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# Alteration of Axonal Microfilaments Alters Schwann Cell Lipid Metabolism

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## ABSTRACT

To investigate the importance of the neuronal cytoskeleton in Schwann cell metabolism, three agents acting on the microfilaments (cytochalasin D, brevine, and phalloïdin) were injected into the endoneurium of rat sciatic nerve. Sciatic nerves were removed 24 h later and separated into two pieces: the first one was the injection site and the second was from nerves located distal to the injection site. The pieces of nerve were incubated with [$^{14}$C] galactose for 3 h. At the site of injection, [$^{14}$C] incorporation into monogalactosyldiacylglycerol (MGDG) was perturbed by the three agents, whereas in the distal part, sulfatides and phosphatidylserine were affected by cytochalasin D and brevine. These results show that the three compounds acting on microfilaments have a different effect on Schwann cell metabolism, depending on whether incubated Schwann cells were directly in contact with the toxin or they were only in contact with the axons affected by the toxin. In the latter case, axonal microfilaments seem to be involved in the regulation of Schwann cell metabolism.

**Index Entries:** Microfilaments; myelin; Schwann cells; intercellular communication; cytochalasin D; brevine; phalloïdin.

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## INTRODUCTION

In the peripheral nervous system, the Schwann cells are responsible for the formation and maintenance of the myelin sheath. But the overriding dependence of the Schwann cell and its myelin sheath on an intact axon, demonstrated by the many studies on Wallerian degeneration, suggests the presence of some axonal signal. Intercellular communication in the peripheral nervous system has been demonstrated by experiments in vivo. Weinberg and Spencer (1976) cross-anastomosed myelinated fibers with nonmyelinated fibers and showed that axonal signals determine whether Schwann cells in a mature nerve will make myelin, thus demonstrating the existence of a signal between both types of cell.

Little is known about the axonal factor and/or the axonal subcellular structures involved in the maintenance of myelin. Some studies have shown the importance of retrogradely-transported trophic factors in myelin sheath maintenance (Heumann et al., 1987), and others studies using tissue culture have shown that axon-Schwann cell membrane contact is very important (Jessen et al., 1987; Mirsky et al., 1980; Poduslo, 1985; Griffin et al., 1987). Others studies (Grafstein and Forman, 1980) have demonstrated a role for axonal transport of factor through the axonal cytoskeleton. Our previous study showed an alteration of cerebroside turnover in Schwann cells after perturbation of axonal microtubules (Souyri et al., 1988). To test the involvement of axonal microfilaments in intercellular communication, we examined the effect of microfilament perturbation on lipid metabolism of Schwann cells. Three toxins were used for this purpose: two of them (cytochalasin D and brevine) shorten actin filaments in vivo and increase gel fluidity in vitro (Bryan and Kurth, 1984; Bader et al., 1986) and the third (phalloidin) rigidifies microfilaments in vivo and decreases actin gel fluidity in vitro (Frimmer, 1987). Cytochalasin D was chosen because it is the only cytochalasin that does not interfere with glucose metabolism (Prentki et al., 1979). It binds to F actin about five times more tightly than the other cytochalasins (Flanagan and Lin, 1980). Brevine is a 90 kD protein purified from chromaffin cells. It is not a toxin, but rather a regulatory protein requiring $Ca^{2+}$. It binds tightly to one end of the actin filaments, thus preventing their polymerization. Brevine resembles the cytochalasins by binding with high specificity to the end of the actin filament. Phalloïdin is a toxic bicyclic peptide produced by the mushroom, *Amanita phalloides*, whose major pharmacological action in mammals seems to be the formation of stable actin filaments by accelerating the rate of actin polymerization (Wehland et al., 1977).

## MATERIALS AND METHODS

### Materials

D-[U-$^{14}$C] galactose (210 mCi/mmol) was from CEA (Saclay, France); cytochalasin D, phalloïdin, and lipid standards were from Sigma

(France). All solvents were from Merck (Germany). Brevine was a gift from M. F. Bader (Strasbourg, France).

### *Animals*

Each experiment was performed at least three times with four female Sprague-Dawley rats (160–200 g). Animals were anesthetized with 200 μL of a mixture of 85% ketamine (Pharmacia, France) and 15% Largactil (Specia, France) by intraperitoneal injection.

