

# Novel Inhibitory Action of Tunicamycin Homologues Suggests a Role for Dynamic Protein Fatty Acylation in Growth Cone-mediated Neurite Extension

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**Abstract.** In neuronal growth cones, the advancing tips of elongating axons and dendrites, specific protein substrates appear to undergo cycles of posttranslational modification by covalent attachment and removal of long-chain fatty acids. We show here that ongoing fatty acylation can be inhibited selectively by long-chain homologues of the antibiotic tunicamycin, a known inhibitor of N-linked glycosylation. Tunicamycin directly inhibits transfer of palmitate to protein in a cell-free system, indicating that tunicamycin inhibition of protein palmitoylation reflects an action of the drug separate from its previously established effects on glycosylation. Tunicamycin treatment of differentiated PC12 cells or dissociated rat sensory neurons, under conditions in which protein palmitoylation is inhibited, produces a prompt cessation of neurite elongation and induces a collapse of neuronal growth cones. These growth cone responses are rapidly reversed by washout of the antibiotic, even in the absence of protein synthesis, or by addition of serum. Two additional lines

of evidence suggest that the effects of tunicamycin on growth cones arise from its ability to inhibit protein long-chain acylation, rather than its previously established effects on protein glycosylation and synthesis. (a) The abilities of different tunicamycin homologues to induce growth cone collapse vary systematically with the length of the fatty acyl side-chain of tunicamycin, in a manner predicted and observed for the inhibition of protein palmitoylation. Homologues with fatty acyl moieties shorter than palmitic acid (16 hydrocarbons), including potent inhibitors of glycosylation, are poor inhibitors of growth cone function. (b) The tunicamycin-induced impairment of growth cone function can be reversed by the addition of excess exogenous fatty acid, which reverses the inhibition of protein palmitoylation but has no effect on the inhibition of protein glycosylation. These results suggest an important role for dynamic protein acylation in growth cone-mediated extension of neuronal processes.

**P**ROTEIN modification by the covalent attachment of fatty acids is a ubiquitous process whose biological significance remains obscure (Grand, 1989; James and Olson, 1990; Schmidt, 1989). Two major classes of fatty acylation have been identified—cotranslational, stable linkage of myristic acid (14 carbons) to the NH<sub>2</sub> terminus of certain proteins, and posttranslational attachment of palmitic acid (16 carbons) and other long-chain fatty acids. The latter modification can be subdivided into early posttranslational acylation and a later component, independent of protein synthesis, that is both dynamic and sensitive to extracellular stimuli (Bourguignon et al., 1991; Huang, 1989; James and Olson, 1989; Magee et al., 1987; Skene and Virag, 1989; Staufenbiel, 1988) and is particularly prevalent in proteins known to be involved in cellular adhesion, signaling, and growth regulation (James and Olson, 1990). This dy-

namic, posttranslational turnover of fatty acyl residues, by analogy to the reversible phosphorylation of proteins, may serve to control or modify proteins subserving a variety of cellular functions.

One well-defined site of posttranslational protein acylation is the motile growth cone apparatus that form the advancing tips of elongating neuritic processes (Skene and Virag, 1989) and mediate the extension of axons and dendrites both in neuronal development and in nerve regeneration (Harris et al., 1987; Sretavan and Reichardt, 1993). Elongation proceeds through cycles of filopodial or lamellipodial extension, consolidation of selected filopodia by membrane insertion and cytoskeletal assembly, and retraction of unconsolidated filopodia (Forscher and Smith, 1988; Goldberg and Burmeister, 1989; Okabe and Hirokawa, 1991). Regulation and guidance of nerve growth are mediated by growth cone interactions with a broad array of specific ligands in the local environment (Jessell, 1988; Mason, 1985), including many that enhance growth mechanisms and others that are proposed to guide or direct neurite extension through spatially

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restricted inhibition of growth cone activities (Baier and Bonhoeffer, 1992; Davies et al., 1990; Moorman and Hume, 1990; Patterson, 1988; Raper and Kapfhammer, 1990; Schwab and Schnell, 1991). Because growth cones from developing brain have been shown to carry out active protein fatty acylation independent of neuronal cell bodies (Skene and Virag, 1989), we were particularly interested in investigating the role of fatty acylation in mechanisms of growth cone-mediated axonal elongation.

Elucidation of biological roles for palmitoylation in growth cone functions, or in other cellular processes, has been hindered by the lack of an inhibitor that would permit direct experimental manipulation of this modification in intact cells. The nucleoside antibiotic tunicamycin (TM)<sup>1</sup> (see Fig. 1 A) has been used extensively to investigate the role of protein N-linked glycosylation in protein processing, receptor activation, viral multiplication and such cellular processes as division, differentiation, and adhesion (reviewed in Elbein, 1984; Hemming, 1982). However, in some cases TM also has been found to interfere with the intracellular targeting of non-glycosylated proteins, including the appearance in a membrane fraction of newly synthesized GAP-43, a major component of neuronal growth cones (Perrone-Bizzozero and Benowitz, 1987). Because membrane association of GAP-43 follows the posttranslational transfer of palmitate residues (Chapman et al., 1992; Skene and Virag, 1989), we considered the possibility that TM might disrupt GAP-43 processing by interfering with long-chain acylation. Structural comparison shows that TM, which comprises a family of closely related compounds, does bear some overall similarity to fatty acyl coenzyme-A, the acyl donor for protein fatty acylation (see Fig. 1 A). This raises the possibility that one or more of the TMs might act as a general competitive inhibitor of protein acylation.

## Materials and Methods

### Materials

TM homologues were prepared by HPLC (Mahoney and Duksin, 1980) from whole TM (Boehringer Mannheim Corp., Indianapolis, IN) by separation on a 4.6 × 250-mm Dynamax column packed with 5 μm Microsorb-C8 (Rainin Instr. Co., Woburn, MA) eluted isocratically with methanol/H<sub>2</sub>O (68:32, HPLC grade) at 40°C. For experiments, TM and homologues were dissolved at 1 mg/ml in 10 mM NaOH, and the concentration confirmed by absorbance at 260 nm after dilution into culture medium (0.12 A.U. = 10 μg/ml). The dissolved drugs were stored at -80°C between experiments, and used within one month of preparation.

<sup>3</sup>H-palmitoyl-coenzyme A was prepared from [<sup>3</sup>H]palmitate (60 Ci/mmol; Dupont NEN, Boston, MA) by incubation with 1 U/ml long-chain acyl:coenzyme A transferase (Boehringer Mannheim Corp.) in 100 μl of 0.05% Triton X-100, 1 mM coenzyme A (CoA; Boehringer Mannheim Corp.), 5 mM ATP, 5 mM MgCl<sub>2</sub>, 10 mM Tris, pH 7.5. Reactions were terminated after 1–2 h by addition of 1 ml chloroform/methanol (1:1), and product was isolated in the upper phase after addition of 0.5 ml chloroform and 0.275 ml H<sub>2</sub>O. After drying, label was redissolved in methanol, aliquoted into reaction tubes, and dried in a Speed-Vac centrifugal evaporator (Savant Instr., Inc., Farmingdale, NY). Analysis by TLC showed ≥95% radiochemical purity.

For addition to cell cultures, radioactive and non-radioactive fatty acids were dissolved in DMSO and added to medium at a final concentration of 0.1% (vol/vol) DMSO, with brief bath sonication for higher concentrations of fatty acid (>10 μM).

1. *Abbreviations used in this paper:* CoA, coenzyme A; DRG, dorsal root ganglion; HE, 10 mM Hepes/1 mM EDTA; PPO, 2,5-diphenyloxazole; TM, tunicamycin.

## Growth Cone Preparation and Labeling

Intact growth cones were prepared from 3–5-d neonatal Sprague-Dawley rat pups by the Ficoll flotation method of Gordon-Weeks (Gordon-Weeks, 1987, 1991; Gordon-Weeks and Lockerbie, 1984). These growth cones were labeled by resuspending in Hepes-buffered DME containing 100 μCi/ml [<sup>3</sup>H]palmitate (60 Ci/mmol) for 20 min at 37°C. Labeling was quenched with 10 volumes of chloroform/methanol (1:1). Washed membranes were prepared from intact growth cones by lysis in 10 mM Hepes/1 mM EDTA, pH 7.4, (HE) followed by centrifugation at 140,000 g for 30 min. Pellets were washed sequentially with 1 M NaCl in HE (two washes) and then again with HE (one wash), with careful trituration through a 22-gauge hypodermic needle. Washed membrane protein concentration was adjusted to 1 mg/ml and solubilized in 0.1% (wt/vol) Lubrol-PX (Sigma Chemical Co., St. Louis, MO) in 100 mM KCl, 20 mM NaCl, 0.1 mM MgCl<sub>2</sub>, 20 mM Hepes, pH 7.4, for 60 min on ice. Insoluble material was pelleted as above. Aliquots (100 μl) of supernatant were added to tubes containing dried <sup>3</sup>H-palmitoyl-CoA and TM, mixed vigorously, incubated at 37°C for 20 min, and labeling reactions were quenched by the addition of 10 volumes chloroform/methanol (1:1).

## Cell Culture and Labeling

PC12 cells were propagated in DME supplemented with 5% horse serum and 5% supplemented calf serum (GIBCO BRL, Gaithersburg, MD). Neuronal phenotype was induced by treatment with 50 ng/ml nerve growth factor (NGF; tissue culture grade, Boehringer-Mannheim Corp.) for not less than 4 d or more than 14 d. Cells for labeling were washed free of serum and incubated with labeled precursors of protein myristoylation ([<sup>3</sup>H]-myristate, 50 μCi/ml), long chain acylation ([<sup>3</sup>H]palmitate, 1 μCi-1 mCi/ml), glycosylation ([<sup>3</sup>H]mannose, 50 μCi/ml, all from Dupont NEN), or synthesis ([<sup>35</sup>S]cysteine, 50–500 μCi/ml) (Amersham Corp., Arlington Heights, IL) for 2 h (total cellular protein labeling) or 1 h (all figures shown) at 37°C in 1 ml DME supplemented with 10 mM Hepes, pH 7.4, 50 ng/ml NGF, and 0.5 μg/ml fatty acid-free BSA (Sigma Chemical Co.). Labeling reactions were quenched by adjusting the medium to 10% (wt/vol) trichloroacetic acid ([<sup>3</sup>H]mannose and [<sup>35</sup>S]cysteine), or replacing the medium with methanol ([<sup>3</sup>H]myristate and [<sup>3</sup>H]palmitate; Juguelin and Cassagne, 1984; Schmidt and Lambrecht, 1985). In early experiments (see data in Fig. 3), intact cells were pretreated with TM for 60 min before addition of [<sup>3</sup>H]palmitate, but this was subsequently found to be unnecessary (see Fig. 3 C, inset) and label and inhibitor were thereafter added together.

