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Differential distribution of (Na,K)-ATPase α isoform mRNAs in the peripheral nervous system

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mRNA transcripts for 3 isoforms of the α subunit of (Na,K)-ATPase have been peviously identified in the nervous system (designated α1, α2 and α3). In order to study the localization and expression of the different α isoforms in the peripheral nervous system, we prepared probes from the unique 3′ untranslated region of α1 cDNA, and from the translated region of α3 cDNA. These probes were used in dot blot and in situ hybridization assays of rat spinal cord, dorsal root ganglia (DRG), and sciatic nerve. Within the ventral horn of lumbar spinal cord, α1 mRNA was found in a discrete set of laterally placed motor neurons, while α3 was found in all the identified neurons of the spinal cord, including those motor neurons containing α1. In the lumbar DRG, α3 was uniformly distributed in DRG neurons, while α1 was abundant in some neurons but little or none was found in other neurons. Satellite cells contained neither isoform. Schwann cells in sciatic nerve were labeled with the α1 probe in a perinuclear distribution, but contained no detectable α3. Dot blot analysis showed α1 and α3 in spinal cord and DRG, but only α1 in peripheral nerve. These results imply that: (1) the riboprobes are able to distinguish α1 from α3 isoform mRNAs under in situ hybridization conditions, (2) both α1 and α3 isoforms of (Na,K)-ATPase are found in neurons, (3) some motor and sensory neurons contain abundant α3 with little or no detectable α1, while others contain abundant α1 and α3, (4) no neurons in the lumbar spinal cord or DRG contain detectable α1 in the absence of α3, and (5) Schwann cells contain the α1 isoform but no detectable α3 isoform mRNA.

INTRODUCTION

(Na,K)-ATPase is the membrane-bound enzyme which is responsible for maintaining ionic homeostasis within cells through the active transport of Na^+ and K^+[19]. The function of (Na,K)-ATPase is crucial in creating the electrochemical gradient required for the electrical excitability of nerve cells[1]. The Na^+ gradient is also critical for regulation of cytosolic content of H^+ and Ca^{2+} through the respective Na^+ antiporters, and for glucose and amino acid uptake through Na^+ symporters[1]. Thus, the enzyme may be subject to different regulatory characteristics in different types of cells.

The enzyme consists of two subunits. The α, or catalytic, subunit (Mr 112 kDa) contains the Na^+ dependent phosphorylation site and amino acids required for ouabain binding[3]. The β subunit (Mr 35 kDa) is a glycoprotein required for enzyme activity although its specific function is unknown. mRNAs for 3 different isoforms of the α subunit, termed α1, α2 and α3 have been identified in brain[22]. The 3 isoforms are products of 3 different genes[25], and expression is developmentally regulated in a tissue-specific manner[11], with α2 and α3 predominating in the brain[11,13]. The isoforms are differentially regulated by hormones in myocyte cultures[12]. There is little information concerning different catalytic properties of the isoforms. In the rodent, α1 is more resistant to inhibition by ouabain than are α2 and α3[20,21,23] and α3 in rodent pineal gland has a higher affinity for Na^+ than has α1[17]. In rat adipocytes, insulin increases the affinity of α2 for Na^+ (Ref. 7). These observations suggest that the isoforms may be adapted for different regulatory characteristics in different cells or subcellular regions.

In order to formulate and test hypotheses regarding specific functional characteristics of the isoforms, one requirement is to define the pattern of isoform expression in individual cells of the nervous system, and to determine whether individual cells express one or multiple isoforms of (Na,K)-ATPase. The principal information regarding (Na,K)-ATPase mRNA expression in nervous system concerns brain[4,6,14]. There are no previous studies of peripheral nerve or spinal cord.

In order to study the cell-specific expression of the different isoforms in the peripheral nervous system, we produced riboprobes for α1 and α3 mRNAs, and used

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these probes in dot blot and in situ hybridization studies of spinal cord, dorsal root ganglion (DRG) and peripheral nerve.

MATERIALS AND METHODS

Production of riboprobes

Cloned rat α isoform cDNAs, graciously provided to us by Dr. Robert Levenson (Yale University) and by Dr. Jerry Lingrel (University of Cincinnati College of Medicine) were used to prepare subclones containing part of the 3′ untranslated region of the α1 isoform cDNA, and from the translated region of α3 isoform cDNA. A 203 bp BamH1-Pst1 restriction fragment from the 3′ end of the α1 cDNA and a 342 bp Pst1-EcoR1 fragment containing a portion of the translated region of the α3 cDNA were subcloned into the vector pGEM4. ^{35}S-labeled riboprobes were prepared by transcription of the linearized clones in the presence of ^{35}S-labeled UTP for in situ hybridization studies. ^{32}P-labeled probes for use in dot blot studies were prepared in a similar manner by transcription in the presence of [^{32}P]UTP. Northern blot analysis showed that both probes recognize transcripts about 4 kb in size in RNA isolated from rain (data not shown), in agreement with previous descriptions of the size of α isoform mRNAs[5]. Because the 3′ untranslated regions of the α isoforms are each different, the α1 riboprobe is completely specific for α1 relative to the other isoforms[16]. The translated region of the α3 isoform used to produce the riboprobe has 70% homology with α1. However, the in situ and dot blot evidence presented below indicates that under our hybridization conditions, the α3 riboprobe does not recognize α1. We attempted to produce a riboprobe specific for α2 but were unable to transcribe any of the fragments we cloned into the pGEM vector.

