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Non-Antagonistic Relationship Between Mitogenic Factors and cAMP in Adult Schwann Cell Re-Differentiation

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Abstract

The expression of myelination-associated genes (MGs) can be induced by cyclic adenosine monophosphate (cAMP) elevation in isolated Schwann cells (SCs). To further understand the effect of known SC mitogens in the regulation of SC differentiation, we studied the response of SCs isolated from adult nerves to combined cAMP, growth factors, including neuregulin, and serum. In adult SCs, the induction of MGs by cAMP coincided with the loss of genes expressed in non-myelin-forming SCs and with a change in cell morphology from a bipolar to an expanded epithelial-like shape. Prolonged treatment with high doses of cAMP-stimulating agents, as well as low cell density, was required for the induction of SC differentiation. Stimulation with serum, neuregulin alone, or other growth factors including PDGF, IGF and FGF, increased SC proliferation but did not induce the expression of MGs or the associated morphological change. Most importantly, when these factors were administered in combination with cAMP-stimulating agents, SC proliferation was synergistically increased without reducing the differentiating activity of cAMP. Even though the initiation of DNA synthesis and the induction of differentiation were mostly incompatible events in individual cells, SCs were able to differentiate under conditions that also supported active proliferation. Overall, the results indicate that in the absence of neurons, cAMP can trigger SC re-differentiation concurrently with, but independently of, growth factor signaling.

Keywords

neuregulin; forskolin; growth factors; myelination; proliferation

INTRODUCTION

Schwann cells (SCs) are a unique cell type in their capacity to respond to cyclic adenosine monophosphate (cAMP) because elevation of intracellular cAMP levels both enhances the rate of G1-S progression stimulated by polypeptide growth factors (Raff et al., 1978a,b) and induces MG expression (Pleasure et al., 1985), therefore mimicking the action of axonal signals (Jessen et al., 1991). Thus, with regard to proliferation, cAMP-stimulating agents synergistically increase the mitogenic potency of neuregulin (Dong et al., 1997; Monje et al., 2006; Rahmatullah et al., 1998; Salzer and Bunge, 1980), and also that of platelet-derived growth

factor, PDGF (Davis and Stroobant, 1990; Kim et al., 2001), basic fibroblast growth factor-2, FGF-2 (Dong et al., 1997), insulin, insulin-like growth factor-1, IGF-1 (Stewart et al., 1996) and transforming growth factor- β , TGF- β (Ridley et al., 1989; Stewart et al., 1991). Other studies have revealed that cell permeable cAMP analogs and forskolin, a potent direct activator of the adenylyl cyclase (AC), also induce the expression of MGs, including the main peripheral nerve myelin glycoprotein P₀, both in early post-natal SCs (Jessen et al., 1991; Mirsky et al., 1990; Sobue et al., 1986) and in SC-derived cell lines (Bansal and Pfeiffer, 1987).

Several of the studies on the induction of MG expression in cultured postnatal SCs have shown that serum or purified growth factors, including neuregulin, FGF-1/2 and TGF- β 1/2/3, block the effect of forskolin as an inductive signal for the expression of key MGs (Cheng and Mudge, 1996; Morgan et al., 1991, 1994). Consistent with these observations, either the addition of neuregulin or the persistent activation of the extracellular signal regulated kinase (ERK) cascade has been shown to promote SC de-differentiation and demyelination in myelinated SC–neuron co-cultures (Harrisingh et al., 2004; Zanazzi et al., 2001). It has also been observed that the activation of the neuregulin receptor, ErbB2, is functionally linked to demyelination in response to axotomy (Guertin et al., 2005) and leprosy bacilli infection (Tapinos et al., 2006).

In contrast, and somewhat paradoxically, recent evidence suggests that axonal neuregulins and ErbB activation in SCs are signals required for normal SC myelination (Garratt et al., 2000; Nave and Salzer, 2006) and that specifically neuregulin-1 type III signaling is connected with the induction of the myelinating phenotype of SCs and the regulation of myelin thickness *in vivo* (Chen et al., 2006; Michailov et al., 2004; Taveggia et al., 2005). Interestingly, recent studies have shown that specific ablation of ErbB2 in adult SCs had no apparent effect on the maintenance of myelinated fibers *in vivo* (Atanasoski et al., 2006).

We initiated this study in order to better understand the relationship between cAMP and growth factors in the control of proliferation and differentiation of adult-derived SCs. SCs from adult rat sciatic nerves rapidly de-differentiate under conventional culture conditions and the cells expanded *in vitro* exhibit the expression of markers typically associated with a pre- or non-myelinating SC phenotype. Adult SCs are fully competent to re-differentiate into myelinating SCs if contact with axons is re-established both *in vitro* (Morrissey et al., 1991) and *in vivo* (Pearse et al., 2004). We began by investigating the conditions that stimulate the differentiation of these cells into a myelin-related phenotype in an axon-free environment. We observed that SC differentiation was dependent on cell density and on long-term and persistent stimulation with doses of cAMP-stimulating agents higher than those required for the enhancement of growth factor-induced proliferation, and that this occurred regardless of the presence of serum, neuregulin or other mitogenic factors in the culture medium. However, proliferating and differentiating cells belonged to essentially non-overlapping SC subpopulations. Overall, we conclude that the initial events involved in the re-differentiation of SCs into a myelinating phenotype are mostly independent of mitogenic signaling.

