Transforming Growth Factor- β_1 and Forskolin Modulate Gap Junctional Communication and Cellular Phenotype of Cultured Schwann Cells

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Following peripheral nerve injury, Schwann cells undergo a series of cellular alterations that are thought to assist the regenerative process. Some of these changes are stimulated by the local release of cytokines and mitogenic factors. To test the hypothesis that cytokine regulation of gap junctional communication between cells helps to coordinate Schwann cell responses, cultured rat Schwann cells, from sciatic nerve, were utilized to study phasic changes induced by transforming growth factor- β_1 (TGF β_1), a cytokine released after nerve injury, or forskolin in combination with bovine pituitary extract (F-BPE), known for its mitogenic effects *in vitro*. In mitotically quiescent cultures, TGF β , significantly decreased both electrical and dye coupling mediated by gap junctions. Single-channel analysis revealed that cultured Schwann cells expressed gap junctions with two distinct channel sizes of about 26 pS and 44 pS. TGF β , treatment reduced coupling due to both populations of channels. Exposure to TGF β , had a minimal effect on proliferation but significantly altered cellular morphology; cell bodies became flattened with multipolar processes within 72 hr. Additionally, immunolabeling for both low-affinity nerve growth factor receptor (L-NGFR) and glial fibrillary acidic protein (GFAP) were reduced, suggesting increased differentiation. In contrast, treatment with F-BPE significantly enhanced both electrical and dye coupling and stimulated Schwann cell proliferation. Additionally, cell bodies became more rounded with polarized, cytoplasmic processes contiguously aligned with adjacent cells. F-BPE reduced immunolabeling for L-NGFR but increased expression of both GFAP and the major peripheral myelin protein, P₀. These data indicate that TGF β , and/or F-BPE induce phenotypic changes in Schwann cells, including the coordinated regulation of proliferation and modulation of intercellular communication via gap junctions. Such mechanisms may underlie phasic responses that orchestrate recovery from nerve injury, indicating that

Schwann cell gap junctions may be critical for peripheral nerve function.

[Key words: forskolin and bovine pituitary extract (F-BPE), gap junctions, intercellular communication, peripheral nerve, Schwann cell proliferation, Schwann cell differentiation, transforming growth factor- β (TGF β)]

Schwann cells are a multifunctional class of non-neuronal cells that play an important role in the maintenance and regeneration of peripheral nerves. In the mature nervous system there are two phenotypically and functionally distinct Schwann cell types, myelin-forming and non-myelin-forming cells. Differentiated Schwann cells can reenter the cell cycle and can alter their phenotype after nerve injury. The degree of phenotypic flexibility is particularly evident when looking at temporal changes in myelinating Schwann cells following nerve injury. Soon after injury, previously mitotically quiescent Schwann cells distal to the site of the insult and removed from their axonal contact, begin to proliferate (Spencer et al., 1981; Pellegrino et al., 1982) and to form linear arrays (bands of Büngner) within the surrounding endoneurial tube (Bunge, 1987). Furthermore, expression of the major peripheral myelin proteins, P₀ and myelin basic protein, rapidly declines (Le Blanc et al., 1987; Gupta et al., 1988; Trapp et al., 1988), whereas expression of proteins normally found in non-myelin-forming cells, such as low-affinity nerve growth factor receptors (L-NGFR) and glial fibrillary acidic protein (GFAP), is increased (Taniuchi et al., 1986; Jessen et al., 1990). These phenotypic changes are believed to be essential for nerve regeneration. Once the nerve regenerates, Schwann cells decrease expression of L-NGFR (Taniuchi et al., 1988), reexpress myelin-specific genes (Le Blanc et al., 1987; Mitchell et al., 1990), stop proliferating, withdraw their multipolar processes from the environment, reestablish a one-to-one relationship with the axon, and begin to remyelinate the nerve (Spencer et al., 1981).

Many Schwann cell responses to injury are stimulated by the release of cytokines and mitogenic factors. For example, after nerve injury, transforming growth factor- β (TGF β) is secreted into the local environment by injured dorsal root ganglion neurons (Rogister et al., 1993), invading macrophages (Assoian et al., 1987), and non-neuronal cells (Ridley et al., 1989; Constam et al., 1992). Northern and Western blot analyses have shown that rat Schwann cells contain TGF β mRNA and may secrete the protein through an autocrine feedback mechanism (Ridley et al., 1989). The TGF β family of cell signaling molecules has

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been implicated in wound repair in many organ systems and includes at least five distinct isoforms of TGF β (Roberts et al., 1990). TGF β receptors are expressed by many different cells (Wakefield et al., 1987), and the cytokine may either inhibit or stimulate proliferation, depending on the cell type and the environmental milieu (Moses et al., 1985). Although it has been reported that TGF β either enhances (Eccleston et al., 1989; Ridley et al., 1989; Rogister et al., 1993) or has no effect (Mews and Meyer, 1993) on proliferation of cultured Schwann cells and osteoblasts (Centralla et al., 1987; Robey et al., 1987), the cytokine inhibits cell division of most nontransformed cell types including astrocytes (Baghdassarian et al., 1993; Hunter et al., 1993), hepatocytes, keratinocytes, B and T lymphocytes, embryonic fibroblasts, endothelial cells, and several epithelial cell types (for review, see Sporn et al., 1987).

The mitogenic effects of nerve injury on Schwann cells can be mimicked *in vitro* by agents that elevate levels of cAMP, such as forskolin (Raff et al., 1978a,b; Porter et al., 1986), which reversibly binds to adenylate cyclase and activates protein kinase A (Seamon and Daly, 1981). In addition to these mitogenic effects, forskolin treatment induces differentiation of cultured Schwann cells to a premyelinating state (e.g., expression of myelin related proteins; Morgan et al., 1991). Schwann cell myelination, however, depends on withdrawal from the cell cycle and is determined by signals that suppress proliferation (Jessen and Mirsky, 1991; Morgan et al., 1991). Schwann cell proliferation is also stimulated *in vitro* by neu differentiation factor- β (NDF β), which is an alternatively spliced product of the same gene as the glial growth factors (Marchionni et al., 1993). However, NDF β does not appear to elevate levels of cAMP (Yoshimura et al., 1993). The mitogenic effects of NDF β are, instead, thought to be mediated by tyrosine phosphorylation of the p185^{erbB2} tyrosine kinase receptor on the Schwann cell surface (Cohen et al., 1992; Marchionni et al., 1993).