Dot blotting

RNA dot blotting was carried out essentially as described previously[2]. Total cellular RNA was isolated from spinal cord, DRG, and peripheral nerve using a commercially available kit (RNAzol B, Cinna/Biotecx Laboratories, TX), and spotted on GeneScreen membranes. RNA was isolated from kidney as reported earlier[6]. Hybridization to ^{32}P-labeled RNA probes was carried out at 70 °C with prehybridization, hybridization, and subsequent washing of filters as described previously[6], except that these procedures were all carried out at 70 °C. Autoradiograms of the filters were produced on Kodak XAR film exposed at −80 °C.

In situ hybridization

In situ hybridization was performed essentially as described by Watson[24]. Briefly, animals were sacrificed by decapitation and 6 μm paraffin sections of lumbar spinal cord, sciatic nerve and lumbar DRG were deparaffinized, covered with an aliquot of hybridization buffer containing approximately 5×10^4 cpm/μl of ^{35}S-labeled RNA probe and sealed with coverslips. Sections were hybridized for 21 h at 55 °C. After removal of the labeled probe, the sections were rinsed twice with 2× SSC at room temperature for 30 min, once with 50% formamide in 2× SSC at 55° for 30 min, treated with 50 μg/ml RNase A for 30 min at 37 °C, washed with 0.5 M NaCl, 10 mM Tris, 1 mM EDTA for 30 min at 60°, and finally rinsed with 2× SSC, water and a graded series of alcohols. The slides were then dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water) and exposed at 4 °C until the desired grain density was obtained. Sections were counterstained either with hematoxylin and eosin or with Toluidine blue, and analyzed both with dark field and phase contrast optics.

In order to determine whether individually identified neuronal cells contained both isoforms, 1-μm cryo semi-thin sections of perfused but unembedded tissue were cut using a Reichert-Jung cryo ultramicrotome and alternate sections were hybridized with either the α1 or the α3 probe using the protocol described.

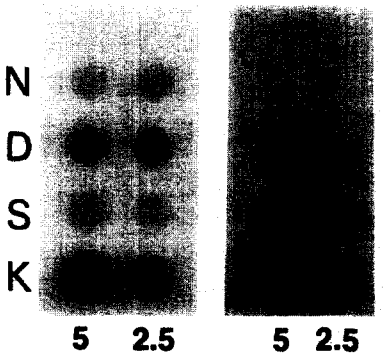

Fig. 1. Dot blot of nerve (N), DRG (D), spinal cord (S) and kidney (K) with ^{32}P-labeled probe. Left: α1; right: α3; 5 and 2.5 μg total RNA as indicated.

EM immunocytochemistry

In order to confirm that the cellular distribution of α isoform mRNAs determined by in situ hybridization corresponded to the distribution of α isoform proteins, we performed immunocytochemistry with an antiserum against α subunits. The production and characterization of the rabbit polyclonal antibody against denatured catalytic (α1 and α2/α3) subunits purified from bovine brain (Na,K)-ATPase have been described[6,15]. Post-embedding electron microscopic immunocytochemistry with secondary antibodies conjugated to colloidal gold was carried out on LR White-embedded tissue as previously described[8,9].

RESULTS

Dot blot analysis indicated the presence of both α1 and α3 in DRG and spinal cord. α1 but not α3 was found in kidney and in peripheral nerve (Fig. 1).

In situ hybridization of the DRG with both α1 and α3 showed intense labeling of DRG neuronal perikarya with both probes (Fig. 2). Silver grains representing binding of the riboprobe were found over neuronal cell bodies (Figs. 2 and 3) but were not found over satellite cells in the ganglion (Fig. 3).

α1 mRNA was present in most but not all DRG neurons (Fig. 3). In contrast, all ganglion cell neurons appeared to contain α3. Analysis of 1 μm serial semi-thin sections showed that many neurons contained both α1 and α3, while some neurons contained a large amount of α3 but little or no α1. We were unable to identify any neurons that contained α1 but not α3.

