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Myosin Light Chain Kinase Inhibitors Induce Retraction of Mature Oligodendrocyte Processes*

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Mature oligodendrocytes emit numerous myelinating processes. Force generating molecules are required for process outgrowth and spreading. We have analyzed the effect of the myosin II light chain kinase inhibitors ML-7 and ML-9 in cultured oligodendrocytes. Both drugs affect oligodendrocyte cell shape, provoking a retraction of high order processes. Our results suggest that the adhesion of the myelinating processes to the substrate depends on MLC phosphorylation, thus likely implicating myosin IIA.

KEY WORDS: Myosin II; MLCK; ML-9; ML-7; myelin; oligodendrocyte processes.

INTRODUCTION

Oligodendrocytes are the myelinating cells of the central nervous system, their differentiation being a complex multistep mechanism whose molecular clues start to be unveiled. Briefly, oligodendrocyte precursor cells proliferate and migrate, then stop dividing and differentiate extending numerous myelinating processes. Oligodendrocyte process outgrowth has been followed in vivo by confocal analysis and three-dimensional reconstructions (1). Initially, premyelinating oligodendrocytes emit multiple cytoplasm projections with radial orientation that diminish in number as axon myelination starts. Fully mature cells bear a few sparsely branched processes that connect cell bodies to the myelin internodes. This branching is restricted to two dimensions in cultured cells, which otherwise perfectly recapitulate differentiation in vivo (2).

Structural cytoskeleton elements and force generating molecules are required for process outgrowth and adhesion to substrate of the large myelin membranes. A candidate molecule to accomplish such roles is the actin-associated motor myosin II. Pharmacological studies revealed that nonmuscle myosin II is required for cell contractility, migration, adhesion, and spreading of amoeba, as well as vertebrate normal and tumor cells. Furthermore, myosin II molecules are believed to participate in neural growth cone motility and filopodia movement (3–7). Using molecular strategies, Wylie et al. (8,9) demonstrated that myosin II motors are required for neurite outgrowth and adhesion to substrate of neuroblastoma cells. Recent studies have shown that myosin II molecules are located at the cell body and leading edge of mature oligodendrocyte processes (10,11), opening an interrogation on the functional significance of this distribution. We have undertaken the analysis on its relevance in oligodendrocyte morphology by means of pharmacological inhibition of myosin II in cultured rat cells.

Myosin II is an hexameric protein that generates mechanical force on ATP hydrolysis by the ATPase

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activity located in the two identical myosin heavy chains (MHC). Two subunits of 15 and 20 kDa are associated to each MHC. The 20-kDa subunit is termed myosin light chain (MLC) or regulatory light chain (RLC), since it bears regulatory properties. Thus, the myosin II motor depends on the phosphorylation state of the MLC, becoming active on phosphorylation of Ser 19 by the action of a specific protein kinase, the myosin light chain kinase (MLCK) (12 and references therein). Drugs that inhibit this enzyme have been widely used to explore the role of myosin II in several cell systems (3,5,11,13–19). We have investigated the effect of the MLCK inhibitors ML-7 [1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine] and ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine] on cultured oligodendrocytes and concluded that myosin II activity is required for maintaining a branched morphology in vitro.

EXPERIMENTAL PROCEDURES

Oligodendrocyte Culture. Oligodendrocytes were prepared from brains of Wistar rats 4–6 days old, as described elsewhere (20), with minor modifications. Briefly, tissue was chopped and digested with trypsin and DNase I for 30 min at 37°C. After trypsin inactivation with fetal calf serum and mechanical disruption by forcing the preparation through nylon meshes (100 and 40 μm were used sequentially), cells were isolated by Percoll (Sigma) gradient centrifugation. Then, three washes were performed using the following buffer: 8 g/L NaCl, 0.4 g/L KCl, 0.09 g/L $HPO_4Na_2.7H_2O$, 0.06 g/L H_2PO_4K, 6 g/L HEPES, and 1 g/L glucose, pH 7.2. Cells were resuspended in defined medium and plated onto glass covers of 12 mm diameter and 0.15 mm thickness coated with 0.01% poly-L-lysine. The defined medium used was DMEM/Ham F12 containing 2.4 mg/ml sodium bicarbonate, 3.58 mg/ml HEPES, 0.146 mg/ml glutamine, 2.5 mg/ml glucose, mg/ml fatty acid–free BSA, 50 μg/ml transferrin, 5 μg/ml insulin, 5.2 ng/ml sodium selenite, 0.11 mg/ml sodium piruvate, 2.5 ng/ml biotin, 0.7 μg/ml hydrocortisone, 10 ng/ml T_3, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% FBS, pH 7,4. Cells were incubated at 37°C and 5% CO_2, and the medium was replaced completely the day after the preparation. Half of the volume was replaced twice a week during the remaining culture time, to keep it conditioned.