MATERIALS AND METHODS

Materials

N⁶ 2'-O-dibutyryladenosine-3',5'-cyclic monophosphate (db-cAMP), 8-(4-chlorophenylthio) adenosine-3',5'-cyclic monophosphate (CPT-cAMP), and 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate (CPT-cGMP) were from Biolog (Axxora LLC, San Diego, CA). Recombinant heregulin- β 1_{177–244} (herein referred to as “neuregulin”) was from Genentech (South San Francisco, CA). Recombinant PDGF-BB, IGF-1, FGF-2, and TGF- β were from R&D Systems (Minneapolis, MN). Defined fetal bovine serum (FBS) was from HyClone (Logan, UT). Forskolin, cholera toxin and 2',3'-cyclic nucleotide 3'-

phosphodiesterase (CNPase) antibody were from Sigma (St. Louis, MO). S100 and glial fibrillary acidic protein (GFAP) antibodies were from DAKO (Carpinteria, CA). Antibodies against myelin-associated glycoprotein (MAG), proteolipid protein (PLP), protein zero (P₀), myelin basic protein (MBP), peripheral myelin protein 22 (PMP22), and neurofilament (NF) were from Chemicon (Temecula, CA). N-cadherin and β -catenin antibodies were from BD Biosciences (San Jose, CA). Bromodeoxy-uridine (BrdU) antibodies and DNAase were from Amersham (Piscataway, NJ). [³H]-thymidine and Solvable™ were from Perkin-Elmer (Boston, MA). Hybridoma cells for p75 neurotrophin receptor (p75^{NGFR}) were from American Type Culture Collection (ATCC, Manassas, VA). O1 and O4 hybridoma cells were a gift from Dr. M. Schachner and anti-NCAM antibodies were from Dr. V. Lemmon.

Primary Cultures of Rat SCs and Transduction with GFP Lentiviral Vectors

Rat SCs were prepared from adult sciatic nerves by a modification of a reported method (Morrissey et al., 1991). Nerve segments were explanted in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% FBS and depleted of fibroblasts by sequential transplantation to new dishes. Explants were dissociated with 0.25% dispase and 0.05% collagenase and cells were plated on a poly-L-lysine (PLL) substrate. Cells were further purified of contaminating fibroblasts by incubation with anti-Thy 1.1 antibodies (ATCC) followed by rabbit complement. Cells were expanded in DMEM-10%FBS supplemented with 2 μ M forskolin, 20 μ g/mL bovine pituitary extract, and 10 nM neuregulin (expansion medium). Experiments were performed using cells from passage 2 to 4 (2–8 population doublings) that were >98% SCs based on immunostaining with anti-S100.

Cultures of post-natal rat SCs were established by a modification of a reported method (Brookes et al., 1979). Briefly, sciatic nerves from postnatal day 1 rats were dissected and dissociated sequentially with 0.1% collagenase and 0.25% trypsin. The resulting cell suspensions were purified of contaminating fibroblasts by including 10 μ M cytosine arabinoside in the culture medium (DMEM-10% FBS) for 3 days. Post-natal SCs were further purified, grown and expanded as described for adult SCs.

SC cultures were transduced at an early passage with a lentiviral vector expressing the green fluorescent protein (GFP) under the control of the cytomegalovirus promoter. The generation and use of GFP lentiviral constructs in SCs was described previously (Blits et al., 2005).

Co-Cultures of SCs and Dissociated Dorsal Root Ganglion Neurons (DRGNs)

DRGNs dissected from rat embryos on the 15th day of gestation were dissociated with 0.25% trypsin (37°C, 45 min) followed by gentle trituration. The resulting cell suspension was plated on air-dried collagen-coated aclar dishes (50,000 cells/dish) and maintained in Neurobasal medium with B27 supplement (Invitrogen) and 10 ng/mL nerve growth factor for 3 weeks. Cultures were purified by 1–3 consecutive treatments with the anti-mitotic agent 5-fluoro-2' deoxyuridine (10 μ M). These conditions allow the establishment of a pure population of DRGNs with an extensive network of radiating axons extending all over the available surface (Eldridge et al., 1987).

To assay the interaction of SCs and DRG axons, SCs (left untreated or treated with db-cAMP for 3 days) were labeled with Cell Tracker Green (Molecular Probes, Eugene, OR), removed from the culture dish by trypsin digestion and seeded on top of a network of pure DRGNs (25,000 SCs/dish). Overnight after plating, cells were fixed for confocal or electron microscopy analysis. Labeling with Cell Tracker, a dye incorporated exclusively by living cells, indicated no signs of reduced metabolic activity in cAMP-treated cells.

Cell Lines

D6P2T cells (ATCC) were routinely grown on PLL-laminin-coated dishes in DMEM medium containing 10% FBS.

Proliferation and Differentiation Assays

Sub-confluent SC cultures growing on PLL-laminin-coated 24-well plates (50,000 cells/well, unless otherwise noted) were pre-synchronized in G1/G₀ by progressively depriving the cells of mitogens and serum prior to stimulation. Specifically, cells were grown for 2 days in DMEM-10% FBS followed by 1 day in HEPES-buffered DMEM containing 1% FBS (non-proliferating medium). The presence of a non-mitogenic concentration of FBS (0.1–1%) was essential for cell attachment and survival.

The incorporation of [³H]-thymidine, or alternatively the incorporation of the thymidine analog BrdU, into nuclear DNA was assayed as a measure of S-phase entry. Cells were exposed to medium containing [³H]-thymidine (0.25 µCi/well), or BrdU (1 µM), present throughout the incubation period. Samples were assayed in triplicate in each experimental condition. Unless otherwise noted, mitogenic concentrations of growth factors were used for all stimulation experiments: 10 nM neuregulin, 20 ng/mL PDGF-BB, 50 ng/mL IGF-1, 20 ng/mL FGF-2 and 20 ng/mL TGF-β. Three days after stimulation, cells were washed with phosphate buffer (PBS), lysed with Solvable™ (300 µL/well) and analyzed for the incorporation of tritium by liquid scintillation counting or alternatively, processed for the immuno-detection of incorporated BrdU. For this, cells were fixed sequentially with paraformaldehyde and methanol and immuno-stained for SC markers as described below. Cells were then treated with DNAase for 2 h at RT in the presence of anti-BrdU (1:100) followed by incubation with Alexa 594-conjugated secondary antibodies (Molecular Probes).

For the differentiation experiments, cells were plated and stimulated under culture conditions identical to the ones described for proliferation assays. Differentiated SCs were identified as those expressing high levels of myelin markers, as detected by immunofluorescence microscopy.

