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Activity of Cyclic AMP Phosphodiesterases and Adenylyl Cyclase in Peripheral Nerve after Crush and Permanent Transection Injuries*

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Recent studies demonstrate that cAMP levels are tightly controlled during demyelination and remyelination in Schwann cells as cAMP decreases to 8–10% of normal following both sciatic nerve crush or permanent transection injury and only begins to increase in the crushed nerve after remyelination (Poduslo, J. F., Walikonis, R. S., Domec, M., Berg, C. T., and Holtz-Heppelmann, C. J. (1995) *J. Neurochem.* 65, 149–159). To investigate the mechanisms responsible for this change in cAMP levels, cAMP phosphodiesterase (PDE) and adenylyl cyclase activities were determined before and after sciatic nerve injury. Basal cAMP PDE activity in soluble endoneurial homogenates of normal nerve was 34.9 ± 1.9 pmol/mg of protein/min ($\bar{\chi}$ ± S.E.; n = 10). This activity increased about 3-fold within 6 days following both injuries. Basal PDE activity remained elevated in the transected nerve, but declined to 70 pmol/mg of protein/min in the crushed nerve at 21 and 35 days following injury. Isozyme-specific inhibitors and stimulators were used to identify the PDE families in the sciatic nerve. The low K_m cAMP-specific (PDE4) and the Ca^{2+}/calmodulin-stimulated (PDE1) families were found to predominate in assays using endoneurial homogenates. The PDE4 inhibitor rolipram also increased cAMP levels significantly after incubation of endoneurial tissue with various isozyme-specific inhibitors, indicating that PDE4 plays a major role in determining cAMP levels. PDE4 mRNA was localized by *in situ* hybridization to cells identified as Schwann cells by colabeling of S100, a Schwann cell specific protein. Adenylyl cyclase activity declined following injury, from 3.7 pmol/mg of protein/min in normal nerve to 0.70 pmol/mg/min by 7 days following injury. Both decreased synthesis and increased degradation contribute, therefore, to the reduced levels of cAMP following peripheral nerve injury and are likely critical to the process of Wallerian degeneration.

The interactions between Schwann cells (SCs)[1] and axons in the peripheral nerve are very precisely controlled. Contact with an axon induces numerous changes in SC biology including proliferation (1, 2), production of basal lamina (3–7), wrapping of myelin loops around the axon (8), the expression of myelin specific proteins, such as P_0 and the myelin basic proteins (9–16), and the increased synthesis of lipid membrane components (17–20). The maintenance of the myelin-forming phenotype is dependent on the continued contact between the axon and SCs, as any interruption of this association leads to a reversion of Schwann cells from a myelinating to a nonmyelinating phenotype. Loss of contact leads to myelin breakdown, down-regulation of myelin genes (14, 16, 21), proliferation of SCs (22–24), and the expression of characteristic nonmyelinated SC markers, such as nerve growth factor receptor, L1, and glucocerebroside (19, 25, 26). Thus, Schwann cells exhibit a remarkable capacity to alter their morphology and cellular function as a result of contact with axons.

The precise signals induced in Schwann cells due to axonal contact remain poorly defined. Previous work indicates a role for the adenylyl cyclase/cAMP-dependent pathways using SCs in culture (27, 28). Cyclic AMP elevation can at least partially mimic the presence of the axon on cultured nonmyelinating SCs. In these studies, embryonic or neonatal Schwann cells are isolated and expanded *in vitro* by the addition of forskolin, a stimulator of adenylyl cyclase (29, 30), and glial growth factor (31). When these mitogens are removed and the Schwann cells re-exposed to forskolin, they re-express low levels of myelin genes (15, 32), myelin-specific membrane components galactocerebroside and sulfatide (33–36), and display a more mature, flattened phenotype (32).

Studies relating cAMP content to myelination have been conducted by our laboratory using the *in vivo* models of crush and permanent transection injury of the rat sciatic nerve. In these models, the injury leads to degeneration of axons in the distal segment of the nerve. The loss of axonal contact with SCs leads to demyelination. In the permanently transected nerve, axonal regrowth is prevented, and Schwann cells remain in a nonmyelinating state. Following crush injury, degeneration and demyelination occur as in the transected nerve, but axons are allowed to regrow and are subsequently remyelinated.

Cyclic AMP content was measured at various time points following both injuries and compared with myelin gene expression (37). The levels dropped to 10% of normal by 3 days following both injuries, concurrently with the decrease in P_0 gene expression, and remained very low in the nonmyelinating transected nerve through 35 days after injury. In the crushed nerve, cAMP levels did increase again starting at about 21 days after injury, but reached only 27% of normal by 35 days after injury, well after P_0 re-expression in the crushed nerve, which was 60% of normal expression by 14 days and 100% of normal by 21 days following injury. Remyelination, therefore, seems to precede cAMP recovery and suggests that cAMP may not play a large role in inducing the process of myelination in the peripheral nerve.

The importance of cAMP in inducing myelin transcripts in

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[1] The abbreviations used are: SC, Schwann cell; PDE, phosphodiesterase; CaM, calmodulin; 8-MeoM-IBMX, 8-methoxymethyl-1-methyl-3-(2-methylpropyl)-xanthine; AC, adenylyl cyclase; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline.

endoneurial segments was further tested using agents that elevate cAMP. Forskolin alone increased cAMP significantly in the normal nerve and in the regenerating crushed nerve, but failed to increase levels in the transected nerve. Cyclic AMP could only be elevated in the transected nerve by treatment with 3-isobutyl-1-methylxanthine, a general inhibitor of phosphodiesterases (PDEs), as well as with forskolin (37). This indicates that PDE inhibition is necessary to elevate cAMP levels in nonmyelinating endoneurium following injury and suggests that PDEs may play an important role in inactivating this second messenger when Schwann cells are nonmyelinating. Treatment of endoneurial explants from normal, crush, and transection injured nerves with these cAMP elevating agents failed to increase myelin gene expression, further confirming the insufficiency of this messenger in inducing myelin gene expression *in vivo* (37). Furthermore, recent studies by Suter *et al.* (38) have identified two promoters on the peripheral myelin protein-22 gene. Promoter 1 is primarily active *in vivo* for peripheral myelin protein-22. A TCAG sequence is found near the peripheral myelin protein-22 transcription initiation site under the control of promoter 1. The same sequence is found at transcription initiation sites in other myelin genes, such as the P_0 gene (25), myelin basic protein gene (39), and proteolipid protein gene (40), which suggests a common control mechanism. A second promoter controlling peripheral myelin protein-22 expression is preferentially activated in cultured SCs in response to forskolin, and thus the control of myelin gene expression in cultured SCs may not mimic the control mechanisms *in vivo*, and may confirm our data that cAMP is not sufficient for myelin gene expression in the nerve.