Drug Treatment. Cells were plated at 50,000 or 250,000 cells per cover. ML-7 and ML-9 were solubilized in dimethylsulfoxide (DMSO) at 10 mM and stored at −20°C. Inhibitors were diluted conveniently at the moment of use. The medium was carefully removed, and fresh medium containing the drugs was added for 2–3 h. For the untreated control cells, medium was replaced with fresh medium plus the corresponding amount of DMSO. No effect of the DMSO was observed whatsoever.

Immunostaining. After a gentle wash in PBS, cells were fixed in prewarmed 4% paraformaldehyde for 10 min at 37°C and then washed three times in PBS and processed immediately or stored overnight at 4°C. Cells were permeabilized during 10 min in 0.1% Triton X-100 and blocked with 10% nonfat milk supplemented with 10% normal goat serum for 1 h. A polyclonal antibody anti-MBP 644 (gift of Dr. David Colman, Mount Sinai School of Medicine, New York, NY) diluted 1:100 was used, followed by an anti–rabbit antibody conjugated to Cy3, diluted 1:100 (Jackson ImmunoResearch Laboratories). A mouse anti-tubulin antibody from Sigma was used diluted 1:100, followed by a secondary antibody conjugated to FITC, diluted 1:100. All incubations were 1 h long. Three washes (10 min each) with PBS–0.05% Tween-20 were performed after the incubation steps with either primary or secondary antibodies. F-actin was stained in permeabilized cells by using rhodamine-conjugated phalloidin (Sigma) diluted 1:400. Finally, the covers were mounted in FluorSave (Calbiochem) or in PBS-glycerol and sealed with nail polish. Immunostainings were observed with a Nikon Eclipse E600 microscope with ×40 and oil immersion ×100 objectives. Micrographs were taken with a Nikon FDX-35 camera using Kodak 400 VC and TMY 100 films. Negatives were scanned with a Nikon LS-2000 scanner and contrast and brightness were digitally adjusted using the Adobe Photoshop 5.0 software. Oligodendrocyte processes were manually counted using 6 × 8 inches black-and-white prints.

RESULTS

In the present study we have worked with oligodendrocytes maintained for 12 days in vitro. After this culture time, immunostaining for myelin basic protein (MBP), a marker of mature oligodendrocytes, revealed cells with distinct morphology (Fig. 1 A–C). The presence of highly branched processes is observed in nearly all MBP-positive cells at this time in vitro (Fig. 1B). A reduced number of cells presented a less differentiated morphology as shown in Figure 1A. These cells displayed a lower ramification degree in their processes that show a uniformly decreasing diameter toward the tip. Finally, a fraction of cells bearing myelin-like membranes consisting of flattened extensions of plasma membrane with abundant MBP staining is present (Fig. 1C). A nonhomogeneous distribution of MBP is observed in this membrane sheet, crossed by minor veins rich in actin (Figs. 1 and 2 and reference 21). It is accepted that this cell domain in vitro represents compacted myelin in vivo, where the bilayers are in close apposition (21).

Figure 2 shows the distribution of tubulin and F-actin in mature oligodendrocytes. Microtubule organizing center and stress fibers are not apparent (Fig. 2 and references 11, 21, and 22). As reported before, tubulin is abundant in the cell body and processes but is barely detected at the thin villi (10,21,22). In contrast, microfilaments are numerous at the most distal processes that support the myelin membrane. Given this subcellular distribution, we investigated the relevance of actomyosin-generated tension in oligodendrocyte process morphology. Mature cells were treated with the MLCK inhibitors ML-7 and ML-9, fixed, and stained as indicated in Experimental Procedures. The complexity of the oligodendrocyte arbor was evaluated by counting the number of primary, secondary,

