 10.7 ($n = 11$)
cAMP analogues	51 ± 5.7 ($n = 22$)
IBMX ($100 \mu\text{M}$)	9 ± 3.5 ($n = 8$)
Control Experiments	
Beta subunit of CTx ($150 \text{ ng}\cdot\text{ml}^{-1}$)	0 ($n = 4$)
Ethanol (0.1%)	0 ($n = 2$)
Butyrate	0.3 ± 0.40 ($n = 3$)
GGF ($1.8 \mu\text{g}\cdot\text{ml}^{-1}$)	0.2 ± 0.10 ($n = 4$)
Calf serum (10%)	0 ($n = 4$)
0–1 d in vitro (no additions)	42 ± 3.0 ($n = 3$)
5 d in vitro (no additions)	0 ($n = 1$)
8 d in vitro (no additions)	0.1 ± 0.10 ($n = 10$)

Schwann cells were removed from 5 d rats and cultured for 5 d before addition of drugs that elevate cAMP or other agents. The cultures were examined with P_0 antibodies either 3 d after the addition of drugs, or in control experiments, cells were examined after 24 h, 5 d, or 8 d in vitro, without addition of drugs. Cyclic AMP analogues, db-cAMP and 8 bromo-cAMP, were used together each at a concentration of 5×10^{-4} M during d1 of treatment and at 5×10^{-5} M during d2 and d3. Butyrate was used at a total concentration of 5×10^{-4} M during d1 of treatment and at 5×10^{-5} M during d2 and d3. In each experiment (n) a minimum of 400 cells were counted, a minimum of 200 cells from each of duplicate coverslips. The number of P_0 positive cells is expressed as a percentage of total Schwann cells identified by morphology with phase-contrast illumination for the number of experiments indicated. In parallel cultures $>95\%$ of the cells were S100-positive Schwann cells. For data on proliferation in CTx, forskolin, and cAMP analogues see Table II.

pected if expression was dependent on cAMP elevation. These results and results from several control experiments are summarized in Table I, and the typical appearance of cells induced to express P_0 is illustrated in Fig. 1.

P_0 induction could be detected using either polyclonal or monoclonal P_0 antibodies, although the MAbs generated a more speckled and less intense labeling pattern. The percentage of cells induced to express P_0 varied quite widely from experiment to experiment. The highest percentage seen in individual experiments was 84 and 85% after exposure to forskolin and CTx, respectively, whereas the average percentage was considerably lower (Table I). When drugs were withdrawn, P_0 expression declined to undetectable levels (data not shown).

As previously reported, a characteristic morphological change in many of the Schwann cells from a bipolar shape to cells of a more flattened morphology which were less phase bright, was induced by CTx, forskolin, and cAMP analogues (Fig. 1) (51). The induced cells were notably larger in size and more flattened than cells in the same culture which remained uninduced. This difference in responsiveness of individual cells to elevation of cAMP remains unexplained.

No P_0 was seen in control experiments with the beta subunit of CTx, ethanol, butyrate, GGF, or calf serum or in experiments with serum-free media alone (Table I).

P_0 Protein Induced by Elevation of cAMP Can Be Detected in an Immunoblot

When cells were exposed to agents that elevate cAMP or mimic its effect, a clear band of P_0 protein was detectable

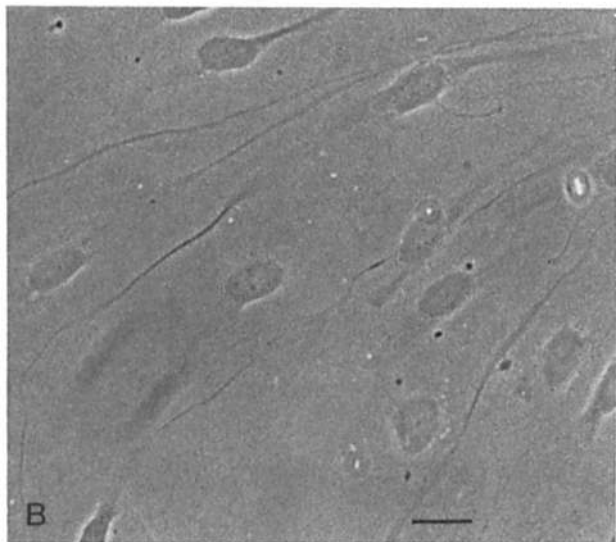
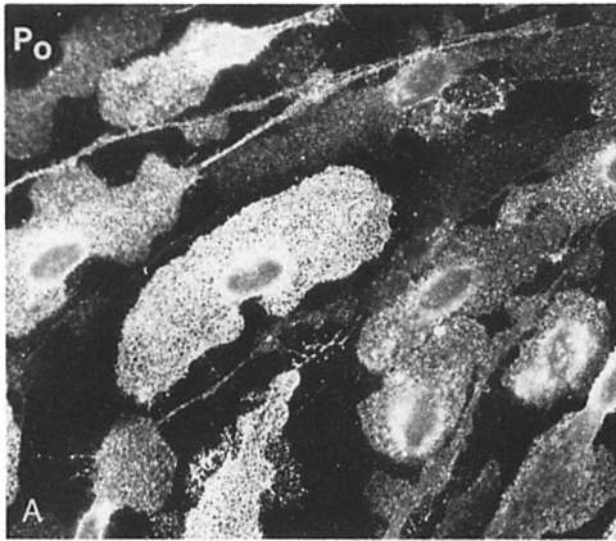


