47

Cytochemical localization of Ca^{2+}-ATPase activity in peripheral nerve

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We used an electron microscopic cytochemical method to determine the localization of Ca^{2+}-ATPase in rat peripheral nerve. We found that reaction product occurred along most cytoplasmic membranes in the dorsal root ganglia (DRG). Unmyelinated axons demonstrated reaction product on the axolemma diffusely along their length. Myelinated fibers, in contrast, had reaction product limited to the axolemma in the paranodal region. Internodal axolemma never showed reaction product and nodal axolemma was only occasionally stained, usually in sections reacted for the maximum times. Schwann cell plasma membranes uniformly showed reaction product. The restricted localization of Ca^{2+}-ATPase to the paranodal region of myelinated fibers suggests that calcium efflux may occur principally at those sites.

INTRODUCTION

Most cells maintain internal cytoplasmic calcium at 10^{-7} M, at least in part through the activation of a plasma membrane-associated calcium-stimulated, magnesium-dependent ATPase (Ca^{2+}-ATPase). Ca^{2+}-ATPase was originally identified in erythrocytes[14], and has since been found in microsome and synaptic plasma membrane fractions of rat brain[8,12,17]. It has been localized by biochemical methods to the smooth endoplasmic reticulum (SER) in synaptosomal preparations from brain[4], and to the plasma membrane of the squid giant axon[6].

The development of an electron microscopic (EM) cytochemical method for Ca^{2+}-ATPase localization[1] has shown that in various oriented cells the distribution of Ca^{2+}-ATPase is asymmetric; it is found along the apical border of tracheal epithelial cells[2,18], but along the basal margin of renal tubule cells and toad bladder epithelial cells[1,5]. Using the same method a restricted ultrastructural localization has been identified within retinal receptor cells[15], and changes with

functional changes in the cell have been defined in crustacea during moulting[10].

Neurons show extreme regional structural and functional specialization, and calcium plays a critical role in triggering a variety of functions, both at the nerve terminal and within axons[11,13,15]. Regulation of intracellular calcium is achieved by limited membrane permeability balanced by sequestration and extrusion of calcium. In order to better understand these processes at the ultrastructural level, we undertook to determine the distribution of Ca^{2+}-ATPase in the peripheral nerve of the rat by EM cytochemistry.

MATERIALS AND METHODS

Male Sprague–Dawley rats 175–200 g were used in all experiments. The animals were anesthetized with chloral hydrate and perfused through the heart with 0.1 M cacodylate buffer containing 4% paraformaldehyde. The sciatic nerves and dorsal root ganglia (DRG) were removed and kept in the same fixative at 4 °C for 4 h. Glutaraldehyde was not used

48

because it decreases Ca^{2+}-ATPase activity at the EM level[18]. Fixed specimens were washed at 4 °C in 0.1 M cacodylate buffer (pH 7.2) overnight.