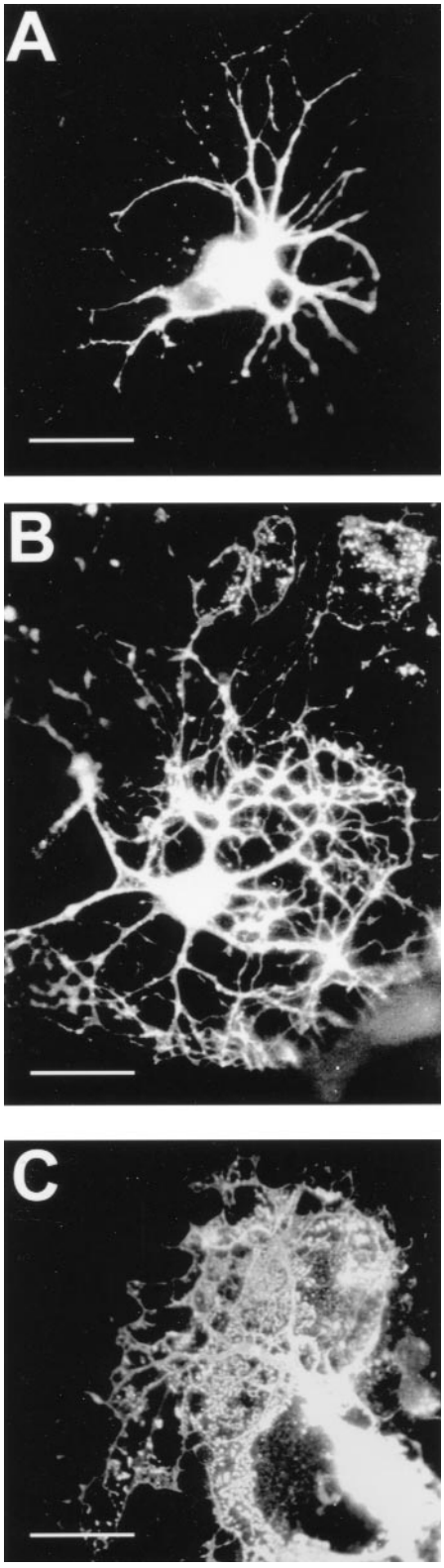

Fig. 1. Immunostaining for MBP in cultured rat oligodendrocytes. Cells were grown on poly-L-lysine–coated covers for 12 days. A minor proportion of cells bears a partially differentiated morphology (A). Most of the cells show highly branched processes (B). Membrane-like structures, loaded with MBP are present in a large fraction of cells (C). Bars, 20 μm.

tertiary, and quaternary processes, as described (10). Immunofluorescence micrographs of cells stained for MBP, F-actin, or tubulin were used for this purpose. The staining observed with tubulin and actin-specific reagents was differential along a given process and between processes of different order. The tubulin signal decreases toward the tip of a primary process and also as the process order increases, being barely detectable at quaternary or higher order processes (Fig. 2). In contrast, F-actin detected by phalloidin staining was highly abundant in tertiary, quaternary, and higher order processes. Consistently with this distribution, the number of high order branches determined by tubulin staining is lower than the value obtained from micrographs of phalloidin-stained cells (Fig. 3).

For the studies reported here, we preferred to determine the actual number of processes by immunolabeling of MBP, which neatly labels the cell perimeter, all the processes and the myelinating membranes with similar intensity. In addition, MBP staining allows the analysis of oligodendrocytes growing on top or near astrocyte clusters, which are negative for MBP and positive for tubulin and actin. A minor drawback is that the visualization of the fine veins at the myelin-like membranes was not possible after MBP staining, thus leading to underestimation of the number of processes of high order when comparing with cells stained for F-actin (Fig. 3).