Conventional Immunofluorescence and Confocal Microscopy

Cultures were fixed sequentially with 4% paraformaldehyde-PBS (10 min) and −20°C methanol (5 min), blocked in 5% normal goat serum-PBS, incubated overnight with primary antibodies (1:200, 4°C) and then for 1 h with Alexa-conjugated secondary antibodies (1:400, RT). O1 and O4 labeling was done by incubating living cells with hybridoma culture supernatant (30 min, RT) prior to paraformaldehyde fixation. Stained cultures were mounted with Vectashield containing the nuclear dye DAPI (Vector Labs, Burlingame, CA) and analyzed by conventional or confocal fluorescence microscopy. Digital images from fluorescence microscopy were artificially colorized, processed and arranged for presentation using Adobe Photoshop V7.0 and Adobe Illustrator CS3. For cell quantification analysis, pictures from random fields were taken at low magnification and the percentage of positively labeled cells was determined in reference to the total number of cells (DAPI staining). Cells were classified as positive or negative for the expression of markers in reference to non-treated controls. At least 500 cells were analyzed/condition.

For confocal imaging, co-cultures of Cell Tracker Green-labeled SCs and DRGNs were fixed with paraformaldehyde and axons were immuno-stained for the specific marker NF using Alexa-647-conjugated secondary antibodies. Confocal microscopy was performed on a Carl Zeiss Laser Scanning Microscope, LSM 510. Argon (488 nm) and helium-neon (633 nm) lasers were used for the visualization of SCs and axons, respectively.

Transmission Electron Microscopy (TEM)

Cells growing on PLL-laminin-coated glass cover slips (SCs) or on collagen-coated aclar dishes (SC-DRGN co-cultures) were fixed overnight in 2% glutaraldehyde-100 mM sucrose and then rinsed in 0.15 M phosphate buffer before post-fixing for 1 h with 2% OsO₄. Subsequently, cells were rinsed, dehydrated in graded ethanol solutions and embedded in Embed (Electron Microscopy Sciences, Hatfield, PA). Thin sections obtained with a Leica Ultracut E microtome were stained with uranylacetate/lead citrate for examination in a Philips CM-10 transmission electron microscope.

RESULTS

cAMP Elevation Is Sufficient to Re-Establish a Differentiated Myelin-Related Phenotype in Isolated Adult SCs: Induction of MG Expression, Morphological Changes, and Functional Properties

Cyclic AMP has been recognized as one of the main inductive signals for MG expression in postnatal SCs (Jessen et al., 1991). As shown in Fig. 1, SCs isolated from adult sciatic nerves and expanded *in vitro* also responded to an elevation of cAMP with an increase in the expression of markers characteristic of myelin-forming SCs. For these studies, SCs were first progressively deprived of mitogens and serum to allow the cells to return to quiescence without undergoing apoptosis, and then treated for 3 days with db-cAMP, a membrane permeable analog of cAMP. Under these culture conditions, which do not support SC proliferation, we detected a dramatic increase in the expression of protein and lipid markers that define the early (CNPase and the sulfatide antigen, O4), middle (MAG and the specific galactocerebroside antigen, O1) and late (P₀, PMP22, PLP and MBP) phases of SC differentiation into myelin-forming cells (see Fig. 1). In addition, we have recently described the up-regulation of mRNA expression of two key enzymes involved in myelin galactolipid metabolism, including a novel fatty acid 2-hydroxylase, which clearly parallels the increase in P₀ mRNA expression upon db-cAMP treatment (Maldonado et al., 2008). As shown in Fig. 2, our results also indicated that db-cAMP stimulation reduced the expression of non-myelin genes (NMGs), i.e., those typically associated with either proliferating or immature SCs, such as N-cadherin and β -catenin (Gess et al., 2008), or with non-myelin-forming SCs, such as N-CAM, GFAP and p75^{NGFR}. The expression of S100, a calcium-binding protein identified as a general SC marker, was unaffected by db-cAMP treatment (see Fig. 2).

The elevation of cAMP also induced a dramatic cell shape transformation from a bipolar to an expanded flat epithelial-like morphology, consistent with previous observations on postnatal SCs (Morgan et al., 1991; Sobue et al., 1986). Conventional fluorescence (Fig. 1 and Fig 2) and TEM analysis (see Fig. 3) revealed that db-cAMP treatment dramatically increased the size of the cytoplasm and of the nucleus, and stimulated the appearance of both extensive vacuoles and abundant thin plasmalemmal extensions resembling epithelial microvilli. The presence of intracellular vacuoles gave the cytoplasm a characteristic reticulate appearance (Cheng and Mudge, 1996; Morgan et al., 1991; Sobue et al., 1986). Vacuoles and microvilli were absent in non-treated SCs (Fig. 3, left panels). TEM studies further revealed that vacuoles increased progressively over time, both in number and in size, after the initial exposure to db-cAMP (Fig. 3, right panels). At 3 days post-stimulation, cells exhibiting two large vacuoles, one on each side of the nucleus, were typically found in the cAMP-treated populations (Fig. 3, lower right panel). TEM analysis indicated no signs of mitochondrial or nuclear degeneration in SCs containing vacuoles (Fig. 3, middle panel). In addition, labeling of SCs with the vital dye Cell Tracker followed by immuno-localization of myelin markers confirmed that the development of vacuoles in cells expressing high levels of MGs was not associated with an impaired metabolic condition of the cells (see Fig. 4). Indeed, cAMP-treated SCs showed a

highly euchromatic nuclear content, indicative of a transcriptionally active cell type (Fig. 3, right panels).

Prolonged treatment with db-cAMP did not prevent the ability of SCs to align following parallel patterns when growing in monoculture (see Fig. 4) or to readily associate with and extend processes along DRG axons (see Fig. 5), features previously associated with fully functional and healthy growing cultured SCs (Porter et al., 1986). Interestingly, cAMP-differentiated SCs had a tendency to extend multiple processes along multiple axonal bundles, in clear contrast to the characteristic spindle-shaped morphology observed in non-treated SCs under identical co-culture conditions (Fig. 5, upper panels). Ultrastructural analysis further revealed that a close apposition of the plasma membrane of cAMP-treated SCs with the membrane of the axons, a typical feature that defines the early SC–axon interaction, developed shortly after initiating co-culturing (Fig. 5, lower panels).