Figure 1. Induction of P_0 in Schwann cells treated with CTx. P_0 -positive Schwann cells cultured from 5 d rat sciatic nerve. Cells were cultured on a laminin substrate and treated with CTx as described in Methods, and labeled with P_0 antibodies after 3 d of treatment. Cells were viewed with (A) fluorescein optics to visualize P_0 , (B) phase-contrast optics. Note the flattened shape of the Schwann cells and the P_0 immunofluorescence distributed throughout the whole area of the cell. Bar, 20 μ M.

by immunoblotting (Fig. 2). The extract of freshly excised sciatic nerve from a 5-d rat used as a positive control showed a greater intensity of P_0 protein than the cultured induced cells, even though less total protein was loaded on the gel track. On prolonged exposure of the immunoblot to x-ray film, a very faint band was also visible in the extract of cells cultured for 8 d in the absence of agents which elevate cAMP or mimic its effects, even though no label was visible in immunofluorescence experiments (not shown). This probably indicates that cells from 5-d rats, many of which have already begun to make myelin, continue to synthesize very low residual levels of P_0 in the absence of axonal contact (39).

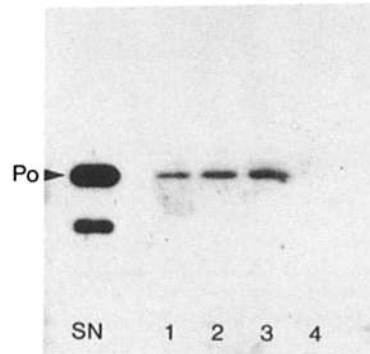


Figure 2. Immunoblot of P_0 induction in Schwann cells. Procedure as described in Materials and Methods. (SN) Extract of 5 d rat sciatic nerve, 25 μ g loaded. Lanes 1-3, extracts from dissociated Schwann cell cultures from 5 d rat sciatic nerve treated with agents that elevate or mimic cAMP. Lane 1, cAMP analogues; lane 2, CTx, lane 3, forskolin, lane 4, no treatment; 31 μ g of extract loaded in each lane. Note that at the P_0 position, which is arrowed, an immunoreactive band is visible in the sciatic nerve lane and in each of lanes 1-3, but not in lane 4 (control). In the sciatic nerve extract a lower breakdown product of P_0 is also visible.

Elevation of cAMP in Cultured Schwann Cells Mimics the Inversion in Protein Expression Characteristic of Myelination In Vivo

When a Schwann cell is induced to form myelin in vivo the induction of myelin specific proteins, such as P_0 , is followed by down-regulation of a set of proteins that includes GFAP, N-CAM, A5E3, and NGFR (21, 23, 26, 27). This inversion of protein expression does not occur in non-myelinating cells, and is reversed on removal of the myelinating cells from axonal contact. Therefore, in the absence of drug treatment, essentially all the cells in our cultures expressed GFAP, N-CAM, A5E3, and NGFR as reported previously (23), in addition to being P_0 negative. We found that elevation of cAMP induced the myelination-specific combination of protein up- and downregulation in many cells, since the expression of GFAP, N-CAM, A5E3, and NGFR was strikingly suppressed in a subset of the P_0 positive cells (Figs. 3, 4, and 5). The results obtained using the 217c antibody to detect the NGFR (15) were confirmed using the 192-IgG monoclonal, which also detects the NGFR (53) (data not shown). The proportion of P_0 -positive cells that assumed this more complete myelin phenotype was larger when P_0 was induced in the presence of serum or GGF although the total number of P_0 cells was smaller under those conditions (see below).

Induction of the Myelin Phenotype and Cell Division Are Negatively Correlated

The ability of cAMP to induce myelin-related differentiation under conditions allowing proliferation was compared with the induction seen under conditions where DNA synthesis did not occur. The mitogenic response to cAMP elevation was regulated in three ways: (a) by including or excluding serum; (b) by including or excluding GGF; (c) by varying cell density in the presence of serum. Myelin-related differentiation was monitored by P_0 antibodies and immunohisto-