Following a nerve lesion or crush, only a localized region of the endoneurial tube is disrupted and becomes a target for mitogenic and differentiating factors released by invading macrophages and other reactive cells. However, Schwann cells distal to the injury react in an orchestrated, phasic fashion (Spencer et al., 1981; Oaklander et al., 1987). The signal(s) that triggers this response and the mechanism underlying the propagation of information remain elusive. The release of cytokines by macrophages (Ignatius et al., 1986; Baichwal et al., 1988), anterograde fast axonal transport (Oaklander et al., 1987) and release of factors, or the loss of target-derived factors previously retrogradely transported through the intact axon, have been implicated in initiating the proliferative response. Alternatively, the direct exposure to myelin breakdown products or to molecules (e.g., heparin-binding protein) on the distal axolemma of the degenerating nerve may coordinate the response (Ratner et al., 1988; Knight et al., 1993). In this study we investigated the possibility that gap junctions may be involved in modulating Schwann cell responses after nerve injury by providing a direct pathway between cells for metabolic and ionic intercellular communication, or cell coupling.

Gap junctions constitute a highly conserved family of proteins that form dodecameric channels between cells through the interaction of two hemichannels, each one of which is composed of six connexin subunits within the membranes of the two cells (for review, see Dermietzel and Spray, 1993; Hall et al., 1993). Gap junctions are permeable to small molecules, up to 1 kDa (Simpson et al., 1977), allowing the direct cell-cell diffusion of

ions, amino acids, and second messenger molecules (Lawrence et al., 1978; for review, see Bennett et al., 1991). Recently, we characterized the electrophysiologic properties of Schwann cell gap junctions *in vitro* (Chanson et al., 1993). In the present study, we examined the effects of TGF β_1 or forskolin and bovine pituitary extract (F-BPE) on the strength of intercellular coupling, via gap junctions, between cultured Schwann cells removed from their axonal contact, and address the question of whether phenotypic changes that occur after nerve injury are altered in parallel with changes in intercellular coupling. The results presented in this article provide evidence that TGF β_1 did not stimulate proliferation and markedly decreased intercellular coupling. Conversely, following treatment with F-BPE, proliferating cells showed high levels of intercellular coupling. The coordinated modulation of proliferation and coupling strength may provide a mechanism for the induction of functional changes that these cells undergo after nerve injury.

Materials and Methods

Sciatic nerve Schwann cell culture. Schwann cells were obtained from 3–4 d postnatal rats by a modification of the method originally described by Brookes (Brookes et al., 1979). Under aseptic conditions 30 sciatic nerves were dissected and minced following removal of the adjacent connective/vascular tissue and epineurium. Nerves were treated three times for 15 min each at 37°C in calcium/magnesium-free Puck's Saline G supplemented with 0.25% trypsin (GIBCO, Frederick, MD) and 0.03% collagenase (GIBCO). The preparation was centrifuged, and the pellet was resuspended in Dulbecco's modified Eagle's medium (Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO), 100 U/ml penicillin, and 50 μ g/ml streptomycin and 10% heat-inactivated calf serum (GIBCO; DMEM-CS). The suspension was triturated 15 times using a Pasteur pipette and then passed through a 60 μ m Nitex filter and centrifuged. The pellet was resuspended in DMEM-CS and plated onto a Falcon 60-mm-diameter tissue culture dish (Becton Dickinson, Lincoln Park, NJ) pretreated with 10 μ g/ml poly-D-lysine (PDL; Sigma). Cultures were maintained at 37°C in humidified atmosphere of 5% CO₂/95% O₂. On day 3 after plating, actively proliferating fibroblasts were eliminated by exposing cultures to 10 μ M cytosine arabinoside (Sigma) in fresh media for 48 hr. On day 5, cells were washed and maintained in DMEM-CS containing either 2 μ M forskolin (F; Calbiochem, La Jolla, CA) and 10 μ g/ml bovine pituitary extract (BPE; Sigma) (similar to studies described by Raff et al., 1978b; Porter et al., 1986) or 50 ng/ml neu differentiation factor- β (NDF β ; used to expand cells only in experiment depicted Fig. 3B; provided by Dr. A. Welcher, AMGEN, Thousand Oaks, CA) to stimulate Schwann cell proliferation. Cultures were expanded by passaging when confluent monolayers were obtained (every 4–7 d). For all experiments, Schwann cells were subcultured no more than six times, were maintained *in vitro* for no longer than 6 weeks, and were withdrawn from mitogen stimulation for 1 week prior to assays.

Recombinant simian transforming growth factor- β_1 (TGF β_1 ; provided by Dr. D. Twardzik, Bristol Myers and Squibb, Seattle, WA) was stored at –90°C until experimental application. The purity of the affinity-purified cytokine and its biologic activity were previously described (Gentry et al., 1987; Chalazonitis et al., 1992). For all experiments, TGF β_1 was used at a final concentration of 10 ng/ml (except for dose response analysis), NDF β at a final concentration of 50 ng/ml, and F-BPE at a final concentration of 2 μ M (F) and 10 μ g/ml (BPE). Experimental factors and medium were replenished every 3 d.

Thymidine incorporation. Confluent dishes of Schwann cells were washed several times and maintained for 1 week in the absence of mitogens. Cells were then dissociated with 0.1% trypsin and seeded at about 30% confluence onto 24-well culture plates (area = 22 mm² well; Nunclon or Falcon). Treatments commenced 24 hr after replating. After addition of TGF β_1 , NDF β , or F-BPE, each experimental well was exposed to 2 μ Ci of ³H-thymidine for the final 6 hr of culture. Following incubation, the cells were washed with cold PBS, lysed with solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), and removed from the culture surface by scraping. Cell suspensions were then applied to a vacuum unit and filtered through Whatman glass microfibre filters (GF/B, 2.1 cm, Fisher

Figure 1. Effects of TGF β or F-BPE on L-NGFR (mAb 217c) and S100 protein immunoreactivity. Cultures were expanded in the presence of F-BPE and were withdrawn from mitogens for 1 week prior to replating onto chamber slides. Cells were treated for 72 hr with TGF β_1 (10 ng/ml) or F (2 μ M)-BPE (10 μ g/ml) and viewed with phase-contrast optics (top row) to visualize morphology and rhodamine optics to view mAb 217c (L-NGFR) immunoreactivity. Sister cultures were examined with fluorescein optics to visualize S100 immunoreactivity. Note the higher levels of L-NGFR in the control group compared to the TGF β_1 and F-BPE groups. All cells were immunoreactive for S100. Also, notice the dramatic effects of TGF β_1 or F-BPE on cell morphology. Scale bar, 100 μ m.