Overall, these results indicate that cAMP treatment of isolated adult SCs is sufficient to induce the re-appearance of a differentiated SC phenotype that involves the induction of MG expression, the reduction of NMG expression and the acquisition of an epithelial-like shape.

The Re-Appearance of a Differentiated SC Phenotype Is Specific to cAMP and Requires Prolonged and Persistent Exposure to cAMP-Inducing Agents

We next investigated the cell culture conditions required for the differentiation of adult SCs in response to cAMP. For these and all subsequent studies, we analyzed the expression of MAG, as a myelin protein marker, and/or O1, as a myelin lipid marker (Bansal et al., 1989), because their expression was negligible or undetectable in non-stimulated SCs, but dramatically increased upon cAMP treatment, facilitating the identification of positive vs. negative cells and quantitative analysis. Time course studies revealed that the induction of MG expression required prolonged exposure to db-cAMP (Fig. 6A), in contrast to the synergistic induction of DNA synthesis under similar culture conditions (Monje et al., 2006). Moreover, db-cAMP was effective as a differentiating agent in a dose-dependent manner and only when given at concentrations in the range of 200 μ M to 1 mM (Fig. 6A). These concentrations were at least 10–100 times higher than those required to synergistically cooperate with neuregulin to induced S-phase entry (Monje et al., 2006). Regardless of the concentration and the type of cAMP-inducing agent used, the appearance of O1- or MAG-expressing SCs was observed 3 days after the initial exposure to cAMP (Fig. 6A). Even though cAMP-induced morphological changes persisted long after the initial stimulation (Fig. 6B), the expression of MGs was strongly down-regulated 5–6 days after the initial exposure to cAMP. High levels of MG expression could be maintained for prolonged periods of time, at least 10 days after the initial stimulation, if fresh cAMP analogs were repeatedly added to the culture medium (Fig. 6B) or if new medium containing cAMP analogs was frequently (e.g., every 3 days) replaced (not shown). Importantly, although the majority of the SCs in the cAMP-treated populations exhibited an enlarged morphology with prolonged exposure (Fig. 4 and Fig 6B), the expression of MGs was only induced in a fraction of the cells, ranging from ~30–40% to ~70–80% depending mainly on the concentration and the type of cAMP-inducing agent used and, as we show below, the initial density of the cultures.

Our results further indicated that the induction of MG expression in SCs was specifically dependent on intracellular cAMP because (1) equivalent concentrations of the cyclic nucleotide CPT-cGMP, a cell permeable non-hydrolysable analog of cyclic-GMP, could not mimic the effect of the non-selective analogs of cAMP, db-cAMP (Fig. 6A) or CPT-cAMP (Fig. 6C) and (2) both forskolin and cholera toxin, a non-reversible activator of the AC stimulatory G α s subunit, induced the expression of MGs (Fig. 6C, shown only for O1 expression) and changes in cell shape (not shown). However, the induction of detectable levels of MG expression required the addition of doses of forskolin, 20 μ M or higher (Fig. 6C), which were at least 10

times higher than those required to synergistically induce neuregulin-dependent S-phase entry (Monje et al., 2006), consistent with previous reports (Sobue et al., 1986; Yamada et al., 1995).

The Re-Appearance of a Differentiated SC Phenotype Is Independent of the Presence of Serum, Neuregulin, or Other Known Mitogenic Factors

Available evidence has suggested an antagonistic effect of serum and several growth factors on cAMP-induced MG expression (Cheng and Mudge, 1996; Morgan et al., 1994). However, in cultures of isolated adult SCs, we observed that the induction of MGs (O4, O1, MAG and P₀) by cell permeable analogs of cAMP was not reduced by the presence of 10% FBS (defined quality) added to the culture medium 1–3 days before (not shown) or at the time of cAMP stimulation (see Fig. 7). Consistent with these results, the presence of 10% FBS did not interfere with db-cAMP's ability to reduce the expression of NMGs (GFAP and p75^{NGFR}) or induce an epithelial-like morphology (Fig. 7A). Interestingly, the addition of FBS alone at concentrations up to 20%, though inducing cell enlargement to some extent, was insufficient to induce the expression of MGs (Fig. 7A).

We next studied the cooperating effects of FBS and cAMP on the stimulation of S-phase entry, since defined FBS contains a mitogenic component for adult SCs. In these and all subsequent studies on SC proliferation, the incorporation of [³H]-thymidine, assessed as a measure of DNA synthesis, was monitored under identical experimental conditions as for the detection of myelin markers. The results indicated that the mitogenic component of FBS synergistically cooperated with cAMP-stimulating agents to increase S-phase progression in SCs (Fig. 7B, lower panel) without affecting cAMP's ability to induce differentiation (Fig. 7B, upper panel). The inability of serum to alter the expression levels of MGs was independent of the type of cAMP-stimulating agent used, as we confirmed our results by using two different cell permeable analogs of cAMP, db-cAMP and CPT-cAMP (Fig. 7B), and also a differentiating concentration of forskolin (50 μ M, not shown).

Because FBS contains a complex mixture of growth factors that are potential mitogens for SCs, including PDGF and insulin, we next investigated the effect of purified growth factors on DNA synthesis and MG expression, in the absence and presence of cAMP analogs or forskolin. Treatment of mitogen-starved SCs with physiologically active concentrations of different polypeptide growth factors, including neuregulin, which displayed the highest potency, and also PDGF-BB, IGF-1, insulin (not shown), and FGF-2, induced S-phase entry of cultured adult-derived SCs. As expected, treatment of SCs with cAMP-stimulating agents synergistically enhanced G1-S progression initiated by all the aforementioned growth factors (Fig. 8C, lower panel). However, co-stimulation of SCs with growth factors did not change the differentiating activity of db-cAMP (see Fig. 8) or forskolin (not shown) as determined by examining the levels of O1 expression (Fig. 8C, upper panel), GFAP expression or cAMP-induced morphological changes (Fig. 8, shown only for neuregulin). In addition, these growth factors, when administered alone, were inactive at inducing MG expression or changes in cell shape comparable to those induced by cAMP (Fig. 8, shown only for neuregulin).

