Localization of Sodium/Potassium Adenosine Triphosphatase in Multiple Cell Types of the Murine Nervous System with Antibodies Raised Against the Enzyme from Kidney

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Abstract

This report describes the development and characterization of a battery of highly specific antibodies to sodium/potassium (Na⁺ + K⁺)-ATPase and their use in localizing this enzyme in nervous tissue. The immunolabeling characteristics of polyclonal antibodies and monoclonal antibodies (Schenk, D. B., and H. L. Lettret (1983) Proc. Natl. Acad. Sci. U. S. A. 80: 5281-5285) raised against rat renal (Na⁺ + K⁺)-ATPase were compared. The interspecies cross-reactivity of the polyclonal anti-rat antibodies was examined by determining their binding to purified rat, eel, or dog enzyme. The immunostaining characteristics of the IgG fraction of the polyclonal antibody preparations, their affinity-purified derivatives, and the monoclonal antibodies were compared. The results obtained with each of these were similar, providing information about where focal concentrations of the enzyme exist within nervous tissue. The IgG fraction of the polyclonal antibody preparations provided the most sensitive probe, facilitating localization of the (Na⁺ + K⁺)-ATPase in the tissue sections from various regions of the nervous system.

(Na⁺ + K⁺)-ATPase-like immunoreactivity was observed along the plasmalemma of α-motor neurons and at the nodal axolemma of myelinated axons from the central or peripheral nervous system. It was determined that the absence of labeling for the enzyme along the paranodal or internodal regions of the axolemma was not an artifact due to a limited accessibility of antibody to these regions. Some central nervous system glial cells demonstrated abundant amounts of plasmalemmal and intracellular (Na⁺ + K⁺)-ATPase-like immunoreactivity. These cells were identified as astroglia by positive labeling of cells in serial sections for glial fibrillary acid protein immunoreactivity in the soma and radial processes in optic nerve, or velous processes in the cerebellum. Astrocyte processes overlying the nodal axolemma also stained positively for the enzyme. (Na⁺+K⁺)-ATPase-like immunoreactivity was not observed in association with oligodendroglia cell bodies or their processes forming myelin sheaths. In contrast, the plasmalemma of myelinating Schwann cells showed greatest immunoreactivity in the region of the node of Ranvier. Although a focal concentration of immunoreactivity was observed along node- and paranode-associated regions of Schwann cells, a lower level of uniform staining was noted along the entire Schwann cell surface membrane. The significance of the distribution of enzyme determined in these studies is considered with respect to functional significance in regions of focal concentration. The results of the tissue survey and characterization of antibody specificity reported here now enable an assessment of the distribution of (Na⁺ + K⁺)-ATPase in developmental and pathological animal models.

The sodium/potassium-activated adenosine triphosphatase (Na⁺ + K⁺)-ATPase in various tissues have employed biochemical, autoradiographic, cytochemical, and immunocytochemical techniques (see DiBona and Mills, 1979, and Ernst and Mills, 1980, for reviews). Recently, these techniques have been used to investigate the subcellular distribution of (Na⁺ + K⁺)-ATPase in the nervous system of a number of different species (Wood et al., 1977; Broderson et al., 1981). Activity of (Na⁺ + K⁺)-ATPase significantly influences neuronal excitability by affecting the resting membrane potential of neurons (Thomas, 1972) and maintaining sodium and potassium gradients required for action potential propagation (Bonting, 1970). In addition, the enzyme may also play a role in the uptake of potassium ions by glial cells during periods of intense neuronal activity (Henn et al., 1972; Walz and Hertz, 1982).

Previous studies localizing (Na⁺ + K⁺)-ATPase in various tissues have employed biochemical, autoradiographic, cytochemical, and immunocytochemical techniques (see DiBona and Mills, 1979, and Ernst and Mills, 1980, for reviews). Recently, these techniques have been used to investigate the subcellular distribution of (Na⁺ + K⁺)-ATPase in the nervous system of a number of different species (Wood et al., 1977; Broderson et al., 1978; Svedahe, 1979; Schwartz et al., 1981; Vorbrodt et al., 1982; Siegel et al., 1984; Stahl and Baskin, 1984; among others). A heterogeneous distribution of (Na⁺ + K⁺)-ATPase along the plasmalemma of different cells has been reported by these investigators. For example, the enzyme is concentrated at the inner segment of retinal photoreceptors and along the axolemma of myelinated axons at the node of Ranvier (although limited accessibility of the immunocytochemical reagents