Our previous study indicated that cAMP levels change dramatically following injury. Cyclic AMP levels primarily reflect the net balance between synthesis and degradation of this messenger. The present study quantifies adenylyl cyclase (AC) specific activity before and after injury to assess how changes in cAMP synthesis contribute to the changing levels of this second messenger. ATP was quantified in normal and injured nerves to determine whether changes in the concentration of the AC substrate could alter cAMP levels. In addition, an increase in PDE activity may wholly or partially be responsible for the declining cAMP levels found during Wallerian degeneration, as PDE inhibition was required to elevate cAMP following injury. The changes in PDE activity following injury were measured, and the specific isozymes present in normal sciatic endoneurium and those that were up-regulated following injury were identified. Finally, *in situ* hybridization with a PDE4-specific probe was performed to demonstrate the expression of this enzyme by Schwann cells.

MATERIALS AND METHODS

Animal Surgery—Animal surgery was conducted on male Sprague-Dawley rats (200–225 g) under deep pentobarbital anesthesia. The sciatic nerves were exposed and injured just below the sciatic notch, either by crushing for 5 s with smooth forceps or by permanent transection with the ligated ends reflected 180° and sutured to adjacent muscle to prevent axonal reentry (12). The distal segment was removed at various time points following injury and the epineurium and perineurium, which consist largely of fibers of connective tissue and epithelial cells, were removed by the microdissection technique of Dyck *et al.* (41). This technique allows exclusive study of the endoneurium, which consists of the neural elements (axons, Schwann cells, and myelin) and supporting connective tissue. All animal use procedures were in strict accordance with the NIH "Guide for the Care and Use of Laboratory Animals" and were approved by the Mayo Animal Care and Use Committee.

Phosphodiesterase Activity—PDE activities were determined by measuring the degradation of [^{3}H]cAMP in a two-step procedure (42–44). Isolated endoneurium from normal or injured nerve was homogenized in 20 mM TES, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 μM leupeptin, 0.1 μM pepstatin, 0.1 μM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100 (45). In studies measuring Ca^{2+}/CaM stimulated activity, the buffer included complete protease inhibitor mixture without EDTA (Boehringer Mannheim), supplemented with 0.1 μM leupeptin and 0.1 μM phenylmethylsulfonyl fluoride, as we found this family of PDEs extremely sensitive to proteolysis. The homogenate was spun at 198,000 $\times$ *g* for 30 min. The supernatant and the pellet were separated. The pellet was resuspended in homogenization buffer and the protein concentrations of both the supernatant and pellet were measured using a Pierce bicinchoninic acid protein assay (46). Basal PDE activity was measured by adding 2.5 μg of protein in 10 μl of homogenization buffer to 100 μl of incubation mixture (containing 50 mM TES buffer, pH 7.4, 0.1% bovine serum albumin, 2 mM EGTA, 10 mM $MgSO_4$, and 0.5 μM total cAMP, with 2.5 pmol of thin layer chromatography purified [^{3}H]cAMP) (42) and incubated at 30 °C for 7.5 min. In experiments examining the presence of specific PDE[2] isozymes, various PDE type-specific inhibitors and activators were included in the incubation mixture and compared with the basal activity of matched samples without these diagnostic agents. In testing for the presence of the Ca^{2+}/calmodulin (CaM)-dependent PDEs (PDE1), the incubation mixture also contained 10 μg/ml CaM and 2.01 mM $CaCl_2$, to obtain 10 μM free Ca^{2+} based on the chelation constant for EGTA (47). Matching samples containing Ca^{2+}/CaM as well as 10 μM Type I-specific PDE inhibitor 8-methoxymethyl-1-methyl-3-(2-methylpropyl)-xanthine (8-MeoM-IBMX) (Biomol Research Laboratories) were also tested. The presence of cGMP-stimulated PDEs (PDE2) was tested by including 5 μM cGMP in the incubation mixture (48–51) along with 10 μM 8-MeoM-IBMX and 3 μM cilostamide (*N*-cyclohexyl-*N*-methyl-4(1,2-dihydro-2-oxo-6-quinohyloxy)butyramide; a kind gift from Otsuka Pharmaceuticals, Osaka, Japan). The contribution of the cGMP-inhibited isozyme toward total PDE activity was measured by including 3 μM cilostamide (52, 53). Similarly, the activity of the low K_m cAMP-specific isozyme was determined by the addition of 3 μM rolipram (4-(3-cyclopentyloxy-4-methylphenyl)-2-pyrolidone; a gift from Smith Kline Beecham Pharmaceuticals, King-of-Prussia, PA) (52, 54) or 3 μM denbufylline. Following incubation, the samples were quickly frozen in a dry ice and acetone slurry to stop the reaction. Fifty μl of PDE buffer (50 mM TES, pH 7.4, 10 mM $MgSO_4$, 1 mM EGTA) was added, and the samples boiled at 95 °C for 90 s to inactivate the PDEs. Upon cooling, 50 μl of 1 mg/ml snake venom (*Ophiophagus hannah*) was added, and the mixture incubated for 30 min to convert the nucleotides to nucleosides. One ml of cold carrier (containing 0.1 M each of adenosine, adenine, hypoxanthine, and inosine) was added, and the samples were added to QAE-Sephadex A-25 columns. The [^{3}H]adenosine was eluted with 20 mM ammonium formate. The eluate was counted in a scintillation counter, and the counts converted to picomoles of cAMP hydrolyzed/mg of protein/min. Hydrolysis was kept to less than 10% of the substrate and linearly proportional to incubation time and enzyme protein.

PDE assays were conducted on the supernatant and the 198,000 $\times$ *g* pellet from normal endoneurium and at various times following injury. The homogenization buffer contained 0.1% Triton X-100 to promote solubilization of PDEs (55), so most activity should be localized to the soluble fraction. Activity in the pellet from normal endoneurium was 27% of total normal PDE activity, falling to just 20% of total activity by 35 days after crush injury and 7% of total activity at 35 days following transection. Thus the majority of PDE activity was found in the soluble fraction. Preliminary studies found no isozyme uniquely located to the pellet, so further studies on the endoneurial PDE isozymes were conducted solely on the soluble fraction.