The effect of the myosin inhibitors ML-7 and ML-9 was evaluated after 12 days of culture in vitro. On treatment with these drugs, the complexity of the oligodendrocyte arbor was markedly reduced. The number of quaternary and higher order processes decreased near to one fifth of the value observed in control cells (Fig. 3). This is consistent with the abundance of F-actin at this cellular regions, since MLCK inhibitors are expected to disrupt actomyosin forces. The retraction of secondary and tertiary processes, rich in tubulin, was also observed, although to a lesser extent. On treatment with ML-7 and ML-9, oligodendrocytes lost the flattened architecture of the cell branches (Figs. 4 and 5). Cellular material accumulated in rounded spots distributed along the processes, interspersed at distances similar to the normal space between branching points (Fig. 4, B and C, arrows). A possible interpretation of these structures is that their represent collapsed higher order processes. This morphology clearly differentiates from that of less mature cells also present in lower amounts in the cultures used (Fig. 1A). At early stages, the ramification degree of the arbor is lower (Fig. 1A), but no material suggestive of retracted higher order branches is accumulated at the cell processes, which display instead a quite uni-

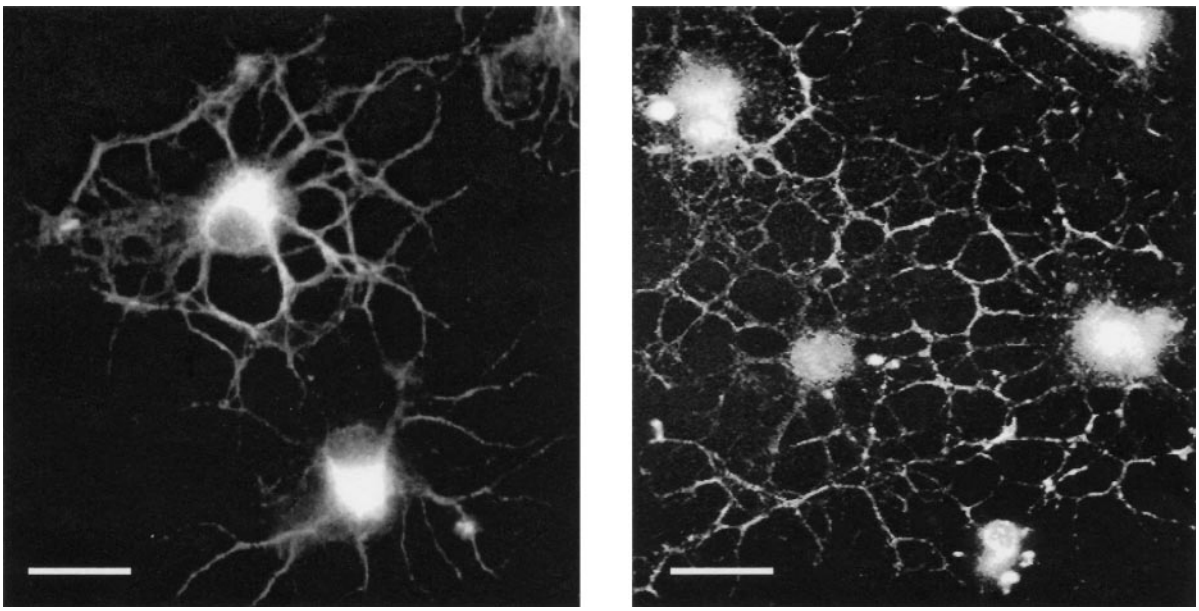

Fig. 2. Distribution of cytoskeletal components in cultured oligodendrocytes. Tubulin (left) is present at cell body and major processes. F-actin (right) is abundant at the distal processes and thin villi. Bars, 20 μm.

form diameter. All this indicates that the morphology induced by MLCK inhibitors (Figs. 4 and 5) does not represent an arrest in cell differentiation but rather a change in the shape of mature cells.

It is known that myosin II participates in adhesion to substrate in several cell systems. Primary cultured oligodendrocytes have a variable cell body size and no significant changes in cell body area were observed upon drug treatment. However, a tendency to cell detachment was confirmed. Moreover, exposure to inhibitors usually leads to a disruption of the myelin-like membranes present in the more mature cells. A variable number of clustered "holes" were observed in these structures (Fig. 5B), thus resulting in a smaller net area of the myelin-like membrane domain. These discontinuities and the collapsed cytoplasm accumulated at the processes are likely a consequence of disruption of normal adhesion to substrate.

The changes in cell shape showed in Figures 4 and 5 are quite distinct from that induced by anti-cytoskeleton drugs (10,21–23). The appearance of rounded cytoplasm accumulations scattered along the processes was never described as resulting from the action of microfilament or microtubule depolarizing or stabilizing agents and thus is specific for MLCK inhibition. Finally, the morphology observed on drug treatment suggests that cell viability is not compromised. In case of cell toxicity, oligodendrocytes undergo either vacuolization of the cytoplasm or detachment of the cell body leaving a halo of latticed processes on the substrate (not shown). No such signals were observed during 3-h treatment with MLCK inhibitors.