Dorsal root ganglion (DRG) cultures were prepared by a modification of the technique of Lindsay (Lindsay, 1988) from adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) after a unilateral sciatic nerve crush performed as previously described (Schreyer and Skene, 1991; Skene and Shooter, 1983) under ketamine/chloral hydrate anaesthesia in accordance with National Institutes of Health guidelines. After at least 48 h, animals were killed by CO<sub>2</sub> asphyxiation, and the fourth and fifth lumbar ganglia from the operated side were excised, cut open, and collected in 1.5 ml L14 medium (GIBCO BRL) containing N1 supplements (5 μg/ml transferrin, 5 μg/ml insulin, 16 μg/ml putrescine, 30 nM sodium selenite, 20 nM progesterone, all from Sigma Chemical Co.) and 50 ng/ml NGF in 35-mm tissue culture dishes (Corning Inc., Medfield, MA) on ice. Ganglia were washed twice in 1.5 ml prewarmed medium, then incubated for 90 min at 37°C in a further 1.5 ml F14 containing N1 additives, NGF and 3.0 μg/ml collagenase (type XI), followed by trituration through a flamed pasteur pipette. Cell dissociation was completed by 15 min with 0.3 mg/ml trypsin (type XII-S), 10 min with 0.1 mg/ml deoxyribonuclease I (type IV), 5 min in 5 mM EDTA and finally further trituration. Dissociated cells were transferred to a 15-ml conical tube, adjusted to 0.3 mg/ml soybean trypsin inhibitor (all from Sigma Chemical Co.) and 5% horse serum, and pelleted. Cells were washed twice with 10 ml medium with gentle centrifugation between, before being plated in F14 medium containing N1 additives and 5% horse serum at a density of one-fifth to one-tenth ganglion/35-mm dish, precoated with polyornithine (Sigma Chemical Co.) and laminin (Upstate Biotechnology, Lake Placid, NY) according to manufacturers' recommendations. Labeling with [<sup>3</sup>H]-palmitate was carried out as for PC12 cells.

## Protein Analysis

Cells labeled with [<sup>3</sup>H]mannose or [<sup>35</sup>S]cysteine were scraped into 10% (wt/vol) trichloroacetic acid and left on ice for 1 h. Insoluble material was pelleted at 12,000 g for 15 min, followed by sequential washes with 10% and 1% trichloroacetic acid, and ether. The remaining material was solubilized in 1% SDS in 100 mM Tris.HCl, pH 7.4, with heating, and radioactiv-

ity determined by liquid scintillation counting. For total protein labeling of cells with [<sup>3</sup>H]myristate or [<sup>3</sup>H]palmitate, cells were scraped into methanol, transferred and adjusted to chloroform/methanol/H<sub>2</sub>O (5:5:1). After extraction for 1 h, insoluble material was pelleted at 12,000 g for 15 min, followed by sequential 1 h washes (3–6) in chloroform/methanol/H<sub>2</sub>O until no further radioactivity was extracted, then two washes with chloroform/methanol (2:1), solubilization and counting as above. Data given were from 5 ([<sup>3</sup>H]myristate), 4 ([<sup>3</sup>H]palmitate, [<sup>3</sup>H]mannose), or 3 ([<sup>35</sup>S]cysteine) independent experiments.

For analysis of specific cellular proteins, labeled cells were scraped into methanol, transferred to polypropylene tubes, and adjusted to chloroform/methanol/H<sub>2</sub>O (5:5:1). After pelleting as above, the proteins were redissolved in sample buffer containing 1% SDS, 10 mM Tris, pH 7.4, and 20% glycerol with heating, and separated on 12% SDS/polyacrylamide gels. Two-dimensional gel analysis was carried out with ampholytes in the range 3–10 and 4–6 (Bio Rad Laboratories, Hercules, CA) at a ratio of 2:1. For immunoprecipitation of labeled GAP-43 from washed growth cone membranes, material was solubilized in 0.1% Lubrol-PX in HE, and incubated overnight at 4°C with the monoclonal antibody 9-IE12 (Schreyer and Skene, 1991) preadsorbed onto agarose-immobilized goat antimouse IgG (HyClone Laboratories, Logan, UT). Proteins were fixed in the gel with 25% isopropanol/10% acetic acid and permeated with 2,5-diphenyl-oxazole (PPO; Sigma Chemical Co.; 1% wt/vol in 15% ethanol/30% xylenes/55% acetic acid) for fluorography. The identification of the major <sup>3</sup>H-palmitoylated protein in growth cones as GAP-43 has been published elsewhere (Skene and Virag, 1989), and the identity of SNAP-25 and GAP-43 in DRG neurons was confirmed by the migration position on two-dimensional gels (Hess et al., 1993). The major labeled protein in PC12 cells was shown to be SNAP-25 by two-dimensional gel analysis, while GAP-43 labeling in PC12 cells varied considerably with culture conditions, and could not always be seen.

### Lipid Analysis

Cells labeled with [<sup>3</sup>H]-palmitate were extracted with chloroform/methanol/H<sub>2</sub>O as described above. Aliquots of the first organic extract (1/10 of total volume) were dried in Speed-Vac, redissolved in chloroform/methanol (2:1) and separated by TLC on aluminum-backed silica gel plates (Art.5553; EM Science, Gibbstown, NJ) developed with butanol/acetic acid/H<sub>2</sub>O (5:2:3, Juguelin and Cassagne, 1984). After drying, plates were permeated with PPO (10% wt/vol in ether) for fluorography.

### Quantitation of Radioactivity

PPO-permeated gels or TLC plates were exposed for various lengths of time at -70°C to x-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, NY) preflashed to an optical density of 0.1 A.U. After development, bands were identified by reference to protein molecular weight or lipid standards, and quantitated by transmittance optical density on a Technology Resources image analysis system.

For experiments using solubilized protein acylating activity from growth cones, the labeling of protein was expressed as the ratio of the amount of label incorporated into GAP-43 compared with the amount remaining in palmitoyl-CoA at the end of the incubation in the same sample. This was done as lipid analysis showed a substantial accumulation of labeling precursor, <sup>3</sup>H-palmitoyl-CoA, in the presence of high concentrations of TM, presumably due to disruption of incorporation into lipid. Therefore, in these experiments, the ratio of product/precursor was used to adjust for more efficient protein labeling due to precursor protection. The unadjusted range of labeling inhibition by TM can be seen by comparing lanes 2 and 3 in Fig. 1 B.

### Time-Lapse Video Microscopy

Cells for video recording were plated on polyornithine/laminin-coated 35-mm tissue culture dishes. PC12 cells were differentiated with NGF as described above. DRG cultures were taken 8 h–3 d after plating. Cells were washed free of serum with the appropriate bicarbonate-free, Hepes-buffered medium containing 50 ng/ml NGF and 0.5 μg/ml fatty acid-free BSA, and N1 additives (DRG cells only). Cells in 2 ml medium were transferred to a temperature controller (Narashige model MS-200D) mounted on an inverted stage microscope (Nikon Diaphot-TMD) and maintained at 37°C. Growth cones were observed with Hoffman modulation contrast optics connected to a charge-coupled device camera (MTI model CCD72S) and recorded on S-VHS tapes using a time-lapse recorder (Panasonic 6750A).

Prints were subsequently made from the tapes using a video printer (Sony UP-3000).

## Results

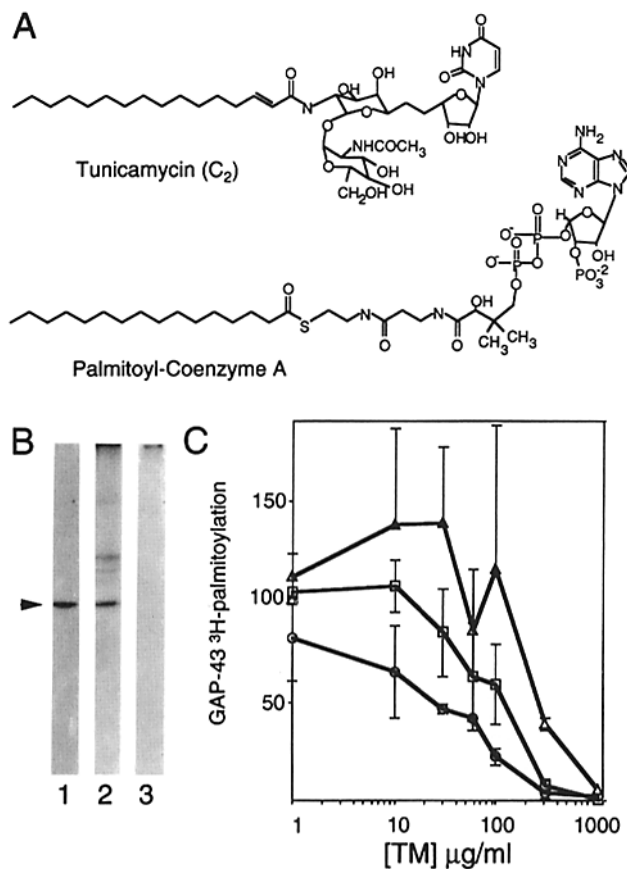
### TM Inhibits Solubilized Protein Acylating Activity from Growth Cones

To explore the possibility that TM could inhibit protein acylation directly, we prepared a detergent-solubilized fatty acyl-CoA:protein acyltransferase activity from neuronal growth cones (Fig. 1 B, lanes 1 and 2). Because these structures occur at the ends of elongating axons and dendrites, at some distance from neuronal cell bodies, they can be isolated as a sub-cellular fraction substantially free of the cellular apparatus for protein synthesis and glycosylation (Gordon-Weeks, 1987, 1991; Gordon-Weeks and Lockerbie, 1984; Pfenninger et al., 1983). Such isolated growth cones can incorporate externally added [<sup>3</sup>H]-palmitate into endogenous protein substrates (Fig. 1 B), the most prominent of which has been identified previously as GAP-43 (Skene and Virag, 1989). Isolated growth cones therefore provide a potential source of protein palmitoylating enzyme(s) for mechanistic studies.

Membranes prepared from isolated neuronal growth cones and extensively washed to remove peripherally bound proteins retain a protein fatty acyltransferase activity, which can be solubilized in nonionic detergent (Fig. 1 B). This activity labels endogenous GAP-43 by transfer of palmitic acid from exogenously added <sup>3</sup>H-palmitoyl-CoA, the immediate precursor of protein acylation (Riendeau and Guertin, 1986). TM caused a dose-dependent inhibition of this final step of GAP-43 palmitoylation (Fig. 1 C).

Immunoprecipitation confirmed the identity of the major endogenous protein palmitoylated in washed growth cone membranes as GAP-43 (Fig. 2). Because virtually all of the GAP-43 remaining associated with these extensively washed membranes would be expected to be palmitoylated before the labeling reaction (Liu et al., 1991; Skene and Virag, 1989), it is likely that active de-acylation of GAP-43 occurs under our reaction conditions. We therefore cannot exclude the possibility that TM interferes with deacylation of GAP-43 to serve as a substrate for reacylation, rather than with the palmitoylating enzyme. The results nevertheless demonstrate that some component of TM can inhibit directly the cycle of protein acylation and deacylation in growth cones, and thus can serve as a pharmacological probe for the biological functions of this dynamic modification in intact cells.