Only one nerve per animal was used for this study, because intraneural injection can subtly affect the contralateral nerve lipid metabolism (Souyri and Bourre, 1989). The left sciatic nerve from each rat was injected 24 h before sacrifice with 1 μL of toxin: $10^{-5}M$ cytochalasin D in 0.1% dimethylsulfoxide (DMSO), 0.06 mg/mL brevine in sodium azide (10 m*M* TRIS + 2 m*M* EGTA + 0.2 m*M* DTT) + 1 m*M* $CaCl_2$; and 1 μg/μL phalloidin diluted in 0.1% ethanol (EtOH).

Because the putative toxins were not directly soluble in 0.9% NaCl, a small percentage of solvent was added: DMSO for Cytochalasin D, sodium azide for brevine, and ethanol for phalloïdin. The control animals used for each drug were injected with the same solvent in 0.9% NaCl. Endoneurial injection was performed with a micropipet 7.5 cm from, and directed toward, the spinal cord. Animals were sacrificed 24 h later and their left sciatic nerve removed, desheathed, and separated into two pieces, measuring 10 mm. The first one was from the injection site (located 7–8 cm from the spinal cord). The second corresponded to the part of the nerve located distal to the injection site (about 8 mm from injection site). All pieces were individually incubated at 37°C in modified Krebs (Pleasure and Towfighi, 1972) for 3 h with [$^{14}$C]galactose (5 μCi/nerve), pH adjusted to 7.4. At the end of the incubation, nerves were rinsed, homogenized, sonicated, and mixed with 15 vol. of chloroform/methanol 1/2 (v/v). After centrifugation (5000 rpm/30 min), the protein pellet was dissolved in 0.1*N* NaOH for counting and protein determination (Lowry et al., 1951). In the meantime, chloroform and water were added to the supernatant to obtain the Folch partition (Folch et al., 1957). The lower phase (total lipids) was analyzed by thin layer chromatography with a mixture of methyl acetate/chloroform/methanol/0.9% KCl (25/25/25/10/9), as previously described (Souyri et al., 1988). Spots were identified with standards, scraped into Ready Solv HP (Beckman), and counted with a Beckman scintillator counter. In some experiments, an aliquot of lipid extract was used for separation of galactolipids and glucolipids by thin layer chromatography using 1% borate-impregnated slides and chloroform/methanol/water/15*M* $NH_4OH$ (280/70/6/1) as developing solvent (Kean, 1966). Comparisons of two sets of data were made using *t*-tests, but comparisons of more than two sets of data were made using analysis of variance (Statistical Analysis System, Statistical Institute, Cary, NC).

## RESULTS

### *Incorporation of [$^{14}$C]Galactose into the Lipids of the Sciatic Nerve Injection Site*

In control nerves, $^{14}$C was preferentially incorporated into galactolipids (GL) and especially into hydroxycerebrosides. Of the total cerebrosides, 97% were galactocerebrosides (CerGal) and only 3% were glucocerebrosides, as revealed by borate thin layer chromatography. MGDG was radiolabeled. Comparison of incorporation of [$^{14}$C]galactose after intraneural injection of cytochalasin D, diluted with 0.1% DMSO, or after injection of 0.1% DMSO alone in saline (0.9%) as control showed the following: cytochalasin D increased total incorporation of radioactivity into the homogenate compared to DMSO, but did not affect the incorporation of radioactivity into the proteins or the total lipids (Table 1). Although cytochalasin D caused a significant decrease of radioactivity incorporated into MGDG ($0.001 < p < 0.01$), the labeling of phosphatidylserine (PS) and phosphatidylethanolamine (PE) ($p < 0.001$) increased by 255 and 287%, respectively (Fig. 1A).

Comparison of incorporation of [$^{14}$C]galactose after intraneural injection of brevine in sodium azide with $Ca^{2+}$ or after injection of sodium azide with $Ca^{2+}$ alone showed an increase of incorporation into proteins and total lipids (Table 1), but the only lipid significantly affected was MGDG, for which the labeling increased by 41%, $0.01 < p < 0.02$ (Fig. 1B).