Scientific, Springfield, NJ) to reduce nonspecific activity selectively. Filters were rinsed with cold PBS and ethanol, and allowed to dry. Radioactivity was assayed by liquid scintillation counting.

Protein assay. Replicate cultures of the cells used for the thymidine incorporation studies were raised in parallel to those described above. At 8, 24, and 72 hr after addition of TGF β_1 and F-BPE, wells were washed with PBS, denatured with 0.05% SDS, and lysates were then scraped into distilled water. Protein content was measured using the Bio-Rad (Bradford; Rockville Center, NY) protein microassay procedure and by averaging duplicate absorbance values obtained from a Gilford spectrophotometer.

Antibodies. A mouse monoclonal antibody (mAb; IgG), 217c, which recognizes L-NGFR (Fields and Dammerman, 1985; Ferrari et al., 1991), was used at a dilution of 1:200. mAb 217c was produced and characterized by Peng et al. (1982) and was a generous gift of K. Fields (Albert Einstein College Medicine) and J. DeVilles (UCLA). A mouse mAb (IgG $_1$) to glial fibrillary acidic protein (GFAP; Boehringer-Mannheim, Indianapolis, IN) was used at a dilution of 1:3. Rabbit polyclonal antibody to bovine S100 (Dakopatts A/C, Santa Barbara, CA) was used at a dilution of 1:250. Rabbit polyclonal antibody to myelin P $_0$ protein (D'Urso et al., 1990; generous gift of Dr. D. Colman, Columbia University) was used at a dilution of 1:250.

For detection of primary Abs, goat anti-mouse IgG tetramethylrhodamine isothiocyanate conjugate (G-anti-mIgG-TRITC; Southern Biotechnology, Birmingham, AL), goat anti-mouse IgG $_1$ fluorescein isothiocyanate conjugate (G-anti-mIgG-FITC; Boehringer-Mannheim Biochemicals), and goat anti-rabbit Ig fluorescein isothiocyanate con-

jugate (G-anti-rIg-FITC; Fisher Biotechnology, Pittsburgh, PA) were used at 1:100.

Immunocytochemistry. All incubations were done on cultures passaged onto PDL-treated plastic culture chambers (Lab-Tek Chamber Slide, VWR, Plainfield, NJ) and maintained in F-BPE until about 80% confluency. Cultures were withdrawn from F-BPE treatment for 1 week prior to experimental treatments and immunocytochemical analysis. TGF β_1 and F-BPE groups were exposed to the factors for 3 d. For staining of living cells antibodies were diluted in DMEM-CS, and for staining of fixed cells they were diluted in PBS. Staining of living cells was performed at 37°C for 45 min, and that of fixed cells at 4°C overnight. For the detection of cell surface antigens, living cells were incubated with mAb 217c followed by G-anti-mIgG-TRITC and then fixed with 4% paraformaldehyde and coverslipped. For detection of intracellular antigens, cells were fixed and permeabilized with 95% ethanol/1% acetic acid before incubation with the anti-GFAP mAb and G-anti-mIgG-FITC secondary antibody. Alternatively, cells fixed with 4% paraformaldehyde and 0.08% Triton were incubated with rabbit anti-P $_0$ or rabbit anti-S100 followed by the G-anti-rIg-FITC secondary antibody. Negative controls for each experimental condition consisted of appropriate dilutions of nonimmune serum with secondary antibodies or secondary antibodies alone. Cells were viewed and photographed with an Olympus BHS microscope (New York/New Jersey Scientific, Middlebush, NJ). Representative photographs were obtained under conditions of fixed exposure and development and reflect the results obtained from at least four separate experiments.

Electrical coupling. For electrophysiological experiments, confluent

dishes of Schwann cells were washed several times to remove any residual F-BPE and maintained in DMEM-CS for 1 week. Cells were then dissociated with 0.1% trypsin and plated at low density onto glass coverslips pretreated with PDL. Dishes were treated with TGF β_1 or F-BPE 24 hr after replating. For electrophysiological recordings, coverslips were fixed into a chamber installed on the stage of an inverted Nikon microscope (Diaphot TMF, Melville, NY) to allow superfusion of the cells with a pH 7.2 solution containing 7 mM CsCl, 0.1 mM CaCl₂, 160 mM NaCl, 10 mM HEPES, and 0.6 mM MgSO₄. The dual whole-cell voltage-clamp technique (Neyton and Trautmann, 1985; White et al., 1985) was used to measure junctional conductance between pairs of Schwann cells. Patch electrodes (2.5–7 M Ω) were pulled and heat polished on a P-87 Flaming-Brown puller (Sutter Instruments, San Rafael, CA) and were filled with a pH 7.2 solution containing 135 mM CsCl, 0.5 mM CaCl₂, 2 mM Na₂ATP, 3 mM MgATP, 10 mM HEPES, and 10 mM EGTA. CsCl was used to reduce activity of nonjunctional channels. Current and voltage recordings were acquired, with filtering at 1–2 kHz, using two patch-clamp amplifiers (Axopatch-1D, Axon Instruments, Sunnyvale, CA), and were recorded on a four-channel chart recorder (Gould Instruments, Madison, WI) and stored on video tape using pulse code modulation (VR-100A, Instrutech Corporation, Minneola, NY) for off-line data analysis. After the dual whole-cell configuration was achieved, both members of a pair were voltage clamped at a common holding potential of 0 mV. To measure macroscopic junctional conductance (g_j), 10 mV pulses of 300 msec duration were alternately applied to each cell to generate a transjunctional driving force (V_j). This protocol induced a current both in the pulsed cell and in the nonpulsed cell. While the latter current is only junctional (I_j), the current appearing in the pulsed cell is the sum of I_j and nonjunctional currents. Junctional conductance was then calculated by dividing I_j by the amplitude of the V_j pulse. In poorly coupled cell pairs, gating of single gap junction channels could be detected. When a driving force was elicited across the junctional membrane, currents flowing through these channels could be discriminated from other membrane channels as step-like changes of opposite polarities but identical amplitudes recorded simultaneously in both current traces. To determine single gap junction channel conductance, the amplitudes of single-channel current transitions were measured using a digitizing tablet and customized software. Amplitude values were then divided by the applied V_j and, for each experiment, these events were converted into step-amplitude frequency histograms. Histograms were fitted to Gaussian distributions to obtain the mean $\pm$ SEM of the measured single-channel conductances. Plots were generated using software (SIGMAPLOT, Jandel Scientific) capable of curve fitting by least squares with any explicit user function.