The amount of TM required to produce a 50% inhibition of palmitoylation in the solubilized preparation under our standard reaction conditions was relatively high (>250 μg/ml), but decreased as the amount of added <sup>3</sup>H-palmitoyl-CoA was reduced (Fig. 1 C). Although these data are not adequate to establish a competitive mechanism of inhibition, it does illustrate that the inhibitory effect of TM on protein palmitoylation depends on the concentration of the fatty acyl donor. The local concentration of palmitoyl-CoA in intact growth cones in vivo or in vitro is not known, but fatty acyl-CoA's in most cells are present in only trace amounts (Molap-arast-Saless et al., 1988). Even the lowest concentration of labeled palmitoyl-CoA used in our reactions, therefore, is likely to exceed the concentration of endogenous palmitoyl-



**Figure 1.** TM directly inhibits a solubilized protein acylating activity from neuronal growth cones. (A) Comparison of the structures of TM (top, homologue C<sub>2</sub>) and palmitoyl coenzyme A (bottom, in extended configuration). TM comprises a family of closely related homologues that differ in the fatty acyl side chain; homologue C<sub>2</sub>, one of the more abundant homologues with a 16-carbon acyl chain, is illustrated. (B) Incorporation of <sup>3</sup>H-palmitate into specific proteins by intact isolated growth cones from neonatal rat brain (lane 1) and by a detergent-solubilized preparation from washed growth cone membranes (lanes 2 and 3). Protein palmitoylating activity evident in the solubilized preparation (lane 2) is strongly inhibited in the presence of 1 mg/ml TM (lane 3). Intact growth cones were labeled with 1.7 μM <sup>3</sup>H-palmitic acid, and solubilized preparations were labeled with 1.7 μM <sup>3</sup>H-palmitoyl-CoA. Incubations were for 20 min at 37°C. The arrow marks the most prominent substrate for palmitoylation, GAP-43 (Skene and Virag, 1989; and see Fig. 2). Coomassie blue staining indicates that the total amount of the GAP-43 substrate is unaffected by the presence of TM (not shown). (C) Effect of TM on protein palmitoylating activity at three concentrations of palmitoyl-CoA. Solubilized growth cones were treated with initial concentrations of <sup>3</sup>H-palmitoyl-CoA of 17 nM (circles), 170 nM (squares) or 1.7 μM (triangles) for 20 min at 37°C with increasing concentrations of TM, and labeled GAP-43 palmitoylation was quantitated and normalized to palmitoyl-CoA levels as described in Materials and Methods. The results are presented as means ± range for two independent experiments. Analysis by two factor ANOVA (Statview II; Abacus Concepts, Inc., Berkeley, CA) shows that the results at 17 nM and 1.7 μM <sup>3</sup>H-palmitoyl-CoA are significantly different ( $P < 0.05$ ).

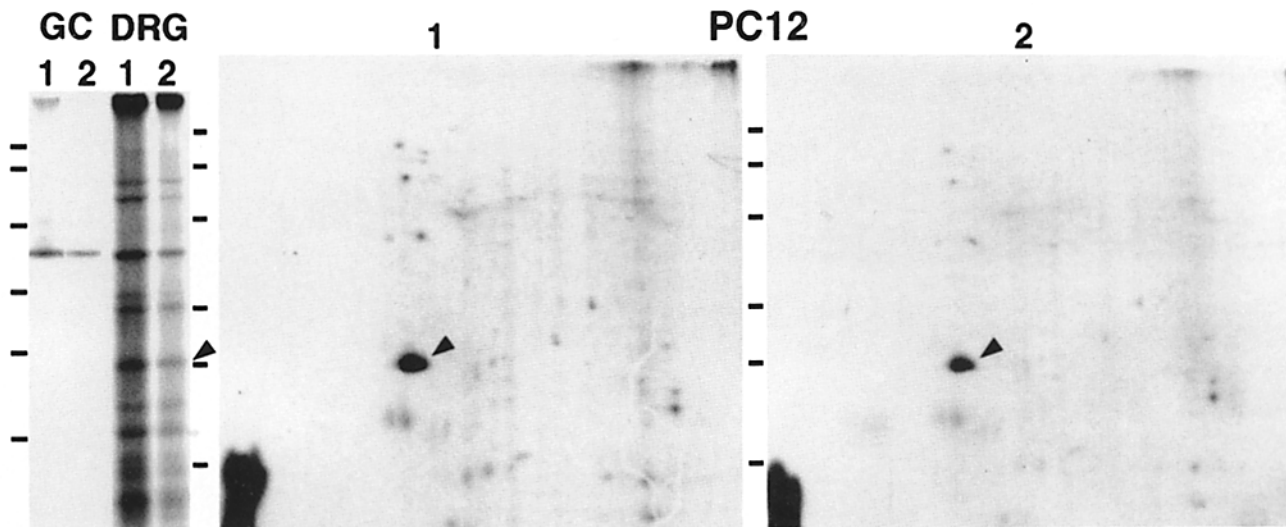
CoA found in growth cones of intact cells, and the amount of TM required to inhibit protein palmitoylation in intact growth cones would be expected to be equal to or lower than that determined with the lowest concentration of exogenous palmitoyl-CoA in the solubilized preparation.

### TM Inhibits Posttranslational Protein Palmitoylation

To characterize the effects of TM on protein acylation in an intact cellular environment, we analyzed the incorporation of palmitic acid into proteins of cultured DRG neurons from adult rats and differentiated PC12 cells (Olson et al., 1985), neuron-like tumor cells that extend processes with active, motile growth cones. Both of these cell types incorporated palmitate into a number of cellular proteins (Fig. 2). Two of these substrate proteins have been identified as GAP-43 and SNAP-25 (Hess et al., 1992, 1993). The relative labeling of these two proteins differs between dissociated adult DRG neurons and PC12 cells (Fig. 2), and among PC12 cells under different conditions (not shown), possibly reflecting differential expression of GAP-43 and SNAP-25 in association with different stages of axonal elongation, arborization and synaptogenesis (Osen-Sand et al., 1993; Oyler et al., 1991; Simkowitz et al., 1989; Skene, 1989). TM at 10 μg/ml inhibited palmitoylation of both of these proteins by 47–65% (Fig. 2). Labeling of other cellular proteins was inhibited to a similar extent, without obvious selectivity for individual substrate proteins (Figs. 2 and 3A). For this reason, the most robustly and consistently labeled proteins in each cell type, GAP-43 in DRG neurons and SNAP-25 in PC12 cells, were used for quantitative analysis.

As in other cells (Bourguignon et al., 1991; James and Olson, 1989; Magee et al., 1987; Skene and Virag, 1989; Staufenbiel, 1988) protein palmitoylation in PC-12 cells has a late posttranslational component, indicated by the large fraction (38%) of total protein labeling by [<sup>3</sup>H]-palmitate that persists after treatment with cycloheximide, a specific inhibitor of protein synthesis (Fig. 3A). Under similar conditions, TM at 20 μg/ml displayed a strong inhibition of total cellular protein glycosylation (70%) and palmitoylation (50%), which were reduced by a larger increment ( $P < 0.02$ , Student's unpaired *t*-test) than could be accounted for simply by the observed inhibition of protein synthesis (10%, data not shown). It has been suggested that translation-dependent and -independent palmitoylation reflect, respectively, the initial processing of proteins in the Golgi and later turnover of fatty acid moieties on proteins at the plasma membrane (Gutierrez and Magee, 1991; Olson, 1988). We found that TM inhibition of the late posttranslational component of palmitoylation (45% at 10 μg/ml TM) was significantly greater than its inhibition of total cellular protein palmitoylation (28%,  $P < 0.05$ , seven independent trials; Fig. 3B). This indicates that, under these assay conditions, the effects of TM on total cellular protein palmitoylation reflect primarily a strong inhibition of the late posttranslational component, with less potent inhibition of acylation occurring in concert with translation.

In either the presence or absence of cycloheximide, TM produced no significant inhibition of [<sup>3</sup>H]-palmitate incorporation into cellular lipids ( $P > 0.05$ , four independent trials; Fig. 3, A and B). A modest inhibition (22%) of protein myristoylation was observed with 20 μg/ml TM (data not shown), but this was not significantly greater than the inhibition of protein synthesis ( $P > 0.05$ ), and may be an indirect consequence of the tight coupling of myristoylation to translation (Buss et al., 1984; Wilcox and Olson, 1987). These results indicate that TM does not interfere equally with all cellular reactions that depend on acyl-CoAs as a fatty acid donor, but is relatively specific for one or more protein



**Figure 2.** TM inhibits general and growth cone protein palmitoylation in intact neuronal cells. Washed growth cone (GC) membrane proteins labeled with  $^3\text{H}$ -palmitoyl-CoA were separated on one-dimensional SDS-PAGE (lane 1) or immunoprecipitated with an anti-GAP-43 monoclonal antibody and run in a parallel lane (lane 2). Intact cultured DRG neurons and NGF-differentiated PC12 cells were labeled with  $3 \mu\text{M}$   $^3\text{H}$ palmitate with or without  $10 \mu\text{g/ml}$  TM for 60 min in culture. Both cell types incorporate label into a range of proteins of different molecular masses (standards from top to bottom: 106, 80, 49.5, 32.5, 27.5, and 18.5 kD). The heavily labeled band in the DRG samples (lanes 1 and 2) migrating in the same position as the growth cone GAP-43 immunoprecipitate has been identified as GAP-43 on two-dimensional gels (Hess et al., 1993), and under these conditions incorporates  $65 \pm 11\%$  less label in the presence of TM (lane 2). The labeled protein migrating just above the 27.5 kD standard in both DRG and PC12 cells (arrowheads) has been identified as SNAP-25 by two-dimensional gel analysis (Hess et al., 1993 and above), and its labeling is inhibited by  $57 \pm 15\%$  in DRG neurons and, in the experiment shown here, by 47% in PC12 cells.

acyltransferase(s) involved in posttranslational palmitoylation.

As in the acyltransferase preparation, TM inhibition of protein palmitoylation in intact cells was dose dependent, although an equivalent inhibition of palmitoylation required a lower dose of TM in intact cells than in the cell-free system (compare Figs. 1 C and 3 C). This may reflect the low intracellular levels of palmitoyl-CoA in intact cells (Fig. 3 A), compared with the substantial amount of palmitoyl-CoA added to the cell-free acyltransferase preparation.