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Materials and Methods

Enzyme purification

The (Na+ + K+)-ATPase was purified from the renal medulla of rats and the electric organ of Electrophorus electricus. Purified canine renal (Na+ + K+)-ATPase was generously gifted by J. Kyte (1971), a modification of the method of Jorgensen and Skou (1969). Eel enzyme was purified by the method of Dixon and Hokin (1978). Enzyme activity was assayed as described by Kyte (1971) except that phoshatidyl-L-serine was not included in the assay mixture. Protein concentrations were determined by the method of Lowry et al. (1951).

SDS-polyacrylamide slab gels were run according to the method of Laemmli (1970). The (Na+ + K+)-ATPase was phosphorylated using the method of Wiskowkar (1971). Eel enzyme was purified by the method of Dixon and Hokin (1978). Enzyme activity was assayed as described by Kyte (1971) except that phosphatidyl-L-serine was not included in the assay mixture. Protein concentrations were determined by the method of Lowry et al. (1951).

Antibody production, purification, and characterization

Antibodies were raised against rat and eel holoenzymes and the α- and β-subunits of the rat enzyme by identical methods. Antibodies were produced in female, white New Zealand rabbits. The rabbits were injected with 100 μg of protein in Freund’s complete adjuvant. Booster injections of 50 μg of protein in Freund’s complete adjuvant at 1- to 3-month intervals. The rabbits were bled 10 to 14 days after booster injections. A portion of the sera was precipitated with ammonium sulfate and passed down a DEAE-cellulose column to partially purify the IgG fraction. Some preparations were further purified by protein A column chromatography. DEAE-purified antibodies were loaded onto the column at 4°C for 1 to 2 days. After washing off excess antibodies by the binding of protein A column chromatography. DEAE-purified antibodies were loaded onto the column at 4°C for 1 to 2 days. After washing off excess antibodies by the method outlined above. The antibody titers and antigen binding specificity were examined by the enzyme-linked immunosorbent assay (ELISA) described below. The antibodies were aliquoted and stored at −20 or −70°C.

The ELISAs were carried out in Linbro/Titer 96 multiwell plates. The indirect ELISA was used to detect the sera and to show the specificity of the antibody for the antigens. Antibodies were raised against the rat enzyme by identical methods. Antibodies were produced in female, white New Zealand rabbits.

Immunochemical procedures

Fixation. Adult Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) and 129 ReJ mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with Nembutal (1 mg/kg, i.p.) and perfused through the heart. Rat Ringer’s solution at 37°C was followed by fixative which was cooled from 37°C to 10°C during the perfusion. Three different fixatives were employed: (1) periodate-lysine-parafomaldehyde (PLP) fixative (McLean and Nohanc, 1974), (2) modified PLP fixative (0.55 mg/liter of sodium periodate, 3.4 g/liter of mono-DL lysine, 4% parafomaldehyde), and (3) 4% paraformaldehyde with different concentrations of glutaraldehyde (0.1, 0.25%, 0.5%, 1%, 1.5%). Tissues were dissected and immersion fixed for an additional hour at 4°C. Qualitatively and quantitatively, staining patterns observed with these fixatives appeared identical.

Preparation of tissue for light microscopy. Spinal roots and spinal cords were incubated in collagenase type IV (1 mg/ml, Sigma Chemical Co., St. Louis, MO) and hyaluronidase, type 1-S (1 mg/ml, Sigma), solutions for 30 min each at 4°C. Tissue was dissected and immersion fixed for an additional hour at 4°C. Tissues were dissected and immersion fixed for an additional hour at 4°C. Qualitatively and quantitatively, staining patterns observed with these fixatives appeared identical.
Affinity of the different polyclonal antibodies for various enzyme preparations was examined using immunoblotting techniques (Fig. 3). Anti-holoenzyme antibodies recognize both the α- and β-subunits (Fig. 3A, lanes 1 to 4). The absence of detectable binding to other proteins in partially purified microsomal preparations (Fig. 3A, lane 4) demonstrated that the anti-holoenzyme preparations are highly specific for (Na\(^+\) + K\(^+\))-ATPase. Occasionally, there was light staining of the track below the α-band. Since this was also present in the blot incubated with the anti-α serum but not the anti-β serum, we interpreted these bands to represent fragments of the α-subunit. The anti-α and anti-β antibodies showed binding only to the appropriate subunit and did not cross-react with the other subunit (Fig. 3B and C). Anti-α antibodies bind to the band corresponding to the α-subunit (Fig. 3B, lanes 1 to 3). No binding to the band corresponding to the β-subunit was detected with the anti-α antisera (Fig. 3B, lanes 1 and 3). Blots incubated with anti-β serum showed staining only of the band corresponding to the β-subunit, with no staining of the α-subunit (Fig. 3C, lanes 1 to 3). Normal rabbit serum did not bind to bands corresponding to either subunit (Fig. 3D, lanes 1 to 3).