Dye coupling. Dye coupling was studied by the technique of scrape loading (El-Fouly et al., 1987). Confluent monolayers of Schwann cells were treated for 72 hr with TGF β_1 or F-BPE after 1 week of mitogen withdrawal. Dishes were then rinsed with PBS and incubated in a solution containing 0.15% Lucifer yellow (Sigma) and several crystals of rhodamine dextran (Sigma) in PBS. Monolayers were subsequently scraped from edge to edge using a mini-glass cutter, and incubated in the dark for 2 min at room temperature. The dishes were then washed several times with PBS and fixed in 4% paraformaldehyde. Cells were considered positive if they exhibited Lucifer yellow fluorescence. Rhodamine-dextran (not gap junction permeant) was used to ensure that loading was comparable between experimental groups and occurred only along the line of the scrape. Scrape-loaded cells were photographed along the scrape under fluorescent illumination using a 10 $\times$ Hoffman objective. Photographs were taken under conditions of fixed exposure time and development. The degree of coupling was assessed visually and by comparing densitometric scan profiles (Computing Densitometer, Molecular Dynamics, Sunnyvale, CA) of photographic negatives across multiple microscopic fields along the scrape region. For each negative, successive vertical zone areas, 3.5 cm wide, parallel to the scrape region were analyzed. Each zone was thus several cells in width. Since only those cells directly contiguous with the scrape were loaded, in instances where there is little or no dye coupling there would be lower overall integrated density even in the first zone on either side of the scrape. In instances where there is dye coupling to neighboring cells, there would be increased optical densities. However, only those negatives where rhodamine-dextran fluorescence in the first zone was the same in all groups, indicating equivalent loading of cells, were analyzed. Five successive regions on each side of the scrape were scanned. Integration of the optical density recorded on film minus background (average optical density at the center of the cell-free scrape region) for pixel area is reported.

Figure 2. Dose-response effects of TGF β_1 on Schwann cell proliferation. Schwann cell proliferative responses to increasing concentrations of TGF β_1 were assessed by measuring ³H-thymidine incorporation. Confluent Schwann cell cultures were maintained in the absence of the mitogen for 1 week and were replated at 30% confluence (about 10⁴ cells/well) onto PDL-treated Falcon multiwell plates. After 24 hr, TGF β_1 (0, 0.5, 5, 10, or 50 ng/ml) was added and plates were assayed after 0, 8, 48, and 72 hr. Cells were pulsed with 2 μ Ci/well of ³H-thymidine for the last 6 hr prior to lysing. Values represent the means of four duplicate determinations for each dose and time point. Error bars indicate SEM. *, TGF β_1 (50 ng/ml) differs from TGF β_1 (10 ng/ml) at $p < 0.05$; **, TGF β_1 (50 ng/ml) differs from all other groups at $p < 0.05$. Statistics were done by ANOVA with Bonferroni post hoc tests.

Statistical analysis. Data were analyzed by ANOVA with the Bonferroni post hoc tests.

Results

Characterization of Schwann cell cultures

The purity of primary Schwann cells was determined on the basis of morphology and immunofluorescent analysis. Unless otherwise stated, cultures consisted of at least 99% Schwann cells, as characterized by the expression of L-NGFR (using the mAb 217c) and S100 proteins. Although TGF β_1 -treated Schwann had a flattened appearance similar to fibroblasts, the two cell types were easily distinguished by immunolabeling with mAb 217c (Fields and Dammerman, 1985); Schwann cells were immunoreactive, whereas fibroblasts were not. Conversely, fibroblasts, but not Schwann cells, were positive for Thy-1.1 (data not shown).

Effects of TGF β_1 or F-BPE on cellular morphology

Untreated Schwann cells displayed a predominantly spindle-shaped morphology with bipolar processes not noticeably oriented to processes of contiguous cells (control, Fig. 1). Within 48 hr TGF β_1 altered Schwann cell morphology, and after 72 hr exposure cells had enlarged and assumed a flattened morphology with multipolar processes projecting from the soma (Fig. 1, TGF β_1). F-BPE caused Schwann cell somas to become rounded with many membranous cytoplasmic projections (Fig. 1, F-BPE, top) after 24 hr. Once cells reached a threshold density, processes

Figure 3. Comparison of proliferative responses in Schwann cells previously expanded either with F-BPE or with NDFβ. Schwann cells were expanded in the presence of F-BPE or NDFβ and then grown in the absence of mitogens for 1 week and replated onto PDL-treated Falcon plastic. After 24 hr, cells were treated with TGFβ₁ (10 ng/ml), F (2 μM)-BPE (10 μg/ml), or NDFβ (50 ng/ml). After 8, 24, and 72 hr exposure, cultures were assayed for ³H-thymidine incorporation. Values represent the mean of four duplicate determinations for each condition and time point. Error bars indicate SEM. *A*, F-BPE expanded Schwann cells. *, NDFβ differs from other groups at $p < 0.01$; **, TGFβ₁ and control groups are significantly lower than other groups at $p < 0.001$; ***, F-BPE differs from other groups at $p < 0.001$. *B*, NDFβ expanded Schwann cells. *, F-BPE and NDFβ groups differ from other groups at $p < 0.001$; **, F-BPE and NDFβ groups differ from other groups at $p < 0.05$. Statistics were done by ANOVA with Bonferroni post hoc tests. Similar results were seen in three independent experiments. Notice that there is little or no autoprofiferation at any of the time points assayed on this substrate.

aligned parallel to those of contiguous cells (Fig. 1, F-BPE, S100).