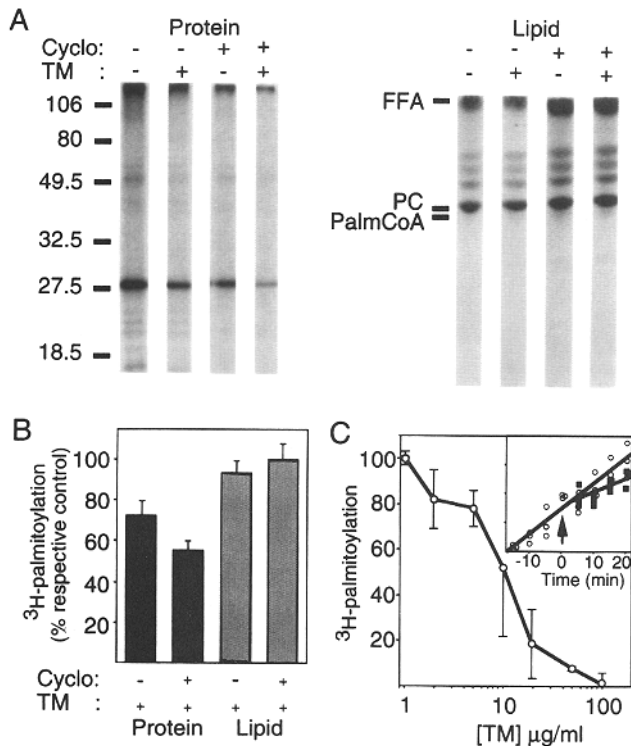
Routine quantitation of TM effects on protein palmitoylation was carried out with 60 min exposure of cells to  $^3\text{H}$ -palmitate to permit uptake and conversion of labeled palmitic acid to palmitoyl-CoA and subsequent incorporation of sufficient label for reliable quantitation. The use of shorter time points resulted in low and variable levels of protein labeling. Nevertheless, we attempted to estimate how rapidly TM was able to exert its inhibitory effect on protein palmitoylation in intact cells. Cells were pre-labeled with  $^3\text{H}$ -palmitate for 20 min, and then labeling was continued in the presence or absence of  $10 \mu\text{g/ml}$  TM for an additional 20 min. In the absence of TM, incorporation of  $^3\text{H}$ -palmitate was linear throughout the 40-min labeling period. Best-fit regression analysis indicates, however, that the rate of incorporation in the presence of TM was reduced by 49% over the 20 min period following addition to intact cells (Fig. 3 C, inset). This reduced rate of palmitoylation at shorter times after TM addition is comparable with the 45% inhibition observed after a standard 60-min incubation (Fig. 3 B). A 49% reduction in palmitoylation over the first 20 min after TM addition indicates that palmitate incorporation must have been inhibited for the majority of the application period. Extrapolation of the best-fit regression curve sug-

gests that the rate of palmitate incorporation may decrease within 5 min after addition of TM to the medium. Thus, while difficulty in quantifying  $^3\text{H}$ -palmitate incorporation after short labeling intervals prevents a precise estimate, TM appears to produce a reduction in protein palmitoylation within 5–10 min of application to cultures of intact cells.

### TM Rapidly Collapses Neuronal Growth Cones

The ability of TM to inhibit posttranslational protein palmitoylation in intact cells makes it a useful reagent for exploring the roles of protein fatty acylation in normal growth cone functions. In culture, the active, motile growth cones of differentiated PC12 cells (Okabe and Hirokawa, 1991) can be followed individually using time-lapse video microscopy (Fig. 4). Within 10 min after addition of TM to such cultures, growth cones ceased to grow forward and “collapsed” to form rounded retraction profiles that are brighter in both modulation-contrast and phase-contrast optics, often with the appearance of multiple varicosities along the neurite shaft. In the continuous presence of TM the neurite would subsequently retract (Fig. 4 A); however, washout of TM resulted in a rapid return of forward mobility (Fig. 4 B).

This rapid growth cone collapse and impairment of neurite elongation in response to TM should be distinguished from a much later (1–6 d) effect of TM on neurite extension, which has been attributed to a prolonged blockade of protein glycosylation (Heacock, 1982; Richter-Landsberg and Duksin, 1983). In addition to its much slower time course, the late inhibition of neurite growth was observed in culture medium containing serum. Previous studies in retinal neurons and non-neuronal cells indicate that TM inhibition of N-linked glycosylation may become apparent only after a lag



**Figure 3.** TM selectively inhibits posttranslational protein palmitoylation in differentiated PC-12 cells. (A) Representative fluorograms of protein and lipid labeling in the presence or absence of 10  $\mu\text{g/ml}$  TM. The posttranslational component of labeling was measured in the presence of 100  $\mu\text{g/ml}$  cycloheximide (*cyclo*). Cells were preincubated with the indicated additions for 60 min before the addition of 3  $\mu\text{M}$  [ $^3\text{H}$ ]palmitate for a further 60 min. Proteins were separated by 12% SDS-PAGE (*left panel*), and lipids were analyzed by thin-layer chromatography (*right panel*). The migration positions of free fatty acid (FFA), phosphatidylcholine (PC), and palmitoyl-CoA (*Palm-CoA*) standards are indicated on the TLC fluorogram. (B) Quantitation of the effect of TM on the incorporation of  $^3\text{H}$ -palmitate into protein (*solid bar*) and lipid (*shaded bar*). The left bar of each pair represents TM inhibition of total cellular incorporation, and the right bar represents inhibition of labeling measured in the presence of cycloheximide. Each bar represents labeling in the presence of 10  $\mu\text{g/ml}$  TM, expressed as a percentage of labeling in corresponding cultures in the absence of TM. Labeling of SNAP-25, the prominent substrate protein migrating close to the 27.5 kD standard was used for quantitation of protein palmitoylation in this and all following experiments with PC12 cells. Label co-migrating with the phosphatidylcholine standard was used to quantitate incorporation of fatty acid into lipid. Results are the means  $\pm$  standard error for seven (protein) or four (lipid) independent experiments. (C) Dose dependency and time course of TM inhibition of protein palmitoylation. The dose dependency of TM inhibition is expressed as a percent of control labeling (no TM). Results are the means  $\pm$  SD for four independent experiments, and were obtained in the absence of cycloheximide. The inset illustrates the time-course of onset of TM inhibition of palmitoylation in the presence of cycloheximide. A series of cell cultures were labeled with [ $^3\text{H}$ ]palmitate for up to 40 min (*open circles*); at 20 min, 10  $\mu\text{g/ml}$  TM was added to some cultures (*arrow*) and labeling was then continued for an additional 20 min (*closed squares*). Triplicate cultures were removed at 5-min intervals for analysis of palmitate incorporation into protein, and the results are plotted in arbitrary densitometry units. Solid lines indicate the best-fit least-squares regression lines for labeling in the presence and absence of TM ( $r = 0.94$  and  $0.66$  for control and TM treated respectively, Statview II, Abacus Concepts Inc.).

of 30 min or more (Heacock, 1982; Takatsuki and Tamura, 1971), and the effect of TM on glycosylation is maintained in the presence of serum and for a long period after removal of TM (Fichard et al., 1990). In contrast, the rapid collapse of growth cones and cessation of neurite extension, within minutes of TM addition, requires serum-free medium and is readily reversed by washout of TM or by addition of serum in the continued presence of TM (Fig. 5). The rapid inhibition of ongoing protein palmitoylation offers one plausible mechanism for the rapid and reversible disruption of growth cones functions by TM.

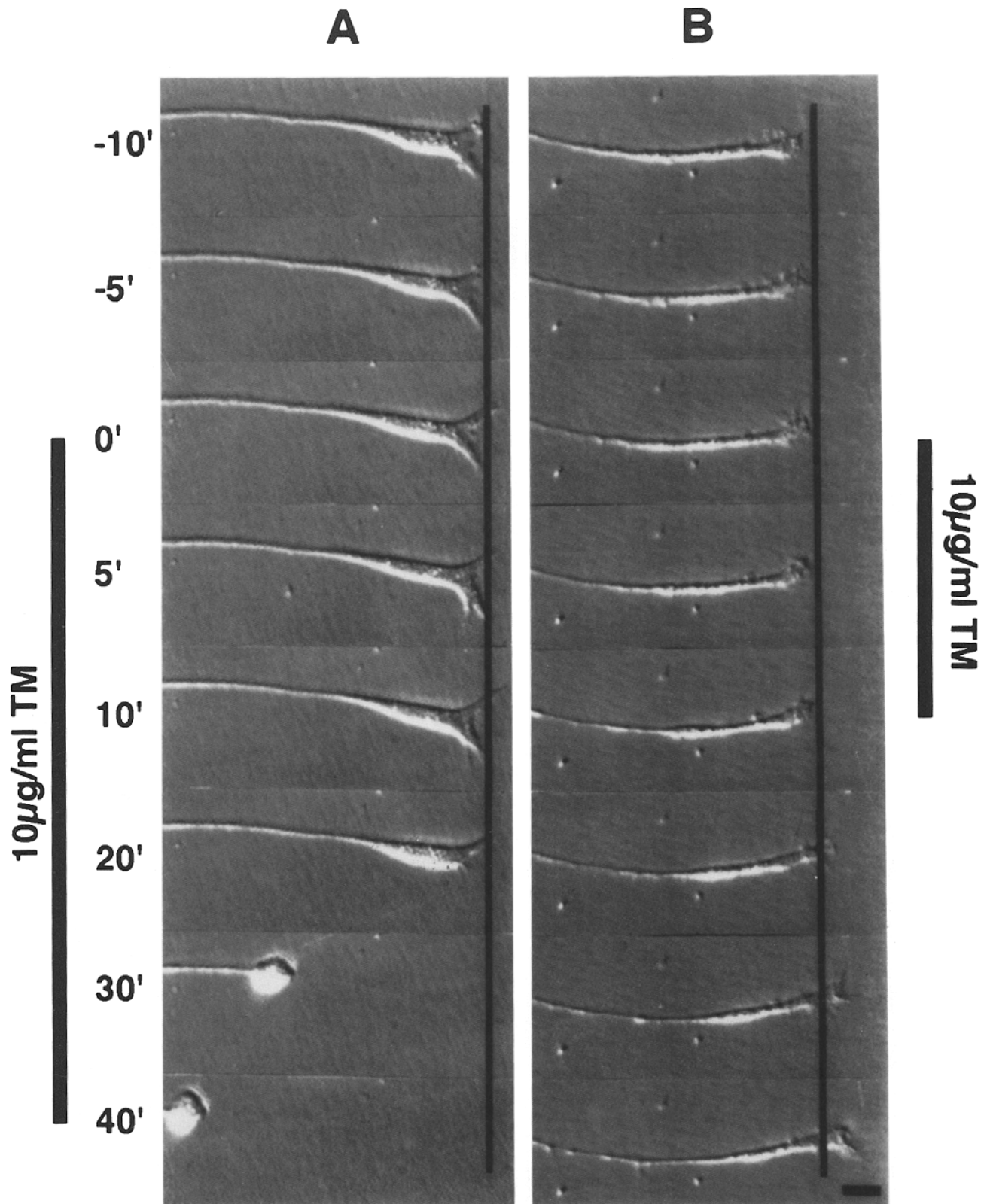
### TM Inhibition of Palmitoylation Can Be Distinguished from Effects on Glycosylation

It would be useful to have more definitive criteria to assess whether growth cone collapse and cessation of neurite elongation after TM treatment are due to the inhibition of protein acylation. The hypothesis that TM acts as a competitive analog of long-chain fatty acyl-CoA (Fig. 1 A) predicts two properties that could be used to distinguish TM inhibition of protein palmitoylation from other actions of the drug, including its established actions on protein glycosylation.