DISCUSSION

Our results suggest that forces generated by myosin II motors underlie adhesion and spreading of the myelinating processes. Pharmacological strategies like the one applied here have been used previously to investigate the role of myosin II in a number of cell functions. BDM (butanodione monoxime), ML-7 and ML-9 are among the most widespread drugs used to this purpose. The first one is an inhibitor of the ATPase activity of myosin II and myosin V (24). In contrast, ML-7 and ML-9 are synthetic naphthalene sulfonamide derivatives that inhibit myosin II light chain kinase by competing with ATP binding (18), thus being more specific for blocking myosin II activity (18,19). Besides activation by MLCK, which specifically phosphorylates the light chain at the Ser 19, myosin II is regulated by phosphorylation of the heavy chain (25, 26). Myosin kinases and phosphatases are in turn under the control of kinases acting upstream (3,6,25,26) and therefore ML-7 and ML-9 might exert an indirect unknown effect on myosin II activity. Nevertheless, in-

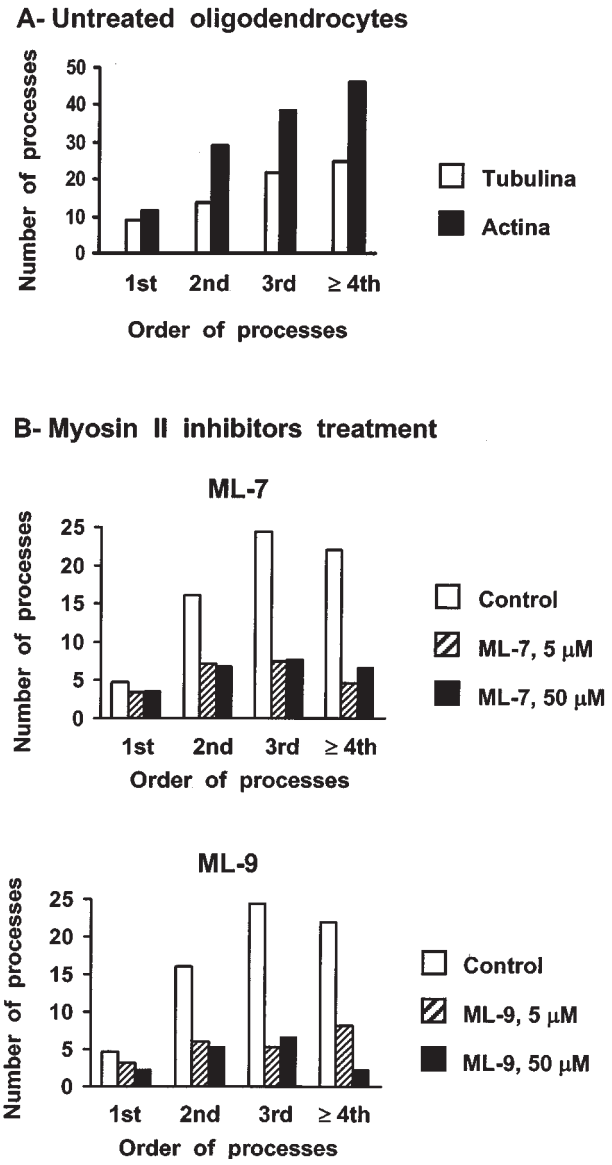

Fig. 3. Quantitation of processes in cultured oligodendrocytes. Cells were stained and processes manually counted on micrographs. A, Cells were stained for tubulin or actin. The actin-specific staining is more sensitive to detect the fine high-order processes, where tubulin is almost absent. B, Cells were treated with MLCK inhibitors at two different doses. The number of processes was determined by MBP immunostaining.

hibition of MLC phosphorylation and concomitant myosin II activity by the action of ML-7 at 20–40 μmol/L or ML-9 at similar doses has been confirmed previously in cultured cells (5,19).