Determining the relative affinities of the anti-α, anti-β, anti-holoenzyme antibodies for the purified α- and β-subunits and the holoenzyme required examination using ELISAs. Results showed that the anti-subunit antisera had high affinity for the subunit for which they had been raised, with very little binding to the other subunit (Fig. 4, A and B). Anti-α antibodies had the highest affinity for the α-subunit, with slightly lower binding to the holoenzyme and little recognition of the β-subunit. Anti-β antibodies had the highest affinity for the purified β-subunit, with a slightly lower binding to the holoenzyme, and little recognition of the α-subunit. The anti-holoenzyme antibodies had the highest binding to the holoenzyme, followed by the β and then the α-subunit (Fig. 4C).

The results of ELISAs and Western blots demonstrated that the anti-holoenzyme, anti-α, and anti-β polyclonal antibody preparations had high affinity for (Na\(^+\) + K\(^+\))-ATPase and the (appropriate) α- and β-subunits. Since the affinity of an antibody for an antigen gives a measure of the specificity of the antibody for that antigen (Sternberger, 1979, p. 15), these results suggested that these antibody preparations are highly specific. The availability of these antibodies, as well as high affinity antibodies against dog and eel (Na\(^+\) + K\(^+\))-ATPase allowed us to examine the degree of cross-reactivity between anti-(Na\(^+\) + K\(^+\))-ATPase preparations raised in various species. The affinity of polyclonal antibodies raised against (Na\(^+\) + K\(^+\))-ATPase from rat, dog, and eel for these proteins was compared by ELISA (Fig. 5). Each antiserum was most specific for its homologous antigen. Rat and dog had intermediate levels of cross-reactivity when compared to results obtained with their homologous antibody-antigen combinations (Fig. 5, A and B). Eel had significant cross-reactivity with rat and dog (Fig. 5, A to C). The results of these experiments justified, in our minds, our efforts to raise specific antibodies against rat (Na\(^+\) + K\(^+\))-ATPase for use in immunocytochemical experiments in rats and mice.

**Localization of (Na\(^+\) + K\(^+\))-ATPase by immunocytochemistry**

Initial immunocytochemical screenings of the monoclonal and polyclonal antibodies were done on rat renal tissue to determine their usefulness in localization studies. The anti-α and anti-β polyclonal antibody preparations revealed limited recognition of the enzyme in rat kidney and optic nerve (data not shown). One possible explanation is that these antibodies are directed primarily at epitopes on denatured portions of each subunit that arose during purification by SDS-PAGE. These results demonstrated that antibodies raised against denatured antigens, although useful biochemically, may not have high affinity for the antigen as found in tissue. Monoclonal and anti-holoenzyme antibodies did recognize (Na\(^+\) + K\(^+\))-ATPase in initial screenings, and these preparations were employed in this investigation. Three different polyclonal anti-holoenzyme antibody preparations, purified by DEAE-cellulose, protein A, or (Na\(^+\) + K\(^+\))-
Figure 1. SDS-polyacrylamide gel of preparations of purified rat renal (Na\(^+\) + K\(^+\))-ATPase. Lane 1, Microsomal preparation; lane 2, purified holoenzyme. The higher molecular weight band corresponds to the \(\alpha\)-subunit. The lower molecular weight band corresponds to the \(\beta\)-subunit and was diffuse, a characteristic of glycoproteins run on SDS-polyacrylamide gel, (Kyte, 1972); see also lane 3, Gel-purified \(\beta\)-subunit; lane 4, gel-purified \(\alpha\)-subunit; lanes 5 and 6, molecular weight standards.