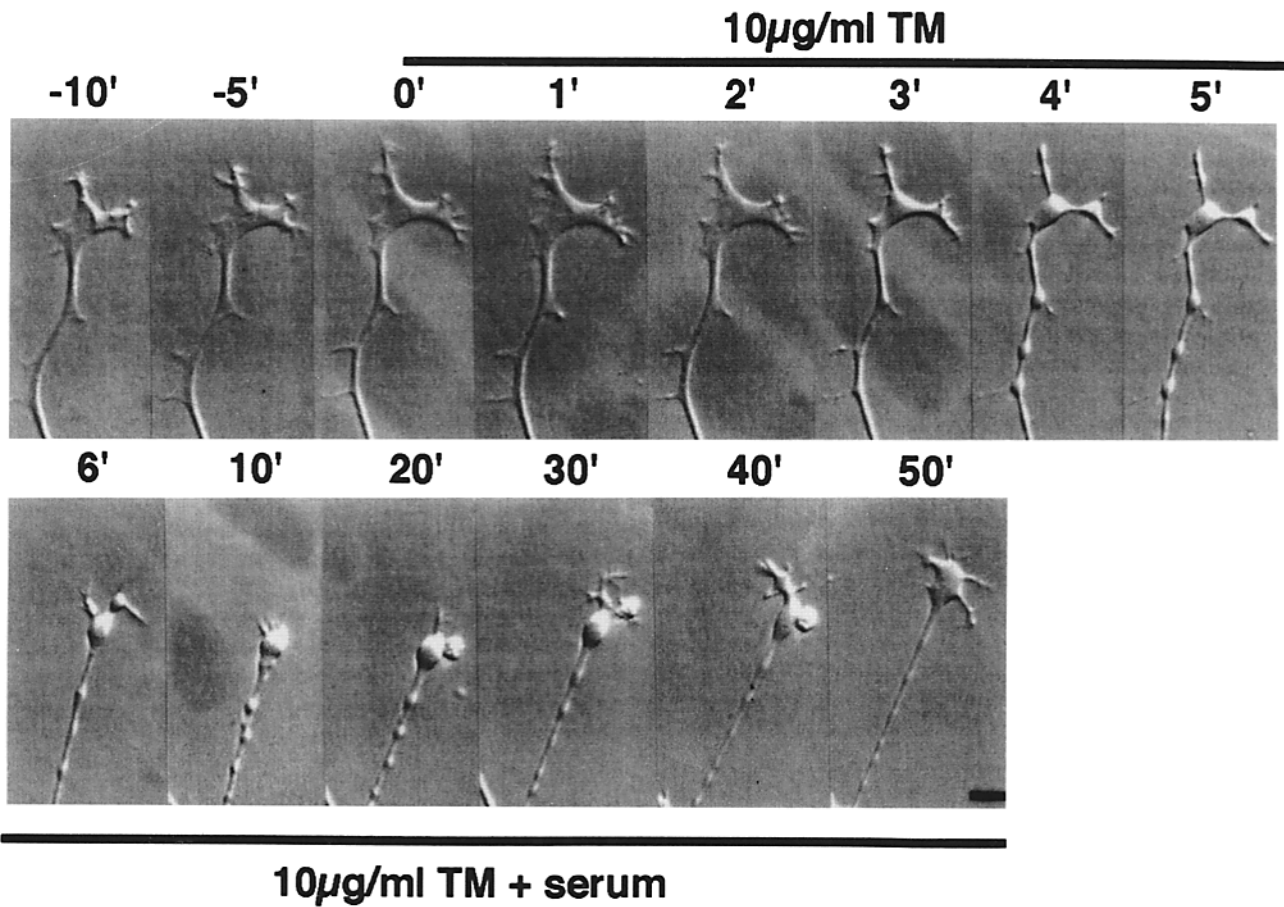
The first distinction is based on the existence of a series of closely related TM homologues. These homologues all share the general structure illustrated in Fig. 1 A, except that each homologue contains a different fatty acyl side-chain. Fractionation of TM by side-chain length produces four families of homologues, with hydrocarbon chains ranging from 14 carbons (TM-A) to 17 carbons (TM-D). Each family contains several homologues that vary in the degree of saturation and branching of the hydrocarbon chain (Mahoney and Duksin, 1980; Takatsuki et al., 1979). Because posttranslational protein acylation exhibits a strong selectivity for fatty acids containing 16 or more carbon atoms (Buss et al., 1984; Grand, 1989; James and Olson, 1990; Olson et al., 1985; Schmidt and Burns, 1989; Wilcox and Olson, 1987), the shorter-chain homologues of TM would be expected to be poor competitive inhibitors of protein acylation. We therefore separated commercial preparations of TM into the constituent homologue families (Mahoney and Duksin, 1980, and see Fig. 9 B) and compared the ability of each fraction to inhibit protein acylation and to produce growth cone collapse. As predicted, the longer-chain fractions TM-C (16-carbon side-chains) and TM-D (17-carbon side-chains), were much more potent inhibitors of protein palmitoylation than the shorter chain families, A and B (14- and 15-carbon side-chains, respectively; Fig. 6 A).

If TM inhibition of protein palmitoylation is due to competition with the acyl-CoA donor, it also should be possible to reverse that inhibition by increasing the local intracellular concentration of palmitoyl-CoA. Although exogenous palmitoyl-CoA does not cross cell membranes, we attempted to increase intracellular palmitoyl-CoA levels by adding a large amount of exogenous palmitic acid. PC12 cells were treated with 10  $\mu\text{g/ml}$  TM in the presence of 1–100  $\mu\text{M}$  palmitate (Fig. 6 B). The higher doses of fatty acid significantly attenuated the inhibition of protein palmitoylation produced by TM ( $P < 0.05$ , 1 vs. 100  $\mu\text{M}$  palmitate). TM inhibition of protein glycosylation was unaffected under these conditions ( $P > 0.05$ ), indicating that the higher concentrations of exogenous fatty acid did not interfere with cellular uptake of TM.

These biochemical data show that selective inhibition of

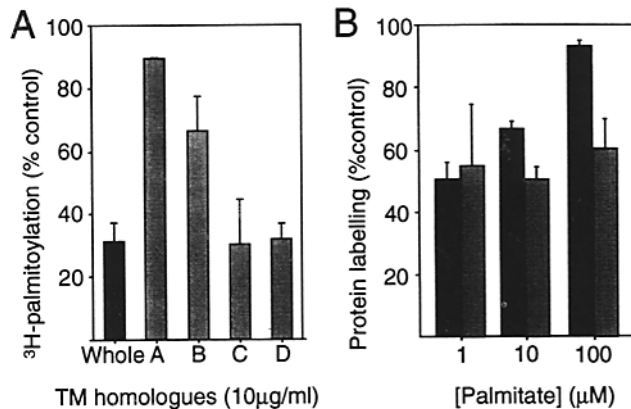


**Figure 4.** TM causes a rapid and reversible collapse and retraction of growth cones. Differentiated PC12 cell growth cones were followed by time lapse video microscopy before and after treatment with 10  $\mu\text{g/ml}$  TM (added at time 0'). (A) In the continuous presence of TM, forward growth quickly stops, followed by collapse of the growth cone to a bright rounded retraction profile and physical withdrawal of the growth cone over a substantial distance. (B) A 10-min treatment of the growth cone prevents forward growth and initiates the rounding up before collapse, but washout of the drug (at time 10') results in a rapid recovery of morphology and forward growth. Bar, 10  $\mu\text{m}$ .



### 10 μg/ml TM + serum

**Figure 5.** The rapid collapsing effects of TM are quickly reversed by addition of serum. A differentiated PC12 cell growth cone was treated with 10 μg/ml TM at time 0', followed by addition of 10% horse serum at 5 min. Between 6 and 10 min after the addition of TM the growth cone retracted substantially, requiring a frame shift between these images. By 15 min after the addition of serum, the growth cone is clearly re-establishing itself, and grows forward rapidly over the next 30 min in the continuous presence of TM. Bar, 10 μm.

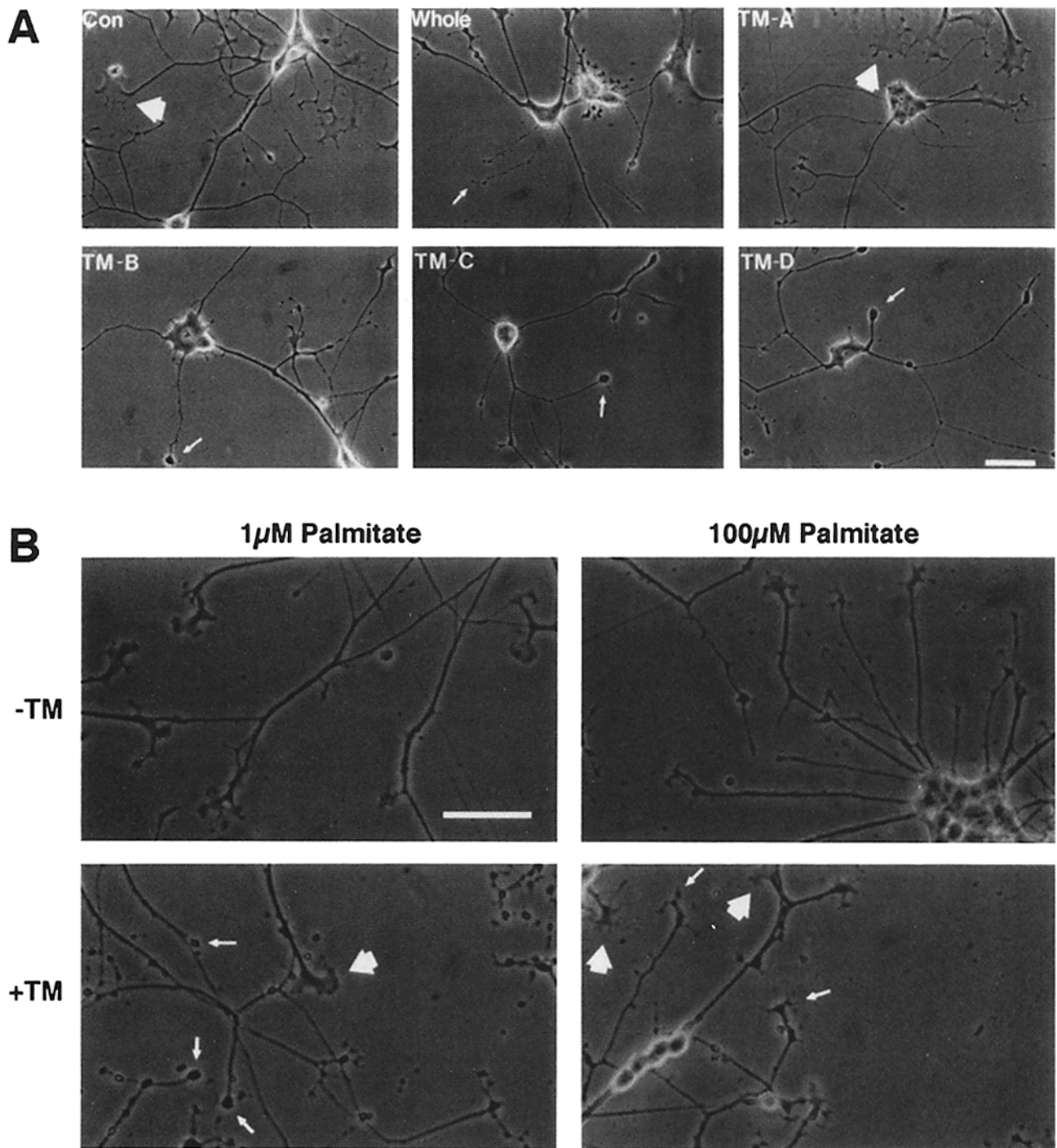


**Figure 6.** TM effects on protein palmitoylation can be distinguished from inhibition of glycosylation. (A) Structural dependence of TM inhibition. PC12 cells were labeled with [<sup>3</sup>H]palmitate in the presence of whole TM or TM homologues containing fatty acyl side chains of 14 (A), 15 (B), 16 (C), or 17 (D) carbons. Protein palmitoylation is expressed as a percentage of the labeling in vehicle-treated control cultures. Results are the means ± range for two independent experiments. (B) Reversal by exogenous fatty acid. Incorporation of [<sup>3</sup>H]palmitate (solid bars) and <sup>3</sup>H-mannose (shaded bars) into PC12 cell proteins was measured in the presence

