Calmodulin distribution in peripheral nerve: an EM immunocytochemical study

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We used electron microscopic immunocytochemistry to study the distribution of calmodulin in rat sciatic nerve. Calmodulin immunoreactivity was found throughout the axoplasmic matrix, but particularly along microtubules. Schwann cell cytoplasm and nuclei demonstrated immunoreactivity, while compact myelin did not. There was particularly intense immuno-gold deposition within Schmidt Lanterman clefts. At the nodes of Ranvier, calmodulin appeared preferentially in the paranodal region, along the apposition of the axolemma to the paranodal loops of myelin and extending into the paranodal loops. The presence of calmodulin immunoreactivity along microtubules supports biochemical and pharmacological evidence of calmodulin involvement in regulating the assembly and phosphorylation of microtubules, and in fast axonal transport along microtubules. The co-localization of paranodal calmodulin immunoreactivity with Ca-ATPase activity demonstrated cytochemically (Mata et al., *Brain Reseach*, in press) supports the notion that the paranodal Ca-ATPase activity may be regulated by calmodulin, and agrees with the in vitro biochemical evidence for Ca-ATPase of other cells.

INTRODUCTION

Calmodulin is an intracellular calcium binding protein which serves as the regulator for a variety of processes, transducing the effect of a slight rise in intracellular cytoplasmic calcium to create specific physiologic effects. To define the functional role of calmodulin in a cell requires that in vitro demonstration of calmodulin-mediated regulation be combined with appropriate ultrastructural localization of the regulating protein to account for its proposed role[17].

In nervous tissue calmodulin-mediated regulation has been demonstrated in vitro for enzymes including cyclic nucleotide phosphodiesterase[5], adenylate cyclase[3], cGMP protein kinase[31], phosphoprotein phosphatase[29] and Ca,Mg-ATPase[23]. Calmodulin involvement in the dynamic regulation of the actin–myosin microfilaments through regulation of myosin light-chain kinase[20], and in the regulation of microtubule polymerization–depolymerization equilibrium[1,19] suggests that it may also play a role in the regulation of the neuronal cytoskeleton.

Immunocytochemical studies of calmodulin localization in central nervous system have yielded conflicting results. At the light microscopic level CaM is seen in neurons, and lesser amounts of immunoreactivity have been described in glial cells[4,21,26,30]. At the ultrastructural level, reports have included descriptions of immunostaining in dendrites particularly along microtubules[4,30] and in axons[4,26], although others have failed to demonstrate the latter distribution[11,28].

But the neuron has a high degree of regional specialization. Axons, particularly myelinated axons, show a heterogeneous distribution of structural and functional macromolecules related to particular functions at limited sites in the axon. In order to help define which of the regulatory processes, demonstrated in vitro as depending on calmodulin, are relevant in nerve, the distribution of calmodulin must be compared to the known distribution of those proteins which might be regulated by calmodulin.

In this paper we report the immunohistochemical distribution of calmodulin on axons of the rat sciatic

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Fig. 1. Longitudinal section of sciatic nerve demonstrates calmodulin immunoreactivity in the nucleus and cytoplasm of the Schwann cell (s), and along the Schmidt-Lanterman cleft (arrows). Calmodulin immunoreactivity in the axoplasm is diffusely distributed, preferentially along microtubules (arrowheads). A, ×10,920; B, ×28,560; C, ×50,400.

nerve as defined at the ultrastructural level, and interpret that data in light of the known regional specialization of nerve.