Figure 2. A representative ELISA comparing the binding activity of experimental (C) and preabsorbed control preparations (○) with normal rabbit serum (○) for the purified holoenzyme preparation. ELISAs measure antibody binding to antigen in units of optical density (O.D.) at 490 nm.

Figure 3. Western blot examining the specificity of the antibodies raised against the purified holoenzyme and the \(\alpha\)- and \(\beta\)-subunits. Lanes 1 and 2 show, respectively, the gel purified \(\beta\)-subunit and the \(\alpha\)-subunit, rerun by SDS-PAGE and transferred to nitrocellulose paper. Lane 3 shows the purified holoenzyme preparation run by SDS-PAGE and transferred to nitrocellulose paper. Lane 4 shows the partially purified microsomal preparation run by SDS-PAGE and transferred to nitrocellulose paper. Anti-holoenzyme antibodies were used in A, anti-\(\alpha\) antibodies were used in B, anti-\(\beta\) antibodies were used in C, and normal rabbit serum was used in D.
Figure 4. ELISAs are a method for evaluating the relative affinities of the anti-α subunit (A), anti-β antibodies (B), and anti-γ antibodies (C) for the purified holoenzyme and subunits. Data were obtained with the anti-α antibodies (A), anti-β antibodies (B), and the anti-γ antibodies (C), using as antigen the gel-purified α-subunit (A), the β-subunit (B), or the purified holoenzyme (C).

Figure 5. ELISAs showing species cross-reactivity between rat, canine, and eel (Na+ + K+)-ATPase and antibodies raised against these enzymes. Results were obtained with polyclonal antibodies raised against the purified holoenzyme from rat (A), dog (B), and eel (C). A, When compared to NRS controls binding to rat holoenzyme was greatest with anti-rat antibodies, intermediate with anti-dog antibodies, and negligible with anti-eel antibodies. B, when compared to NRS controls, binding to canine holoenzyme was greatest with anti-canine antibodies, intermediate with anti-rat antibodies, and negligible with anti-eel antibodies. C, When compared to NRS controls, binding to eel holoenzyme was greatest with anti-eel antibodies, intermediate with anti-rat antibodies, and negligible with anti-canine antibodies.
Figure 6. (Na⁺ + K⁺)-ATPase immunoreactivity in myelinated axons. A to D, Myelinated axons teased from a rat spinal root. A, Brightfield image of an experimental preparation. Bar = 10 μm. B, Corresponding immunofluorescent image of the myelinated axon in A. Note the intense nodal axolemmal staining (arrows). The plasmalemma of Schwann cells is also stained (arrowheads). C, Brightfield image of an absorbed control preparation. D, Corresponding immunofluorescent image of the myelinated axon in C. Note the absence of staining. E, Immunoelectron microscopy of a myelinated axon from rat optic nerve. Immunoreactivity was observed at the node of Ranvier (arrows). Note the heavily stained astrocyte process (see below), which lies in the extracellular space around the node of Ranvier (arrowheads). Mildly treated with Lubrol PX. Bar = 0.25 μm. F, Immunoelectron microscopy of an absorbed control preparation from mouse spinal root. Staining was not observed in these preparations. Bar = 3 μm. G, Immunoelectron microscopy of myelinated axons from mouse spinal root. Immunoreactivity was restricted to the nodal axolemma (arrows) and the Schwann cell plasmalemma (arrowheads). Bar = 1 μm.
ATPase affinity column chromatography, were used. All three yielded similar qualitative and quantitative results and, unless otherwise noted, the results reported have employed DEAE-cellulose column chromatographed preparations.

Although substantial biochemical evidence suggested that both the monoclonal and polyclonal antibody preparations are highly specific for \( \text{Na}^+ + \text{K}^+ \)-ATPase, additional evidence was necessary to establish that these probes are specific for the enzyme in tissue. The best measure of tissue specificity of an antibody preparation is to compare results obtained with other examples in the literature (Stemmerberg, 1979, p. 148). Thus, as the final measure of the specificity of both the monoclonal antibodies and polyclonal antibodies, the data obtained in this study were compared with previously reported results from a number of different species (rat, mouse, Sternarchus, chicken, and goldfish) using a variety of techniques (immunocytochemistry, autoradiography, and cytochemistry). Based on these comparisons, these probes were determined to be highly specific for \( \text{Na}^+ + \text{K}^+ \)-ATPase in tissue and were used to investigate further the distribution of the enzyme along the axolemma of myelinated axons, and in glial cells.