In addition, protein kinase A (PKA) and protein kinase C (PKC) are susceptible to these drugs. The ML-7 Ki for MLCK is 0.3 μmol/L vs. 21 and 42 μmol/L for PKA and PKC, respectively. In contrast,

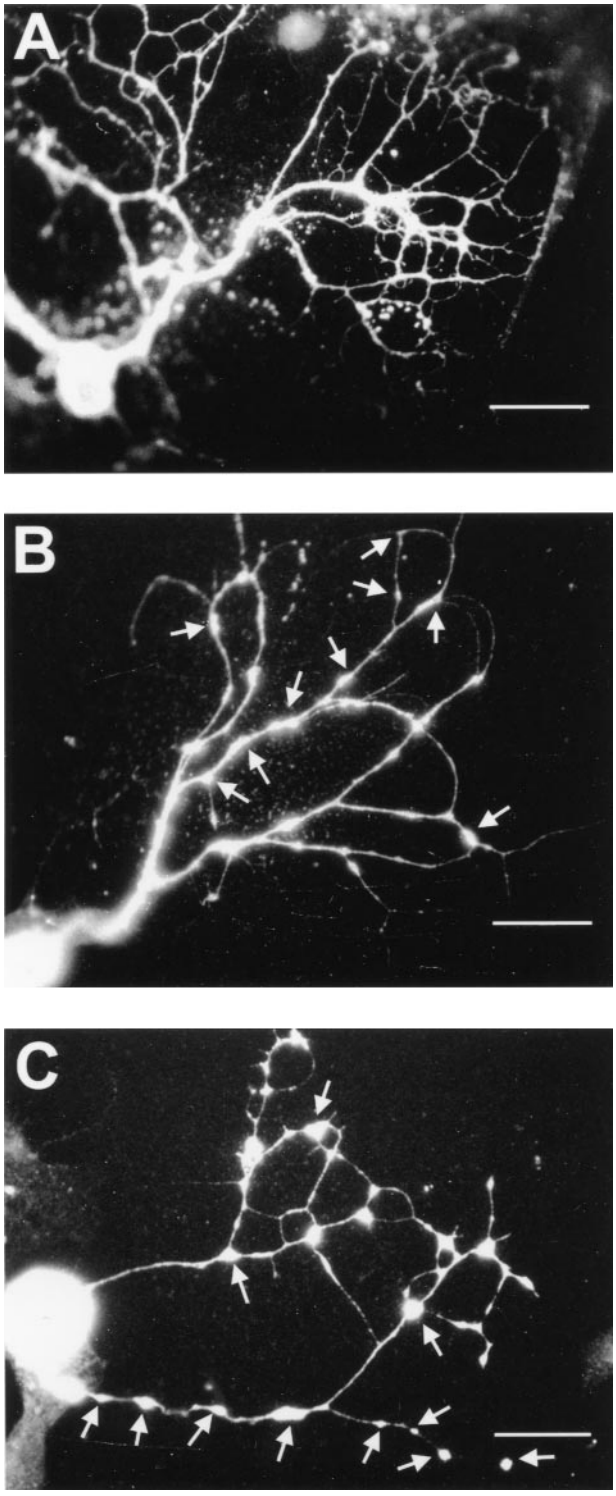

Fig. 4. Treatment with ML-7. Cultured oligodendrocytes were allowed to differentiate for 12 days, then exposed to ML-7 0.05 mmol/L for 3 h and immunostained for MBP. Control cells (A) bear highly-branched processes. Treated cells (B and C) show accumulated material at the branching points of retracted processes (arrows). Bars, 20 μm.