EM immunocytochemistry with a polyclonal antiserum raised against denatured brain catalytic subunits showed immunoreactivity along the plasmalemma of all the DRG neurons (Fig. 4) extending into the axolemma of the

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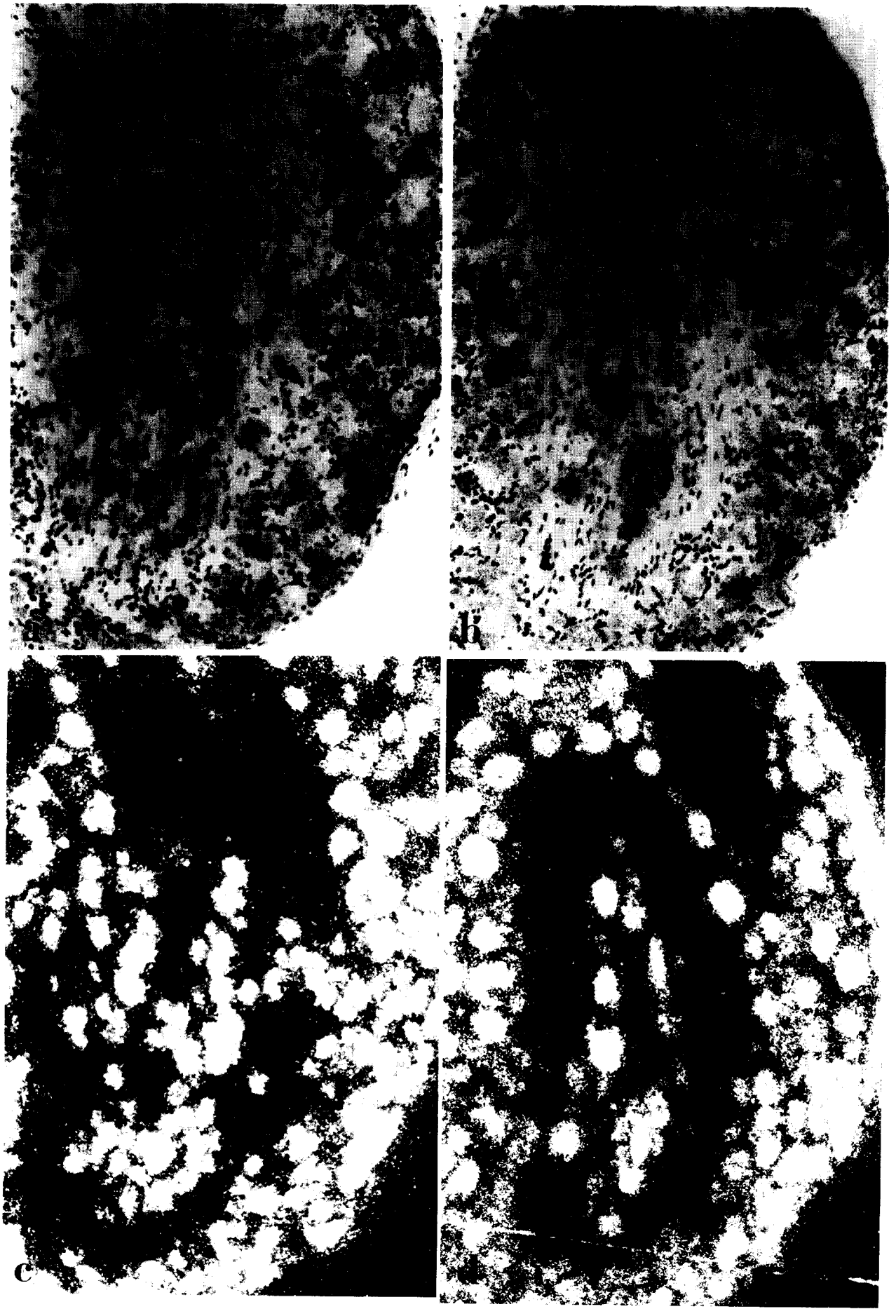
Fig. 2. Brightfield (a and b) and darkfield (c and d) light microscopic pictures of in situ hybridization of DRG with ^{35}S-labeled riboprobes for α1 (a and c) and α3 (b and d). Exposure time 2 weeks, original magnification ×125.