To confirm these results, we investigated in more detail the potential effect of neuregulin on cAMP-induced SC differentiation. For this, we monitored the expression of alternative markers for SC differentiation, including the MGs O4, MAG, and P₀ and the NMG, p75^{NGFR} (see Fig. 8). Overall, the results indicated that the presence of neuregulin did not interfere with the differentiating effects of cAMP, regardless of the concentration of neuregulin (1–200 nM), the type of marker monitored or the type of cAMP-inducing agent used for differentiation, i.e., db-cAMP, CPT-cAMP or 50 μ M forskolin (Fig. 8 and Fig 10, and data not shown).

To investigate whether the origin of the cells could account for the apparent discrepancy between our results and published data, similar experiments were carried out using SCs isolated from newborn rats. By subjecting these cells to treatment with FBS or neuregulin in combination with cAMP analogs, we obtained nearly identical results as for adult SCs, supporting a non-antagonistic action of mitogens on cAMP-induced SC differentiation under defined culture conditions (not shown). We also investigated the potential effects of TGF- β , a reported mitogen for postnatal SCs, and found that it was ineffective in inducing proliferation of adult SCs, either in the absence or presence of cAMP-increasing agents. TGF- β was highly effective at inducing cell aggregation and changes in cell morphology when administered alone; however, and as shown for other growth factors, it did not reduce MG expression when given in combination with cAMP (not shown).

Interestingly, the addition of high doses of cAMP-stimulating agents was sufficient to induce differentiation of SCs growing in medium supplemented with a combination of neuregulin, forskolin (2 μ M), pituitary extract and serum (expansion medium), further supporting the observation that SC differentiation may occur under culture conditions which also support active cell division (not shown). Of note, prolonged treatment of SCs with high doses of cAMP, either in the absence or presence of serum or growth factors, induced cell growth arrest, as differentiated SCs became refractory to proliferate when exposed to mitogenic factors (not shown).

Collectively, these data suggests that the initial events leading to the induction of a myelin-related genetic program in SCs are mostly independent of growth factor signaling.

The Re-Appearance of a Differentiated SC Phenotype Is Dependent Upon Cell Density

We next investigated if we could identify factors contributing to the regulation of MG expression in response to cAMP. We found that the type of culture medium (DMEM, DMEM/F12 or Neurobasal) or the addition of ascorbic acid did not significantly affect the ability of cAMP-stimulating agents to induce the expression of MGs. Likewise, by comparing cells growing on PLL-laminin with cells growing on PLL or laminin alone or the combination poly-ornithine-laminin, we found a non-requirement of a laminin substrate for the induction of MG expression in response to cAMP, as reported previously (Morgan et al., 1991).

Importantly, we observed that one key factor controlling cAMP-induced SC differentiation was cell density. Strikingly, increasing the number of cells/surface area reduced the ability of cAMP-stimulating agents to induce MG expression either in the absence or presence of 10% FBS (Fig. 9A). Most importantly, cAMP was able to induce high levels of MG expression and morphological differentiation of SCs under conditions where the majority of the cells were not contacting neighboring cells (Fig. 9B). In the absence of cAMP stimulation, an increase in cell density did not induce MG expression (not shown). Similar results on the requirement of low cell density for optimal induction of MG expression in response to cAMP were observed in D6P2T cells (Fig. 9C), a schwannoma-derived cell line that exhibits the expression of MGs (Bansal and Pfeiffer, 1987). Overall, these results suggest a non-requirement of cell to cell contact for the initiation of MG expression in SCs. Surprisingly, conditions of sub-confluency were essential for an optimal response of SCs to cAMP as both a proliferative and a differentiating agent.

cAMP Induces Cell Proliferation and Differentiation of Phenotypically Different Subsets of SCs

To better define the effects of cAMP as a proliferative vs. a differentiating agent for isolated adult SCs, we performed a quantitative analysis of individual cells under-going proliferation and differentiation by simultaneously labeling SCs entering S-phase (cells that incorporated

the thymidine analog BrdU) and SCs expressing myelin markers (cells labeled with O1 antibodies). For these experiments, SC cultures were treated with the cAMP analog CPT-cAMP in the absence or presence of neuregulin or 10% FBS to achieve an optimal response in both S-phase entry and MG expression, and treatment was performed for 3 days, the minimum period of time required for the detection of O1 positive SCs. Results from BrdU labeling experiments confirmed the synergistic interaction between cAMP and neuregulin in the control of S-phase entry and further revealed that ~30% of the SC population underwent cell division in the combined presence of neuregulin (or FBS) and CPT-cAMP (Fig. 10A, left panel). Surprisingly, under these culture conditions, cells partitioned into two main sub-populations of either proliferating (BrdU positive/O1 negative) or differentiating (BrdU negative/O1 positive) cells (Fig. 10A, right panel and Fig. 10B, left panel, shown only for neuregulin + CPT-cAMP). However, a small proportion of the cells had double-labeling for O1 and BrdU (Fig. 10, right panels). These SCs were however, low-middle O1 expressors as assessed by immuno-staining intensity, and also exhibited low or moderate changes in cell shape, indicating that the onset of differentiation was most likely delayed in this fraction of the population (not shown). Comparable results were obtained by monitoring the expression of MAG in cultures co-stained with BrdU (not shown).

In addition, cultures treated with neuregulin (or 10% FBS) and CPT-cAMP were triple immuno-stained to detect O1, GFAP and BrdU (Fig. 10B). Interestingly, we observed that more than half of the SCs incorporating BrdU expressed high levels of GFAP (GFAP positive/O1 negative). As shown in Fig. 10B (left panel), BrdU-labeled GFAP positive SCs exhibited a range of different sizes and morphologies, including some enlarged cells with a phenotype similar to the one of O1-expressing SCs. Of note, some of these GFAP-positive proliferating SCs expressed higher levels of GFAP than control non-treated cells (Fig. 10B, arrows).

In agreement with previous observations (Morgan et al., 1991), these results indicate that essentially non-overlapping and phenotypically distinctive SC sub-populations enter cell division or acquire a differentiated myelin-related phenotype in response to cAMP.