Comparison of incorporation of [$^{14}$C]galactose into total lipids and proteins after intraneural injection of phalloïdin solubilized in 0.1% ethanol, or after injection of 0.1% ethanol alone, showed no difference between treated and control nerves, except for a decrease of radioactivity incorporated into MGDG (64%), $0.001 < p < 0.01$ (Fig. 1C).

### *Incorporation of [$^{14}$C]Galactose into Lipids After Incubation of the Distal Part of the Sciatic Nerve*

After cytochalasin D injection, radioactivity incorporation was increased in homogenate, total proteins (Table 2), neutral lipids (NL) and two lipids: sulfatides (Sul) (+128%, $0.01 < p < 0.02$) and phosphatidylserine (+221%, $p < 0.001$). After brevine injection, incorporation of radioactivity into total lipids, proteins (Table 2), neutral lipids, cerebrosides, and phosphatidylethanolamine decreased ($0.02 < p < 0.05$), but increased in phosphatidylserine (+242%, $0.001 < p < 0.01$) and sulfatides (+433%), compared with controls (Fig. 2B). Phalloïdin injection had no effect on incorporation of radioactivity into any of these fractions compared to control. (Table 2 and Fig. 2C).

Table 1
Incorporation of $^{14}C$ into Homogenate, Proteins, and Total Lipids of the Injection Site of Sciatic Nerve 24 h after Endoneurial Injection of Toxins[a]

| dpm/mg nerve | DMSO | CD + DMSO | SA | Brev + SA | EtoH | Phal. + EtOH |
|---|---|---|---|---|---|---|
| Homog. | 749,303 | 782,316[b] | 518,203 | 765,670 | 770,000 | 721,000 |
| Prot. | 44,716 | 38,736 | 21,257 | 27,540[c] | 50,589 | 53,066 |
| TL | 86,369 | 102,195 | 45,456 | 63,255[c] | 88,165 | 85,294 |
| GL/PL | 4.75 | 3.63 | 6.84 | 6.73 | 4.03 | 3.42 |
| GL/NL | 16.04 | 16.58 | 12.03 | 16.18 | 9.43 | 7.62 |

[a]DMSO: dimethylsulfoxide, CD: cytochalasin D; SA: Sodium Azide: Brev.: Brevine; EtOH: ethanol, Phal.: phalloidin; Homog.: homogenate; Prot.: proteins, TL: total lipids; GL: galactolipids; PL: phospholipids. Twenty-four hours after injection of the toxins (CD, Brev, or Phal.) or after injection of the toxins (CD, Brev., or Phal.) or after injection of the toxin solvents (DMSO, SA, or EtOH), sciatic nerves were removed at the site of injection and incubated 3 h with [$^{14}C$] galactose; then the radioactivity of each fraction (H, Prot., and TL) was counted.

[b]$0.01 < p < 0.02$.

[c]$0.001 < p < 0.01$.

## DISCUSSION

Intraneural injection of toxins acting on microfilaments (brevine, cytochalasin D, and phalloïdin) affected the incorporation of radioactivity into MGDG in Schwann cells at the injection site within 24 h. In contrast, except for phalloïdin-injected nerves, incorporation of radioactivity into sulfatides and phosphatidylserine by Schwann cells distal to the injection was increased, compared with control nerves.

[$^{14}C$]Galactose was chosen as a precursor because it preferentially labels cerebrosides, MGDG, and sulfatides, lipids that are specific for the myelin sheath. After a 3-h incubation, [$^{14}C$]galactose is incorporated mainly into the hydroxycerebrosides and MGDG in our experiment. These results are identical with those obtained by Yao and Poduslo (1988). The galactolipid-associated radioactivity is mainly in galactose. Nevertheless, galactose is also metabolized during the incubation, as demonstrated by the presence of radioactivity in molecules without galactose in their formula; for example, the phospholipids (PL) that incorporate a small amount of the total radioactivity.