b
c

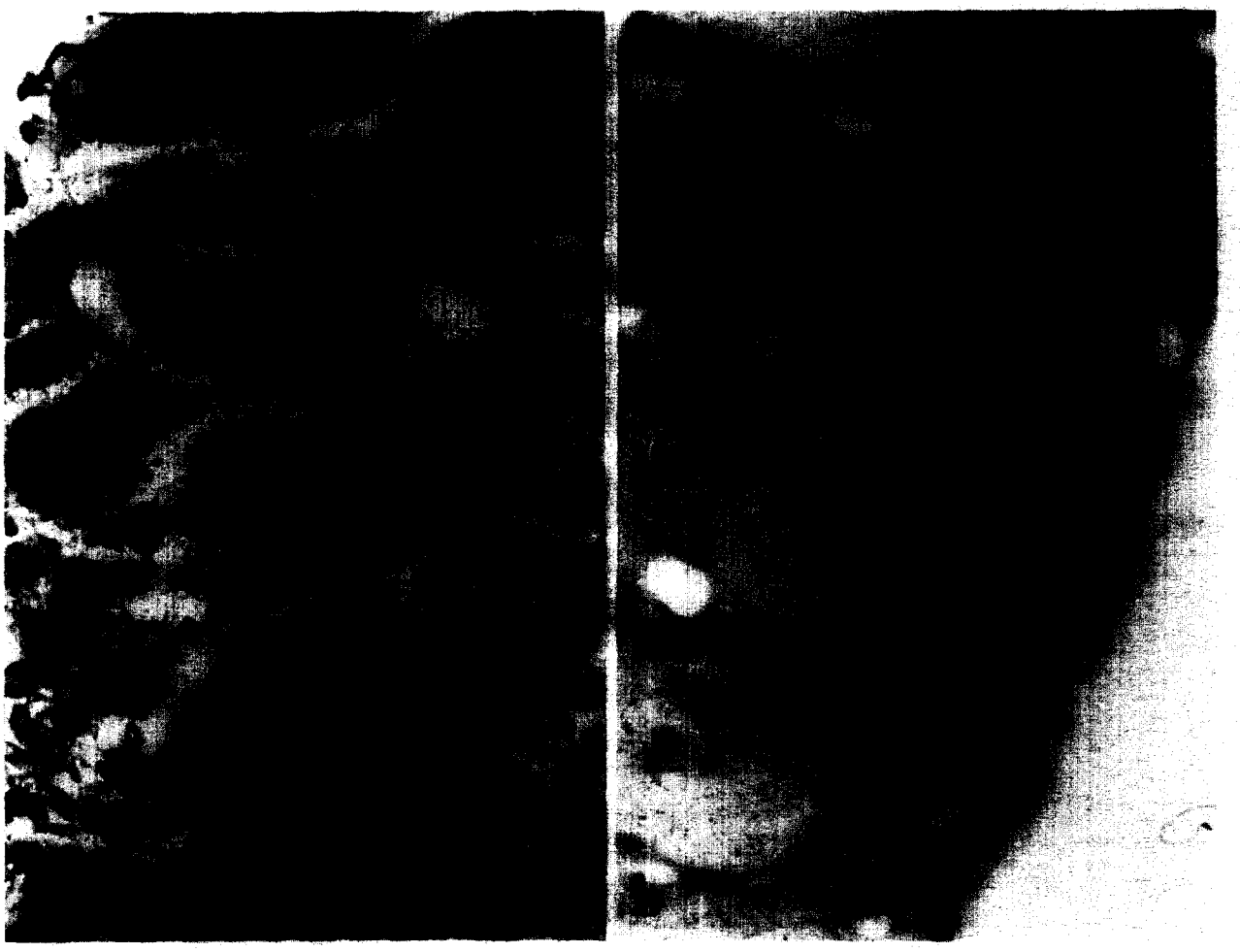


Fig. 3. Brightfield of 6 μm paraffin (a) and 1 μm cryo (b) sections of DRG in situ hybridization with ^{35}S-labeled riboprobe for α1. Many DRG ganglion cells containing little or no α1 mRNA. In b, the absence of grains over satellite cells is very clearly seen. Exposure times 2 weeks (a) and 4 weeks (b), original magnifications ×312 (a) and ×1200 (b).

initial segment of the axon (Fig. 4), but not along the plasmalemma of the satellite cells. This distribution accords with the general cellular distribution of α isoform mRNAs seen with in situ hybridization, and confirms that the α isoform riboprobes are identifying cells which produce α isoform proteins.

In the lumbar spinal cord, abundant α1 mRNA was found in a limited set of motor neuron cell bodies in the ventral horn (Fig. 5). α3 mRNA, in contrast, was found in neurons throughout the dorsal and ventral gray matter of the spinal cord (Fig. 5). One-μm serial semi-thin sections showed that all of the neurons producing α1 also contained α3 mRNA, while many individual cells contained only α3 but little or no α1 (Fig. 6).

EM immunocytochemistry with the same polyclonal antiserum showed immunoreactivity along the plasmalemma of all the neurons in the spinal cord (Fig. 7), corresponding to the widespread distribution of α3 isoform mRNA seen by in situ hybridization, and again confirming in the spinal cord that the α isoform protein distribution agrees with the distribution of α isoform mRNA.

In the peripheral nerve, Schwann cells showed α1 mRNA in a patchy distribution around most Schwann cell nuclei (Fig. 8). This reaction product was seen after 4 weeks exposure, in contrast to the DRG α1 signal which was clear with 3 days exposure. In agreement with the dot blot analysis, no α3 hybridization signal was seen in peripheral nerve. A similar pattern was seen in the white matter tracts of the spinal cord, where α1, but not α3 hybridization signal was found after prolonged exposure (Fig. 9).

DISCUSSION

There are 3 major findings of this study. The first is that α1 mRNA but not α3 mRNA is found in Schwann cells (Fig. 8) and oligodendroglia (Fig. 9). Prior to the identification of the 3 isoforms of (Na,K)-ATPase, Sweadner reported that the kidney isoform (called 'α' but

identical to the currently labeled '$\alpha 1$' isoform) was the predominant glial isoform, based on biochemical identification of (Na,K)-ATPase $\alpha 1$ polypeptide in cultured neonatal astrocytes[20]. A recent immunohistochemical study of optic nerve suggested the presence of $\alpha 1$ and $\alpha 2$ polypeptides in glial cells of the optic nerve[10]. The present in situ mRNA data for peripheral nerve and spinal cord white matter, where $\alpha 1$ but not $\alpha 3$ mRNA is expressed in Schwann cells and oligodendroglial cells, confirm the $\alpha 1$ isoform distribution in oligodendroglia and is the first evidence for $\alpha 1$ mRNA in Schwann cells.