MATERIALS AND METHODS
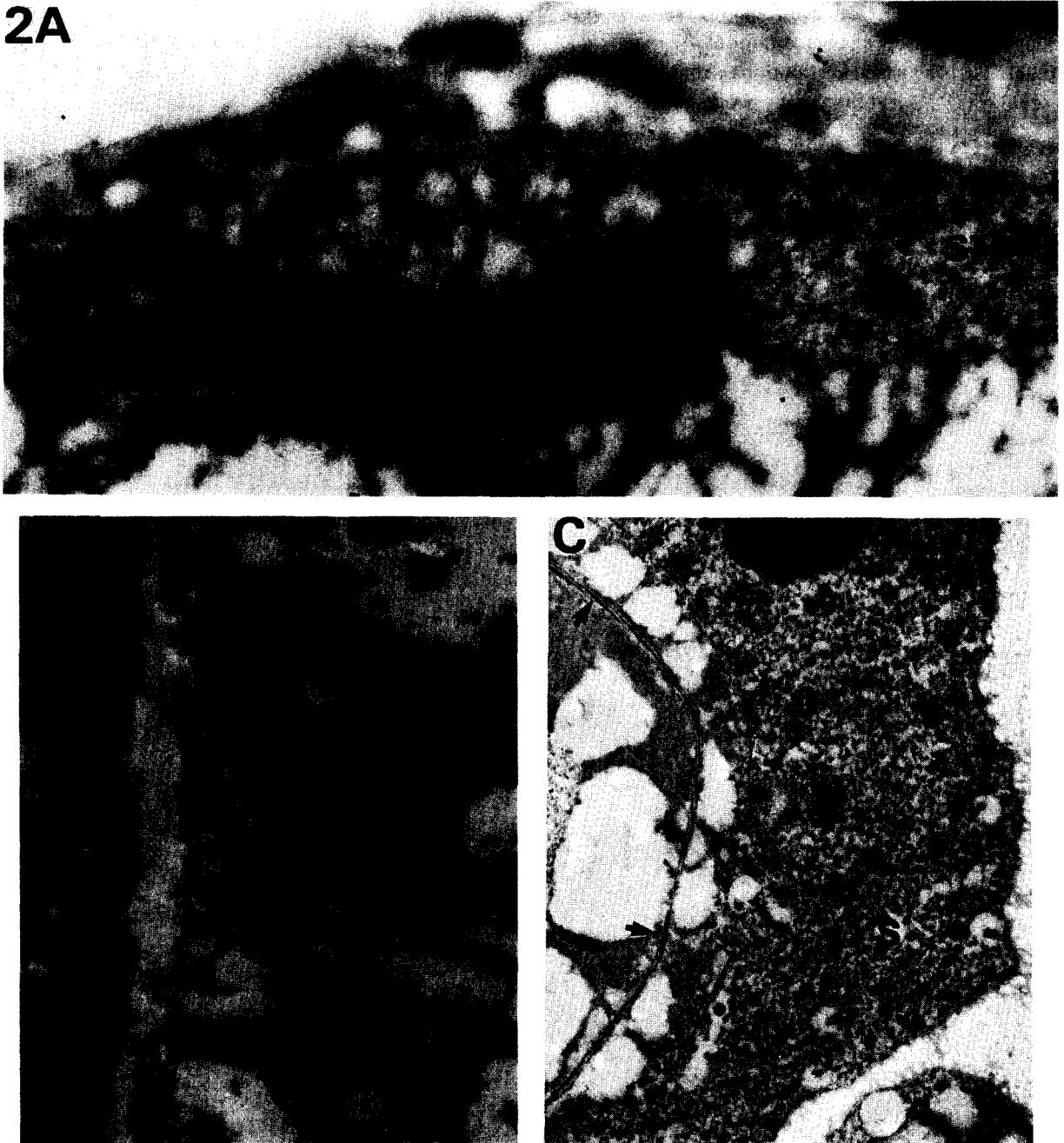
All studies were performed on Sprague–Dawley

Fig. 2. A and B: high-power view of Schmidt-Lanterman clefts show immunoreactivity predominantly in the cytoplasm of the cleft (arrows). In A, the Schmidt-Lanterman cleft can be seen opening into the Schwann cell cytoplasm (s). C: calmodulin immunoreactivity is seen in the nucleus and cytoplasm of a Schwann cell (s), and in the Schmidt-Lanterman cleft (arrows). A, ×37,800; B, ×29,400; C, ×19,320.

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rats weighing 200–250 g. The animals were perfused through the heart with 100 mM phosphate buffer followed by 0.5% glutaraldehyde, 4% paraformaldehyde in the same buffer. Blocks of the rat sciatic nerve were placed in the same fixative for an additional 2 h and then embedded in LR white. Ultrathin sections were exposed to affinity-purified sheep anti-calmodulin (raised against calmodulin from bovine brain, Polysciences) in dilutions from 1:1000 to 1:2000 for 4–72 h, followed by rabbit anti-goat IgG

bound to 15 mM colloidal gold (E-Y Laboratories), and the grids were stained for 20 min with uranyl acetate and examined in a JEOL 100S electron microscope.