For control values, we used the values obtained after injection of the toxin solvent, but these solvents themselves are liable to change nerve metabolism (Donoso et al., 1977; Tashiro and Komya, 1983). Indeed, injection of the solvent alone changes the incorporation of [$^{14}C$]galactose into lipids, compared with saline injection, but it would have been wrong to use as control normal nerves or saline-injected nerves. Nevertheless, we cannot rule out the possibility of interactions between solvent and

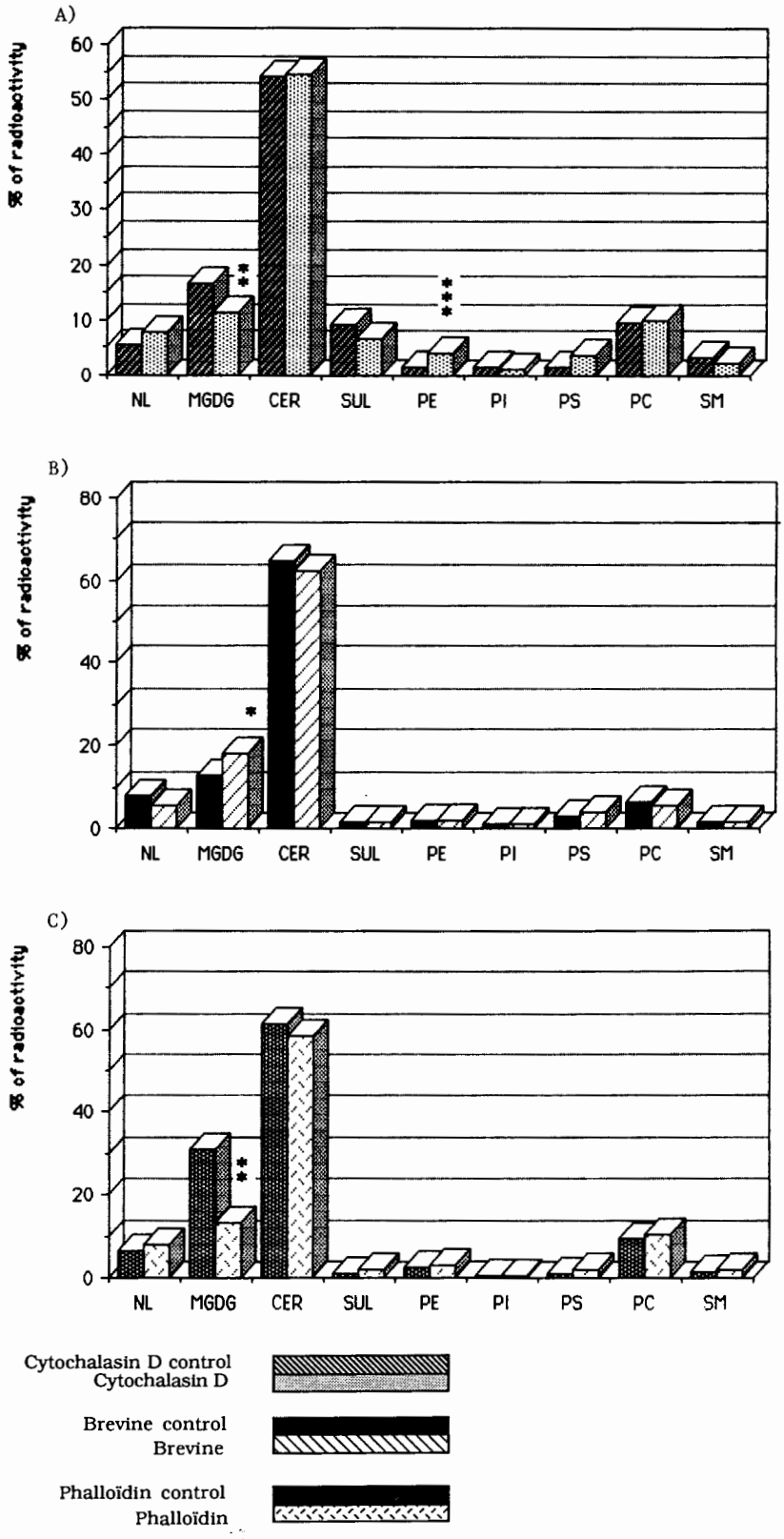

A)
% of radioactivity
60
50
40
30
20
10
0
NL
MGDG
CER
SUL
PE
PI
PS
PC
SM
B)
% of radioactivity
80
60
40
20
0
NL
MGDG
CER
SUL
PE
PI
PS
PC
SM
C)
% of radioactivity
80
60
40
20
0
NL
MGDG
CER
SUL
PE
PI
PS
PC
SM
Cytochalasin D control
Cytochalasin D
Brevine control
Brevine
Phalloïdin control
Phalloïdin