Effects of TGFβ₁ or F-BPE on DNA synthesis and protein content

To determine the effect of TGFβ₁ on Schwann cell proliferation, cultures were withdrawn from mitogens (F-BPE) for 1 week and replated onto PDL-treated Falcon multiwell plates at 30% confluence. After 24 hr, cells were treated with increasing concentrations of TGFβ₁ and assayed for thymidine incorporation (Fig. 2). Untreated Schwann cells (control) were mitotically quiescent when cultured on this substratum, with only low levels of thymidine incorporation. TGFβ₁ did not significantly affect DNA synthesis at concentrations of 0.5, 5, or 10 ng/ml at any of the time points examined. However, TGFβ₁ significantly inhibited proliferation, over time, when applied at a final concentration of 50 ng/ml.

To determine whether the cultured Schwann cells were capable of proliferating in response to a mitogen, cultures were

either reexposed to F-BPE or treated with either NDFβ or TGFβ₁ (Fig. 3*A*). NDFβ treatment significantly elevated levels of DNA synthesis fivefold by 72 hr. Similarly, reexposure to F-BPE significantly increased thymidine incorporation 14-fold. By contrast, treatment with TGFβ₁ (10 ng/ml) failed to alter thymidine incorporation.

To examine the possibility that expansion of cells using F-BPE somehow altered their ability to respond to TGFβ₁, Schwann cell cultures were instead expanded in the presence of NDFβ (see Materials and Methods). After 1 week withdrawal of this mitogen, cultures were replated and either reexposed to NDFβ or treated with either F-BPE or TGFβ₁ (Fig. 3*B*). NDFβ or F-BPE significantly elevated Schwann cell DNA synthesis; thymidine incorporation increased fourfold after 72 hr. However, the F-BPE-induced mitogenic response observed with NDFβ-reared cells appeared to be less pronounced than that of F-BPE-reared cells after 72 hr (compare Fig. 3*A,B*), suggesting some differences in cells expanded in different ways. Nevertheless,

treatment with TGF β_1 (10 ng/ml) failed to alter thymidine incorporation in either of the cultures.

Finally, to examine effects of TGF β_1 or F-BPE on Schwann cells already in a proliferative state, mitogen withdrawn cultures were established on a PDL substratum in Nunclon plates (Fig. 4). There were significant levels of DNA synthesis in untreated Schwann cells (control). Compared to untreated cultures, there was a fourfold increase in thymidine incorporation (Fig. 4A) and a twofold increase in total protein (Fig. 4B) following 72 hr exposure to F-BPE or to F-BPE in combination with TGF β_1 . TGF β_1 and F-BPE cotreatment did not alter the stimulatory effects of F-BPE on either DNA synthesis or total protein. Although TGF β_1 alone had no effect on Schwann cell proliferation, it significantly increased total protein content (Fig. 4B), corresponding to the increased cytoplasmic surface area of the cells (Fig. 1).

TGF β_1 or F-BPE decreased expression of L-NGFR in cultured Schwann cells

The effects of TGF β_1 and F-BPE on expression of several markers of differentiation were compared. Using mAb 217c, Schwann cells were examined for the presence of L-NGFR, which is found on nonmyelinating and cultured Schwann cells, but not on myelinating Schwann cells (DiStefano and Johnson, 1988; Lemke and Chao, 1988; Jessen et al., 1990). Compared to controls, L-NGFR immunoreactivity was dramatically decreased after 72 hr treatment with either TGF β_1 or F-BPE (Fig. 1). Under all conditions, Schwann cells were immunoreactive for S100 (Fig. 1), a protein that is present specifically in mature myelinating and nonmyelinating cells (Jessen and Mirsky, 1991).

Effects of TGF β_1 or F-BPE on expression of GFAP and P₀

Both nonmyelinating and short-term cultured Schwann cells express GFAP, but downregulate expression of P₀, the major protein of peripheral myelin (Lees and Brostoff, 1984), when cultured without neurons (Mirsky et al., 1980; Lemke and Chao, 1988). In the present study, untreated cultured cells were immunoreactive for GFAP, but not P₀ (Fig. 5). Cells treated for 72 hr with TGF β_1 , however, showed decreased immunostaining for GFAP, and minimal labeling for P₀ (Fig. 5). By contrast, after treatment with F-BPE, cells labeled intensely for both GFAP and P₀ (Fig. 5).

Effects of TGF β_1 or F-BPE on electrical coupling between Schwann cell pairs

To examine the effects of various treatments on Schwann cell gap junctional coupling, macroscopic junctional conductance, g_j , between cell pairs was measured (Fig. 6). For untreated cell pairs, g_j was low (for representative recording in Fig. 6, g_j = 500 pS). Whereas g_j was almost abolished in the presence of TGF β_1 after 48 hr (for representative recording in Fig. 6, g_j = 0 pS), it was markedly enhanced after treatment with F-BPE (for representative recording in Fig. 6, g_j = 2100 pS). Functional electrical coupling, therefore, was significantly decreased by treatment with TGF β_1 , whereas it was markedly enhanced by treatment with F-BPE.

To quantify the temporal changes in electrical coupling, g_j was measured between Schwann cell pairs at various times after treatment with TGF β_1 or F-BPE (Fig. 7). TGF β_1 treatment decreased g_j in a time-dependent manner (Fig. 7A). Before treatment (control), g_j averaged 483 ± 85 (SEM) pS and decreased