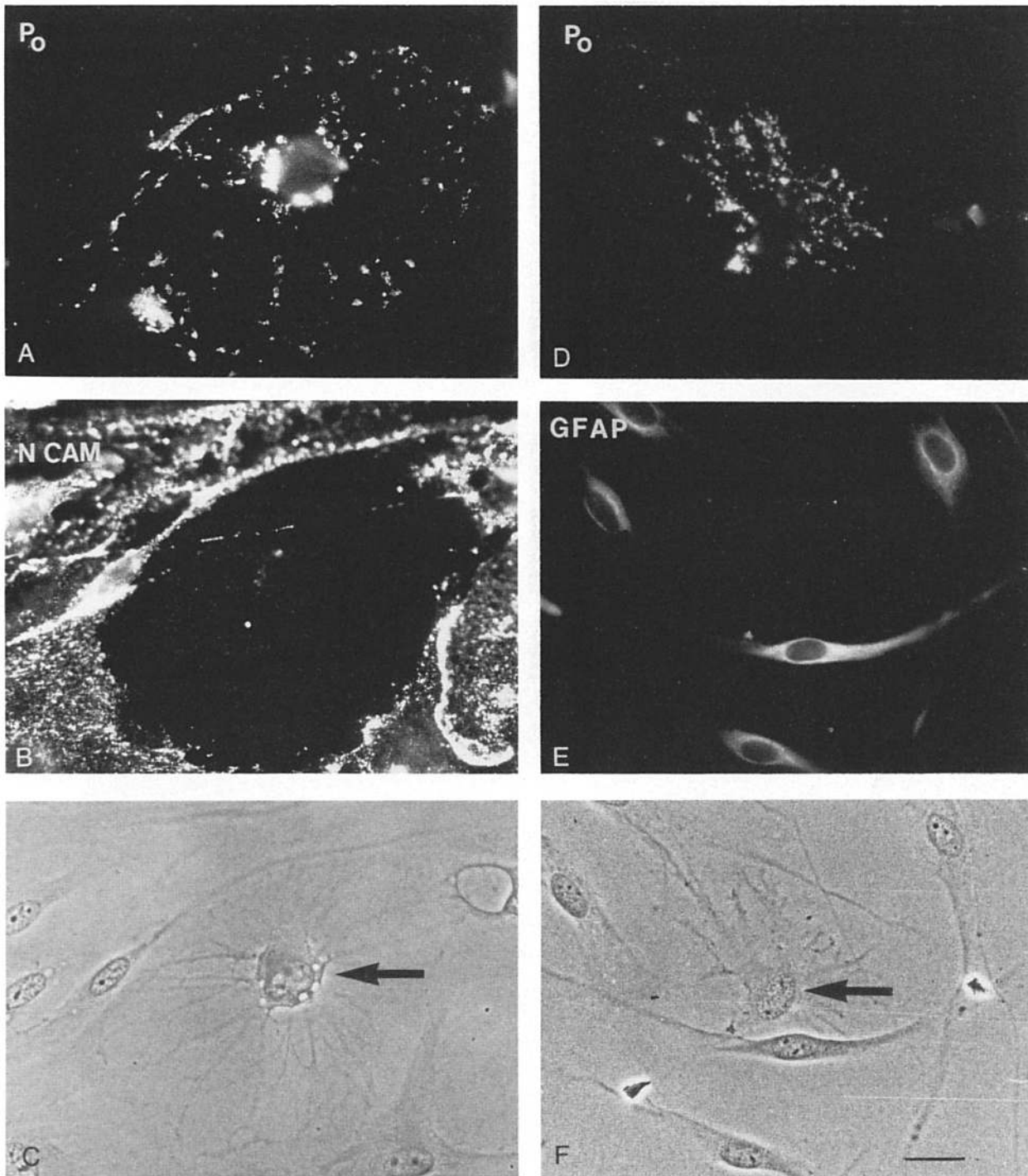


Figure 3. Induction of a myelin-related phenotype in cultured Schwann cells. Double-label immunofluorescence using mAbs to P_0 and N-CAM antibodies (A-C), or mAbs to P_0 and GFAP antibodies (D-F). Schwann cells from 5 d sciatic nerve were treated with cAMP analogues for 3 d under conditions where both induced and uninduced cells were present in reasonable numbers. Cultures were viewed with (A and D) fluorescein optics to visualize P_0 ; (B and E) rhodamine optics to visualize N-fluorescein optics to visualize P_0 ; (B and E); or (C and F) phase-contrast optics. Note that the P_0 -positive cell seen in A is unlabeled with antibodies to N-CAM (B), while surrounding P_0 -negative cells are intensely N-CAM positive, and that the P_0 -positive cell seen in D is GFAP negative in E, whereas surrounding P_0 -negative cells show typical GFAP immunofluorescence. Note that both the P_0 -positive cells have assumed a flattened morphology, seen in C and F. Bar, 20 μ M.

chemistry, and DNA synthesis was assessed by the BrdU method. A negative correlation between induction of the myelin phenotype and cell division was observed in every case. The results are summarized in Table II and Fig. 6.

Serum. The ability of cAMP to promote Schwann cell growth depends on the simultaneous activation of at least one other second messenger pathway by agents such as serum or GGF, and the mitogenic response to cAMP elevation is low or