protein acylation by long-chain TM homologues, and reversal of this inhibition by exogenous palmitate, provide two criteria that can be used to distinguish cellular effects attributable to disruption of protein palmitoylation from those due to inhibition of glycosylation. Still photography of living cultures allowed us to evaluate the morphological responses of growth cones to TM in the same PC12 cell cultures used for the biochemical analyses. With regard to both criteria, morphological growth cone collapse was closely correlated with the inhibition of protein palmitoylation (Fig. 7). The short-chain A series of TM homologues, which include potent glycosylation inhibitors (Duksin and Mahoney, 1982), evoked no apparent growth cone collapse (Fig. 7 A); the B series of homologues produced less extensive morphological

and absence of TM (10 μg/ml) at three concentrations of palmitic acid. Protein labeling in the presence of TM is expressed as a percentage of control labeling at the same concentration of palmitic acid. TM inhibition of palmitoylation is largely reversed by increasing concentrations of exogenous palmitate, while the inhibition of glycosylation is not statistically different at different palmitate concentrations. Results are means ± SD for three independent experiments.





**Figure 7.** Collapse of PC12 cell growth cones is selective for long-chain homologues of TM, and is reversed by palmitate. (A) Long chain TM homologue families selectively collapse growth cones. Phase-contrast photographs taken 15 min after the addition of vehicle (NaOH, final concentration 0.5 mM, Con) or the individual TM fractions (10 μg/ml) to cultures in one of the experiments quantitated in Fig. 6 A. Large arrows mark characteristic growth cone profiles, which are dark and spread in the absence of TM, but form characteristic phase-bright collapsed profiles after addition of TM (small arrows). Note the increased appearance of collapsed growth cones with the longer-chain TM homologues, and the appearance of varicosed processes after treatment with TM-C and D. (B) Exogenous palmitic acid attenuates the effect of TM on growth cone morphology. Cells from cultures quantitated in Fig. 6 B were treated without (top) or with (bottom) 10 μg/ml TM in the presence of 1 μM (left) or 100 μM (right) palmitate, and photographed after 15 min. The overall occurrence of TM-collapsed growth cones (small arrows) was reduced in the presence of the higher concentration of palmitate, and more spread growth cones (large arrows) were observed. There is also a more frequent occurrence of varicosed processes after treatment with TM in the presence of the lower concentration of palmitate. Bars, 25 μm.

responses than unfractionated TM. In contrast, the long-chain C and D series of homologues, which were the most potent inhibitors of protein palmitoylation, elicited a more pronounced collapse of PC12 cell growth cones than unfractionated TM applied at the same concentration (10  $\mu\text{g}/\text{ml}$ , Fig. 7 A).

Exogenously applied palmitic acid also appeared to attenuate growth cone collapse induced by TM (Fig. 7 B), despite an increase in collapse evoked by high concentrations of exogenous fatty acid alone. Counts of rounded or collapsed growth cone profiles in PC12 cell cultures showed that, at a concentration of only 1  $\mu\text{M}$  exogenous palmitate, TM increased the frequency of collapsed growth cones from 7% in the absence of TM to 93% 15 min after addition of 10  $\mu\text{g}/\text{ml}$  TM. When the same concentration of TM was added in the presence of 100  $\mu\text{M}$  palmitic acid, only 59% of growth cones exhibited a collapsed morphology. This attenuation of TM-induced growth cone collapse occurred despite some adverse responses of growth cones to high concentrations of exogenous fatty acid alone (36% collapsed in the absence of TM).

These initial observations of PC12 cells indicate that TM-induced growth cone collapse can be dissociated from TM effects on glycosylation, but is tightly linked to the inhibition of protein palmitoylation. This in turn suggests that dynamic protein palmitoylation is required for some steps in growth cone activity and, by implication, in neurite elongation.

#### *TM Impairs Axon Regeneration by Primary Neurons*

To explore more fully the potential role of dynamic protein palmitoylation in neurite growth, we used time-lapse video analysis of DRG neurons *in vitro*. Despite their utility for biochemical studies, PC12 cells do not adequately reflect some biologically important forms of axon elongation. When DRG neurons are axotomized to elicit axon regeneration *in vivo* and are subsequently explanted to tissue culture, the cultured neurons uniformly exhibit a rapid regrowth of neurites that can extend for several millimeters *in vitro*. The elongation rate and lengths of these neurites resembles the regeneration of axons *in vivo*, which is accompanied by induction of the major palmitoylated growth cone protein, GAP-43 (Basi et al., 1987; Hoffman, 1989; Schreyer and Skene, 1991) and a partial reduction in expression of SNAP-25 (Redshaw and Bisby, 1985). Thus, cultured adult DRG neurons more accurately reflect aspects of axon growth required for *de novo* pathway formation and nerve regeneration *in vivo*, and their rapid growth *in vitro* allows a more rapid and unambiguous quantitation of effects on growth cone function than with PC12 cells.

We used time-lapse video microscopy to select vigorously extending growth cones in DRG cultures and to examine the effects of TM on their morphology and growth. These growth cones responded to addition of TM with rapid morphological collapse, cessation of forward growth, and retraction, followed by morphological and functional recovery upon washout of the drug (Fig. 8 A). Movements of growth cone filopodia and the rapid, bidirectional transport of vesicles within the regenerating neurites (Hollenbeck and Bray, 1987) continued at concentrations of TM required to bring about growth cone collapse.

At a given concentration of TM, DRG growth cones typically respond more quickly than those of PC12 cells. This

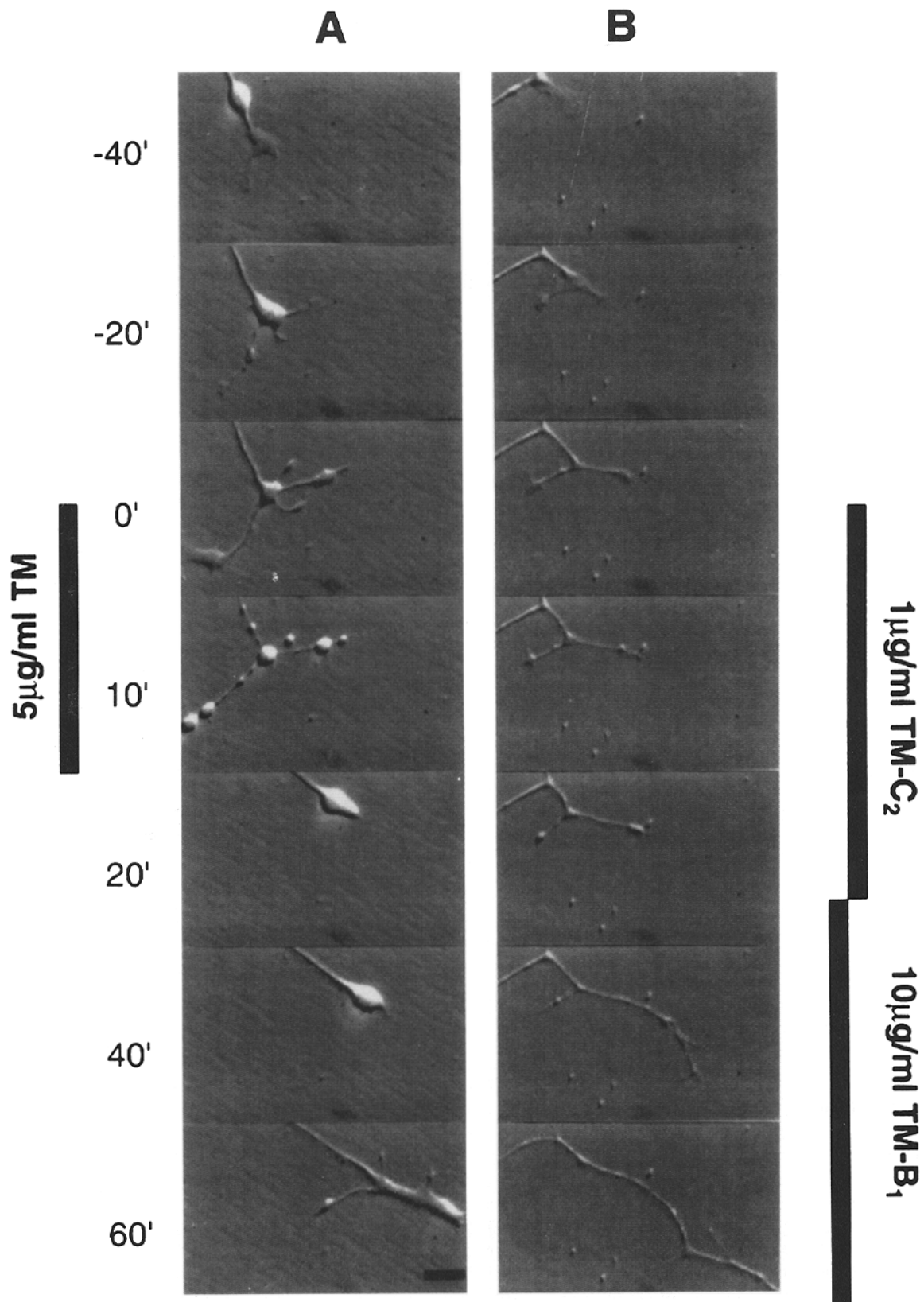
may indicate that the growth cones of more rapidly elongating neurites are more susceptible to the effects of TM. Responses to TM were apparent within 3–5 min of application, even in growth cones up to 2.4 mm from the neuron cell bodies, at the ends of the longest DRG neurites examined. This suggests that the effects of TM are unlikely to be due to disruption of protein synthesis or glycosylation in the neuron cell bodies. Furthermore, DRG growth cone morphology and neurite extension were maintained for at least 2 h in the presence of cycloheximide (Fig. 8 B and additional data not shown) to inhibit protein synthesis and thus glycosylation. Both the TM-induced collapse of growth cones and subsequent recovery can occur in the continuous presence of cycloheximide (Fig. 8 B) and are thus independent of *de novo* protein synthesis.