Treatment of Sciatic Endoneurium with PDE Isozyme Inhibitors—The distal segments of normal, and crushed or transected nerves 21 days following injury were removed, desheathed, and minced into 2–3-mm slices. The slices were incubated for 30 min at 37 °C in a Krebs-Ringers II solution (110 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.4 mM $MgSO_4$, 4 mM $NaHCO_3$, 12.8 mM glucose, 12.6 mM Na_2HPO_4, and 3.1 mM NaH_2PO_4) containing 30 μM rolipram, cilostamide, or 8-MeoM-IBMX and with or without 100 μM forskolin (Calbiochem). Following incubation, the slices were homogenized in 400 μl of cold 4 mM EDTA, pH 7.0. Aliquots were removed for DNA quantification. The remaining

[2] In this paper, the PDE isozymes are classified according to the system proposed by Beavo (52, 84). In this system, PDE1 refers to the family of PDE isozymes whose activity is dependent on Ca^{2+} and calmodulin; PDE2 is the family stimulated by micromolar concentrations of cGMP; PDE3 are isozymes with low K_m for cAMP and are inhibited by cilostamide and cGMP but not inhibited by rolipram; PDE4 are isozymes with high affinity and selectivity for cAMP and are inhibited selectively by rolipram.

homogenate was boiled for 3 min and the precipitated proteins centrifuged at 15,800 × *g* for 5 min in a refrigerated Eppendorf microfuge. The supernatant was collected and assayed for cAMP using a kit from Amersham. Fifty μl of [^{3}H]cAMP (0.025 μCi: 0.9 pmol) was added to 50 μl of supernatant, followed by 100 μl of cAMP-binding protein dissolved to the manufacturer's specifications. The tubes were vortexed and kept on ice for 2 h. One-hundred μl of suspended charcoal was then added to adsorb any unbound cAMP. The tubes were centrifuged to pellet the charcoal, and 200 μl of this supernatant was removed and placed into scintillation vials for counting. The counts were compared with a standard curve constructed with known amounts of unlabeled cAMP, and the results expressed as picomole of cAMP/μg of DNA.

In situ Localization of PDE4 mRNA—First strand cDNA was synthesized from sciatic nerve total RNA with random hexanucleotide primers using a cDNA synthesis kit (Boehringer Mannheim) following the manufacturers directions. Polymerase chain reaction was performed in a 50-μl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, and 0.25 mM deoxynucleotides, 1 unit *Taq* polymerase, and 100 ng of cDNA. One μM rat PDE4/4B-specific primers (sense, 5′-GGCTGGGTACTCCCATAATCGG-3′; antisense, 5′-GTAGAGAGGAGAACGTGGCGTTG-3′) complementary to bases 1090–1111 (sense) and 1275–1296 (antisense) (56) were added. This region is located near the beginning of the conserved catalytic region and avoids the alternatively spliced regions near the 5′ end. The PDE4 isogenes are approximately 70% identical in this region (57). Other PDE families have a much lower homology in this region. Preliminary polymerase chain reaction studies amplifying this region using primers specific for PDE4D were not successful. The polymerase chain reaction product of PDE4B was thus used for *in situ* hybridization. The polymerase chain reactions were performed for 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 25 cycles. The amplified product was ligated into pCRII plasmids (Invitrogen) and transformed into INVaF cells. Plasmids from individual colonies were isolated by miniprep (Qiagen) and sequenced. Digoxygenin-labeled probes were created by SP6 (antisense) and T7 (sense) run-off transcription using a Boehringer Mannheim kit. Labeling efficiency was verified by dot-blot analysis of serially diluted labeled transcripts. Dilutions giving equivalent signals for sense and antisense were used for *in situ* probing of sciatic nerve sections.

In situ hybridization was conducted on sections of normal and 4-day transected sciatic nerves. Sciatic endoneurial segments were fixed in fresh 4% paraformaldehyde/PBS overnight followed by a PBS wash. The tissue was dehydrated in a series of baths of increasing ethanol concentrations followed by a xylene wash. The dehydrated tissue was embedded in paraffin, sectioned in 5-μm sections, starting approximately 1 cm from the injury site, and baked on slides overnight at 45 °C. The sciatic sections were deparaffinated by washing in xylene for 14 min followed by a series of 4-min EtOH baths of decreasing concentrations (100 to 50%). The sections were then treated with 0.2 N HCl for 20 min followed by water and PBS rinses followed by incubation in 1 μg/μl proteinase K for 30 min at 50 °C. After PBS and 2× SSC washes, the tissues were incubated in 0.1 M triethanolamine with 0.25% acetic anhydride for 10 min. Prehybridization was conducted by incubation in hybridization buffer (containing 2× SSC, 10% dextran sulfate, 1 × Denhardt's solution, 50% formamide, and 0.1 mg/ml salmon sperm DNA) for 2 h at 45 °C. Hybridization buffer containing diluted digoxygenin-labeled probes was then added. Coverslips were applied and the section incubated for 24 h at 45 °C. The sections were washed in SSC, and blocked in 100 mM Tris-NaCl with 1% bovine serum albumin for 30 min. The samples were then incubated with anti-digoxygenin antibodies conjugated with alkaline phosphatase. Color development in NBT/BCIP for 5 min followed.

S100 Immunolocalization—S100 was localized immunohistochemically in sections previously stained for PDE4 mRNA location. Immunohistochemical steps were used for S100 localization as the antidigoxygenin antibody treatment and color development steps during PDE4 *in situ* hybridization were not RNase free, and the unhybridized S100 mRNA could become degraded and unsuitable for *in situ* hybridization. Endogenous peroxidase activity was blocked by incubation of sections of 0.6% H_2O_2/MeOH for 30 min. Nonspecific binding sites were then blocked by incubation in 20% normal goat serum, PBS, 0.05% Tween 20, pH 7.4. The sections were incubated overnight in S100 primary antibody (Biogenex) diluted 1:15 in 10% normal goat serum/PBS. Following a rinse in PBS, a biotinylated goat anti-rabbit antibody was added and incubated for 1 h. Streptavidin/peroxidase was then added for 1 h, followed by color development in tetrahydrochloride 3,3′-diaminobenzidine in 0.05 M Tris with 0.3% H_2O_2 for 5–10 min.

DNA Quantification—DNA was quantified by the method of Lipman (58). Two-μl aliquots of sample homogenate were added to 2 ml of assay buffer (containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1.5 μM Hoechst Dye number 33258), and the fluorescence measured using a TK-100 fluorometer (Hoefer Scientific Instruments). Calf thymus DNA (Sigma) was used as a standard. The presence of PDE inhibitors was not found to interfere with DNA determination.