Neuronal cells. Neuronal \( \text{Na}^+ + \text{K}^+ \)-ATPase immunoreactivity was observed on the nodal axolemma and on the somal plasmalemma of neurons. Myelinated axons from the spinal roots of rats and mice and the optic nerve of rats demonstrated significant \( \text{Na}^+ + \text{K}^+ \)-ATPase immunoreactivity along the axolemma only at the node of Ranvier (Fig. 6). Absorbed control preparations showed that this result was specific for \( \text{Na}^+ + \text{K}^+ \)-ATPase (Fig. 6, D and F). Similar reports of nodal axolemmal \( \text{Na}^+ + \text{K}^+ \)-ATPase have been communicated by Wood et al. (1977) in Sternarchus telencephalon and in mouse cerebellum (Siegel et al., 1984). Cytochemical studies have reported \( \text{Na}^+ + \text{K}^+ \)-ATPase in association with the plasmalemma of dendrites (Broderson et al., 1978; Inomata et al., 1983; Nasu, 1983) but not the plasmalemma of neuronal somata (Inomata et al., 1983).

Glial cells. Glial \( \text{Na}^+ + \text{K}^+ \)-ATPase immunoreactivity was observed in astrocytes and Schwann cells but not in oligodendrocytes, using both polyclonal and monoclonal antibody preparations. Fibrous and protoplasmic astrocytes including their processes showed significant amounts of plasmalemmal and intracellular \( \text{Na}^+ + \text{K}^+ \)-ATPase immunoreactivity. Experiments in cross-sections and longitudinal sections of rat optic nerve showed glial cells heavily stained for \( \text{Na}^+ + \text{K}^+ \)-ATPase (Fig. 9 to 11). The identification of these cells as astrocytes was accomplished by staining adjacent sections in a series of cross-sections with antibodies against \( \text{Na}^+ + \text{K}^+ \)-ATPase or GFAP (Fig. 9). The results obtained demonstrated that cells positively labeled for \( \text{Na}^+ + \text{K}^+ \)-ATPase (Fig. 9A) were also GFAP immunoreactive (Fig. 9B), thus identifying these cells as astrocytes (Eng and DeArmond, 1981). There is little \( \text{Na}^+ + \text{K}^+ \)-ATPase-like

![Figure 7](image_url)
immunoreactivity in these cells when incubated with antibodies preabsorbed with (Na\(^+\) + K\(^+\))-ATPase (Fig. 9C). Immunoelectron microscopic examination of optic nerve also showed abundant and diffuse intracellular staining of fibrous astrocytes in the parenchyma as well as those closely applied to capillaries (Fig. 10). Heavily labeled astrocytic processes were also observed in the extracellular space overlying the node of Ranvier (Fig. 6E). Immunoperoxidase results employing the monoclonal antibody 9B1 also demonstrated heavy intracellular staining in fibrous astrocytes (Fig. 11). Monoclonal antibody 9A5 had a similar pattern of immunoreactivity but required higher concentrations (data not shown). The results observed with these monoclonal antibodies were similar to that obtained with the polyclonal antibody preparations. More detailed localization of the intracellular site (Na\(^+\) + K\(^+\))-ATPase concentration will require non-enzymatic labels (such studies utilizing ferritin- and colloidal gold-conjugated antibodies are in progress).

Experiments in the cerebellum of rats and mice showed (Na\(^+\) + K\(^+\))-ATPase-like immunoreactivity in thin or velate processes of glial cells in the granule cell layer (Fig. 12A). Immunoelectron microscopy demonstrated abundant intracellular reaction product within these velate processes (Fig. 12B). These processes are characteristic of protoplasmic astrocytes as visualized with high voltage electron microscopy in Golgi-stained preparations (Palay and Chan-Palay, 1974). To determine whether these processes arose from astroglia, we examined them after reaction with anti-GFAP antibodies. These velate processes were GFAP immunoreactive, verifying that they were astrocyte processes (Fig. 12C). Other investigators have also reported (Na\(^+\) + K\(^+\))-ATPase in astroglia. (Na\(^+\) + K\(^+\))-ATPase immunoreactivity in the plasmalemma of pericapillary and periterminal astrocytic processes has been reported previously by Wood et al. (1977). The enzyme has also been detected in the plasmalemma of Muller cells in guinea pig retina by cytochemistry (Ueno et al., 1981). However, Broderson et al. (1978), Inomata et al. (1983), and Nasu (1983) have reported an absence of the enzyme in glial cells using cytochemical techniques.