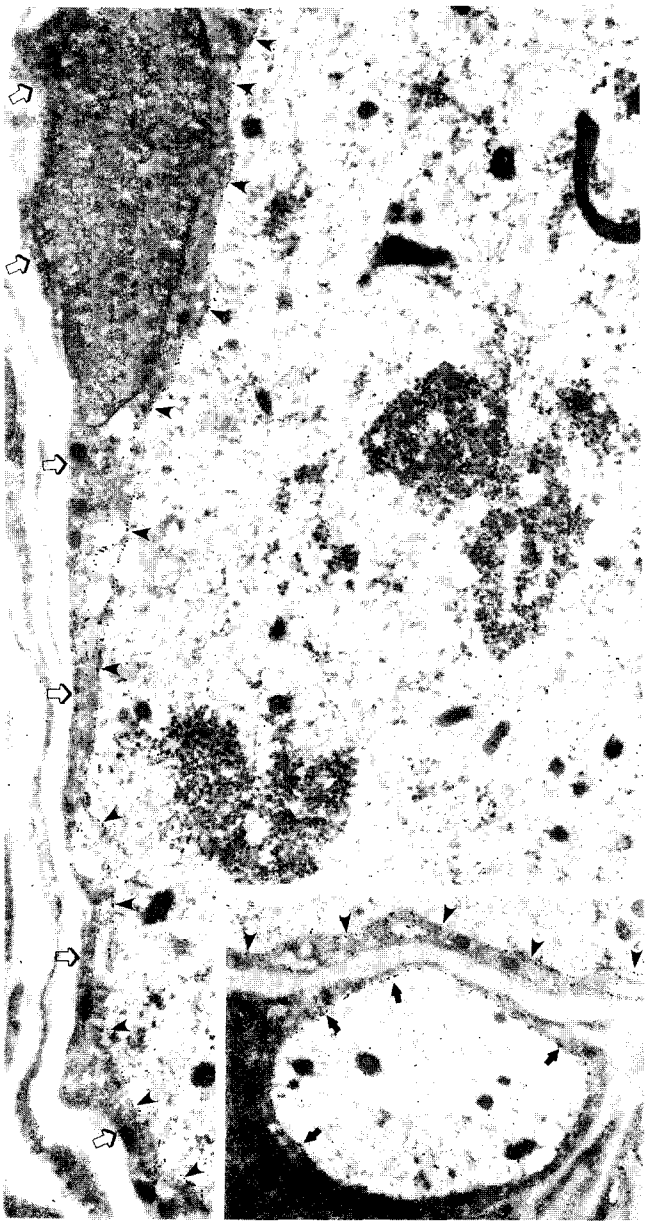


Fig. 4. EM immunocytochemistry with colloidal gold using a polyclonal antiserum against denatured combined α subunits. (Na,K)-ATPase immunoreactivity is seen along the plasma membrane of the DRG neuronal perikaryon (arrowheads) and along the axolemma of the initial segment of the axon (inset, arrowheads). There is no immunoreactivity along the plasmalemma of the satellite cell (open arrows). ×14,600; inset ×11,800.

The second major finding is the expression of both $\alpha 1$ mRNA and $\alpha 3$ mRNA in neurons. Previous data have suggested that $\alpha 1$ mRNA is found in embryonic DRG[14] and that $\alpha 3$ mRNA is specific for brain and spinal cord of fetal and adult rat[14]. There are no previous in situ studies of adult spinal cord, ganglia, or peripheral nerve for comparison. Our data are the first evidence that both the $\alpha 1$ and the $\alpha 3$ mRNA isoforms are found in neurons of the DRG and of the gray matter of the spinal cord of adult rats. Thus, while the glial isoform may be $\alpha 1$, the $\alpha 1$ isoform in the nervous system is not exclusively glial.

The $\alpha 1$ probe was created from the unique untranslated region of the $\alpha 1$ cDNA. This region has no sequence homology with the cDNAs of other isoforms of (Na, K)-ATPase and therefore its binding is specific for $\alpha 1$ mRNA. The $\alpha 3$ probe was created from a cDNA segment containing the translated region, and therefore has some sequence homology with both $\alpha 1$ and $\alpha 2$. However, the $\alpha 3$ signal under the conditions employed in this study in fact does not represent cross-reaction with $\alpha 1$ mRNA in either dot blot or in situ hybridization analyses. In dot blot analysis, the $\alpha 3$ probe did not hybridize with the kidney mRNA (Fig. 1), in agreement with previous data showing that kidney contains only $\alpha 1$ isoform[21]. In in situ hybridization analysis, the exclusive presence of one or the other isoform in different cell types within the same sections demonstrates the specificities of the probes. Thus, $\alpha 1$ only was seen in Schwann cells of the peripheral nerve (Fig. 8), and abundant $\alpha 3$ was found in neurons which did not show detectable $\alpha 1$ signal (Fig. 3).