Controls for the histochemical reaction included deletion of the anti-calmodulin antibody, or pre-absorption of the anti-calmodulin antibody independently with purified calmodulin from bovine brain (Sigma) and from bovine testis (Polysciences).
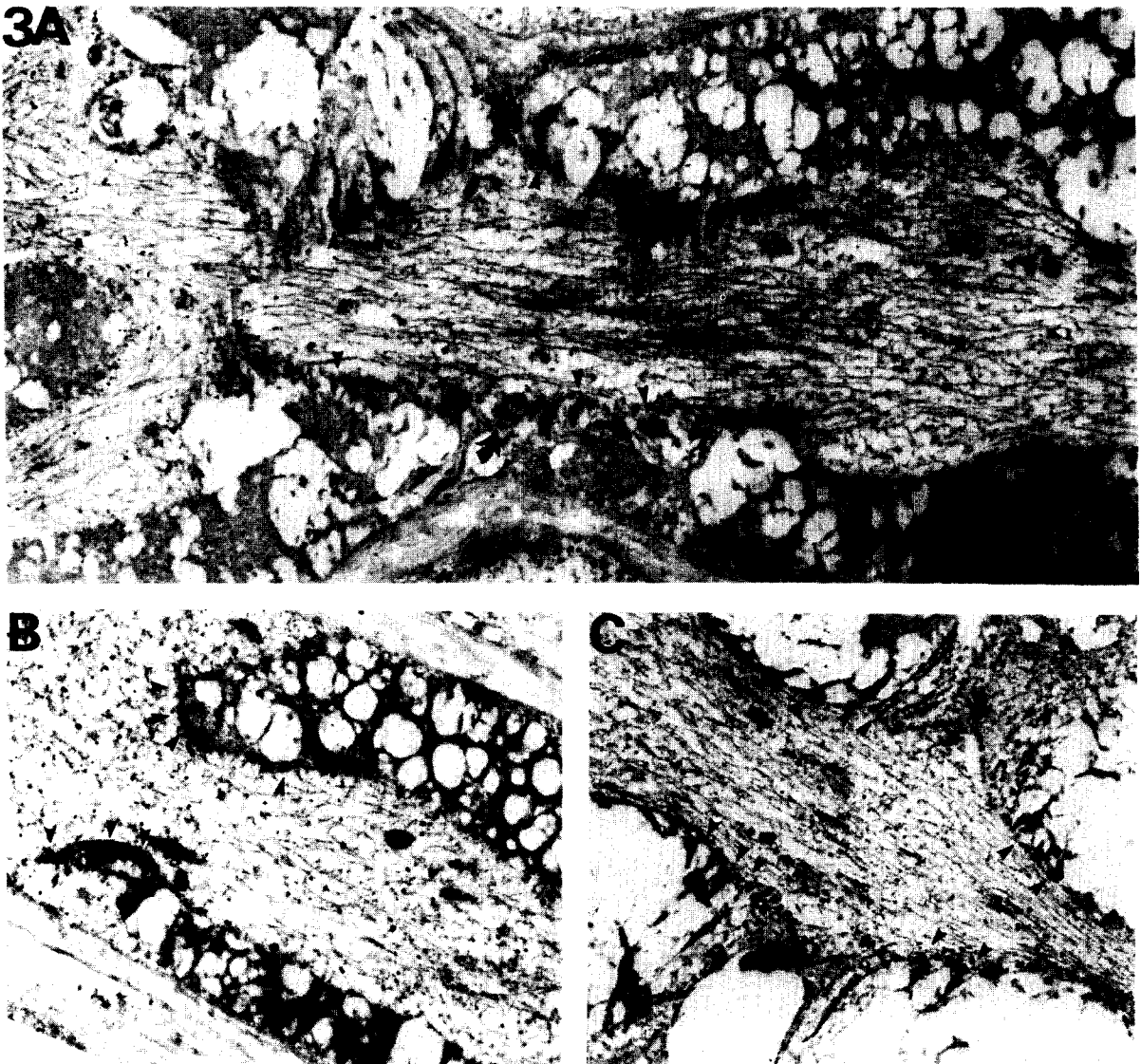

Fig. 3. At the node of Ranvier immunogold deposition is seen particularly in the paranodal region (arrowheads) while the nodal axolemma shows little or no immunoreactivity (arrows). A, ×16,128; B, ×18,480; C, ×14,700.