Some glial cells in rat optic nerve were observed that were not (Na\(^+\) + K\(^+\))-ATPase immunoreactive (Fig. 13A). Although they were not stained with uranyl or lead salts, these cells could be identified as oligodendrocytes. They could be distinguished from astrocytes, based on their characteristic morphology (Peters et al., 1976). They exhibited dense and eccentric nuclei, dark cytoplasm, a well developed rough endoplasmic reticulum, and few cytoplasmic fibrils or glycogen granules (Fig. 13B). Thus, it appears that oligodendrocytes in the rat optic nerve have little intracellular or plasmalemmal (Na\(^+\) + K\(^+\))-ATPase-like immunoreactivity. Labelling along the outer myelin sheath of oligodendrocytes was not noteworthy (Fig. 6E). (Na\(^+\) + K\(^+\))-ATPase immunoreactivity has been reported by others in association with the somal plasmalemma of oligodendroglia (Wood et al., 1977) and on their outer myelin sheath (Wood et al., 1977; Schwartz et al., 1981).

In contrast to the results observed in oligodendrocytes, the myelinating cell type of the PNS, the Schwann cell, showed plasmalemmal (Na\(^+\) + K\(^+\))-ATPase immunoreactivity. Immunofluorescence experiments on teased spinal roots from rats with polyclonal antibodies demonstrated continuous (Na\(^+\) + K\(^+\))-ATPase immunoreactivity along the outer loops of the Schwann cell process (Fig. 6B). Monoclonal antibodies 9A5 and 9B1 (Fig. 14) also demonstrated similar results in teased single fibers from the spinal root of rats. Immunoelectron microscopy of mouse spinal root showed staining of the terminal loops overlying the nodal axolemma (Fig. 6G). These results were not surprising since the Schwann cell, unlike the oligodendrocyte, has a cytoplasm-filled terminal wrapping of the myelin sheath. Cytochemical examination of (Na\(^+\) + K\(^+\))-ATPase in peripheral nerves of mice showed an absence of the enzyme in the microvilli of Schwann cells overlying the node of Ranvier (Vorbrodt et al., 1982).

**Epithelial cells of the choroid plexus.** Epithelial cells of the choroid plexus from the fourth ventricle of rats had positive (Na\(^+\) + K\(^+\))-ATPase immunoreactivity exclusively along the apical surface of the cell. Absorbed controls did not demonstrate significant immunoreactivity (Fig. 15). Similar results have also been reported employing autoradiography (Quinton et al., 1973) and immunocytochemistry
Figure 9. Immunoperoxidase experiment on serial sections of rat optic nerve, demonstrating (Na\(^+\) + K\(^+\))-ATPase and GFAP immunoreactivity co-localized in the same cells. A, (Na\(^+\) + K\(^+\))-ATPase-like immunoreactivity in glial cells. Note the positive labeling (arrows) of the somas and processes around capillaries (c). B, GFAP-immunoreactive cells in a serial section (arrows). These cells were astrocytes. C, Preabsorbed controls had little (Na\(^+\) + K\(^+\))-ATPase-like immunoreactivity. (Normal rabbit serum controls appeared similar. The contribution of peroxidase-containing granules (Kumamoto, 1961) to staining in the experimental preparations was negligible (data not shown.)) Nomarski differential interference contrast optics. Bar = 2 \(\mu\)m.
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Figure 10. Immunoelectron microscopy of fibrous astrocytes from a longitudinal section of rat optic nerve. Note the abundant intracellular staining. A, Lower magnification of processes extending from an astrocyte (a), heavily stained for (Na\(^+\) + K\(^+\)) ATPase and surrounding a capillary (c), and an oligodendrocyte (o). Bar = 2 \(\mu\)m. B, A higher magnification of the pericapillary astrocytic process in A. End-feet of the process (e) were also heavily stained. Bar = 1 \(\mu\)m.

(Masuzawa et al., 1984; Siegel et al., 1984). Cytochemical techniques have reported (Na\(^+\) + K\(^+\))-ATPase in both regions. Milhorat et al. (1975) concluded that (Na\(^+\) + K\(^+\))-ATPase was confined to the basolateral plasmalemma of choroid epithelial cells based on their results. Other investigators (Masuzawa et al., 1981) have reported (Na\(^+\) + K\(^+\)) ATPase in the microvillar processes and basal, but not lateral, plasmalemma of choroid plexus cells using cytochemical methods.