These data also demonstrate our third principal finding, namely, that individual neurons in DRG and spinal cord may contain abundant $\alpha 3$ and little or no $\alpha 1$ mRNAs, while some neurons contain an abundance of both $\alpha 1$ and $\alpha 3$. So far, no neurons containing abundant $\alpha 1$ without $\alpha 3$ have been identified. It remains to be seen whether neurons in other regions of nervous system in rodent contain $\alpha 1$ without $\alpha 3$ and to determine whether the distribution of isoforms is related to the electrophysiologic or neurotransmitter characteristics of those neurons[17]. It should be noted that while it is valid to compare the amount of labeling between cells or regions using a single probe hybridized under identical conditions, it is not possible to infer the relative amounts of different mRNAs by comparison of the amount of labeling produced by two different probes. In addition, with a single probe, the absence of detectable signal above background implies that the amount of mRNA in that cell type is below the limit of detection using this method but does not mean that there is *none* of that particular α isoform mRNA in the cell.

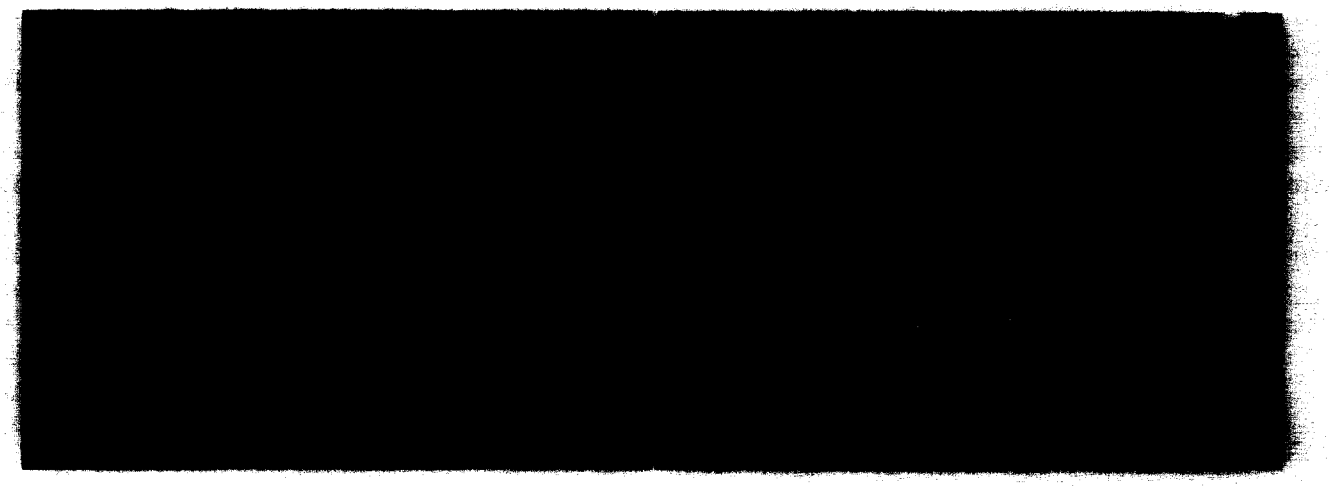


Fig. 5. Darkfield of spinal cord in situ hybridization with ^{35}S-labeled riboprobes for α1 (a) and α3 (b). α1 is found exclusively in laterally placed anterior horn cells (a, arrowheads) while α3 is found in neurons throughout the gray matter of spinal cord (b). 2 week exposure, original magnification ×30.

We also found that the amount of α1 mRNA in individual Schwann and oligodendroglial cells was much less than that found in individual neurons. With identical probes, the α1 mRNA signal from neurons could be readily seen within 3 days of exposure in 6-μm sections of DRG or spinal cord, but 3 to 4 weeks of exposure were