#### *Requirement for Dynamic Protein Palmitoylation in Axon Regeneration*

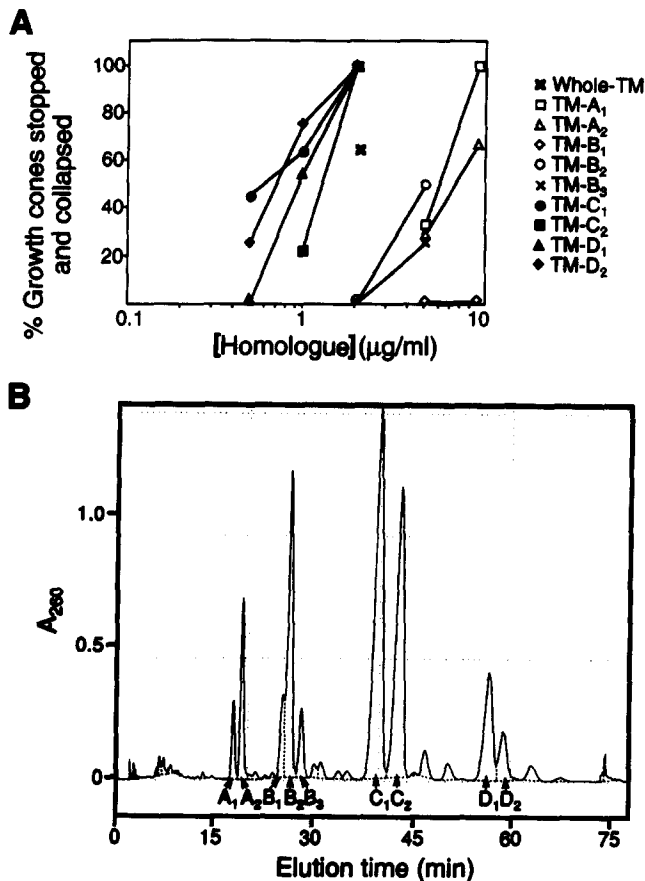
Late posttranslational protein palmitoylation has been measured in DRG neurons, and the two most prominent proteins labeled by incorporation of [ $^3\text{H}$ ]-palmitate have been identified on two-dimensional gels as GAP-43 and SNAP-25 (Hess et al., 1993). Due to the small number of cells in primary DRG cultures compared with PC12 cultures, extensive biochemical characterization of the TM effect on DRG neurons has not been carried out. Palmitoylation of both major substrate proteins, however, is strongly inhibited by 10  $\mu\text{g}/\text{ml}$  TM (Fig. 2) in either the presence or absence of cycloheximide (data not shown). To determine whether the inhibition of ongoing protein palmitoylation is responsible for the disruption of axon regeneration by TM, we explored the structural dependence of the growth cone responses and their reversal by exogenous palmitate.

Time-lapse video microscopy of DRG neurites permitted us to observe the morphologies of individual DRG growth cones and to evaluate individual neurite elongation before and after application of TM. For structure: function analysis, all TM homologues of known structure were screened at different concentrations for their ability to impair growth cone activity. In these assays, each growth cone that stopped elongating after application of TM also exhibited morphological collapse. However, at sub-maximal concentrations of each homologue, we often observed signs of morphological collapse in growth cones that continued elongating during the 10–20-min assay period. To compare the efficacy of individual TM homologues on growth cone activity, we expressed the potency of each homologue as the percent of active growth cones that both collapsed and ceased forward growth in the 10–20 min period immediately following application at different concentrations (Fig. 9 A). The potencies of the individual homologues sorted according to acyl chain length, with relatively little variation among individual members of each family. The long-chain C and D homologues were, on average, an order of magnitude more potent than the shorter-chain A and B homologues. Whole TM, containing a mixture of all homologues (see Fig. 9 B), gave an intermediate effect in this assay (Fig. 9 A).

The structure: function relationship of these TM homologues with regard to growth cone inhibition is clearly distinct from that described previously for protein glycosylation and synthesis. In particular, inhibition of glycosylation and protein synthesis exhibit no systematic variation with chain



**Figure 8.** TM causes a rapid, reversible collapse of regenerative DRG growth cones, even in the absence of protein synthesis. (A) Addition of 5  $\mu\text{g/ml}$  TM (at time 0') to cultures of adult DRG neurons causes rapid collapse and retraction. After 13 min the TM is washed out. After retracting further in the ensuing few minutes, the growth cone re-establishes and extends at a rate comparable with that observed before TM treatment. (B) In the continuous presence of 100  $\mu\text{g/ml}$  cycloheximide to inhibit protein synthesis, a DRG growth cone extends actively until addition of TM. Upon treatment with 1  $\mu\text{g/ml}$  TM-C<sub>2</sub>, the rapid extension of this growth cone is arrested for 20 min. When the C<sub>2</sub> homologue is replaced by a shorter-chain homologue (B<sub>1</sub>) at much higher concentration (10  $\mu\text{g/ml}$ ), the growth cone recovers and rapidly extends for at least 40 min. Bar, 10  $\mu\text{m}$ .



**Figure 9.** Potency of purified TM homologues on DRG growth cones sorts according to acyl chain length. (A) Actively extending DRG growth cones were followed by time-lapse video microscopy before and after treatment with purified TM homologues at concentrations between 0.5 and 10  $\mu\text{g/ml}$ . The percentage of growth cones exhibiting morphological collapse and cessation of forward growth at each concentration is plotted for each homologue. The data represent the results from 19 growth cones in seven separate cultures (whole-TM), 11 growth cones in six cultures (A<sub>1</sub>), 13 growth cones in eight cultures (A<sub>2</sub>), nine growth cones in five cultures (B<sub>1</sub>), eight growth cones in five cultures (B<sub>2</sub>), 24 growth cones in six cultures (B<sub>3</sub>), 26 growth cones in 11 cultures (C<sub>1</sub>), 19 growth cones in 11 cultures (C<sub>2</sub>), 25 growth cones in 10 cultures (D<sub>1</sub>), and 12 growth cones in 8 cultures (D<sub>2</sub>). (B) Representative preparative-HPLC trace of homologue separation (0.5 mg whole TM loaded), with previously identified homologues marked.

length (Duksin and Mahoney, 1982; Duksin et al., 1982). Furthermore, growth cones that have been collapsed by a long-chain TM homologue can recover in the presence of shorter-chain homologues and continue growing for several hours (Fig. 8 B and further data not shown). The structural requirements for TM effects on growth cone collapse, therefore, are well correlated with the predicted and observed order of potency for the inhibition of protein acylation, but not with the other known biochemical actions of these compounds.

To quantitate the ability of exogenous fatty acid to attenuate the effects of TM on DRG neurite extension, we used video microscopy to analyze the responses of a large number of DRG growth cones to TM in the presence or absence of exogenously applied palmitic acid. The frequency of sponta-

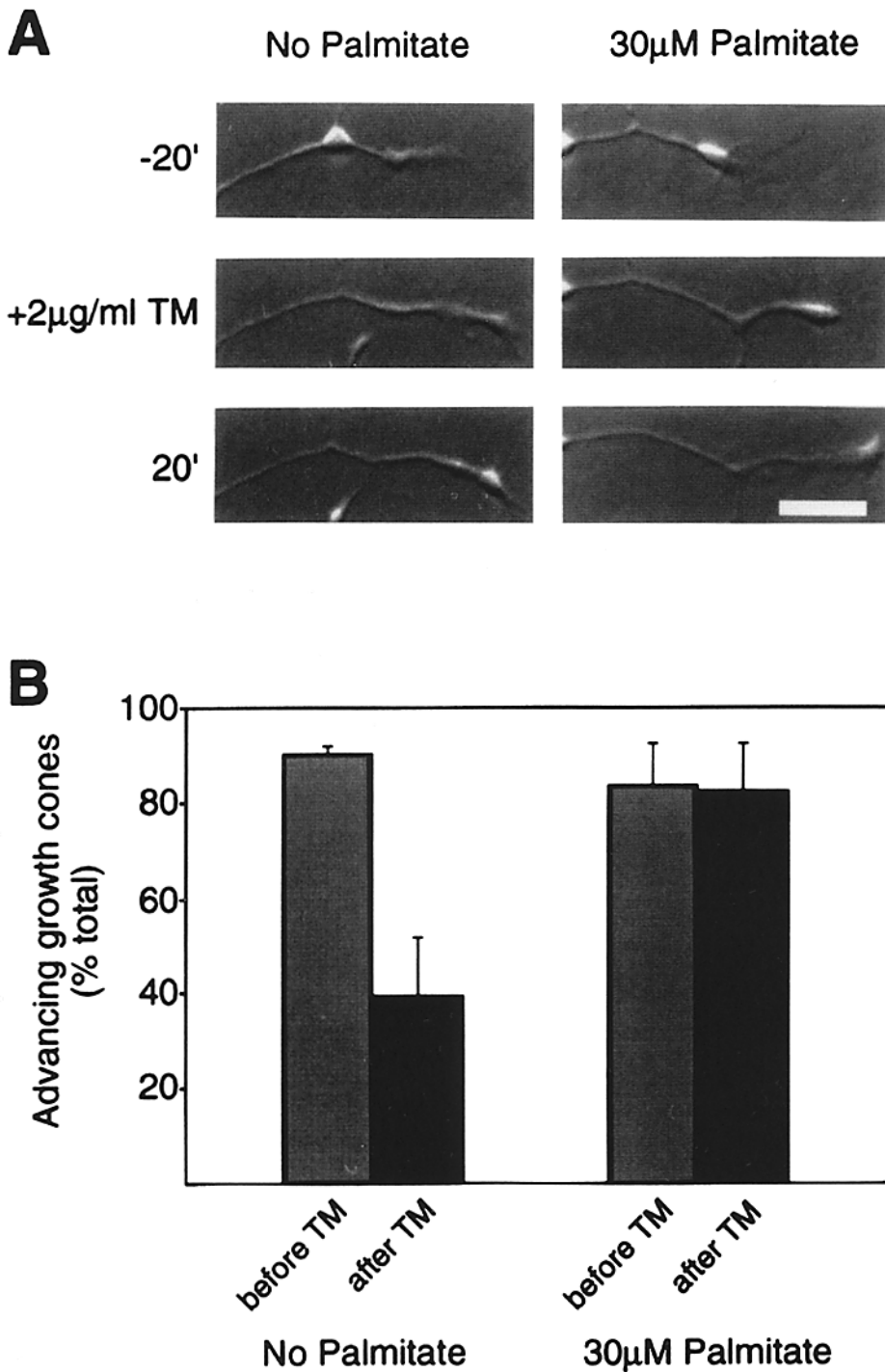
neous stopping was not statistically different in the presence or absence of exogenous palmitate. We used a dose of TM (2  $\mu\text{g/ml}$ ) which, in the absence of added palmitate, stopped growth in 60% of the neurites observed (Fig. 10). In the presence of 30  $\mu\text{M}$  palmitate, only 18% of growth cones stopped growing within 20 min after treatment with the same dose of TM. This is the same frequency at which growth cones exhibited spontaneous cessation of growth during a 20-min control observation period, before treatment with TM (Fig. 10).

## Discussion

The results show that some homologues of tunicamycin can inhibit the dynamic posttranslational fatty acylation of proteins in intact cells. The ability of TM to inhibit protein palmitoylation in a cell-free acyltransferase preparation shows that the inhibition of protein fatty acylation is a direct action of TM, and not a secondary consequence of the well-established effects of TM on N-linked glycosylation and protein synthesis. The precise mechanism by which TM homologues inhibit protein palmitoylation is not known, but the preferential inhibition by long-chain TM homologues, and the ability of excess palmitoyl-CoA to reverse this inhibition in a cell-free assay, are consistent with a model in which TM competes with fatty acyl-CoA substrates for binding to one or more protein acylating enzymes. Reversible inhibition of protein long-chain acylation establishes a new biochemical action of TM that can be exploited to investigate the biological roles of this dynamic protein modification in various eukaryotic cells.