Table 2
Incorporation of $^{14}C$ into Homogenate, Proteins, and Lipid Extract of the Distal Part of the Sciatic Nerve 24 h after the Proximal Injection of Toxins[a]

| dpm/mg nerve | DMSO | CD + DMSO | SA | Brev + SA | EtOH | Phal. + EtOH |
|---|---|---|---|---|---|---|
| Homog. | 658,150 | 1,194,619 | 743,550 | 464,724 | 571,861 | 622,475 |
| Prot. | 44,096 | 100,348[b] | 46,844 | 21,377 | 41,460 | 40,772 |
| TL | 149,676 | 226,978 | 101,866 | 30,672[d] | 57,840 | 70,153 |
| GL/PL | 4.70 | 4.03 | 5.14 | 4.31 | 5.39 | 5.10 |
| GL/NL | 11.98 | 9.62 | 10.94 | 17.95 | 15.79 | 13.58 |

[a]DMSO: dimethylsulfoxide, CD: cytochalasin D; SA: sodium azide: Brev.: brevine; EtOH: ethanol; Phal.: phalloidin; Homog.: homogenate; Prot.: proteins, TL: total lipids; GL: galactolipids; PL: phospholipids. Twenty-four hours after injection of the toxins (CD, Brev, or Phal.) or after injection of the toxin solvents (DMSO, SA, or EtOH), the part of sciatic nerves distal to the injection was removed and incubated 3 h with [$^{14}C$]galactose. Then, the radioactivity of each fraction was counted.

[b]$0.01 < p < 0.02$.

[c]$0.001 < p < 0.01$.

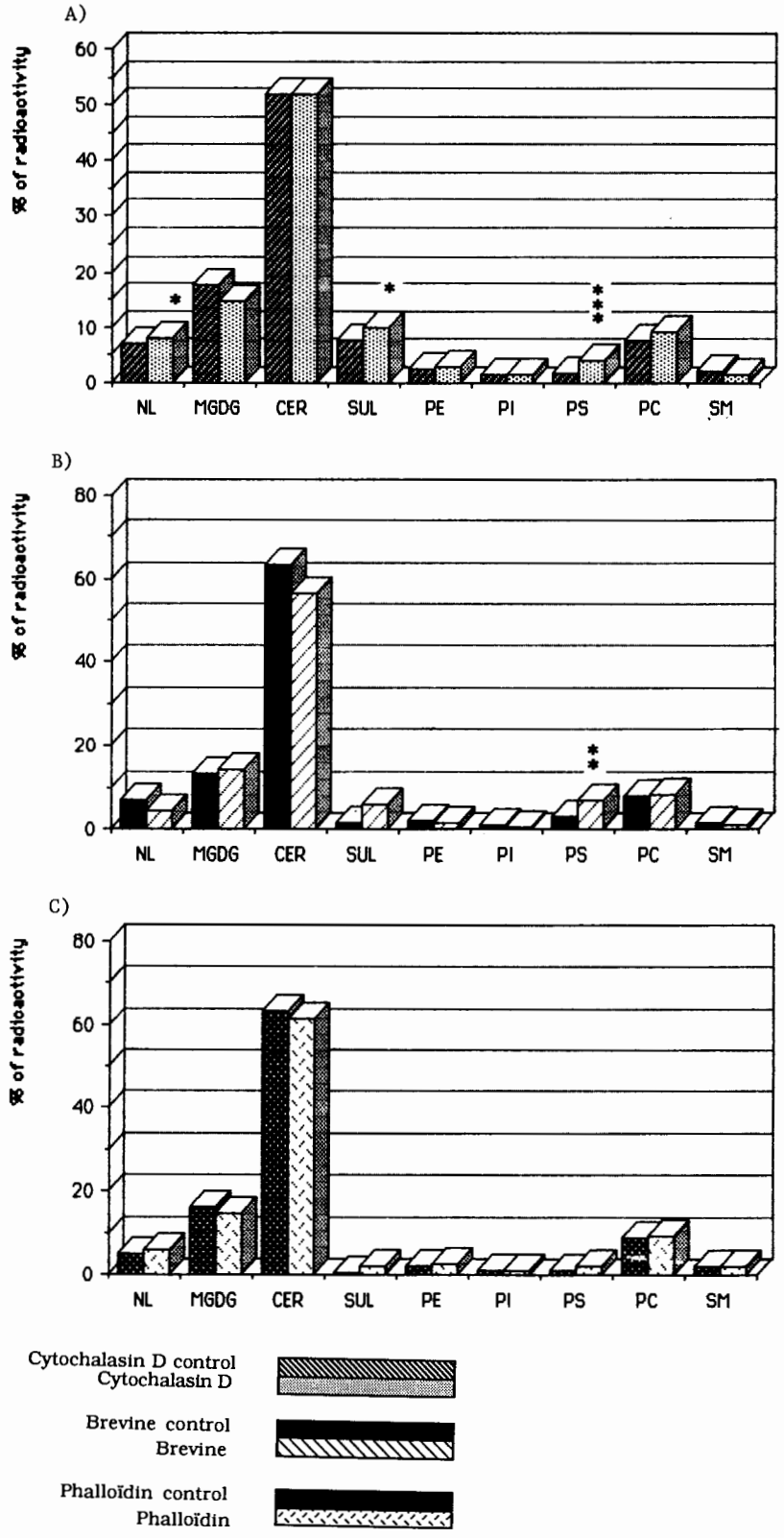
[d]$p < 0.001$.