Adenylyl Cyclase Assay—AC activity was measured in sciatic endoneurial homogenates from normal uninjured rats and 7 days following crush injury. Isolated endoneurium were homogenized in 50 mM triethanolamine buffer, pH 7.4, 5 mM $MgCl_2$, 0.5 μg/ml leupeptin, and 0.5 mM Pefabloc (Boehringer Mannheim). The homogenate was centrifuged for 20 min at 8,000 × *g* at 4 °C. The pellet and supernatant were separated, and the pellet resuspended in homogenization buffer. Protein concentrations of both the supernatant and pellet fractions were measured using a bicinchoninic acid assay (Pierce) with bovine serum albumin as standard. AC activity was measured using a two-column method (59–61). Seventy μl of incubation mixture (containing final concentrations of 50 mM TEA, pH 7.4, 5 mM $MgCl_2$, 0.1 mM cAMP, 0.1 mM ATP, 1 mg/ml bovine serum albumin, 5 mM creatine phosphate, 2.5 units/100 μl of creatine phosphokinase, 5 mM dithiothreitol, 500 μM 3-isobutyl-1-methylxanthine, 500,000 cpm of [α-^{32}P]ATP and with or without 100 μM forskolin) was added to each tube. 130–300 μg of protein in 30 μl of homogenization buffer was added to the tubes and incubated at 30 °C for 20 min. The tubes were then removed, 100 μl of stop solution (containing 50 mM triethanolamine, pH 7.4, 2% SDS, 40 mM ATP, 1.4 mM cAMP, and approximately 80,000 cpm/100 μl of [^{3}H]cAMP to measure recovery) was added, and the tubes placed on ice until they could be boiled for 90 s. The [^{32}P]cAMP formed was then separated from the remaining ATP using the sequential double column method. The sample was first placed onto a Dowex AG 50W-X12 column (0.8 × 3.0 cm) that had been conditioned with 1 M HCl and washed with H_2O. The samples were washed with 2 ml of H_2O to remove the majority of the [^{32}P]ATP, and the Dowex columns placed over neutral alumina columns (Activity grade Super 1, Type WN-6; Sigma). The sample was further eluted from the Dowex onto the alumina with 8–10 ml of H_2O. Once the eluate from the Dowex had drained through the alumina, the cAMP was eluted from the alumina with 7 ml of 100 mM Tris, pH 7.5. The samples were counted for both ^{3}H and ^{32}P, and the counts converted to picomole of cAMP/mg of protein/min. Results reported are from the pellet fraction as 87.4 ± 0.3% of the total activity was found in this fraction.

ATP Quantification—ATP was assayed in sciatic nerve homogenates from normal rats and at 21 and 35 days following transection injury. ATP was quantified by the bioluminescent methods of Strehler (62) using an ATP bioluminescence kit (Sigma) according to the manufacturers instructions. One nerve was homogenized in 400 μl of cold sterile H_20, and a small aliquot was removed for protein determination as described above. Four-hundred μl of cold 0.8 N $HClO_4$ was added, and the sample rehomogenized. The sample was spun 15,800 × *g* for 15 min. The supernatant was removed, and 400 μl of 0.8 N KOH was added to neutralize the acid. The sample was centrifuged again at 15,800 × *g* and 400 μl of 0.3 M Na_2HPO_4 was added to the supernatant. This sample was diluted 1:5 in H_2O for ATP determination. ATP standards were diluted in an identical solution, and the results were normalized to total protein.

RESULTS

PDE Activity in Rat Sciatic Endoneurium following Injury—The PDE specific activity assayed under basal conditions (with no inhibitors or modulators added) in the supernatant of normal endoneurial homogenate was 34.9 ± 1.9 pmol/mg of protein/min ($\bar{\chi}$ ± S.E.; n = 10) (day 0, Fig. 1). This activity significantly increased by 2 days following both crush and transection injuries, and continued to increase 3–3.5-fold over normal to 105–129 pmol/mg of protein/min within 6 days of both injuries. In the nonmyelinating transected nerve, PDE activity remained elevated, at 105–110 pmol/mg/min at 21 and 35 days following injury. In the crushed nerve, on the other hand, PDE activity declined as remyelination occurred, falling from a high of 128.8 ± 11.7 pmol/mg/min at 6 days to 76.1 ± 11.7 pmol/mg/min by 21 days following injury. PDE activity remained at this level through 35 days following crush injury.

Identification of the Specific PDE Isozymes using Modulators and Inhibitors—PDE activity was measured on paired samples to compare activity with and without stimulators or inhibitors. The PDE1 isozyme is a Ca^{2+}/CaM-stimulated isozyme. The

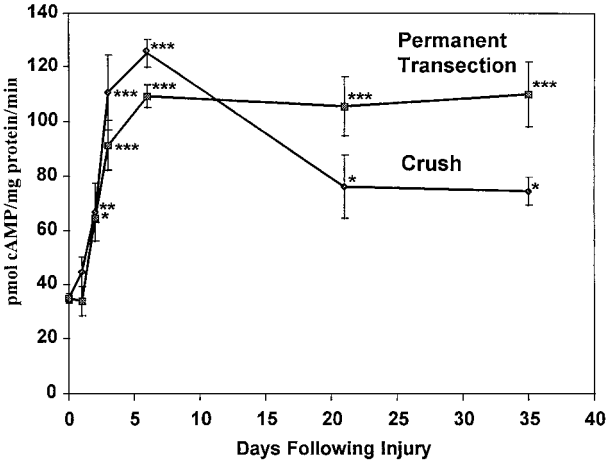

FIG. 1. **Basal cAMP phosphodiesterase activity following crush and transection injury to rat sciatic nerve.** Basal activities using 0.5 μM cAMP as substrate were assayed in soluble fractions of normal nerves (Day 0) and following injury without added inhibitors or modulators in media containing 2 mM EGTA. ($\bar{\chi}$ ± S.E.). * Values significantly different from normal by t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Days 1–3: $n = 3$ per time point; all others: $n = 7$–10 per time point).