Ca^{2+}-ATPase activity was demonstrated using the technique described by Ando[1]. Vibratome sections were placed for 10–25 min at 37 °C in a standard incubation medium consisting of 250 mM glycine-KOH buffer (pH 9.0) containing 3 mM ATP, 10 mM $CaCl_2$, 4 mM lead citrate (in 50 mM KOH) and 8 mM levamisole. After incubation the sections were rinsed in 0.1 M cacodylate buffer (pH 7.4), post-fixed with cacodylate buffered 1% OsO_4, dehydrated in a
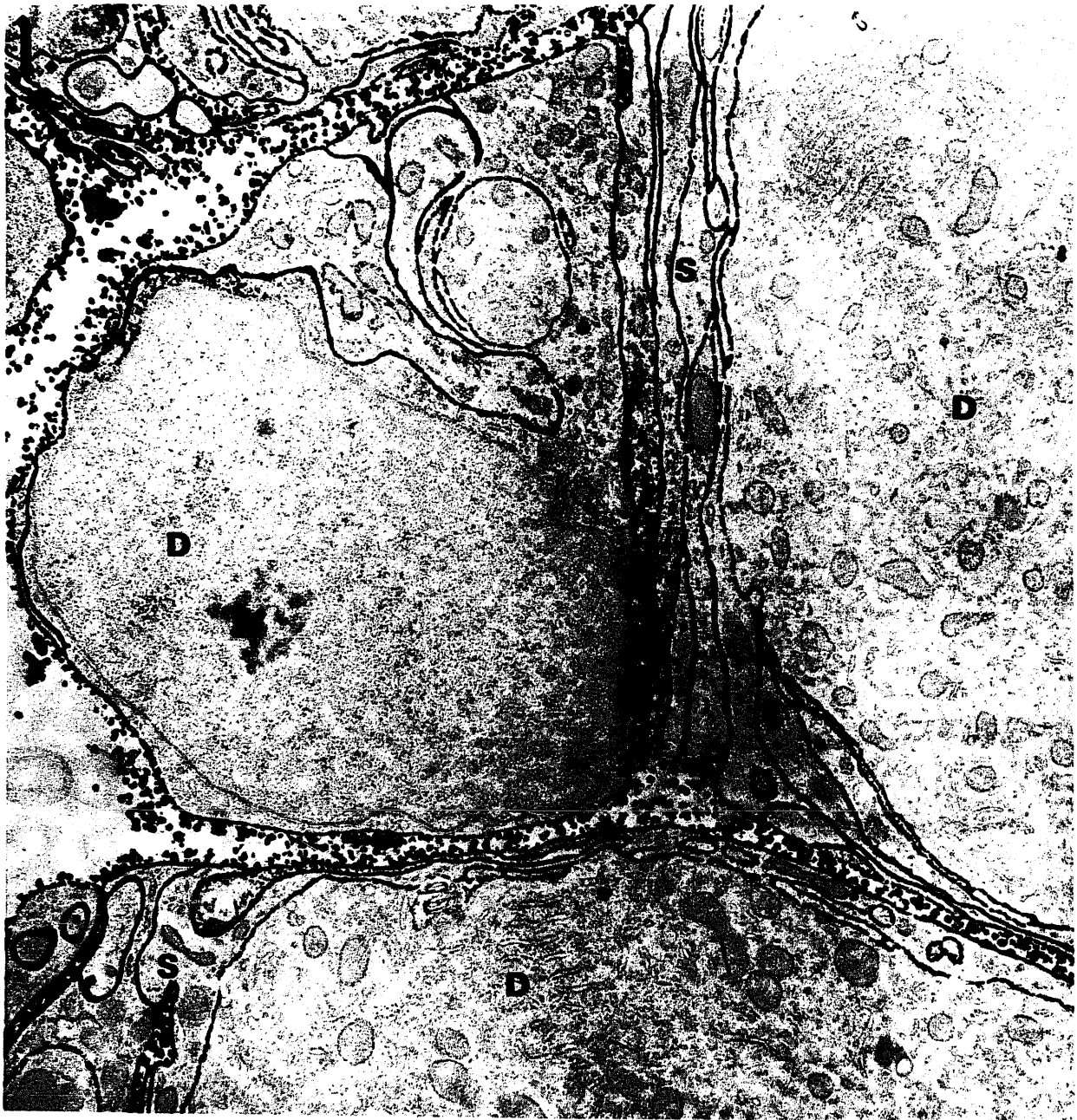

Fig. 1. High-power view of DRG neurons showing the distribution of Ca^{2+}-ATPase reaction product along the plasma membrane of both the DRG neurons (D) and the satellite cells (S). ×17,000, stained with urnayl acetate, reaction time 15 min.

graded series of ethanols and propylene oxide and embedded in Epon–Araldite. Ultrathin sections were examined using a JEOL 110S electron microscope. Some sections were examined unstained, others were post-stained with uranyl acetate.

Three controls to demonstrate the specificity of the reaction product were performed: (1) $CaCl_2$ was deleted and 10 mM EGTA added to the reaction mixture; (2) ATP was omitted from the incubation medium; and (3) 10 mM vanadate was added to the incubation medium.

RESULTS

Thin sections of rat DRG and sciatic nerve prepared as described were reacted for cytochemical staining of Ca^{2+}-ATPase. Reaction product was seen predominantly along the cell membrane of DRG neurons and satellite cells (Fig. 1). It was also seen along the cytoplasmic membrane of other cells including vascular endothelial cells and pericytes (not shown). Intracellularly the Ca^{2+}-ATPase was found on mitochondria, both in the outer mitochondrial membrane and within the cristae, although in
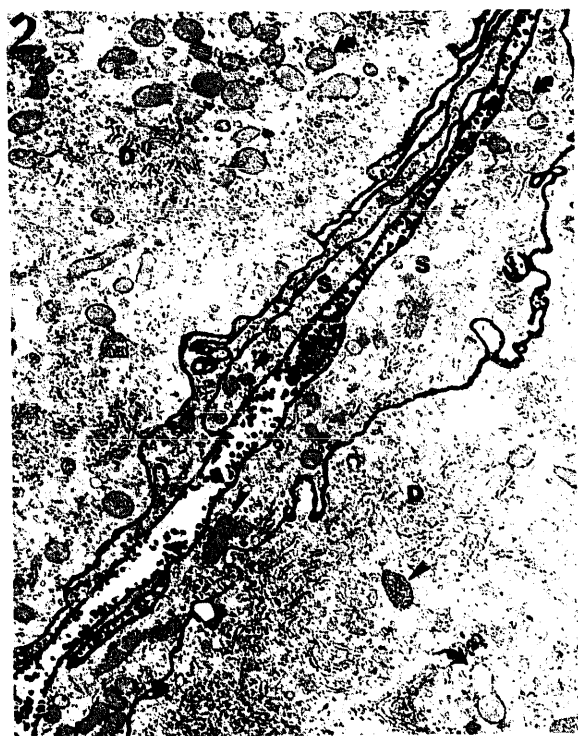