toxin. After intraneural injection of toxin, the incubation of the proximal and distal parts is a simple model for studying both the direct effect of the toxins on Schwann cells and the indirect effect of the toxin through its action on the axons.

### *Results Obtained for the Injection Site*

In the part of the nerve directly in contact with the toxin, the only common metabolic alteration was the perturbation of the incorporation of [$^{14}C$]galactose into MGDG. Klein and Mandel (1976) have shown that MGDG accounts for 2.2% of lipid weight in rat sciatic nerve. The percentage of incorporation we found is quite considerable, which confirms the results of Yao (1986). This MGDG could be associated with myelination and the wrapping of the myelin sheath (Curatolo and Neuringer, 1986). Our experiment does not show whether this effect of the three drugs on

Fig. 1. Effect of cytochalasin D, brevine, and phalloidin on the labeling pattern of lipids in sciatic nerve injection site, shown as the percentage of [$^{14}C$]galactose incorporated into lipids after incubation for 3 h. All the toxins were injected intraneurally 24 h before incubation of nerves with the precursor. (A) Results after injection of $10^{-5}M$ cytochalasin D in 0.1% DMSO (in 0.9% NaCl); controls were injected with 1 μL 0.1% DMSO in 0.9% NaCl. (B) Results after injection of 0.06 mg/mL brevine in sodium azide + 1 m*M* $CaCl_2$; controls were injected with 1 μL sodium azide + 1 m*M* $CaCl_2$. (C) Results after injection of 1 μg/μL phalloidin in 0.1% ethanol (in 0.9% NaCl); controls were injected with 1 μL of 0.1% ethanol in 0.9% NaCl. Each experiment included four rats, and at least three experiments of the same schedule were performed (to-total tal number of animals >12). Values significantly different from controls are indicated: * = $0.01 < p < 0.02$; ** = $0.001 < p < 0.01$; *** = $p < 0.001$. Abbreviations used: NL: neutral lipids; MGDG; monogalactosyldiacylglycerol; Cer: cerebrosides; Sul: sulfatides; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin.


A)
% of radioactivity
60
50
40
30
20
10
0
NL
MGDG
CER
SUL
PE
PI
PS
PC
SM
B)
% of radioactivity
80
60
40
20
0
NL
MGDG
CER
SUL
PE
PI
PS
PC
SM
C)
% of radioactivity
80
60
40
20
0
NL
MGDG
CER
SUL
PE
PI
PS
PC
SM
Cytochalasin D control
Cytochalasin D
Brevine control
Brevine
Phalloïdin control
Phalloïdin

the incorporation of radioactivity into MGDG is owing to an effect of these toxins on the microfilaments of the Schwann cells or on the enzymes responsible for MGDG synthesis. Antonow et al. (1984) treated C-6 glial cells with cytochalasin D and showed an inhibition of CNPase, the myelin specific enzyme. They associated microfilaments with myelination. More recently, Wilson and Brophy (1988) showed interactions between CNPase and the cytoskeleton by western blotting. The cytochalasin D in our experiment could lead to a decrease of synthesis of the myelin constituents with an increase of the nonspecific lipids.