RESULTS

Within axons, calmodulin immunoreactivity was seen diffusely throughout the axoplasmic matrix. The distribution was homogeneous and did not demonstrate the gradients which we have previously found for the distribution of calcium in myelinated axons. Immunogold labeling was deposited preferentially along microtubules and occasionally the axolemma (Fig. 1).

In Schwann cells, calmodulin immunoreactivity appeared in the cytoplasm and in the nucleus (Figs. 1A, 2C). There was no immunoreactivity along the membranes forming compact myelin, but we found consistently intense immunogold deposition within the Schmidt-Lanterman clefts (Fig. 2). This distribution appeared within the cytoplasm of the cleft, not in the membranes.

At the nodes of Ranvier calmodulin immunoreactivity was seen predominantly in the paranodal region, along the junction of the paranodal axolemma and the paranodal Schwann cell membrane (Fig. 3). With the resolution of this technique it is not possible to determine whether that immunoreactivity is axolemmal or within the paranodal loops. At greater distances from the center of the axon, however, it was clear that the immunoreactivity was found in the paranodal loops of myelin formed by the Schwann cell (Fig. 4).
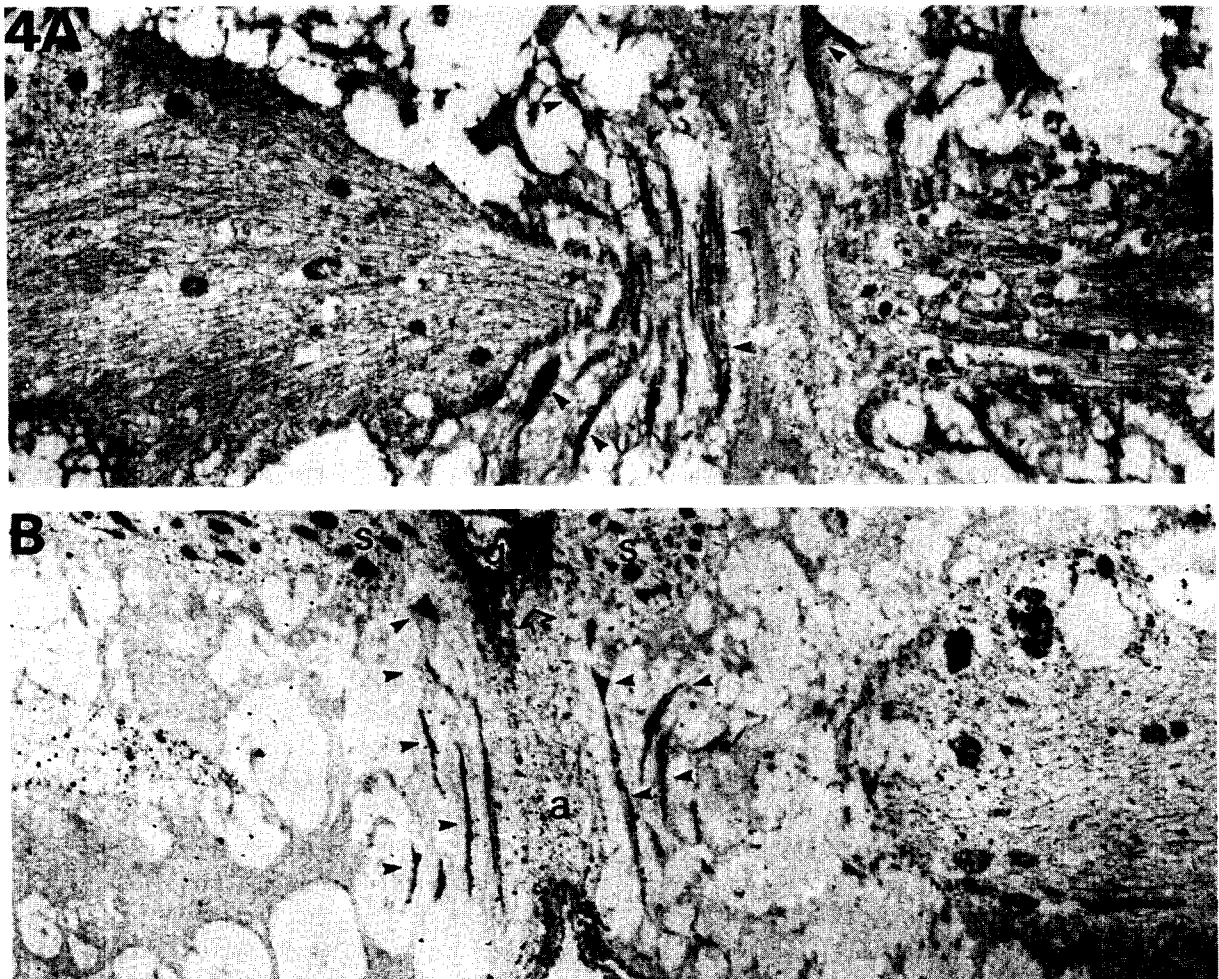
Although immunolabeling is not strictly quantita-