Fig. 2. Lesser amounts of staining can be seen within the DRG neurons (D) and satellite cells (S), principally along the outer mitochondrial membrane (arrows) and in the cristae of the mitochondria (arrowheads). ×15,725, stained with uranyl acetate, reaction time 15 min.

Fig. 3. Within the nerve, Ca^{2+}-ATPase reaction product was found uniformly along the length of the axolemma of unmyelinated axons (a), and on the plasma membrane of the Schwann cells surrounding myelinated fibers (arrowheads). ×17,000, stained with uranyl acetate, reaction time 15 min.

amounts much less than that seen on the cell membrane (Fig. 2).

In the nerve itself, staining was found along the length of unmyelinated fibers, and along the outer membrane of Schwann cells (Figs. 3 and 4). In myelinated fibers staining was seen along the outer membranes of the Schwann cells, and along the axolemma predominantly in the paranodal region (Fig. 5). It was not possible to determine whether the ATPase is associated with the axolemma, or with the paranodal loops of myelin apposed to the axolemma. There was no staining of the internodal axolemma beneath the myelin sheaths, and in general no staining of intracytoplasmic or axoplasmic organelles. However, in sections with more dense reaction product or in sections incubated for the longest periods of

time precipitate could be seen within the axoplasm of unmyelinated fibers around the membrane of intracellular organelles, in the nodal membrane of myelinated axons, and occasionally within the nodal axoplasm as well (Fig. 5).

Control sections, prepared as described in Methods showed no staining in any of the distributions described above (Fig. 6).

DISCUSSION

The EM method for cytochemical localization of Ca^{2+}-ATPase utilizes lead citrate to trap the phosphate liberated from ATP in the presence of calcium to form electron-dense lead phosphate deposits. Levamisole is used to block non-specific alkaline phos-

Fig. 4. Cross-section of nerve showing reaction product in the axolemma of unmyelinated fibers (a) and along the outer Schwann cell plasma membrane of the Schwann cell surrounding the unmyelinated fibers (arrows). ×16,000, stained with uranyl acetate and lead citrate, reaction time 25 min.