Discussion

This paper describes the development and characterization of polyclonal antibodies to rat (Na\(^+\) + K\(^+\))-ATPase. These antibodies cross-react in varying amounts with dog and eel (Na\(^+\) + K\(^+\))-ATPase. These probes, along with the anticatalytic monoclonal antibody preparations (previously characterized by Schenk and Leffert, 1983, and Schenk et al., 1984) raised against rat renal (Na\(^+\) + K\(^+\))-ATPase have demonstrated immunocytochemically that they bind to (Na\(^+\) + K\(^+\))-ATPase in rat nervous tissue. The results with these different probes were similar, showing regions of focal concentration of the enzyme. (Na\(^+\) + K\(^+\))-ATPase-like immunoreactivity was observed along the plasmalemma of neurons and at the nodal, but not the paranodal or internodal, axolemma of myelinated axons of the CNS and PNS. Astroglia had abundant plasmalemmal and intracellular (Na\(^+\) + K\(^+\))-ATPase-like immunoreactivity. The plasmalemma of Schwann cells were positively labeled, particularly in the paranodal region. The soma and myelin of oligodendroglia were not positively stained for the enzyme.
Interspecies and isoynme cross-reactivity. Results obtained with ELISAs demonstrate that there is limited cross-reactivity between polyclonal antibodies raised against (Na\(^+\) + K\(^+\))-ATPase from one species for the enzyme from another species. These data suggest that even in mammals as closely related as the dog and rat there are variabilities in enzyme structure. This suggests that results from xenogenic immunocytochemical studies must be interpreted with caution. In this study, a careful comparison between rats and mice showed no qualitative differences in the distribution of (Na\(^+\) + K\(^+\))-ATPase in neurons and glia. This suggests a high degree of cross-reactivity between these anti-rat (Na\(^+\) + K\(^+\))-ATPase antibodies and the mouse enzyme, allowing these probes to be used in the future in various mutant mouse models.

The immunocytochemical data suggest that both the polyclonal and monoclonal preparations recognize isoynmes of (Na\(^+\) + K\(^+\))-ATPase found in the nervous system (Sweadner, 1979; Urayama and Nakao, 1979; Rubin et al., 1981). Sweadner (1979) has reported two principal forms of the higher molecular weight subunit of the enzyme, referred to as \(\alpha\) and \(\alpha^+\), in the brains of a number of species. Only the \(\alpha^+\) form appears to be present in axolemmal tissue fractions from myelinated axons from the CNS (excluding synapticosomal fractions), even though some neurons apparently synthesize both forms (Specht and Sweadner, 1984). Glial cells were found to contain only the \(\alpha\)-subunit which appeared very similar to the form found in kidney. In this study, polyclonal antibody preparations required higher concentrations to detect the axolemmal form of the enzyme. One interpretation of these data suggest that only a subset of the polyclonal antibodies raised against the glial/renal form cross-reacts with high affinity for the axolemmal form of the enzyme. Previous reports have also demonstrated cross-reactivity between antibody preparations raised against non-nervous system (Na\(^+\) + K\(^+\))-ATPase and brain enzyme in the same species (McCans et al., 1975; Jean and Albers, 1976; see Note added in proof).

Figure 7. Immunoperoxidase results in longitudinal sections of rat optic nerve employing monoclonal antibody 9Bl. Heavy intracellular staining was present in the processes of glial cells (arrows), including those around capillaries (C). Nomarski differential interference contrast optics. Bar = 15 \(\mu\)m.