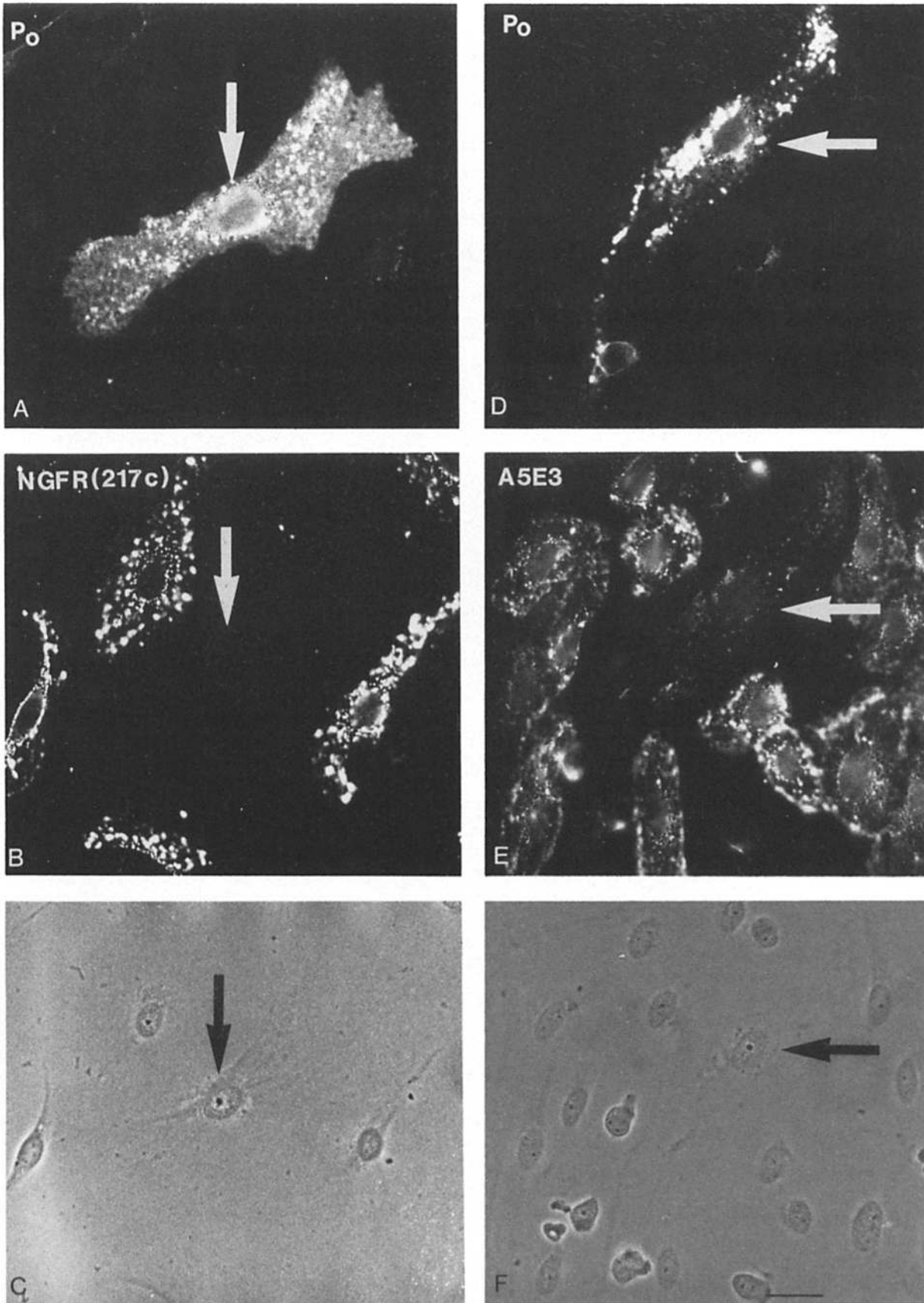


Figure 4. Induction of a myelin phenotype in cultured Schwann cells. Double-label immunofluorescence using antibodies to P_0 and antibodies to NGFR (detected by the 217c mAb) (A–C); or antibodies to P_0 and A5E3 antibody (D–F). Schwann cells from 5 d sciatic nerve, cultured in high density in serum, were treated with cAMP analogues for 3-d as described in Fig. 3. Cultures were viewed with (A and D) fluorescein optics to visualize P_0 ; (B and E) rhodamine optics to visualize NGFR (B) or A5E3 antigen (E); or (C and F) phase-

absent in defined medium (43; Stewart, H. J. S., R. Mirsky, and K. R. Jessen, unpublished). We therefore tested the ability of cAMP analogues to induce P_0 expression after 3 d and 4 d of exposure to cAMP analogues in the presence of 0% (defined medium), 0.5 and 10% calf serum (Fig. 6). This showed that as the amount of serum was increased, the number of P_0 -positive cells fell very substantially, suggesting a strong negative correlation between proliferation and myelin-related differentiation. Using double label immunofluorescence we then measured simultaneously the number of cells synthesizing DNA, and the number of cells undergoing myelin-related differentiation in cultures under different conditions. Cultures exposed to cAMP analogues, CTx, or forskolin for 3 d in defined medium on the one hand, and in the presence of 10% calf serum on the other, were compared (see Table II, first four columns). The data demonstrate that there is a strong inverse relationship between P_0 expression and DNA synthesis.

Glial Growth Factor. To test alternative means of inducing cell division in cultures treated with cAMP elevating agents, we used GGF in serum-free defined medium (Stewart, H. J. S., R. Mirsky, and K. R. Jessen, unpublished). This greatly increased DNA synthesis and, as in the case of serum, dramatically lowered the percentage of cells that are induced to make P_0 (see fifth and sixth columns of Table II).