Tellam and Frieden (1982) showed that brevine and cytochalasin in vitro have the same effect on the microfilaments, but brevine (Bader et al., 1986) exists in the cells and regulates, with fodrin, the polymerization state of the microfilaments. Legrand et al. (1986) showed by immunocytochemical staining of the brain that neurons and astrocytes are devoid of brevine, whereas oligodendrocytes are intensely stained. They suggest that brevine could play a key role in the formation of myelin, and this is compatible with our result showing increased incorporation of [$^{14}$C]galactose into MGDG.

The intraneural phalloïdin injection leads to a decreased incorporation of radioactivity into MGDG. Ellisman (1984) suggested that the cytoskeleton may act as a substratum for the attachment of transcellular filaments. Thus, if some parts of the filaments are outside the axons, phalloïdin can interact with them. These results, obtained in Schwann cells located at the injection site, show that in these cells, injection of toxins acting on microfilaments can disturb the metabolism of one of the lipids most specific for myelin.

### *Results Obtained for the Distal Part*

The effects of cytochalasin D and brevine on lipid metabolism in the distal part of the nerve are different from those in the proximal part; sulfatides and phosphatidylserine are the two lipids particularly affected. It is not clear why sulfatides, but not cerebrosides, are affected. This difference may be related to different sites within the cells of their biosynthetic enzymes. Microfilaments are not involved in axonal trans-

Fig. 2. Effect of cytochalasin D, brevine, and phalloidin on the labeling pattern of lipids in sciatic nerve distal part, shown as the percentage of [$^{14}$C]galactose incorporated into lipids after incubation for 3 h. All the toxins were injected intraneurally 24 h before incubation of nerves with the precursor. (A) Results after injection of $10^{-5}M$ cytochalasin D in 0.1% DMSO (in 0.9% NaCl); controls were injected with 1 μL 0.1% DMSO in 0.9% NaCl. (B) Results after injection of 0.06 mg/mL brevine in sodium azide + 1 mM $CaCl_2$; controls were injected with 1 μL sodium azide + 1 mM $CaCl_2$. (C) Results after injection of 1 μg/μL phalloidin in 0.1% ethanol (in 0.9% NaCl); controls were injected with 1 μL 0.1% DMSO in 0.9%NaCl. Each experiment included four rats and at least three experiments of the same schedule were performed (total number of animals >12). Abbreviations used: NL: neutral lipids; MGDG: monogalactosyldiacylglycerol; Cer: cerebrosides; Sul: sulfatides; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyeline. Values significantly different from controls are indicated: * = $0.01 < p < 0.02$; ** = $0.001 < p < 0.01$; and *** = $p < 0.001$.

port, but they interact in the relationship between cytoskeleton and plasma membrane in the axoplasm. The microfilaments are anchored in the plasma membrane via spectrin, which itself is associated with membrane phosphatidylserine (Monbers et al., 1980; Haest, 1982). The breakdown of the microfilaments by cytochalasin results in increased metabolism of actin (Tannenbaum and Brett, 1985). It is possible to hypothetize that modifications of axoplasm fluidity and modifications of relationship between axonal cytoskeleton and axolemma could lead to lipid perturbation of Schwann cells located distally. After injection of phalloïdin, the lipid metabolism of the Schwann cells located distal to the site of injection is the same as control. This could mean that phalloïdin cannot penetrate the axonal membrane and cannot react with axonal actin.

In conclusion, 24 h after intraneural injection of cytochalasin D, brevine, or phalloïdin, incubation of the nerves with [$^{14}$C]galactose revealed that: (1) these compounds are able to modify Schwann cell lipid synthesis; and (2) these three compounds have a different effect on Schwann cell metabolism depending on whether incubated pieces of nerve were from the site of injection, i.e., directly in contact with the toxin, or taken from a point distal to the injection site, i.e., in contact with the axons affected by the toxin. In this case, axonal microfilaments seem to be involved in the regulation of Schwann cell metabolism.

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