DISCUSSION

In this study, we have presented evidence indicating that in the absence of axons, cAMP elevation is sufficient to re-establish a differentiated myelin-related phenotype in cultured adult SCs and that the differentiating action of cAMP is not antagonized by the presence of growth factors or serum in the culture medium. This effect is clearly distinct from the synergistic regulation that cAMP exerts on growth factor-dependent SC proliferation under identical culture conditions. Moreover, we confirmed two basic previous observations that define the differentiation of SCs in the absence of axonal contact: (1) A change in gene expression including the upregulation of a complete panel of MGs, concomitant with the down-regulation of genes associated with non-myelin forming and/or proliferating SCs, and (2) A change from a bipolar to an epithelial-like morphology. Neuregulin and other growth factors, while effective in promoting proliferation when given alone or in combination with cAMP-stimulating agents, were inactive as either direct differentiating agents or as modulators of cAMP-induced SC differentiation. The main results of this study are summarized in the diagram shown in Fig. 11.

Our results on the response of adult SCs to cAMP are consistent with similar findings in postnatal SCs showing up-regulation of cell-surface sulfatide (Mirsky et al., 1990), galactocerebroside (Sobue and Pleasure, 1984; Sobue et al., 1986), MAG (Shuman et al., 1988), P₀ and MBP (De Deyne et al., 1994; Jessen et al., 1991; Morgan et al., 1991; Sobue et al., 1986; Yamada et al., 1995) and down-regulation of p75^{NGFR} (Mokuno et al., 1988), N-CAM and GFAP (Morgan et al., 1991). We have now extended these observations by showing that, despite the dramatic change in cell shape that SCs experience after prolonged cAMP

treatment and despite the important remodeling of the SC membrane (e.g., loss of the adhesion molecules N-CAM and N-cadherin), cAMP-differentiated SCs did not show an impaired ability to align with each other when growing in monoculture or to interact with DRG axons.

One important novel observation is that cAMP-induced differentiation of SCs is, under the conditions of our experiments, independent of serum or growth factors. This also implies that the activation of the ERK cascade by neuregulin or other factors may not be sufficient to prevent or reduce MG expression in the presence of differentiating signals such as cAMP, as suggested previously (Harrisingh et al., 2004). This implication is based on our observation that cAMP elevation in SCs does not inhibit, but instead synergistically enhances and also prolongs the duration of ErbB and ERK activation stimulated by neuregulin, a signal that is required for G1-S progression (Monje et al., 2006, 2008). It is possible that the ability of growth factors to reduce MG expression may depend on the particular set of pathways activated, as previously observed (Ogata et al., 2004), or on other extracellular cues. Instead, our observations are consistent with the emerging idea that axonal neuregulins are signals necessary for normal SC myelination (Chen et al., 2006; Taveggia et al., 2005). It is worth mentioning that the expression of axonal neuregulins does not seem to decline over the period of active SC myelination, and that myelinating SCs have been reported to express neuregulin receptors (Guertin et al., 2005; Michailov et al., 2004). Therefore it is likely that not only MG expression but also the process of SC myelination may have to proceed normally in spite of the continuous activation of ErbB signaling, including the activation of the ERK cascade. In addition, it has long been shown that in a co-culture system of SCs and DRGNs, the formation of myelin sheaths not only proceeds normally but also requires the addition of serum to the culture medium (Eldridge et al., 1987; Moya et al., 1980).

In contrast to our observations, previous data have shown an antagonistic effect of neuregulin, FGF, TGF and serum on the expression of P₀ and MBP (Cheng and Mudge, 1996; Morgan et al., 1991, 1994). We have not been able to determine at this point the cause of the apparent discrepancy between the previous and present findings. We have confirmed our results using cAMP-treated SCs isolated from postnatal rat sciatic nerves. In addition, we have compared the effect of different cell permeable analogs of cAMP and forskolin as differentiating agents, and have also monitored the expression of a variety of MG and MNG markers. It is possible that several factors or combinations of factors may contribute to explain the differences observed, including the state of the cells at the time of stimulation and the effect of cell selection in culture. These and other possibilities could be tested in future experiments.

Even though cAMP fulfills the requirements for a candidate signal in the control of SC proliferation and differentiation, the question still remains whether axons stimulate an increase in intracellular cAMP in contacting SCs. So far, a membrane bound form of neuregulin has been the only fully recognized axon-derived molecule controlling multiple aspects of SC function, including cell fate specification, survival, migration, proliferation and myelination (Jessen and Mirsky, 2005; Nave and Salzer, 2006). However, neuregulin, that signals through the activation of ErbB2 and ErbB3 receptor tyrosine kinases in SCs, is not a good candidate to elicit direct AC activation and cAMP production (Britsch, 2007). Because stimulation of SCs with neuregulin was not sufficient to elicit MG expression or appreciable changes in cAMP (Monje et al., 2008), our observations support the concept that neuregulin signaling might control myelination by targeting an event that lies downstream or is independent of the onset of MG expression.

Our results are consistent with previous studies showing that cAMP elevation triggers SC differentiation concomitant to cell growth arrest (Jessen et al., 1991; Morgan et al., 1991), as we have observed that cAMP-differentiated SCs become unresponsive to growth factors to re-initiate DNA synthesis. However, our results indicate that a cessation in proliferation does not

seem to be a sufficient condition *per se* to allow for the differentiation of SCs, as we show here that (1) contact inhibited SCs did not differentiate despite prolonged cAMP stimulation and (2) non-dividing SCs made quiescent by prolonged serum-mitogen deprivation differentiated in response to cAMP elevation under either permissive (addition of neuregulin, serum or other SC mitogens) or non-permissive (no serum or mitogens added) conditions for proliferation. In contrast to previous findings (Jessen et al., 1991), we have observed that the induction of MG expression was not impaired in SCs that establish no apparent contact with surrounding cells. During development, the period of maximal proliferation of SCs coincides with the period of maximal contact between adjacent SCs (Wanner et al., 2006), and the onset of myelination requires SC segregation and the establishment of an intimate association of the membrane of the myelinating SC with that of the ensheathed axon (Webster et al., 1973). In addition, and confirming previous observations (Sobue et al., 1986; Yamada et al., 1995), we have found that the induction of adult SC re-differentiation requires not only much longer exposure to but also higher doses of cAMP-inducing agents than those required for the stimulatory effect of cAMP on cell proliferation, indicating that cAMP controls proliferation and differentiation through a different mechanism of action. This idea is further supported by the observation that cAMP synergistically enhanced growth factor-dependent proliferation (adjuvant effect), whereas it elicited MG expression independently of growth factors (direct effect). Available data has shown a role for the cAMP-activated kinase, PKA, on cAMP-induced SC proliferation (Kim et al., 1997; Monje et al., 2008). However, neither PKA nor neuregulin signaling was required for SC differentiation induced by cAMP (Monje, unpublished).