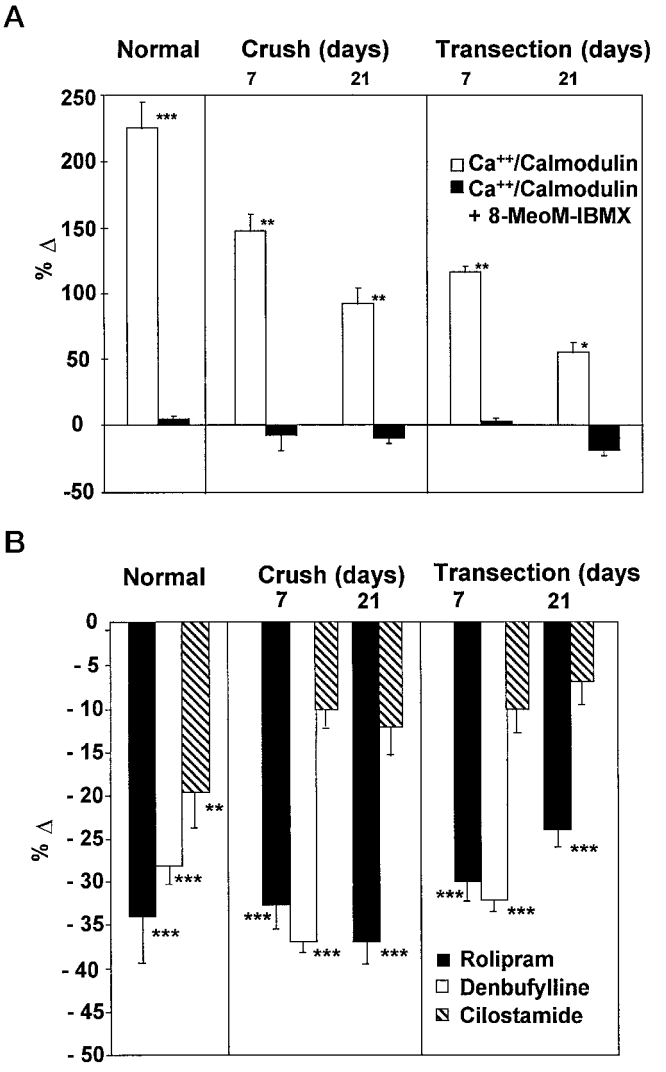

FIG. 2. **The effect of isozyme specific stimulators or inhibitors on cAMP PDE activity before and after injury.** *A*, the effect of Ca^{2+} and calmodulin on phosphodiesterase activity (PDE1). Paired samples were tested with or without 10 μM Ca^{2+}/calmodulin and 10 μg/ml calmodulin. Matching samples with Ca^{2+}/calmodulin and 10 μM 8-methoxymethyl-IBMX were also tested. *B*, the effect of cilostamide inhibition of PDE3 and inhibitors of low K_m-cAMP specific phosphodiesterases (PDE4) on cAMP PDE activity before and after injury. PDE activity was tested on paired samples with or without 3 μM cilostamide, an inhibitor or cGMP-inhibited (PDE3) enzymes, or with or without 3 μM rolipram or 3 μM denbufylline, inhibitors of low K_m cAMP-specific phosphodiesterases (PDE4). Denbufylline was only tested in normal and 7 day injured samples. The % difference of each paired sample ± inhibitor or stimulator was calculated and the mean % difference is shown ($\bar{\chi}$ ± S.E.). *A*, $n = 5$–7; *B*, $n = 7$–10). *, values significantly different from paired samples without inhibitor or stimulator by paired t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

presence of this family was tested by the addition of 10 μM Ca^{2+} and 10 μg/ml CaM to the incubation medium and comparing the activity to paired samples incubated without Ca^{2+}/CaM. The change from basal activity was tested in normal homogenates, and at 7 and 21 days following both crush and transection injury. The addition of Ca^{2+}/CaM more than doubled PDE activity in the normal nerve (Fig. 2*A*). The Ca^{2+}/CaM stimulated specific activity increased 7 days after injury (Table I), but declined in both the crushed and transected nerves by 21 days. The Ca^{2+}/CaM stimulated activity represented a smaller percentage increase in activity after injury (Fig. 2*A*) due to the higher basal PDE activity. The increased PDE activity due to Ca^{2+}/CaM was completely blocked by the addition of the PDE1 specific inhibitor 8-MeoM-IBMX, verifying that the increased activity was due to PDE1 stimulation. Thus the PDE1 family is present and may increase after injury. However, the high activity is not sustained in the nonmyelinating transected nerve.

The presence of the cGMP-stimulated isozyme (PDE2) was tested by incubating identical samples with and without 5 μM cGMP in the incubation mixture. This represents a 10-fold excess of cGMP over cAMP. Cilostamide and 8-MeoM-IBMX were included in samples both with and without cGMP. These inhibitors were included to block the activity of the other PDEs that either are inhibited by cGMP or hydrolyze cGMP (63). Both inhibitors were found to be necessary, as cGMP decreased cAMP hydrolysis if either inhibitor was omitted from the reaction mixture. With the inhibitors present, any change in PDE activity represents the net effect of cGMP on the PDE2 isozymes. Cyclic GMP was not found to stimulate cAMP PDE activity at any time point (data not shown), and thus the presence of PDE2 isozymes could not be demonstrated. As a positive control, stimulation of cAMP PDE activity in total brain homogenate by cGMP was tested. A stimulation of activity by 71% was found (data not shown), similar to previous descriptions (51, 64).

The activity of the cGMP-inhibited isozymes was tested by the inclusion of cilostamide, a specific inhibitor of PDE3 isozymes. In the normal nerve, 3 μM cilostamide (maximum inhibitory dose) decreased PDE activity by 6.8 pmol/mg/min (Table I), a 19% decrease (Fig. 2*B*). The PDE3 activity increased little following injury (Table I), and thus represents a smaller percentage of the total activity following injury, typically from 9 to 11% (Fig. 2*B*). Thus, while the PDE3 family is present in peripheral nerve, it appears to contribute minimally to the overall increase in PDE activity that occurs during demyelination.

The contribution of the low K_m cAMP-specific (PDE4) isozyme to total PDE activity was tested using the selective inhibitors rolipram and denbufylline. Three μM rolipram (maximum inhibitory dose) was added to paired samples and the mean % inhibition is shown (Fig. 2*B*). In normal nerve, rolipram decreased activity by an average of 12.4 pmol/mg/min, a 34% decrease. Following injury, rolipram continued to inhibit activity by about 25 to 43 pmol/mg/min (Table I), a decline from 24 to 37% ($p < 0.001$ at all time points). The specificity of

TABLE I

Activities of phosphodiesterase 1, 3, and 4 in sciatic endoneurium before and after injury

$\bar{\chi}$ ± S.E., pmol/min/mg protein; n = 4-10.