In the past, TM has been used extensively to implicate protein glycosylation in a variety of viral and cellular functions. The present findings suggest that some TM-sensitive functions might profitably be reexamined to assess whether they reflect biological roles of protein palmitoylation rather than glycosylation. Similarly, TM may also prove to be a useful tool for exploring the functional sequelae of posttranslational palmitoylation of proteins such as ion channels and cell-surface receptors (Karnik et al., 1993; Mouillac et al., 1992; Schmidt and Catterall, 1987), heterotrimeric and small GTP-binding proteins (Adamson et al., 1992a; Kuroda et al., 1993; Magee et al., 1987; Parenti et al., 1993), Golgi and vesicular proteins (Hess et al., 1992; Mundy and Warren, 1992; Söllner et al., 1993; Südhof and Jahn, 1991), chloroplast photosystem components (Mattoo and Edelman, 1987), and other substrates in a variety of eukaryotic cells. Although the naturally occurring homologues of TM appear to provide a valuable set of cell-permeable probes for exploring the biological roles of protein fatty acylation, all known homologues of TM also inhibit N-linked glycosylation at the concentrations examined here. However, the rapidity of the inhibition, its preferential expression in the posttranslational component, the selective inhibition of palmitoylation by long-chain TM homologues, and its reversal by exogenous fatty acids, all provide an initial set of criteria to distinguish biological effects attributable to protein acylation from those due to glycosylation. The TM molecule, furthermore, may serve as a reference compound for the design of more selective inhibitors of protein long-chain acylation.

In neurons, TM produces a rapid and reversible collapse of growth cones and cessation of neurite extension. These



**Figure 10.** Exogenous fatty acid attenuates the effects of TM on DRG growth cones. (A) Representative video frames illustrating net growth of DRG neurites during 20 min periods before and after addition of 2  $\mu$ g/ml TM. In the absence of exogenous palmitate (*left*), a growth cone extends rapidly before the addition of TM, and ceases forward extension after addition of drug. A second growth cone growing in from the bottom of the frame also exhibits morphological collapse, but continued to extend in the presence of TM. In the presence of palmitate (*right*), a growth cone continues to extend after TM addition. (B) Quantitation of growth in the presence or absence of TM and palmitate. Net elongation of individual DRG neurites was recorded in the presence or absence of 30  $\mu$ M palmitate for 20-min periods before and after addition of 2  $\mu$ g/ml TM, as in A. The percentage of neurites exhibiting net elongation in each observation period is plotted. In the absence of exogenous palmitate (*left*), the addition of TM substantially reduced the number of elongating neurites ( $P < 0.002$ , Student's unpaired two-tailed  $t$ -test). In the presence of added palmitate, the same dose of TM failed to reduce the number of elongating neurites ( $P > 0.05$ ). The percentage of elongating neurites was determined in four separate cultures for each condition (24–77 neurites per culture), and the bars indicate the means  $\pm$  SD for the four experiments. A total of 159 and 166 growth cones were analyzed in the absence or presence of palmitate respectively. Bar, 10  $\mu$ m.

effects cannot be explained by the previously documented effects of TM on protein synthesis and glycosylation. Neither cycloheximide, an inhibitor of protein synthesis, nor treatment with short-chain homologues of tunicamycin, which include potent inhibitors of glycosylation, mimics the rapid effects of whole TM on neuronal growth cones. Furthermore, if growth cone collapse resulted from depletion of glycoproteins or other proteins required for elongation, recovery of function should require protein synthesis and glycosylation, which is tightly linked to protein synthesis (Elbein, 1984; Hemming, 1982). Yet growth cones that have

collapsed in response to TM are able to recover normal morphology and resume rapid neurite elongation in the continued presence of cycloheximide and short-chain homologues of TM. Finally, TM is an effective inhibitor of glycosylation in the presence of serum in many different cell types, including cultured neurons (Heacock, 1982) and neural tumor cell lines (Richter-Landsberg and Duksin, 1983), but the growth cone responses to TM are rapidly reversed by serum.

On the other hand, both the structural requirements and the sensitivity to exogenous fatty acid of the growth cone

reponses to TM are consistent with effects mediated by a competitive inhibition of protein palmitoylation. Structure: function analysis with 10 individual homologues of TM showed that the ability to alter growth cone activity depends on the length of the fatty acyl moiety of tunicamycin; TM homologues with side-chains of 16 carbons or more are potent inhibitors of neurite extension, while the shorter 14- or 15-carbon homologues are consistently less potent. This bimodal segregation is predicted for competitive inhibition of protein palmitoylation by TM, based on the reported ability of palmitoylating activities in a variety of cell types to recognize palmitoyl-CoA (16 carbon atoms) and a variety of other long-chain acyl-CoAs, but not myristoyl-CoA (14 carbon atoms). The systematic dependence of growth cone responses on the fatty acyl chain length of TM homologues also is well correlated with the observed inhibition of palmitoylation by the homologue families.

The most compelling evidence that growth cone collapse and retraction are due to TM inhibition of protein palmitoylation is the ability of exogenous fatty acid to attenuate or reverse these effects. Although the intracellular levels of free fatty acid and acyl-CoA are low and strongly buffered (Arduini et al., 1992; Molaparas-Saless et al., 1988), high concentrations of exogenous palmitic acid should produce at least a transient increase in intracellular palmitoyl-CoA, which is predicted to compete with TM for binding to protein fatty acyltransferases. Direct labeling studies in PC12 cells confirm that exogenous fatty acid can reverse TM inhibition of protein palmitoylation without preventing TM inhibition of protein glycosylation. Still photography of PC12 cells, and more quantitative video analysis of DRG growth cones, show that the disruption of growth cone functions by TM is largely or completely attenuated by exogenous palmitic acid. The ability of elevated fatty acid levels to reverse the TM inhibition of protein palmitoylation might also account for the reversibility of growth cone collapse in the presence of serum, which contains lipoproteins and free fatty acids.

The characteristics of TM inhibition of neuronal growth cone functions, therefore, are not explained by previously documented effects of TM on N-linked glycosylation and protein synthesis, but are consistent with the predicted and observed properties of TM-mediated inhibition of protein palmitoylation. The rapid collapse of growth cones and inhibition of neurite elongation when palmitoylation is inhibited implies that ongoing fatty acylation of growth cone proteins is important for one or more steps in normal growth cone functions and neurite extension.

Advancing growth cones mediate cycles of filopodial or lamellipodial extension, followed by consolidation of selected filopodia by the assembly of membrane and cytoskeletal elements transported from the neuronal soma. Time lapse observations evinced continued rapid axonal transport in neurites (data not shown) and active extension and retraction of filopodia during TM-induced collapse and retraction. This indicates that the effects of TM did not arise from a gross disruption of the supply of raw material for neuritic assembly, or from general disruption of actin-based motility. The rounding of growth cones and the formation of varicosities early in the response to TM are consistent with a disruption of membrane-cytoskeletal interactions involved in maintaining cell shape, or with a loss of adhesion to the substratum.

It is not clear from the present data which protein substrates for palmitoylation may be most important for normal growth cone activity and neurite elongation. The most prominent substrate for palmitoylation in isolated growth cones (Skene and Virag, 1989), and one of the most prominent substrates in intact DRG neurons (Hess et al., 1993), has been identified as GAP-43. GAP-43 is an abundant constituent of growth cones, transiently expressed in most neurons during developmental axon outgrowth and reinduced during nerve regeneration (Skene, 1989). The most prominent substrate for palmitoylation in PC12 cells, and a second major substrate in DRG neurons is SNAP-25, a component of mature synaptic terminals that also has been proposed to play an important role in late stages of axon growth and synaptogenesis during development (Osen-Sand et al., 1993; Oyler et al., 1991; Söllner et al., 1993). The prominence of these proteins, however, does not exclude the possibility that TM-induced growth cone collapse results from interference with dynamic palmitoylation of additional, less prominent substrates. Proteins known to be palmitoylated in different cell types include several other proteins involved in vesicle trafficking (Adamson et al., 1992a; Adamson et al., 1992b; Cheng and Reese, 1987; Hancock et al., 1990; Pfanner et al., 1989; Söllner et al., 1993; Südhof and Jahn, 1991); membrane receptors (Bourguignon et al., 1991; Moss et al., 1990; Wellner et al., 1987) and ion channels (Okubo et al., 1991; Schmidt and Catterall, 1987); and proteins involved in membrane-cytoskeleton interactions such as vinculin (Burn and Burger, 1987),  $\alpha$ -actinin (Burn et al., 1985; Sobue and Kanda, 1989), ankyrin (Bourguignon et al., 1991), p41 (Schneider et al., 1988), p55 (Ruff et al., 1991), and p21rac (Ridley et al., 1992; Newman and Magee, 1993). It is not known which of these or other substrates are actively palmitoylated during elongation of intact neuronal growth cones, but evidence for multiple palmitoylated substrates in other cells suggests that the contribution of dynamic protein palmitoylation to neurite growth may reflect pleiotropic actions of palmitoylating enzymes on several steps in growth cone advance.

An important role for ongoing protein palmitoylation in growth cone functions implies that the biochemical control of this modification, including extracellular signals that impinge on acylation (Huang, 1989; James and Olson, 1989; Jochen et al., 1991; Mouillac et al., 1992), would have a significant effect on nerve growth in development or regeneration. Although it is not yet clear how palmitoylation is regulated in vivo, growth cones are the targets for a broad array of cues in their local environment that control and direct axon elongation. Dynamic cycles of protein acylation and deacylation now can be viewed as one potential site of action of naturally occurring ligands proposed to guide or modulate axon extension through a mechanism that produces localized growth cone collapse (Caroni and Schwab, 1988; Harris et al., 1987; Keynes and Cook, 1990; Lohof et al., 1992; Raper and Grunewald, 1990; Sretavan and Reichardt, 1993; Walter et al., 1990). Thrombin, for example, which modulates cycles of protein palmitoylation and deacylation in platelets (Huang, 1989), is found in the developing brain (Dihanich et al., 1991) and has been reported to bring about the rapid and reversible collapse of neuronal growth cones (Jalink and Moolenaar, 1992; Suidan et al., 1992). Apolipoprotein E and lipoproteins containing this apoprotein also are extremely

abundant in the developing nervous system and in regenerating nerves, where they can direct the receptor-mediated uptake of free fatty acids and fatty acyl esters into growth cones (Block and Pletscher, 1988; Ignatius et al., 1987; Skene and Virag, 1989). ApoE-containing lipoproteins also have been shown to influence the rate, extent, and branching pattern of neurite outgrowth by DRG neurons (Handelmann et al., 1992). Finally, we have found that nitric oxide can interfere with protein palmitoylation and also produces a rapid and reversible collapse of neuronal growth cones (Hess et al., 1993). The ability of these molecules to influence protein palmitoylation may in some cases contribute to their regulatory influences on neurite growth.

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