Even though cAMP enhanced proliferation and differentiation under similar culture conditions, our studies indicated that the population of dividing SCs did not overlap with the one that differentiated. This result indicates that proliferation and differentiation are indeed incompatible events in individual cells and also raises the interesting question as to whether cAMP signals might be interpreted differently by different subpopulations of cells residing in the SC cultures. Consistent with this interpretation, we have found that not all SCs were able to undergo differentiation into a myelin-like phenotype, even after prolonged treatment with repeated additions of cAMP-stimulating agents, and that surprisingly, a significant proportion of the dividing SCs belonged to a subgroup of cells that did not down-regulate, but instead showed an increased expression, of GFAP. Because GFAP is a well-recognized marker not only for immature SCs but also for differentiated SCs of the non-myelinating SC lineage, further studies are required to explore the possibility that cAMP signaling might exert a role in SC differentiation into a non-myelinating phenotype.

Shared ultrastructural features between cAMP-induced and axon-induced morphological changes of myelinating SCs are the development of microvilli, the increase in the size of the nucleus, and the expansion of the cell membrane. However, the origin of the vacuoles remains elusive as they are not apparently derived from endoplasmic reticulum or Golgi membranes based on immuno-staining with specific markers (not shown). We have observed that the membrane of these vacuoles usually stains positive for myelin protein markers, including MAG and PLP, and that they bear abundant clathrin-coated pits (not shown), suggesting that they most likely are derived from the plasma membrane. Intracellular compartments resembling the ones herein described have been shown in freshly isolated SCs expressing high levels of P₀ (Cheng and Mudge, 1996). However, the functional relevance of the vacuoles is unknown, and we are not aware of a precedent of vacuolization during developmental myelination in SCs. In epithelial cells, vacuoles may occur as separate organelles for the storage of apical membranes (Vega-Salas et al., 1987). In isolated SCs, the vacuoles may arise simply as a consequence of the synthesis of myelin membranes that cannot fold properly because of the absence of axonal contact. Indeed, mature oligodendrocytes accumulate intracellular structures containing myelin when they grow in isolation from axons (Arvanitis et al., 1992).

In conclusion, our studies are consistent with previous findings supporting a role of cAMP as a prominent inducer of the myelinating phenotype in SCs. We provide new evidence that this role can occur coincident with signaling activated by neuregulin, as well as a range of growth factors that are also effective mitogens for SCs, even though we saw no indication that neuregulin itself, other mitogens or serum could induce or influence the expression of MGs in the absence of axons. Elucidation of the precise details of the interaction between cAMP and growth factor signaling in myelin sheath formation remains an important direction for future research.

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Fig. 1.

Increase in the expression of protein and lipid markers typically associated with myelinating SCs in cAMP-treated adult-derived rat SCs. SCs were left untreated (control) or treated with 1 mM db-cAMP for 3 days (db-cAMP) in non-proliferating medium. Staining with specific antibodies was done in live cells (O4 and O1 epitopes) or para-formaldehyde-fixed cells (all remaining markers) and visualized by conventional fluorescence microscopy. Note the specific distribution of myelin lipids and proteins in the membrane and/or cytoplasm of cAMP-differentiated SCs, with the exception of MBP, which localizes exclusively in membrane processes. Staining with MAG antibodies also revealed labeling of the vacuolar membrane in a fraction of the cells (arrows). Arrowheads point to representative SCs containing vacuoles and expressing high levels of myelin-related proteins. Results in this and the following figures were representative of at least 3 independent experiments performed. Scale bar, 50 μ m.

Fig. 2. Reduction in the expression of markers associated with non-myelinating and/or proliferating SCs in cAMP-treated SCs. Experimental conditions were identical to those of Fig. 1. S100 staining serves to emphasize the change in phenotype from an elongated (control) to an epithelial-like shape (db-cAMP). Note that a fraction of the SCs in the db-cAMP-treated populations did not down-regulate the expression of GFAP; those cells were negative for the expression of MGs (arrow). N-Cad, N-cadherin; B-Cat, β -catenin. Scale bar, 50 μ m.

Fig. 3. cAMP-induced morphological changes in adult SCs: characterization by TEM. SCs were left untreated (control) or treated with db-cAMP for the times indicated, as described in Fig. 1. Center panel: general overview and higher magnification (inset) of a representative SC treated with db-cAMP for 3 days, featuring cytoplasm containing numerous vacuoles (V), euchromatic nucleus (N) and extensions of the plasma membrane (M). Note integrity of nuclear double membrane (asterisk) and intracellular organelles (mitochondria, Mt, and rough endoplasmic reticulum, rER) in a SC containing vacuoles. Right panels: Time course of cAMP-induced changes. At 3 day post-stimulation, the vacuoles take up most of the cell's cytoplasm and it is usual to find cells exhibiting two prominent vacuolar compartments, one on each side of the nucleus (lower right panel). Left panels: control SCs exhibiting the typical bipolar morphology (above) and bearing a cytoplasm containing abundant lysosomes and mitochondria but no vacuoles. Scale bars, 1200 nm.

Fig. 4. Prolonged cAMP treatment did not reduce SC metabolic activity or interfere with the tendency of the cells to align following linear patterns in culture. Non-infected (lower panels) or lentivirally infected SCs to overexpress GFP (upper panels) were left untreated (control) or treated with 1 mM db-cAMP (db-cAMP). Three days after stimulation, SCs were photographed live (upper panels, GFP) or vitally labeled with Cell Tracker Green, fixed and immuno-stained for the myelin protein MAG (lower panels). Scale bars, 50 μ m.