	Normal	Crush		Transection	
		7 Days	21 Days	7 Days	21 Days
PDE1	102.8 ± 8.1***	187.1 ± 13.8***	85.4 ± 11.2***	154.6 ± 8.2***	49.6 ± 7.6**
PDE3	6.8 ± 1.4	13.7 ± 2.7	9.1 ± 2.4	11.2 ± 2.5	8.3 ± 2.9
PDE4	12.4 ± 2.7	42.9 ± 6.0***	28.7 ± 4.8**	31.2 ± 3.7***	24.9 ± 3.0**

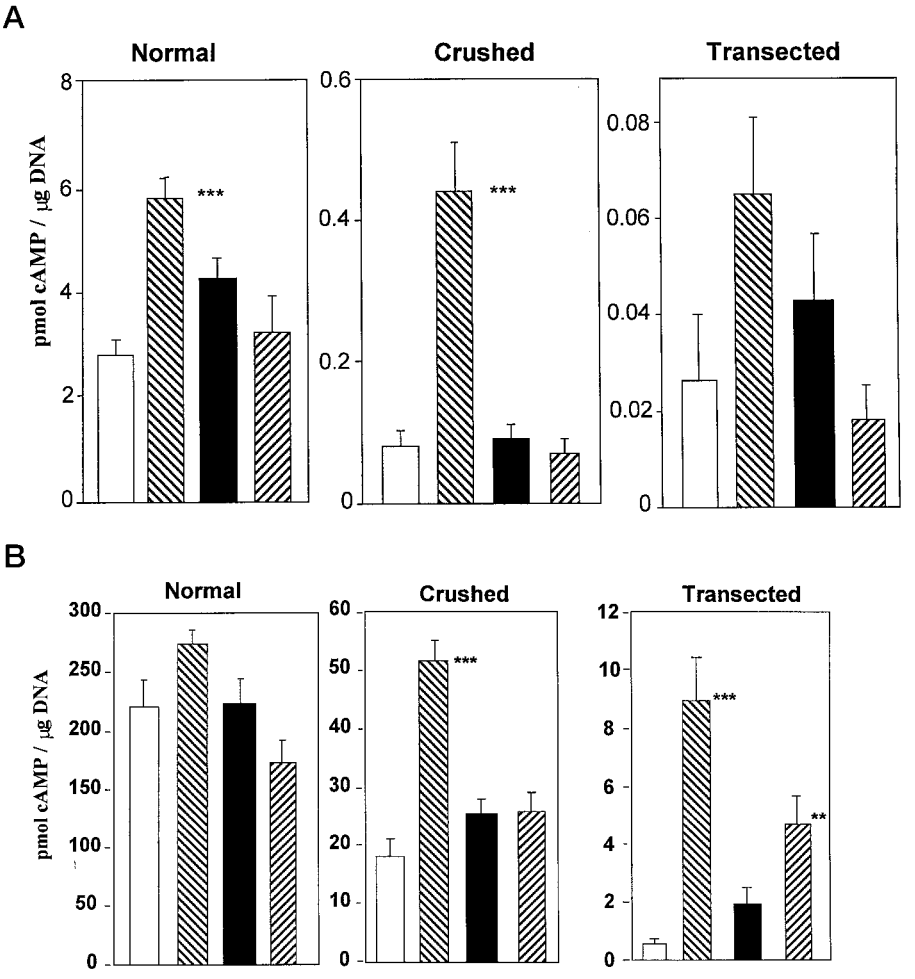

FIG. 3. **The effect of PDE isozyme-specific inhibitors on cAMP accumulation in endoneurial explants from normal nerve and 7- and 21-day crushed or transected nerves under basal (*A*) and forskolin-stimulated (*B*) conditions.** *A*, isolated endoneurial segments were incubated in Krebs-Ringer alone (control) or with 30 μM rolipram, cilostamide, or 8-MeoM-IBMX for 30 min, followed by cAMP quantification. *B*, isolated endoneurial segments were incubated with PDE inhibitors + 100 μM forskolin for 30 min, followed by cAMP quantification (n = 7–12 per time point). **, $p < 0.01$; ***, $p < 0.001$) *versus* Krebs-Ringer by t test. □, Krebs; ■, cilostamide; ▨, rolipram; ▨, 3-MeoM-IBMX.

inhibition by rolipram was verified by also testing PDE inhibition by denbufylline, an unrelated PDE4 inhibitor, in homogenates from normal nerve and 7 days following both injuries (Fig. 2*B*). Thus the low K_m cAMP-specific isozyme is a predominant isozyme present in the rat sciatic nerve, and the increased activity of this family probably largely contributes to the overall increase in PDE activity following injury.

cAMP Changes in Endoneurial Explants due to Inhibition of Specific Isozymes—The role of individual PDE families in hydrolyzing cAMP in sciatic endoneurium explants was tested. The effects on cAMP content due to specific PDE isozyme inhibition under conditions of basal AC activity was tested by incubating endoneurium from normal nerve, and nerves 21 days following crush and transection injury for 30 min in solutions of Krebs-Ringers and 30 μM rolipram, cilostamide, or 8-MeoM-IBMX, an inhibitor of the PDE1 family. The effects of these inhibitors using identical solutions containing 100 μM forskolin (PDE inhibition under AC stimulated conditions) was also tested. The effects of PDE inhibition under basal conditions are shown in Fig. 3*A*. In the normal nerve, cAMP levels were 2.8 ± 0.3 pmol/μg of DNA. These levels were elevated significantly only by rolipram to 5.8 pmol/μg of DNA. Rolipram similarly elevated cAMP to a greater extent than the other inhibitors in the crushed (remyelinating) and transected (nonmyelinating) nerves. A similar effect of rolipram was found following AC stimulation by forskolin. In normal nerve, cAMP levels in endoneurium incubated in Krebs-Ringer and forskolin were 221 ± 22.8 pmol/μg of DNA (Fig. 3*B*). The addition of rolipram slightly increased cAMP to 273 ± 12.6 pmol/μg of DNA. The other inhibitors failed to increase cAMP. In the crushed nerve, rolipram increased cAMP levels from 18.0 ± 3.0 pmol/μg of DNA to 51.5 ± 3.2 pmol/μg of DNA ($p < 0.001$). Cilostamide and 8-MeoM-IBMX increased cAMP in nonsignificant amounts. In the transected nerve, similarly, rolipram increased cAMP from 0.5 ± 0.1 pmol/μg of DNA to 8.9 ± 1.4 pmol/μg of DNA ($p < 0.001$). Cilostamide and 8-MeoM-IBMX increased cAMP by smaller amounts. The rolipram-inhibitable isozymes, therefore, seem to be the predominantly active form in endoneurial explants. PDE activity in the normal nerve is quite low, and thus it is not surprising that the increase in cAMP due to rolipram in normal AC-stimulated nerve was not