Cell Density. The common action of serum and GGF on Schwann cells is to generate conditions under which cAMP elevation increases DNA synthesis and the most likely interpretation of the above results was that a mitogenic response involving cAMP was incompatible with myelin-related differentiation. Alternatively, it was possible that serum and GGF contained factors that directly blocked induction of the myelin phenotype, and that the mitogenic effects of serum and GGF were unrelated to the inhibition of myelin-related differentiation. To test this we took advantage of the observation that Schwann cells show a strong density-dependent inhibition of division. Cells were exposed to agents that elevate cAMP in the presence of 10% calf serum at low plating densities, allowing a strong proliferative response, and at a series of higher densities when proliferation was increasingly suppressed. Cells were plated at densities ranging from 2,500 to 20,000 cells per coverslip and exposed to cAMP analogues for 5 d in the presence of 10% calf serum. A labeling index of $82 \pm 4\%$ was observed at the lowest plating density, and no P_0 expression was seen. At the highest plating density the labeling index observed was 26 ± 6.7 and $21 \pm 8.5\%$ of the cells expressed P_0 . Thus, even in the presence of serum an inverse relationship between proliferation and myelin-related differentiation was revealed. When these data (from a total of 28 individual experiments) were analyzed using Kendall's rank correlation test, a rank correlation coefficient, τ value of -0.48 ($p < 0.001$) was found, indicating a strong negative correlation between DNA synthesis and P_0 expression. Furthermore, of all the conditions tested in the present work, cAMP elevation in high-density cultures with 10% calf serum gave the best induction of the myelin

phenotype in individual cells, i.e., highest levels of P_0 expression as judged by the intensity of immunolabeling, and also downregulation of GFAP, N-CAM, A5E3, and NGFR in the largest proportion of the P_0 -positive cells. Together, these observations exclude the possibility that serum contains factors that directly block cAMP induction of the myelin phenotype, and suggest that serum might in fact contain factors that act together with cAMP to promote maximal expression of the myelin phenotype, provided DNA synthesis is suppressed.

Individual Cells Expressing P_0 Do Not Incorporate BrdU and Are Therefore Not Synthesizing DNA

The results above show that Schwann cell populations responded to elevation of cAMP by a high degree of myelin-related differentiation and low proliferative activity, or by a low level of differentiation and rapid DNA synthesis depending on other conditions in the culture. This suggests that in individual Schwann cells cAMP elevation will either stimulate DNA synthesis or induce myelin-related differentiation, but not both. To test this, DNA synthesis in the P_0 -negative and -positive Schwann cell populations was compared. In these experiments plating density was adjusted to obtain substantial proportions of both BrdU-positive cells and of P_0 -positive cells in the presence of cAMP analogues and 10% calf serum (see previous section). In four experiments the number of P_0 -positive cells averaged $23 \pm 3.7\%$ and the number of cells synthesizing DNA within this population was only $0.9 \pm 0.32\%$, whereas the number of cells synthesizing DNA within the P_0 -negative population was $31 \pm 3.2\%$. This shows that cells acquiring the myelin-related phenotype and dividing cells are largely nonoverlapping populations, suggesting that DNA synthesis and myelin-related differentiation are essentially incompatible in individual Schwann cells.

cAMP Induced 04 Expression Is Not Blocked by Proliferation

In vivo, it is clear that full expression of 04, unlike that of P_0 , is compatible with proliferation during development (29). To test whether 04 expression and DNA synthesis were compatible in cultured Schwann cells these parameters were monitored in cells exposed for 3 d to $2 \mu\text{M}$ forskolin in defined medium and in medium containing GGF. It was found that in defined medium $54 \pm 8.0\%$ ($n = 8$) of the Schwann cells were 04 positive (BrdU positive cells $<5\%$), while in defined medium with GGF $32 \pm 12.0\%$ ($n = 3$) of the cells expressed 04 (BrdU-positive cells $>80\%$). This reduction in 04 expression is not statistically significant. Furthermore, in related experiments we found that a large proportion of individual cells responded to cAMP elevation both by DNA synthesis and 04 expression (Fig. 7). In these experiments, cells were exposed to cAMP analogues in the presence of 10% calf serum, resulting in 04 expression in $88 \pm 3.7\%$ ($n = 3$) of the cells. Of the total cell population

contrast optics. Note that the P_0 -positive cell arrowed vertically in (A) is not labeled with NGFR antibodies (B) while surrounding P_0 -negative cells are strongly labeled with NGFR antibody. The P_0 -positive cell arrowed horizontally in D is likewise very weakly labeled with A5E3 antibody, while surrounding P_0 -negative cells are strongly A5E3 positive. Note that both P_0 -positive cells have assumed a flattened morphology (C, F). Bar, $20 \mu\text{M}$.