Fig. 4. Calmodulin immunoreactivity is seen in the paranodal loops of myelin (arrowheads) about the node of Ranvier in these longitudinal sections cut away from the center of the axon. Note the absence of immunoreactivity along the axonal membrane at the node (open arrow). s, Schwann cell; g, ground substance. A, ×17,640; B, ×14,280.
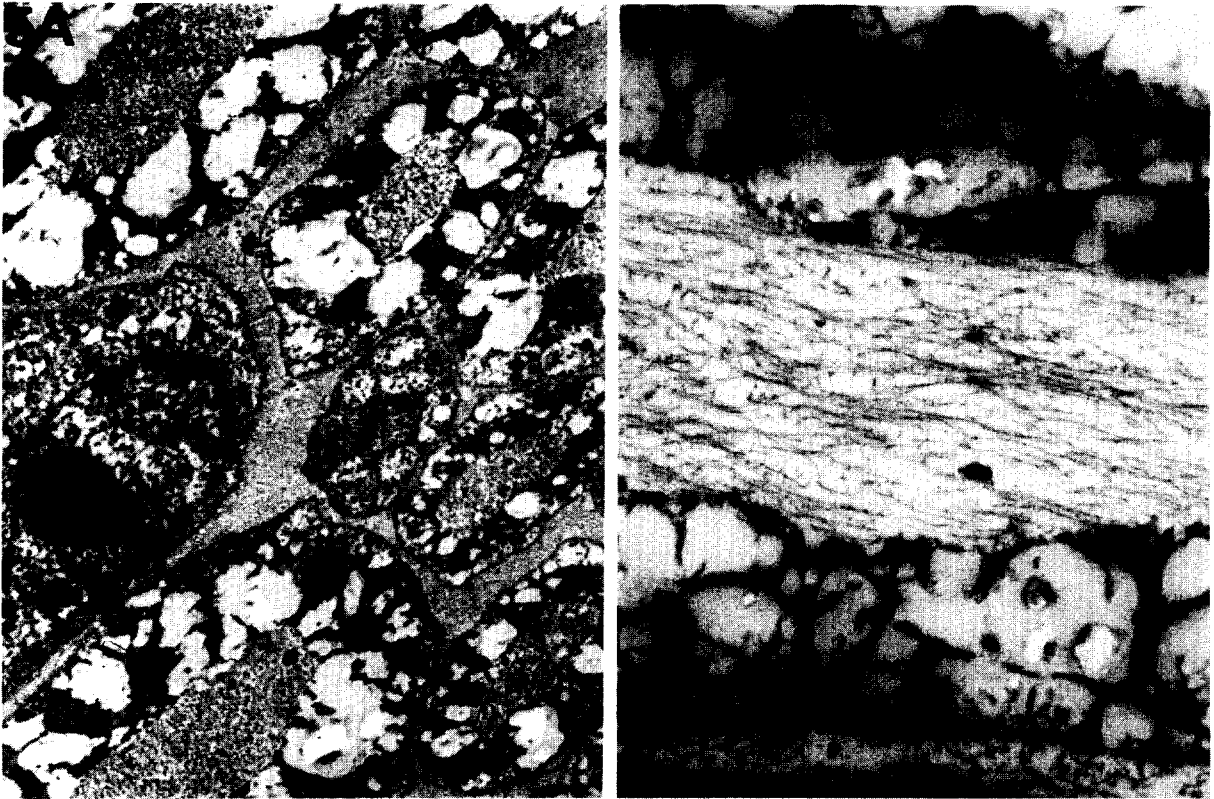
Fig. 5. A: control using preabsorption of the anti-calmodulin antibody (1:2000) with calmodulin (0.4 mg/ml). ×9720. B: control using deletion of the primary antibody. Subsequent steps were carried out as described. ×14,580.