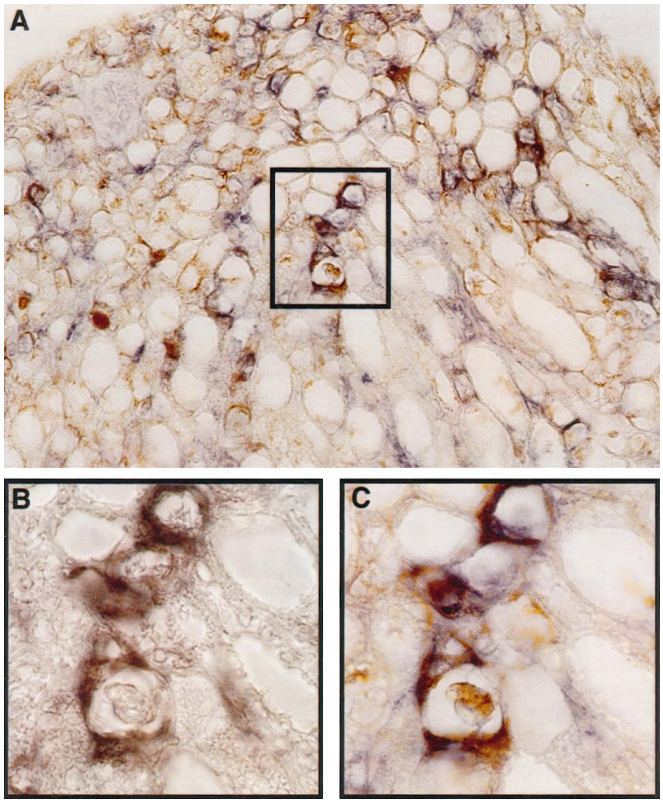

FIG. 4. **Colocalization of PDE4 mRNA and S100 protein in sciatic nerve 4 days following crush injury.** *A*, *in situ* localization of PDE4 (*blue*) and immunohistochemical localization of the SC marker S100 (*brown*) in transverse section of sciatic nerve 4 days following crush injury. Only cells that strongly stained for S100 were also strongly stained for PDE4. (× 40). *B* and *C*, higher magnification (× 100) of the boxed region in *A* showing *in situ* localization of PDE4 (*B*), and the same region after both *in situ* localization of PDE4 and S100 immunohistochemistry (*C*).

significant, as it was after crush and transection injuries when PDE activity is much higher.

In situ Localization of PDE4—To verify that the PDE4 expression is in SCs, PDE4B mRNA was localized in sciatic sections by *in situ* hybridization. The sciatic sections were first probed for PDE4B mRNA location. The sections were then photographed, followed by immunohistochemical localization of S100, an SC specific protein (65) on the same sections.

Numerous cells scattered throughout the sciatic section were heavily stained following *in situ* hybridization of PDE4B mRNA (Fig. 4). The strongest *in situ* labeling would be expected in SCs sectioned through their cytoplasm, where PDE4 mRNA would be located. Other cells in which the plane of the section did not traverse the cytoplasm would not be as strongly stained. The hybridization pattern is consistent with these expectations. The cells which stained darkest for PDE4 also were heavily stained by S100 immunohistochemistry. S100 immunoreactivity was primarily localized to SC cytoplasm, with occasional light staining of SC membranes. In biochemical studies, S100 has been found to associate with cytoplasmic fractions of nerve homogenates, with a small percentage found to be tightly associated with a particulate, membrane-associated fraction (66, 67). Some slight S100 staining was localized to myelin, similar to immunohistochemical localization reported by others at both the light and electron microscopic levels (68–70), where the PDE4 message would not be expected. Nearly all cells that stained for PDE4B also stained for S100. An occasional cell that stained heavily for S100 partially obscured a weaker signal for PDE4, but comparison of pictures taken after PDE4B *in situ* hybridization and prior to S100 immunostaining verifies that PDE4 is found in these cells. Only cells that strongly labeled for S100 gave a strong PDE4 signal, indicating that SCs are the principle source of PDE activity and there is not another sciatic element with extensive PDE expression. *In situ* hybridization using sense strand transcripts did not give any color reaction (data not shown).

AC Activity following Injury—Another factor contributing to the decline in cAMP levels in distal endoneurium following injury could be decreased synthesis by ACs. The activity of AC in the distal endoneurium was measured in membrane fractions from normal nerve and 7 days following crush injury, a time when cAMP is lowest (37). Basal AC activity, without forskolin added, in normal nerve was 3.7 ± 0.3 pmol/mg of protein/min (n = 10). At 7 days following injury, basal activity was just 0.7 ± 0.08 pmol/mg/min (n = 15), only about 19% of normal. Forskolin stimulated AC activity in the normal endoneurium by 4.6-fold, to 17.1 ± 0.8 pmol/mg/min (n = 10), while following injury, forskolin stimulated activity to 5.0 ± 0.6 pmol/mg/min (n = 15), which is 29% of normal forskolin stimulated values. The values for normal nerve both with and without forskolin treatment are very similar to those found by Shindo *et al.* (71). Thus it is evident that a decrease in AC activity and an increase in PDE activity both contribute to the decreased cAMP levels found in the distal endoneurium following injury.

ATP Quantification following Injury—One possible limiting factor in AC activity *in vivo* could be ATP concentration. If AC substrate levels declined following injury, it could contribute to the overall decrease in cAMP levels. ATP concentration was thus determined in normal endoneurium, as well as at 21 and 35 days following transection injury, times when cAMP levels are very low (37). In normal nerve, ATP concentrations were 519 pmol/mg of protein. A similar value was found at 21 days, but by 35 days, ATP levels were somewhat lower at 362 pmol/mg of protein. These fluctuations in ATP are probably not sufficient to cause the decline in AC activity following injury.

DISCUSSION

Cyclic AMP levels have been previously shown to change dramatically following injury to the sciatic nerve (37). The results from the present series of experiments indicate that these variations in cAMP levels are the result of changes in rates of both synthesis and degradation of this second messenger.

A 3–3.5-fold increase in phosphodiesterase activity occurs shortly after injury. This activity remains elevated in the transected nerve, while it declines to a level that is still twice normal activity by 21 and 35 days following crush injury. This increase in PDE activity occurs at the time when cAMP levels are declining following injury (*cf.* Ref. 37; Fig. 1). The continuing elevated PDE activity in the transected nerve correlates well with the low endogenous levels of cAMP that persist following this injury. In the crushed nerve the partial decline in PDE activity that occurs at 21 and 35 days following injury correlates well with the partial recovery in cAMP levels that occur at this time. Cyclic AMP levels did not return to normal levels even at 35 days following injury (37) which may be due to the failure of PDE activity to decline to normal levels at this time point. These data suggest that cAMP levels are tightly regulated following injury.

The predominant isozymes of PDE in the sciatic endoneurium both before and after injury appear to be the low K_m cAMP-specific (PDE4) isozyme and the Ca^{2+}/CaM-stimulated (PDE1) isozyme. The activity of both these families was upregulated following injury, although the contribution of the PDE1 isozyme was slightly lower as a percentage of the total activity after injury. The PDE4 activity increased sufficiently

to maintain a similar percentage of the total activity both before and after injury. This was in contrast to the cilostamide inhibitable (PDE3) activity which was just 19% in normal nerve and 9–11% of basal activity following injury. The presence of the cGMP-stimulated isozymes could not be demonstrated, as there was no increase in PDE activity in the presence cGMP.