Figure 4. Proliferative effects on Nunclon plates after treatment with TGF β_1 and F-BPE. Schwann cells were grown in the absence of mitogens for 1 week and replated onto PDL-treated Nunclon plastic. After 24 hr, cells were treated with TGF β_1 (10 ng/ml), and/or F (2 μ M)-BPE (10 μ g/ml), and assayed after 8, 24, and 72 hr for 3 H-thymidine incorporation and protein (values for DNA synthesis and protein assays are from the same experiment). *A*, Incorporation of 3 H-thymidine (2 μ Ci/well). Values represent the mean of five duplicate determinations for each condition and time point. Error bars indicate SEM. *, TGF β_1 and control groups differ from other groups at $p < 0.01$; **, TGF β_1 and control groups are significantly lower than other groups at $p < 0.001$. Statistics were done by ANOVA with Bonferroni post hoc tests. Similar results were seen in six independent experiments. *B*, Total protein content/well. Values represent the mean of four duplicate determinations. Error bars indicate SEM. *, TGF β_1 differs from Control at $p < 0.05$ and from all other groups at $p < 0.001$. Additionally, control differs from the TGF β_1 -F-BPE and F-BPE groups at $p < 0.001$. Statistics were done by ANOVA with Bonferroni post hoc tests. Similar results were seen in four independent experiments. Notice the significant increase in the total protein content of the TGF β_1 -treated cells.

significantly to 124 ± 38 pS after 8 hr exposure to TGF β_1 . g_j remained significantly decreased with time: even after 72 hr, without additional TGF β_1 , there was a very significant decrease in cell-to-cell coupling (93 ± 29 pS). By contrast, following treatment with F-BPE, there was a significant increase in coupling between some cell pairs after 8 hr (Fig. 7B; heterogeneous response is reflected in the large standard error). However, a more homogeneous and maximal response was observed at 24 hr and thereafter, and g_j significantly increased from 483 ± 85 pS (untreated controls) to 1044 ± 193 pS (Fig. 7B). When cultures were withdrawn from mitogen treatment, g_j decreased to control values within 48 hr. Furthermore, g_j did not differ between cells continuously treated with F-BPE and cells with-

Figure 5. Effects of $\text{TGF}\beta_1$ or F-BPE on GFAP and P_0 immunoreactivity. Immunocytochemical detection of GFAP or P_0 protein. Untreated (control) cultures were positive for GFAP but negative for P_0 . $\text{TGF}\beta_1$ (10 ng/ml)-treated cultures exhibited low levels of immunoreactivity for both GFAP and P_0 . F (2 μM)-BPE (10 $\mu\text{g/ml}$)-treated cultures labeled intensely for both GFAP and P_0 . Scale bar, 100 μm .

drawn from the mitogen for 1 week, reexposed to F-BPE, and recorded within 24 hr (data not shown).

Single-channel analysis of Schwann cell gap junctions

Continuous recording of single gap junctional current activity observed between untreated Schwann cell pairs when a driving force was elicited across the junctional membrane is illustrated in Figure 8A. The abrupt current transitions from the baseline to two distinct levels of opening indicate at least two distinct channel sizes (arrows). No apparent modifications in the size and frequency of events of the two channels were observed after treatment with $\text{TGF}\beta_1$ or F-BPE (halothane was used to partially uncouple cells when g_j was high; data not shown). The distribution of junctional conductance values can be fit by two Gaussian frequency histograms with peaks of 26.2 ± 5.2 ($\pm\text{SD}$) pS and 44 ± 4.4 pS, indicating the presence of two populations of gap junction channels (Fig. 8B).

Schwann cell dye coupling

To examine the extent of gap junctional coupling in confluent Schwann cell cultures, the cell-to-cell transfer of Lucifer yellow (gap junction permeant) was examined after scrape loading. To ensure that loading was comparable among groups, rhodamine-dextran, a gap junction-impermeant dye, was used in parallel with Lucifer yellow. For all treatments, rhodamine-dextran labeling was restricted to the first layer of cells abutting the line of the scrape and was equivalent among all groups (data not shown), indicating equal loading. In control cultures (Fig. 9A), Lucifer yellow diffused from the area of loading to neighboring cells to a distance two or three cells away from the scrape. By contrast, 72 hr of treatment with $\text{TGF}\beta_1$ (Fig. 9B) significantly reduced the apparent spread of dye to no more than one cell diameter away from the scrape. Conversely, after 72 hr exposure to F-BPE (Fig. 9C), the dye diffused to cells six or seven cell diameters distant from the scrape. There was virtually no non-

specific Lucifer yellow cellular uptake by intact cells outside of the scrape region (Fig. 9D).

Densitometric analysis was used to quantitate the apparent changes in coupling, assayed as dye spread away from the line of the scrape (Fig. 9E). Examination of the first zone on each side lateral to the scrape zone, which included the loading region, revealed no significant differences between control and F-BPE-treated cells (seen as comparable optical density values). The decreased $\text{TGF}\beta_1$ integrated optical density values in the first zone (which was several cells in width) on either side of the scrape reflect the minimal transfer of dye to adjacent cells after this treatment. Differences in the lateral spread of dye, or dye coupling, are recognized as broadening or narrowing of the densitometric scan. Averaged scan profiles of cells treated with $\text{TGF}\beta_1$ (solid triangles) show a statistically significant decrease ($p < 0.001$), whereas cells treated with F-BPE (open triangles) show a dramatic increase in the lateral spread of dye.

Discussion

After nerve injury, Schwann cells proliferate and undergo a series of cellular changes followed by a return to the quiescent phenotype after nerve regeneration. A hallmark of Schwann cell responses is the spatiotemporally coordinated nature of changes distal to the site of injury (Spencer et al., 1981; Oaklander et al., 1987). Our observations *in vitro* suggest that sequential changes in gap junctional communication may contribute to coordination of these cellular responses.

cAMP has been suggested as an intracellular signal that regulates both proliferation (Raff et al., 1978a,b) and differentiation (Sobue and Pleasure, 1984; Jessen et al., 1991) of Schwann cells. Consistent with prior investigations, we found that F-BPE stimulated Schwann cell proliferation and resulted in confluent monolayers with cells aligning in parallel whirling arrays (previously reported by Porter et al., 1986) in cultures maintained for up to 6 weeks. Furthermore, L-NGFR expression was de-