Fig. 5. At the node of Ranvier of myelinated axons, Ca^{2+}-ATPase reaction product can be seen predominantly in the paranodal region (arrowheads). Lesser amounts of reaction product can be seen in the nodal membrane (B and C), but no Ca^{2+}-ATPase reaction product is seen in the internodal axolemma or in the compact myelin. A, ×16,000; B, ×12,000; C, ×13,600, all stained with uranyl acetate, reaction time 15 min.
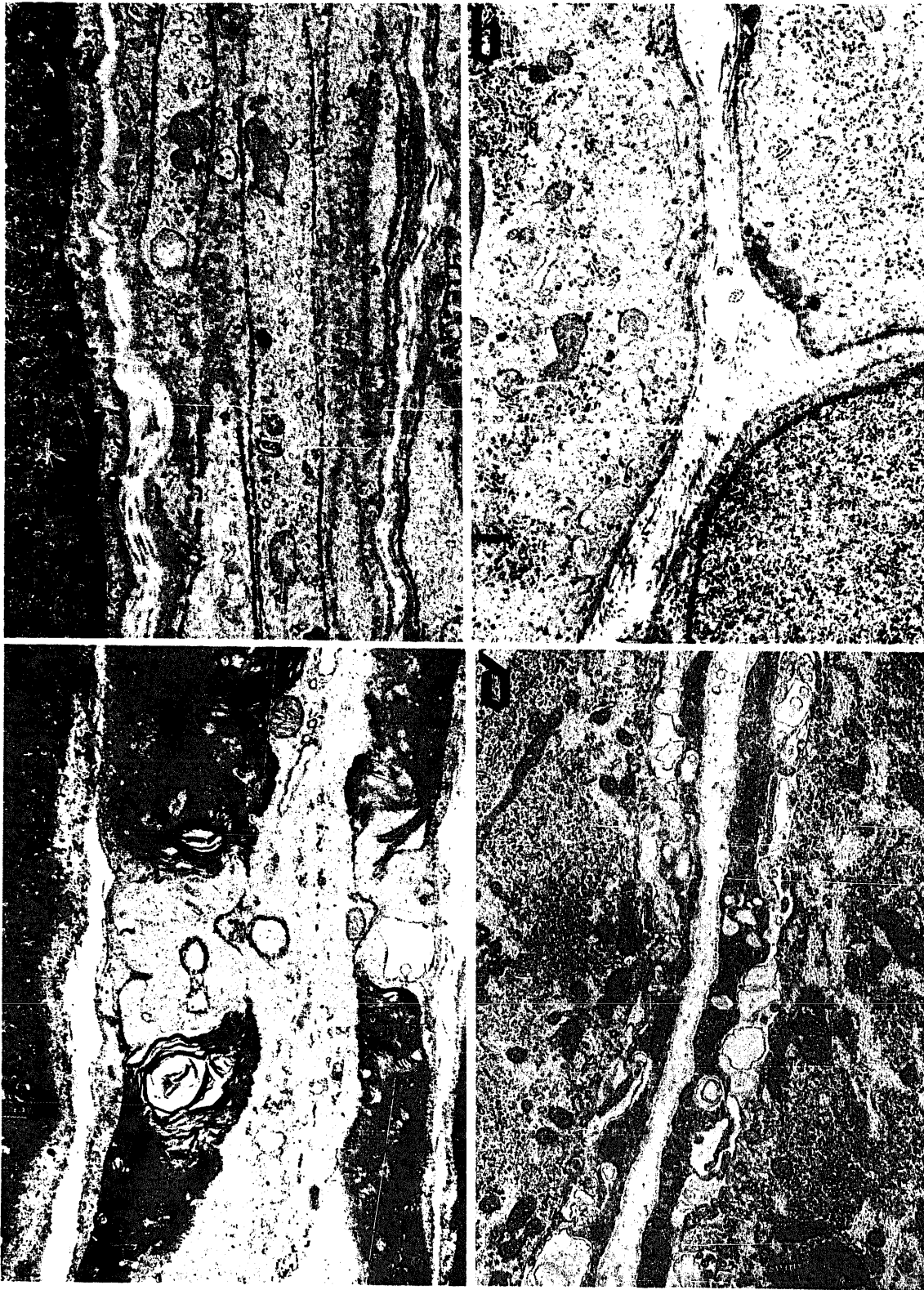
Fig. 6. Control reactions demonstrate the specificity of the staining. a: nerve, reacted identically except for the deletion of ATP from the incubation medium. ×10,000, stained with uranyl acetate, reaction time 25 min. b: DRG, reacted identically but with Ca^{2+} deleted from the incubation medium. ×8000, stained with uranyl acetate, reaction time 15 min. c: myelinated axon at the node of Ranvier, with 10 mM vanadate added to the standard incubation medium. ×12,000, stained with uranyl acetate, reaction time 15 min. d: DRG, with 10 mM vanadate added to the standard incubation medium. ×10,000, stained with uranyl acetate, reaction time 15 min.

phatase. The specificity of the reaction, its substrate dependence, and the identity of the electron dense deposits have been reported in detail by Ando[1]. The method allows the determination of the precise ultrastructural distribution of this calcium pump in cells. We have found that while the enzyme is located diffusely along most cell membranes in the nerve, there is a regionally specific distribution in myelinated axons and their ensheathing Schwann cells. Reaction product was limited to the paranodal regions, and to the outer cytoplasmic membrane of Schwann cells, but was not found within compact myelin nor along the adaxonal membrane.

This distribution of enzyme activity differs from the distribution of Na, K-ATPase defined by immunocytochemistry in myelinated fibers of goldfish optic nerve[16], and in rat central and peripheral nerves[3], where that enzyme activity is limited to the nodal axolemma but is absent from the paranodal regions. It also differs from the distribution of sodium channels in the myelinated fibers of the dorsal spinal nerve of electrophorus, which appear limited to the nodal axolemma when demonstrated by immunocytochemical staining[7]. Our data adds to the growing body of evidence of specialization of the nodal and paranodal membranes of myelinated fibers, and a potential role for localized ionic flux and active ion pumping in the membrane.

We did find lesser amounts of staining in the axoplasm and around organelles in unmyelinated axons and at the node of Ranvier. Occasionally, in the presence of dense reaction product, the nodal membrane was also stained. This most likely represents the presence of lesser amounts of enzyme activity at these sites. We feel differences in penetration are not the principal explanation for the differential localization, because the nodal membrane is more accessible than the paranodal membrane, but it is the latter which stains more readily.

There is good evidence that calcium channels are present in nerve cell bodies and at synaptic terminals[9], but the role of calcium flux in the axon is not yet well understood. While unmyelinated axons have Ca^{2+}-ATPase uniformly along their length, the localized concentration of Ca^{2+}-ATPase in the paranodal region of myelinated fibers suggests that there may be a localized calcium flux in those fibers.

ACKNOWLEDGEMENTS

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