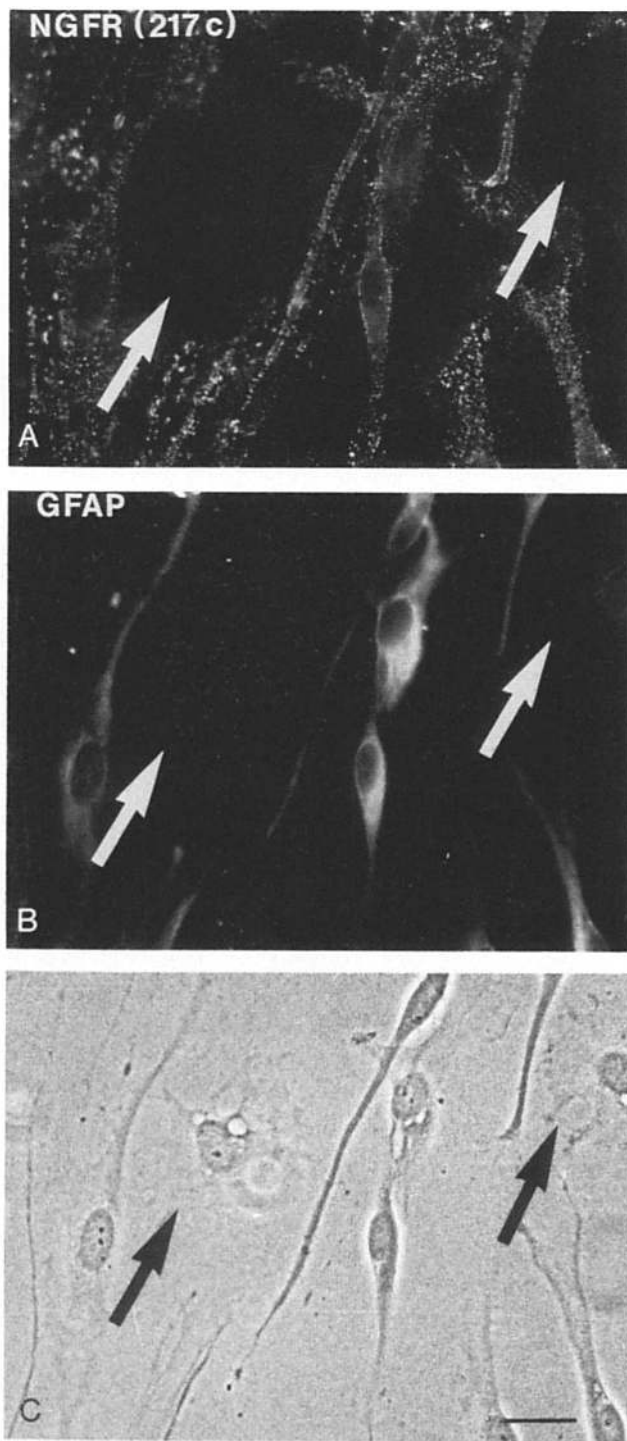


Figure 5. Induction of a myelin-related phenotype in cultured Schwann cells. Double-label immunofluorescence using antibody to NGFR (A) and antibody to GFAP (B). Phase-contrast micrograph shown in C. Culture conditions are as described in Fig. 3. Two NGFR negative cells (arrowed) are surrounded by NGFR-positive cells in (A). The same cells are also GFAP negative (arrowed) in B, while surrounding cells are GFAP positive. Both of the arrowed cells have a typical flattened morphology (C). Bar, 20 μ M.

72 \pm 8.3% incorporated BrdU, whereas 64 \pm 6.4% within the 04 positive population were also synthesizing DNA.

The Role of Laminin

Laminin-coated substrates were not obligatory for induction of the phenotype, since cells cultured on polylysine-coated coverslips or tissue culture plastic could be induced to make P_0 in response to elevation of cAMP. In a representative experiment, 63% of cells plated on laminin and 42% of cells plated on polylysine expressed P_0 after a 3-d exposure to cAMP analogues. Extracellular matrix from bovine corneal endothelial cells was also effective although in this case the cells assumed a process-bearing morphology rather than the flattened sheetlike morphology more typical of cells on laminin (data not shown).

Discussion

We have demonstrated that in short-term Schwann cell cultures elevation of intracellular cAMP induces expression of the major myelin protein P_0 and, in the same cells, suppresses expression of a set of proteins all of which are normally suppressed during myelination *in vivo*. We find that there is an inverse relationship between induction of the myelin phenotype and DNA synthesis. In contrast, the lipid antigen 04, which is also induced by cAMP elevation, is readily expressed in cells synthesizing DNA.

The Relationship between cAMP, Proliferation, and Differentiation

An inverse relationship between proliferation and differentiation has not been directly demonstrated previously in Schwann cells. It is significant that it was observed only with respect to myelin-related differentiation. This accords well with *in vivo* development where 04 appears at E16 in proliferating cells, but the formation of the first myelin wrap, which coincides with the onset of high levels of P_0 expression, is a postmitotic event. Clearly, the effects of cAMP on cultured Schwann cells are quite similar to the effects of the axon-associated signals which regulate 04 expression and myelination since both induce 04 expression in proliferating cells and myelin-related differentiation in quiescent ones.

The existence of an axon-associated mitogen has been convincingly demonstrated in *in vitro* experiments (46). It is not clear to what extent this mitogen drives Schwann cell proliferation in embryonic nerves or whether other Schwann cell mitogens, also defined *in vitro*, play a part. These other mitogens include GGF, PDGF, TGF β , and FGF (9, 12-14, 47). Agents that elevate cAMP levels synergize with all of these polypeptide factors, possibly via upregulation of growth factor receptors (57). Furthermore, in serum-free medium none of these growth factors stimulate Schwann cell division except when used with agents that elevate cAMP (Stewart, H. J. S., R. Mirsky, and K. R. Jessen, unpublished). Interestingly, mitogenic axonal membranes have been reported to elevate intracellular cAMP in Schwann cells (45), see, however, Meador-Woodruff et al. (28) for a contrary result.