Figure 6. Evaluation of junctional conductance between Schwann cell pairs. Cultured Schwann cells were withdrawn from mitogens for 1 week, treated with $\text{TGF}\beta_1$ (10 ng/ml) or F (2 μM)-BPE (10 $\mu\text{g}/\text{ml}$), and examined for differences in electrical coupling. Representative chart records for individual cell pairs from Figure 7 are shown. Both cells of a pair were held at a common holding potential of 0 mV throughout the recordings and hyperpolarizing voltage pulses were applied alternatively to the two cells. The application of a voltage pulse to one cell (V_1), for example, induced current changes in the two paired cells (I_1 and I_2). The downward deflection seen in the pulsed cell (I_1) reflects the sum of junctional and nonjunctional currents. The upward deflection seen in the nonpulsed cell (I_2) represents the junctional current, indicating the occurrence of electrical coupling. Similar observations were made when a voltage pulse was applied to the other cell of a pair (V_2). Again, junctional current (I_1) was detected in the nonpulsed cell. The ratio between the junctional current measured in one cell (upward deflection in I_1 , I_2) and the voltage pulse applied in the other cell gives macroscopic junctional conductance (g_j). Note the decrease in g_j after $\text{TGF}\beta_1$ treatment, and the increase after F-BPE treatment, as compared to control. At higher gain in the $\text{TGF}\beta_1$ recordings, the transient capacitive artifacts elicited by the pulses can be seen in the current traces. Calibration: 2 sec, 20 mV; Control and F-BPE, 50 pA; $\text{TGF}\beta_1$, 10 pA.

creased and P_0 expression was enhanced, as previously reported and indicative of cellular differentiation (Morgan et al., 1991). In contrast to some prior studies that looked at the effects of forskolin alone (Jessen et al., 1991), however, we found that forskolin increased expression of GFAP when combined with bovine pituitary extract. The concurrent expression of P_0 and GFAP is not typically observed (Jessen and Mirsky, 1991). Since P_0 is expressed by myelin-forming Schwann cells and GFAP is expressed by nonmyelinating and proliferating Schwann cells (Jessen and Mirsky, 1991), our observations suggest that F-BPE induced only partial transition to the myelinating phenotype. This is consistent with the conclusion of others that no single signal is sufficient to induce functional differentiation and that cells must definitively exit the cell cycle to differentiate fully (Jessen and Mirsky, 1991; Morgan et al., 1991).

Since the mitogenic effects of nerve injury on Schwann cells can be mimicked *in vitro* by agents that elevate levels of cAMP (Appenzeller and Palmer, 1972; LeBlanc et al., 1992), forskolin has been used, in combination with other factors, to stimulate proliferation and to obtain large populations of pure Schwann cells. However, there is still some controversy as to whether cAMP is elevated in Schwann cells following injury (Meador-Woodruff et al., 1984). Nevertheless, there is evidence that $\text{p185}^{\text{erbB2}}$, a putative receptor for the glial growth family of proteins (which includes NDF β and other isoforms that are Schwann cell mitogens; Marchionni et al., 1993), is upregulated on the surface of Schwann cells during Wallerian degeneration and also by exposure to forskolin (Cohen et al., 1992), which potentiates

Figure 7. Quantification of the effects of $\text{TGF}\beta_1$ or F-BPE on junctional conductance. Schwann cells were withdrawn from mitogens for 1 week and replated at low density onto glass coverslips. After 24 hr, cells were treated with $\text{TGF}\beta_1$ (10 ng/ml) or F (2 μM)-BPE (10 $\mu\text{g}/\text{ml}$). **A**, $\text{TGF}\beta_1$ reduced cell coupling in a time-dependent manner. Before treatment (control) junctional coupling was 483 ± 85 pS ($n = 43$) and decreased dramatically to 188 ± 78 pS ($n = 5$) after 2 hr exposure to $\text{TGF}\beta_1$. *, By 8 hr, $\text{TGF}\beta_1$ decreased g_j significantly ($p \leq 0.03$) to 124 ± 38 pS ($n = 12$) and g_j remained significantly ($p \leq 0.02$) lower than control after 24 hr (43 ± 23 pS; $n = 9$), 48 hr (33 ± 20 pS; $n = 10$), and 72 hr (93 ± 29 pS; $n = 16$) exposure. Error bars indicate SEM. **B**, After 8 hr exposure to F-BPE, junctional conductance between Schwann cell pairs was elevated from 483 pS $\pm$ 85 ($n = 43$) before treatment (control) to 888 pS $\pm$ 340 ($n = 10$). **, G_j was significantly ($p < 0.005$) elevated after 24 hr (1044 pS $\pm$ 193; $n = 27$) of treatment with F-BPE.

the mitogenic response of glial growth factor (Davis and Stroobant, 1990). However, previous exposure to mitogens, such as F-BPE or NDF β , during Schwann cell culturing may alter normal cellular responses, and our observations, thus, may not accurately reflect normal Schwann cell physiology. For example, the decreased proliferative response to F-BPE of Schwann cells originally reared in the presence of NDF β versus F-BPE may indicate altered responses. Nevertheless, the reversible nature

Figure 8. Activity of single gap junction channels as recorded in a weakly coupled Schwann cell pair. *A*, Continuous recording of the junctional current measured when one cell of the pair was hyperpolarized to -40 mV, whereas the other cell was kept at a constant potential of 0 mV. This procedure allowed the detection of single gap junctional activity as visualized by abrupt current transitions from the base line to two distinct levels of opening (upward transitions). Data were filtered at 50 Hz. Arrows demark examples of the two channel sizes in this recording. Calibration: 2 sec, 2 pA. *B*, Frequency histograms of conductance values calculated from each current transition and grouped in bins of 4 pS. The distribution of values was well fit by two Gaussian relations (solid line) with two peaks of 26 ± 5 pS and 44 ± 4 pS (mean $\pm$ SD, $n = 4$ experiments). This indicates that currents were carried by two populations of channels with distinct unitary conductances.

of expanding Schwann cells with F-BPE is suggested by previous reports that rat sciatic nerve Schwann cells cultured in the presence of forskolin and glial growth factor return to quiescence following removal of the mitogenic stimulus (Porter et al., 1986; Morrissey et al., 1991). Additionally, these Schwann cells retained the ability to myelinate dorsal root ganglion neurites, both *in vitro* (Porter et al., 1986; Morrissey et al., 1991) and *in vivo* (Feltri et al., 1992), and to support regeneration of processes from embryonic rat retinal explants (Morrissey et al., 1991).