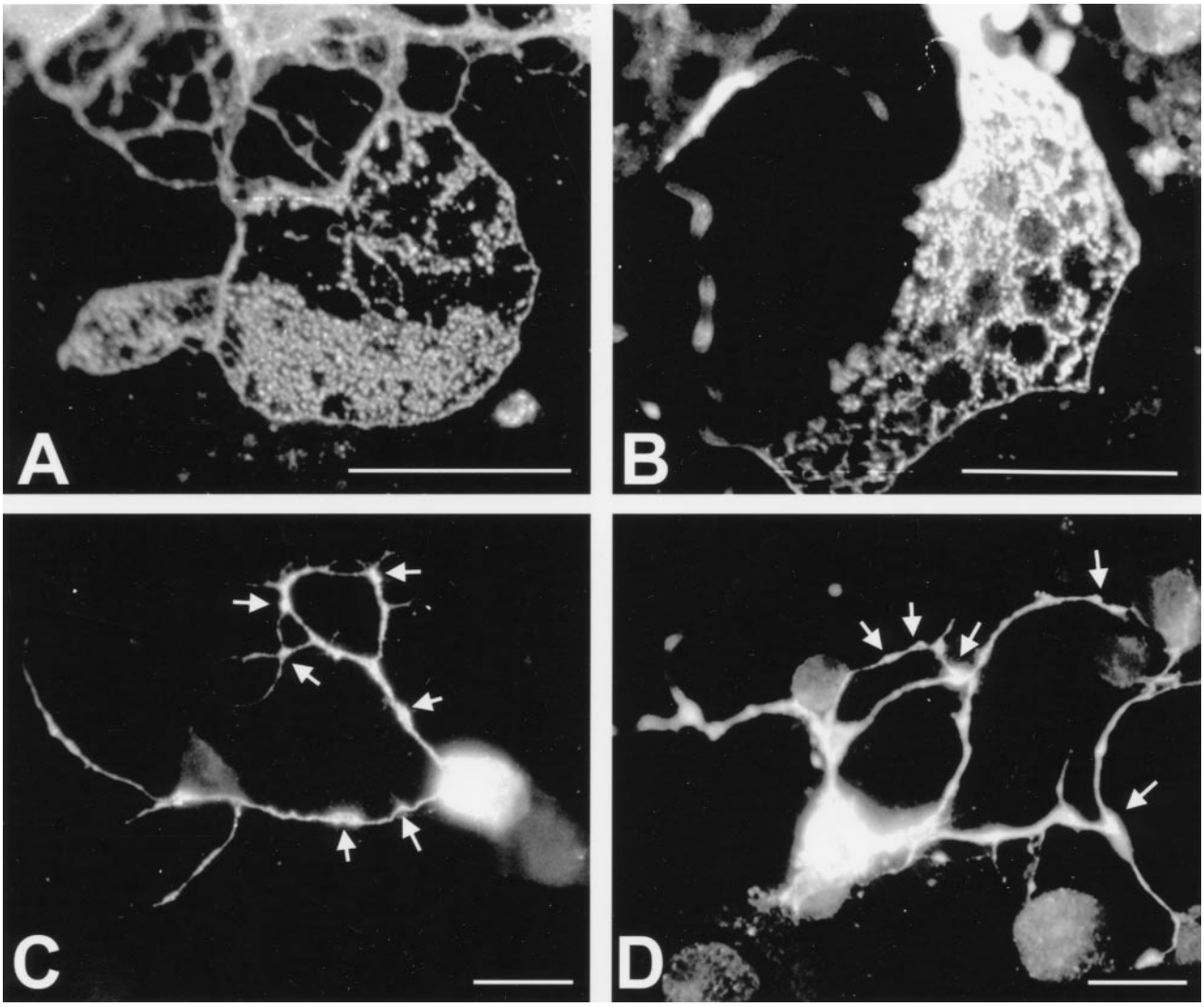

Fig. 5. Treatment with ML-9. Cultured oligodendrocytes were allowed to differentiate for 12 days, then exposed to ML-9 0.05 mmol/L for 3 h and immunostained for MBP. The presence of myelin-like domains is observed in control cells (A). Drug treatment disrupts this structure (B) and provokes retraction of the processes, with accumulation of cell material as in Figure 4 (arrows). Bars, 20 μm.

the ML-9 Ki for MLCK is 3.8 μmol/L vs. 32 μmol/L for PKA and 54 μmol/L for PKC, thus being less selective than ML-7 (18,19). Therefore, a minor effect on PKA and PKC activities should be considered in our studies with the 50 μmol/L dose. The relevance of the PKA and PKC signaling in oligodendrocytes has been studied mostly during the early differentiation of precursor cells. It has been suggested that PKA inhibits mitotic activity, whereas PKC is involved in proliferation and inhibition of differentiation (27–31). The relatively scarce studies on the role of PKC at later differentiation stages seem to suggest that activation of this kinase induces process outgrowth and myelination by mature oligodendrocytes. Likewise, the differentiation of the oligodendroglial cell line CG-4 from a bipolar to a mature multipolar stage requires PKC activity (31). Therefore, although a minor contribution of PKC inhibition on oligodendrocyte branch retraction might be possible, it would be negligible at the lower doses used.