Together, the present observations and previous work (20, 22, 32, 50, 51) suggest the possibility that axon-associated signals maintain elevated cAMP levels in Schwann cells,

Table II. The Inverse Relationship between cAMP-mediated P_0 Induction and DNA Synthesis

	Defined medium		Calf serum (10%)		GGF (1.8 $\mu\text{g}\cdot\text{ml}^{-1}$)	
	% P_0 -positive Schwann cells	% Schwann cells synthesizing DNA	% P_0 -positive Schwann cells	% Schwann cells synthesizing DNA	% P_0 -positive Schwann cells	% Schwann cells synthesizing DNA
Forskolin (2-4 μM)	37 \pm 10.7 (n = 11)	3 \pm 2.5 (n = 5)	0 (n = 3)	90 \pm 4.3 (n = 3)	5 \pm 4.0 (n = 3)	81 \pm 1.1 (n = 3)
cAMP analogues	51 \pm 5.7 (n = 22)	2 \pm 1.5 (n = 6)	1 \pm 2.1 (n = 3)	88 \pm 3.7 (n = 3)	15 \pm 4.0 (n = 3)	72 \pm 10.2 (n = 3)
CTx	62 \pm 6.5 (n = 14)	3 \pm 2.8 (n = 4)	0.1 \pm 0.1 (n = 3)	86 \pm 3.4 (n = 3)	4 \pm 1.5 (n = 3)	79 \pm 8.4 (n = 3)

For experimental treatment and cell counting procedure see legend to Table I or Materials and Methods. DNA synthesis was assessed by BrdU incorporation and immunolabeling and is expressed as percentage of Schwann cells showing BrdU positive nuclei. In column 1, the data on P_0 -positive cells are reproduced from Table I for comparison.

starting from a relatively early developmental stage (E15-E16). In the presence of other mitogenic factors this would promote proliferation and trigger O4 expression, but not myelination. Withdrawal from the cell cycle first occurs around birth in cells associated with the larger axons via a mechanism as yet unknown. In these quiescent cells the ongoing cAMP elevation would induce myelination. This implies that myelination *in vivo* may not be triggered directly by a rise in cAMP levels, as previously suggested, but by the secondary action of factors that suppress DNA synthesis in cells in which cAMP levels have already been elevated.

Inhibition of Schwann Cell Proliferation

The mechanisms behind the cessation of proliferation that occurs before the formation of the first myelin wrap (56) could include a lowering of growth factor levels, downregulation of receptors, the presence of active inhibitory molecules, or contact inhibition. Modulation of the levels of growth factor receptors on cultured Schwann cells have been described but it is not clear whether those observations are relevant for growth inhibition *in vivo* (33, 57). The only

identified protein that actively inhibits Schwann cell DNA synthesis in short-term cultured Schwann cells is type 1 collagen (13), whereas growth inhibition of these cells is also caused by an unidentified factor associated with enteric neurons (11), and by protein secreted by cultured Schwann cells (35). In view of the possibility that cAMP levels in Schwann cells in normal nerves are higher than those in cultured cells (present paper and references in introduction), it is interesting that provided cAMP levels are elevated in cultured Schwann cells, γ -interferon acts as a growth inhibitor (14). Furthermore, some neurons are known to express interferon-like activity (25).

Effects of Basal Lamina Components on Induction of the Myelin Phenotype

Studies *in vitro* have shown that basal lamina formation is a prerequisite for myelin formation (6). In our studies exogenous laminin did not seem to be strictly required for induction of the myelin phenotype since it could be induced on a polylysine substrate. Laminin is, however, produced by Schwann cells themselves and treatment with cAMP analogues upregulates expression of laminin in cultured Schwann cells (2, 7) so it is difficult to be definitive on this point. The strikingly flattened shape of induced cells on laminin does suggest that it might play a role in the expansion and flattening of the Schwann cell that is required when the Schwann cell wraps around the axon and makes myelin *in vivo*.

Comparison with Previous Experiments on cAMP Induction and Schwann Cell Differentiation

Our results on induction of the myelin phenotype by elevation of intracellular cAMP complement and extend those of others. The original experiments of Sobue and Pleasure (50, 51) showing that galactocerebroside could be induced in short-term Schwann cells treated with high doses of cAMP analogues or high doses of forskolin suggested a role for cAMP in Schwann cell differentiation. Shuman et al. (49) reported that another myelin-related protein, P_{170K} , could be induced in short-term Schwann cell cultures using 8 bromo-cAMP (5×10^{-4} M to 3×10^{-3} M) or forskolin (100 μM) in medium with 10% calf serum. They were, however, unable to induce P_0 under these conditions. In view of the results reported in the present paper it seems likely that this failure can be traced to a high level of cell division, which