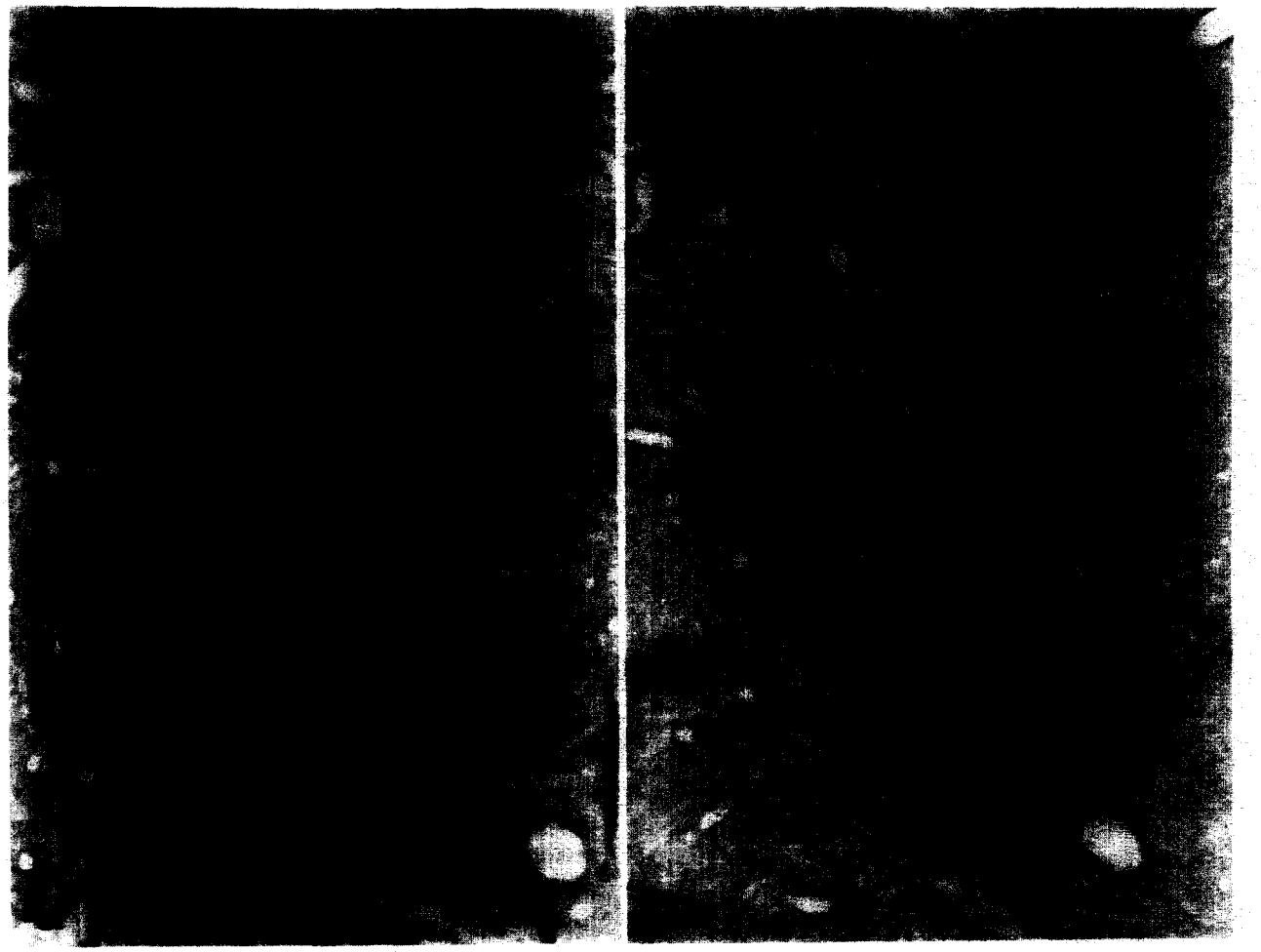


Fig. 6. Brightfield of 1 μm serial sections of spinal cord hybridized with ^{35}S-labeled riboprobes for α1 (a) and α3 (b). Some neurons labeled with α1 also contain α3 (arrowheads, in a and b). Other neurons containing little or no α1 label are heavily labeled with the α3 probe (arrows, in a and b). 4 week exposure, original magnification ×500.