The importance of the PDE4 family in hydrolyzing cAMP in sciatic endoneurium was verified by studies using intact endoneurial tissue. Incubation of endoneurial explants with type-specific inhibitors showed that PDE4 inhibition caused a greater elevation in cAMP than inhibition of the other isozymes. Thus the activity of this isozyme has a major role in hydrolyzing cAMP in intact endoneurium. The elevation in cAMP by rolipram in the presence of forskolin in the normal nerve was greater than the response to other inhibitors, but was not significant as it was following injury (Fig. 3). PDE activity in normal nerve was lower than in the injured nerve, and thus inhibition of PDE activity in the normal nerve would likely not have as great an effect on cAMP levels as it would following injury.

Another determining factor of cAMP levels is the rate of synthesis by adenylyl cyclases. We, therefore, measured AC activity in normal nerve and 7 days following crush injury, when cAMP levels are very low (37). AC activity declined to about 19% of normal by 7 days following injury. Thus a decline in synthesis as well as an increase in hydrolysis contributes to the low levels of cAMP following injury.

Numerous changes occur in the distal endoneurium following injury. Axonal degradation occurs rapidly, concomitant with infiltration of the distal segment by macrophages (72, 73), the transformation of the SCs from a myelinating to a nonmyelinating state, and proliferation of SCs (22, 24). Thus the cAMP changes reported by Poduslo *et al.* (37) reflect the sum of the changes in cAMP levels of all the constitutive elements remaining in the endoneurium, and the results of the present study also must be interpreted with regard to the changes occurring in the cellular elements.

The change in AC activity may occur in several different cellular elements of the endoneurium. The activity changes may reflect altered AC activity in SCs, as axolemmal constituents have been proposed to stimulate SC ACs (2). The total activity, however, probably also reflects declines in axonal AC. The role of the axon with regard to AC transport has previously been investigated. Bray *et al.* (74) observed that constrictions for 20 h around chicken sciatic nerve led to accumulations of AC activity by 266% immediately proximal to the constriction, while the AC activity immediately distal to the constriction changed little in 20 h. The accumulation proximal to the constriction was interpreted as being the result of blocked axonal transport of AC. Longer term studies were not conducted, but if AC transport is blocked, it would be expected that AC activity in the distal segment would decline. Carlsen (75) and Carlsen and Anderson (76) similarly showed an accumulation of AC activity proximal to a ligature places around frog sciatic nerve. Thus the decline in AC activity that we have demonstrated may be due in part to the loss of the axon with its contributing AC activity distal to the crush injury. However, there may also be changes in AC expression and activity that occur in the cellular elements in the distal segment. A down-regulation of AC activity may accompany the increased PDE activity to lower the cAMP concentration in some of the remaining cellular elements.

The principle source of PDE activity is due to PDE expression in SCs. This is indicated by several lines of evidence. First, *in situ* localization of PDE4 mRNA (Fig. 4) demonstrated prominent staining of SC, identified by costaining for S100, a SC specific protein. There were no cells that were heavily labeled for PDE4 that were not also stained for S100. Furthermore, SCs account for 85–90% of the cells present by 35 days following transection (77), thus the PDE activity probably largely reflects the activity of SCs.

Macrophages infiltrate the distal segment, and thus could contribute to an increased total PDE activity. However, at 35 days following transection, endoneurial macrophages account for only 1–3% of total cell area (77), so these cells probably do not contribute significantly to the total PDE activity. Furthermore, the differences that are seen between the transected nerve and the remyelinating crushed nerve 21 and 35 days following injury make it likely that the changes in PDE activity reflect changes in SCs as they remyelinate, and not changes due to macrophages. Further studies to eliminate the contribution of macrophages have been conducted using explants of sciatic nerve. Sciatic endoneurium from normal nerve was incubated from 1 to 4 days *in vitro*. This paradigm allows axonal degeneration and SC dedifferentiation to occur without an invasion by circulating macrophages (78). Cyclic AMP levels were measured in these explants cultured for 1–4 days and compared with cAMP changes that occur following crush injury *in vivo*.[3] The levels of cAMP *in vivo* decline following crush injury to just 11% of normal by 4 days following injury (37). Similarly, cAMP levels dropped in endoneurial explants to 33% of normal by 4 days of culturing. The similar decline in cAMP levels observed *in vivo* and in endoneurial explants suggests that invading macrophages do not contribute to the increased PDE activity observed *in vivo*.

It is not known what mechanisms may be controlling PDE activity in SCs. The up-regulation of PDE activity may be intrinsically controlled during differentiation of SCs. There is also mounting evidence that PDE levels can be actively controlled by extracellular ligands. For example, in the C6 glioma cell line, follicle-stimulating hormone leads to an increase in message levels of some forms of PDE4 (56). Insulin, which acts through a receptor tyrosine kinase, also exerts metabolic effects via phosphorylation and activation of PDE3 (79, 80). The up-regulation of PDEs that occurs in the distal nerve after injury may be under some form of hormonal or growth factor influence. As demyelination occurs, some receptor may be up-regulated resulting in increased responsiveness of PDEs to external stimuli. Conversely, cAMP changes in Schwann cells can modulate the expression of growth factor receptors (81), and thus the active regulation of cAMP by PDE activation may affect the response of SCs to growth factors by controlling receptor expression.

A further role for PDEs in the demyelination processes has been suggested in experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (82, 83). Encephalomyelitis is induced by immunization of susceptible animals with central nervous system tissue. This immunization provokes a T-lymphocyte autoimmune attack against central nervous system myelin, which involves participation of cytokines. Rolipram has been demonstrated to suppress the production of cytokines by autoreactive T cells (82). Through this mechanism, rolipram has been found to prevent the lesions found in encephalomyelitis. PDEs may well play a role in demyelination in peripheral nerve as well, either through mechanisms in the SCs themselves, or by altering the influence of other cells on demyelination.

The characterization of PDE activity in the distal endoneurium following injury with the subsequent changes in my-

[3] J. F. Poduslo, *et al*, manuscript in preparation.

elination indicate that PDE activity is related to the processes of demyelination and remyelination. It is possible that the active removal of cAMP in SCs via an up-regulation of PDEs may lead to demyelination. Conversely, a down-regulation of PDE activity may then be an obligate step preceding the process of myelination. The identification of the specific isozymes present will allow us to selectively manipulate their activity to investigate the role of these PDE isozymes in these processes.

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