Fig. 5.

Prolonged cAMP treatment induced SC differentiation without impairing the ability of SCs to extend processes and associate with DRG axons. Cells were treated and labeled with Cell Tracker as described in Fig. 4. SCs were removed from the culture dish and then plated on a pure culture of DRGNs. Cells were fixed 20 h after initiating co-culture and either immunostained with neurofilament (NF) for visualization by confocal microscopy (above), or alternatively processed for TEM analysis (below). Note that cAMP-treated SCs extended several elongated processes along single or multiple axonal bundles and that within 20 h, SCs became embedded in the axonal web and established physical interaction with both the collagen matrix (C) and the axolemma of surrounding axons (ax). No evident ultrastructural changes were identified when comparing the apposition of SC and axonal membranes in control and cAMP-differentiated SCs (lower panels). Higher magnification pictures of selected areas (rectangles) are shown on the right. N, nucleus; SC, SC cytoplasm. Scale bars, 10 μ m (above), 1200 nm (below).

Fig. 6.

Dose, time and specificity characteristics of cAMP-induced MG expression in adult SCs. **A**, **B**: Dependency on the dose and on the time of exposure to cAMP. In **A**, SCs were treated with db-cAMP (as indicated) and the expression of MAG and O1 was evaluated 1–4 days after stimulation. In **B**, SCs expressing GFP were left untreated (control), or treated with a single (cAMP $\times$ 1) or 3 consecutive administrations of db-cAMP every 3 days (cAMP $\times$ 3). Ten days after the initial stimulation, cells were photographed live (GFP, upper panels) or fixed and stained with O1 antibodies (lower panels). Note that SCs retained morphological changes and detectable levels of O1 expression 10 days after receiving a single dose of db-cAMP. **C**. Dependency on cAMP. SCs were stimulated with CPT-cAMP or CPT-cGMP, 250 μ M each, and alternatively, with forskolin or cholera toxin (100 ng/ml), as indicated, and the expression of O1 was evaluated 3 days after stimulation. In these and all subsequent graphs, bar heights are means of triplicate determinations; error bars represent standard deviations. Scale bars, 100 μ m (**A**), 50 μ m (**B**).

Fig. 7. Effect of FBS and cAMP on DNA synthesis and on the expression of representative MGs and NMGs in adult SCs. SCs were treated for 3 days with db-cAMP (1 mM) or CPT-cAMP (200 μ M), in the absence or presence of 10% FBS and evaluated for the expression of the indicated SC markers and the incorporation of ³[H]-thymidine (**B**, lower panel). Representative micrographs (**A**) and quantitative analysis of O1 and MAG expression (**B**, upper panel) are shown. Scale bar, 50 μ m.

Fig. 8.

Effect of neuregulin, other growth factors and cAMP on DNA synthesis and on the expression of representative MGs and NMGs in adult SCs. SCs were treated for 3 days with db-cAMP (1 mM), in the absence or presence of mitogenically active concentrations of the indicated growth factors and evaluated for the expression of SC markers (**A**, **B**) or the incorporation of ³[H]-thymidine (**C**, lower panel). Representative micrographs of immuno-stained SC cultures (**A**) and quantification of the percentage of cells expressing MAG and GFAP (**B**) or O1 (**C**) are shown. In **A**, note that treatment with neuregulin did not induce changes in cell shape when administered alone, or reversed cAMP-induced changes when administered in conjunction to db-cAMP. Neu, neuregulin. Scale bars, 50 μ m.

Fig. 9.

Density dependency of cAMP-induced MG expression. Adult SCs (**A**, **B**) or D6P2T cells (**C**) were plated at different densities, as indicated, and subjected to mitogen and serum deprivation prior to stimulation with db-cAMP (1 mM for SCs, 200 μ M for D6P2T cells) in medium containing 1% FBS (**A–C**) or 10% FBS (**A**, db-cAMP + FBS). Cells were analyzed for the expression of the indicated markers 3 days after stimulation. In **B**, the arrows point to specific MAG positive SCs growing without establishing apparent contact with surrounding cells. O1 expression was negligible in non-treated cells (not shown). PC, phase contrast. Scale bars, 100 μ m (**A**, **B**); 50 μ m (**C**).

Fig. 10.

Non-overlapping SC subpopulations proliferate or differentiate in response to cAMP. **A**, left: Quantitative analysis of proliferating (BrdU positive) and differentiating (O1 positive) SCs in cultures treated with CPT-cAMP alone or in combination with neuregulin. **A**, right: Distribution of proliferating and differentiating SCs in cultures co-treated with neuregulin and CPT-cAMP. **B**, left: Representative micrograph of a SC culture treated with CPT-cAMP and neuregulin. **B**, right: quantitative analysis of dividing cells based on co-immunostaining with BrdU, GFAP and O1 antibodies. In all the experiments, SCs were mitogen and serum deprived and treated with 250 μ M CPT-cAMP, alone or in combination with neuregulin (10 nM) or 10% FBS. Three days after the initial stimulation, cells were fixed and analyzed for the expression

of O1 and BrdU (A), or O1, BrdU and GFAP (B). The arrows point to representative SCs double-labeled with GFAP and BrdU antibodies. No individual BrdU-incorporating cells were identified to co-express O1 and high levels of GFAP (not shown). Scale bar, 20 μ m.

Fig. 11.

Regulation of SC proliferation and differentiation by growth factors and cAMP in the absence of neurons. Treatment of SCs with cAMP-stimulating agents induced a morphological transformation of the cells and an up-regulation of MG expression concomitant to a down-regulation of NMG expression. Growth factors (GFs) or serum did not decrease MG expression, increase NMG expression or revert the cell shape transformation induced by cAMP. However, under similar conditions, cAMP was able to induce a synergistic increase in GF-stimulated DNA synthesis in a fraction of the SC population that did not undergo differentiation into a myelin-like phenotype. These results indicate that whereas cAMP

cooperates with GFs to enhance proliferation, it acts independently of GFs to induce differentiation.