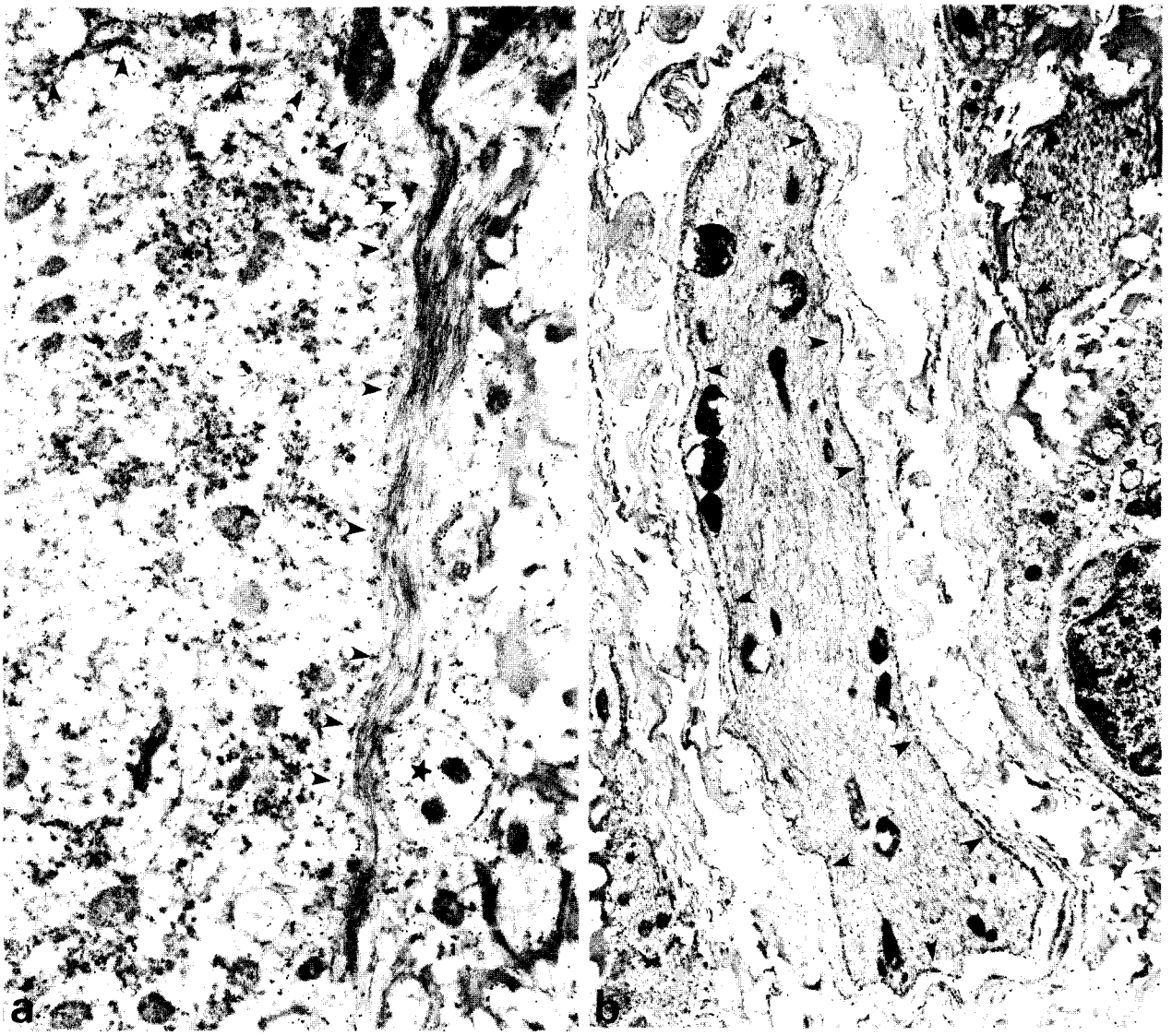


Fig. 7. EM immunocytochemistry with colloidal gold using polyclonal antiserum against denatured combined α subunit shows labeling along the plasma membrane of an anterior horn cell (arrowheads, a), along the axolemma of a myelinated axon of a lower motor neuron exiting the lumbar spinal cord (arrowheads, b), and along a dendrite (asterisk). a: ×16,900; b: ×10,200.

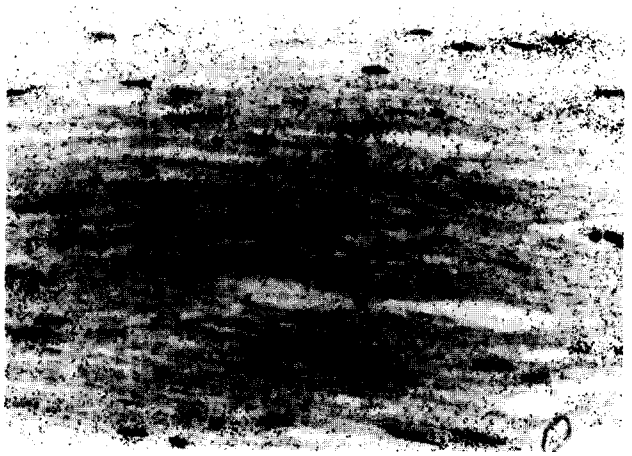


Fig. 8. In situ hybridization of sciatic nerve with ^{35}S-labeled riboprobe for α1 shows prominent labeling of Schwann cells. 4 week exposure, magnification ×360, original magnification ×500.

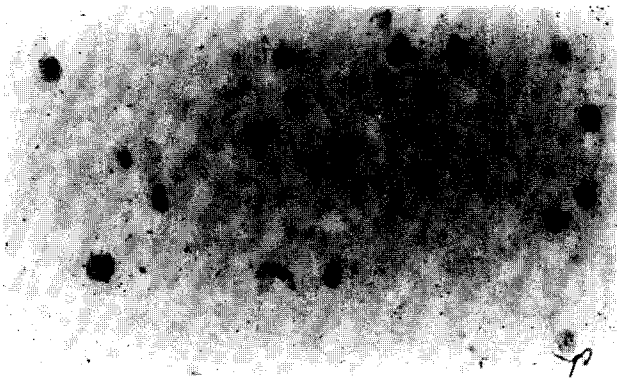


Fig. 9. In situ hybridization of white matter tracts of spinal cord with ^{35}S-labeled riboprobe for α1, exposed for 4 weeks, shows labeling of oligodendroglial cells. Magnification ×260, original magnification ×400.

required for signal detection in either the nerve or the white matter of spinal cord. This suggests that, despite their large membrane areas, Schwann cells and oligodendroglial cells produce little (Na,K)-ATPase mRNA in comparison to neurons.

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