Myosin II has been implicated in cell adhesion in several systems. Drugs with antagonistic effects on actin-myosin contractility such as BDM and ML-7 disrupt focal adhesion in fibroblasts, HeLa, and glial cells, among others (3,6,32,33). Recently, Wylie and Chantler (9) reported that anti–myo II molecular strategies affect neuroblastoma cell adhesion, inducing a reduction of 13% in cell soma diameter. Likewise,

we conclude from our studies that oligodendrocyte adhesion to substrate is affected by MLCK inhibitors, provoking the collapse of myelinating membrane extensions and processes. The retraction of the distal oligodendrocyte domains is consistent with the specific action of these inhibitors on MLCK-dependent actomyosin complexes. It is known that in addition to regulation by MLCK-mediated phosphorylation, myosin II is activated by the Rho kinase (ROCK). It has been suggested that MLCK acts at the peripheral regions of cultured fibroblasts, while ROCK directly phosphorylates MLC at the center of the cells (3). Accordingly, in many examples, MLCK inhibitors seem to affect mostly the peripheral actomyosin and actin cytoskeleton (reference 5, and our unpublished observations in cultured fibroblasts). Nevertheless, we have also observed that the tubulin-rich primary and secondary processes are partially affected, thus indicating microtubule breakdown in this cell domain. Similarly, Song et al. recently reported that microtubules at the oligodendrocyte processes are disrupted upon F-actin depolymerization by the action of cytochalasin B. These observations are also consistent with the accepted interdependence of the microtubule and the microfilament networks (10,22).

In vertebrates, there are two genes coding for myosin II heavy chains. These two highly homologous isoforms named IIA and IIB are present in almost all cell types, MHC IIB being more abundant than MHC IIA in the central nervous system (12). Antisense strategies have been recently used to investigate the relevance of MHC IIA and IIB in the neuroblastoma line Neuro-2A and in neurons from MHC IIB KO animals (8,9). It was found that myosin IIB is essential for neurite outgrowth while myosin IIA is required for cell adhesion and focal contacts, without affecting neurite extension. These results indicate for the first time a differential role for the two myosin II heavy chains and thus, it is expected that a similar phenomenon will be found in other central nervous system cells bearing processes, such as myelinating oligodendrocytes. In these cells, myosin IIA and IIB have a quite distinct distribution (10). Myosin IIB is located preferentially at the leading edges of nascent oligodendrocyte processes and at branching points. In contrast, myosin IIA is located mainly at the perinuclear area and proximal region of main processes, although not totally excluded from the distal processes (10). The pharmacological analysis described here may help distinguish between the physiological role of myosin IIA and IIB molecules in oligodendrocytes. Based on indirect evidence, it has been suggested that myosin IIB is largely insensitive to the phosphorylation state of the MLC (9), this notion being in accordance with recent molecular studies. Namely, while wild-type *Dictyostelium* myosin II is activated by phosphorylation of the light chain, chimeric molecules are not regulated when the responsive domain of the *Dictyostelium* heavy chain is replaced by the corresponding fragment from the mouse myosin IIB (34). All these data suggest that myosin IIB activity would be unaffected by the presence of MLCK inhibitors. Therefore, a likely interpretation of our results is that the disruption of the oligodendrocyte arbor and myelin-like membranes is due to inhibition of myosin IIA molecules. However, the effect of ML-7 and ML-9 on myosin IIB activity has not been evaluated and thus, the inactivation of this motor cannot be completely discarded from our results. Molecular approaches are required to confirm a distinct role for each myosin II isoform. A tempting hypothesis to test is that MHC IIB would be required for oligodendrocyte processes outgrowth and that MHC IIA would participate in a second step, mediating adhesion of the growing myelin.

Finally, the recently described nonconventional myosin VII also has a function in cell adhesion (35). The expression of this novel myosin in oligodendrocytes as well as the effects of ML-9 and ML-7 on its motor activity remains to be addressed. Other authors have shown that the myosin inhibitor BDM blocks the migration of oligodendrocytes precursors in vitro (11). Furthermore, extension and retraction of membranes at the thin villi are supported by actin filaments, thus again implicating myosin motors (2,10). Together with our observations, these results stress the importance of myosin molecules at different stages of myelination.

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