tive, the amount of labeling in the Schmidt-Lanterman clefts and in the paranodal region was much greater than that in other regions, and appeared earlier at higher dilutions of the primary antibody.

Control sections did not demonstrate immunoreactivity (Fig. 5).

DISCUSSION

This is the first study of CaM immunoreactivity in the peripheral nervous system. We found calmodulin immunoreactivity within the axoplasm, confirming the distribution which has previously been described in the CNS (hippocampus, cerebellum[4], and cerebrum[26]) and in rat superior cervical ganglion[22]. We found immunogold deposition within the axons preferentially along microtubules. This localization is in agreement with the biochemical demonstration that newly synthesized calmodulin is transported down axons with the slow transport of microtubules[2,16].

There is biochemical evidence that calmodulin is involved in the regulation of microtubule polymerization by calcium[1]. There is conflicting evidence regarding whether calmodulin binds directly to tubulin[7] or to microtubule-associated proteins[12,24]. A calcium/calmodulin-dependent kinase (CaM kinase II) co-purifies with tubulin[10,27], and has been proposed to regulate microtubule assembly and microfilament−neurofilament interactions through phosphorylation of microtubule-associated proteins[28]. Monoclonal antibodies raised against calmodulin-dependent kinase appear to label microtubules[28] though previous reports with a different monoclonal antibody showed only faint axonal immunoreactivity[18].

In addition to its role in regulating assembly of the cytoskeleton, indirect evidence implicates calmodulin in rapid axonal transport which takes place along microtubules. Calmodulin antagonists inhibit both anterograde and retrograde axonal transport in concentrations that do not disrupt microtubules[6,11]. The

direct localization of calmodulin along microtubules in the current study supports such a role for calmodulin.

It should be noted that we did not see gradients in calmodulin distribution that we have previously seen in calcium distribution[14]. This suggests that those calcium gradients are not a result of gradients in the distribution of the binding proteins.

In the PNS we found that all Schwann cells stained unequivocally in their cytoplasm and nuclei. This is in accord with the virtually universal distribution of calmodulin, though in contrast to the CNS immunoreactivity in glia which has been described as weak[26] or found in only some glial cells[4]. We found particularly intense immunoreactivity in Schmidt-Lanterman clefts. The function of these structures is not known, though they are known to contain microtubules. In our oxalate–pyroantimonate studies of calcium distribution we found a similar distribution of calcium precipitate within the Schmidt-Lanterman clefts[14]. The distribution of calmodulin and calcium suggests that a calcium/calmodulin-mediated function may be carried out by these structures.

We also found calmodulin in the paranodal loops at the node of Ranvier. We have recently demonstrated that Ca-ATPase activity in myelinated fibers (determined by enzyme cytochemistry) has a similar distribution with a particularly dense reaction in paranodal loops[15]. The erythrocyte has a Ca-ATPase on its membrane which is regulated by calmodulin[8] and synaptic plasma membranes isolated from mammalian brain also contain a Ca,Mg-ATPase with a calmodulin-regulatable component[25]. The co-localization at paranodal loops in peripheral myelinated fibers of calcium[14], Ca-ATPase activity[15], and CaM immunoreactivity strongly suggests that at that site small increases in cytosolic calcium may serve to activate the calcium pump through calmodulin.

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