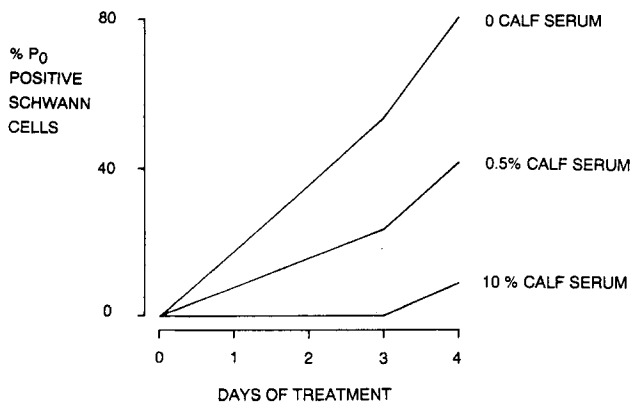


Figure 6. The inverse relationship between P_0 induction and increase in calf serum. This graph shows that P_0 is suppressed in the presence of increasing concentrations of calf serum. Schwann cells were cultured in the presence of cAMP analogues for up to 4 d, and the percentage of P_0 positive Schwann cells at each time point assessed by counting a minimum of 200 cells from each of duplicate coverslips. The results from a single representative experiment are shown.

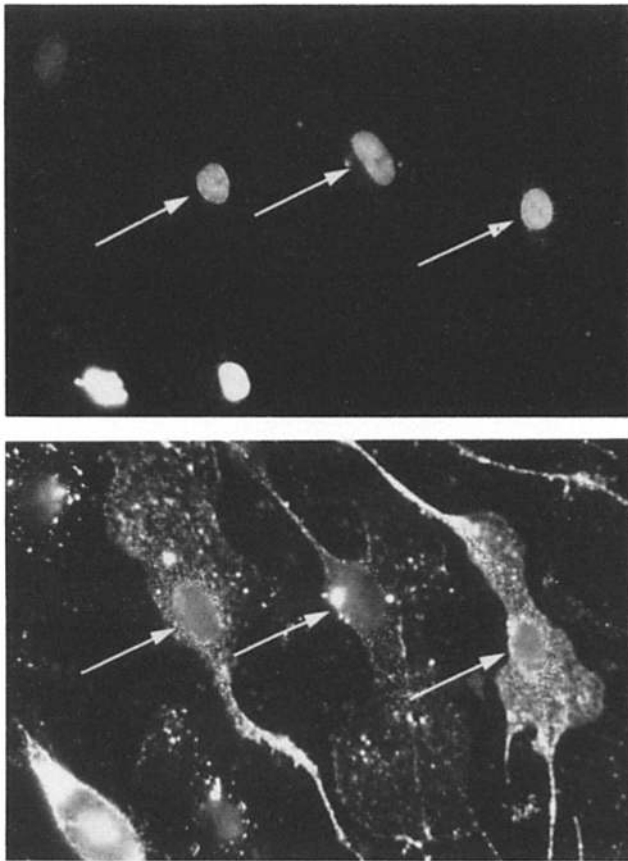


Figure 7. O4 is readily expressed in Schwann cells synthesizing DNA. Double label immunofluorescence using O4 antibody (A) and antibody to BrdU (B). Schwann cells were cultured for 72 h in the presence of cAMP analogues. Three O4-positive cells arrowed in A are also BrdU positive in B, indicating that they can synthesize DNA.

was not monitored in these experiments. Using immunoblotting and Northern blotting techniques, induction of P_0 and P_0 mRNA by 2 μ M forskolin and of myelin basic protein mRNA by 20 μ M forskolin in the presence of 10% FCS has been reported in passaged Schwann cells that have been previously exposed to forskolin and GGF for 3–4 wk (24). The induction of the POU-domain SCIP gene mRNA before induction of P_0 mRNA in the same culture system has been recently suggested as an important step in the series of events by which elevation of cAMP triggers myelin formation in Schwann cells (34). Cell division was not measured in these experiments. It should be noted that exposure of long term secondary Schwann cells to mitogenic doses of forskolin and GGF leads ultimately to constitutive expression of P_0 even when mitogens are withdrawn (24, 40, 41). Constitutive expression of P_0 has also been reported in long-term Schwann cells treated with CTx (54). Thus regulation of P_0 may be less stringent in long-term than in short-term cultured Schwann cells.

In developing nerve, there are no biochemical data on cAMP levels. These would, in any case, be difficult to interpret since cAMP levels in axons, Schwann cells and fibroblastic cells of the endo-, peri-, and epineurium would all contribute to the measured overall level. It has been reported that cAMP levels in the adult rabbit sciatic nerve rise sig-

nificantly in the distal stump of the nerve within 1–6 h after crush injury (1) a situation in which cell proliferation is initiated in previously quiescent Schwann cells, probably in response to macrophage invasion (3, 38).

Conclusions

Elevation of cAMP levels mimics events in Schwann cell development that take place *in vivo* from E16 onwards. These include induction of O4 antigen in the presence of DNA synthesis and induction of the myelin phenotype in quiescent cells. This suggests that in Schwann cells cAMP elevation may be a primary developmental signal, with a more general action than hitherto envisaged. Neither the present data, nor previous work on galactocerebroside and O4 induction, are consistent with the idea that cAMP elevation specifically triggers myelination. Rather, we suggest that secondary signals removing cells from the cell cycle effectively act as the myelination trigger, since this would permit the ongoing cAMP elevation to induce myelin-specific differentiation.

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