For the proliferation assays, both Falcon and Nunc plates, pretreated with PDL, were used to determine the effects of TGF β_1 on Schwann cells. Nunc plates were chosen because there were detectable levels of thymidine incorporation that would facilitate the detection of both decreases and increases in prolifera-

Figure 9. Effects of TGF β_1 or F-BPE on Schwann cell dye coupling. Representative fluorescent micrographs of confluent Schwann cell cultures without treatment (control, *A*), or following 72 hr exposure to either TGF β_1 (10 ng/ml, *B*), or F (2 μ M)-BPE (10 μ g/ml) (*C*). *D*, Area away from the scrape line showing minimal nonspecific uptake of the Lucifer yellow dye. Notice that compared to control, TGF β_1 decreased dye coupling to one cell diameter, while treatment with F-BPE increased dye coupling to up to seven cell diameters away from the scrape. Scale bar, 140 μ m. *E*, Integrated (volume) density scan profiles (optical density vs 0.35 by 2.3 cm increments moving out, bilaterally, parallel to the scrape) of five negative images for each treatment, from the scrape loading experiment shown in *A–C*. *, TGF β_1 differs from control at $p < 0.001$. Statistical results were obtained from the average optical density values, minus background (average value at the center of scrape), of three independent negatives for each experimental condition. Similar results were seen in three independent experiments.

tion. In contrast to the proliferative effects of F-BPE or NDF β , treatment with TGF β_1 did not stimulate proliferation. Additionally, we did not find a synergistic effect when TGF β_1 and F-BPE were combined. This differs from initial reports that the same TGF β_1 isoform stimulates Schwann cell proliferation (Ridley et al., 1989; Rogister et al., 1993) and acts synergistically with forskolin (Ridley et al., 1989). Recently, however, Mews and Meyer (1993) also observed no increases in cell number after treatment with the TGF β_1 isoform. It is possible, however, that cells exposed to a more physiological mitogen, such as axonal plasma membranes, might respond differently to TGF β_1 . Nevertheless, the morphologic changes we observed after TGF β_1 treatment were identical to those reported by others (Chalazonitis et al., 1992; Rogister et al., 1993), indicating similarities in biologic activities. Additionally, TGF β_1 treatment decreased

L-NGFR and GFAP expression, indicative of a more differentiated phenotype. Further, cultured Schwann cells treated with TGF β_1 produced a neuronotrophic factor that supported the survival of dorsal root ganglion sensory neurons (Chalazonitis et al., 1992). Thus, TGF β_1 may be involved in both the repair of peripheral neurons after nerve injury and for Schwann cell redifferentiation following nerve regeneration (e.g., during remyelination).

In both untreated dishes and after treatment with F-BPE, Schwann cell pairs were coupled by gap junctions with two different channel sizes of about 26 pS and 44 pS. These distinct sizes could reflect expression of two different connexins, or they may represent different states of the same connexin, as previously reported for connexin 43 (Moreno et al., 1992). However, the latter possibility appears to be less likely, because the phosphorylation treatments used here did not preferentially affect events of either size. Interestingly, these channels exhibited electrophysiological properties that are unique among the gap junctions characterized to date (Chanson et al., 1993), suggesting that they are new members of the connexin gene family. However, species or tissue differences could also account for some of their unique channel gating properties.

Since the degree to which cells communicate through gap junction channels is dictated by the number of channels in the membrane, the size of the channels, and the fraction of time that the channels are open, the observed changes in intercellular coupling strength after TGF β_1 and/or F-BPE treatments could reflect alterations in any of these properties. In the present study, although the number of functional channels changed (i.e., TGF β_1 decreased whereas F-BPE increased g_j), the size of the channels did not change with different treatments. Additionally, F-BPE treatment increased surface area contact between cells (i.e., processes aligned parallel to those of contiguous cells), which may have increased the number of functional channels without *de novo* synthesis of connexins. This is similar to observations made *in vivo* after nerve injury where proliferating Schwann cells distal to the injury align in parallel arrays (Bunge et al., 1987). Following nerve injury, changes in the strength of coupling between cells could regulate the diffusion of second messengers, such as cAMP, Ca $^{2+}$, or IP $_3$. Such molecules could pass through these channels and serve as the signals that regulate intercellular communication and mitogenesis between neighboring Schwann cells.

The gap junctions examined in this study were presumably localized to the cell membranes of coupled, nonmyelinating cells. This contrasts the recent *in vivo* localization of connexin32 to the lamellar layers of myelinating Schwann cell nodes of Ranvier and Schmidt-Lanterman incisures (Bergoffen et al., 1993). These studies link connexin32 mutations to the demyelinating Charcot-Marie-Tooth disease and implicate this gap junction protein in normal adult nerve function. In association with our findings, this suggests a significant role for the regulation of gap junction expression and strength of coupling between Schwann cells during PNS maintenance and after nerve injury.

In previous studies using normal rat kidney cells, TGF β_1 enhanced both proliferation and cell-to-cell coupling when combined with epidermal growth factor (van Zoelen and Tertoolen, 1991). Conversely, TGF β inhibited both proliferation and intercellular coupling in human primary keratinocytes and also induced differentiation in these cells (Madhukar et al., 1989). These data (measured by Lucifer yellow dye transfer) parallel

the present findings that factors that stimulated proliferation simultaneously enhanced coupling, whereas factors that inhibited or had no effect on proliferation significantly reduced intercellular communication via gap junctions. Furthermore, after injury of cultured endothelial cells there were coordinated responses (Pepper et al., 1989). Immediately following scrape loading, injured endothelial cells were not coupled. However, the cells were well coupled once they began to divide, but uncoupled once confluent. In the present study, the effects of F-BPE on Schwann cell gap junctions was rapid and reversible, suggesting that a signal (such as cAMP) is required not only for proliferation but also to maintain increased coupling (see Kessler et al., 1984; Traub et al., 1987).

Thus, as part of the initial response to injury (perhaps mediated by increases in cAMP or changes in Ca $^{2+}$), Schwann cells could become more coupled to one another, begin proliferating, and alter their phenotype. The enhanced degree of coupling would serve to insure uniform and synchronous responses. After nerve repair has begun, differentiating signals might simultaneously uncouple cells and promote conversion to the differentiated, quiescent phenotype. Understanding of the role of Schwann cells in nerve injury responses will thus require a thorough understanding of the connexins expressed by the cells and of mechanisms regulating functional communication via gap junctions.

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