Figure 11. Immunoperoxidase results in longitudinal sections of rat optic nerve employing monoclonal antibody 9Bl. Heavy intracellular staining was present in the processes of glial cells (arrows), including those around capillaries (C). Nomarski differential interference contrast optics. Bar = 15 \(\mu\)m.
Figure 72. (Na\(^+\) + K\(^+\)-ATPase immunoreactivity in the cerebellum of rats and mice. A, Phase contrast image of immunoperoxidase results of experiments employing anti-(Na\(^+\) + K\(^+\))-ATPase antibodies in mouse cerebellum. Note cytoplasmic immunoreactivity in the delicate cellular processes (arrows). Bar = 3 μm. B, Electron microscopic localization of (Na\(^+\) + K\(^+\))-ATPase in the granule cell layer of the rat cerebellum. Bar = 2 μm. C, Anti-GFAP immunofluorescence was also observed in these delicate processes identifying them as velate processes of astrocytes. Bar = 6 μm.
the question of membrane integrity. The absence of well preserved morphological structures makes it difficult to distinguish between axolemmal and other membranes. Also, it is possible that, in the absence of reported controls, nonspecific interaction in the internodal region could account for the observed immunoreactivity. The use of a monoclonal antibody did not eliminate the possibility of cross-reactivity with other proteins (for example, see Lindstrom et al., 1979, p. 4475), although availability of an anticatalytic monoclonal antibody would have suggested an additional level of specificity. Thus, localization studies done previously have not resolved the question of whether or not (Na+ + K+)-ATPase is concentrated in the internodal and paranodal axolemma.

Our report addressed this controversy directly. The data presented show that the level of the enzyme was undetectable in the internodal and paranodal regions while being readily detectable at the nodal axolemma in the absence of permeability barriers to immunocytochemical reagents. It would be inappropriate to claim that (Na+ + K+)-ATPase was not present in the internodal axolemma because the technique employed might not be sensitive enough to detect small concentrations of the enzyme on the order necessary to compensate for passive leakage of Na and K ions through the axolemma. The data do demonstrate significant differences in the levels of (Na+ + K+)-ATPase-like immunoreactivity in the membranes of the internodal and paranodal regions of the axolemma versus the nodal axolemma itself.

The role of astroglia in ionic homeostasis. This report may contribute toward a better understanding of the relative roles of neuron and glia in maintaining ionic homeostasis in nervous tissue. Early investigations suggested that increased extracellular K was dissipated by diffusion through the extracellular space (reviewed by Kuffler, 1967). Later work suggested that glial cells were passively involved in the uptake of elevated extracellular K (reviewed by Varon.
and Somjen, 1979). However, the ability of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) to catalyze the antiport of Na and K ions across cell membranes suggested that it may play a significant role in the reabsorption of K ions by astrocytes. Studies have described \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) in astrocytes and the ability of this enzyme in cell cultures of astrocytes to contribute to the uptake of elevated concentrations of K ions (Waltz and Hertz, 1982). Early immunocytochemical work in \textit{Stenar-}

chus (Wood et al., 1977) demonstrated the enzyme in astrocyte processes surrounding capillaries and terminals supporting this concept. In a more recent study on mouse brain at the light level employing immunocytochemical techniques, Siegel et al. (1984) were unable to determine whether staining in some regions was attributable to neurons or glia. Data generated using cytochemical methods to determine the location of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) in rats are conflicting. Using this technique, enzyme activity was detected along dendritic plasmalemma but not in association with glial membranes (Stahl and Broderson, 1976; Broderson et al., 1978; Inomata et al., 1983; Nasu, 1983). These results might be interpreted to suggest that neurons, not glia, are the primary cell type involved in maintaining extracellular K ion homeostasis. These inconsistencies between reports on the relative distribution of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) in glial and neuronal cells may be attributable to differences in the techniques employed.

In the present work we addressed this question by re-examining the distribution of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) in neurons and astrocytes in rats and mice using immunocytochemical techniques. The enzyme was detected in the somal plasmalemma and nodal axolemma of neurons. Abundant amounts of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\)-like immunoreactivity was also observed in association with the plasmalemma of the soma and distal processes of astrocytes. The cytoplasm also contained reaction product, suggesting large amounts of the enzyme within these cells, possibly in association with a cytoplasmic membrane system. Some of this immunoreactivity could represent proenzymatic forms of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\). The possibility also remains that the polyclonal antibodies, and even the anticytolytic monoclonal antibodies, are cross-reacting with another antigen, perhaps an ion-binding protein. Astrocytic processes forming pericapillary end-feet demonstrated considerable labeling similar to results reported in \textit{Stenarachus}. In addition, we noted that astrocytic processes overlying the node of Ranvier also exhibited abundant \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\)-like immunoreactivity. This may be particularly important considering the small volume of the perinodal cuff of extracellular fluid where rapid, and frequently repeated ionic or fluid flux would significantly alter ionic concentrations, in particular local sodium and potassium ion gradients. This is particularly interesting since \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\)-like immunoreactivity was not detected on the plasmalemma or outer myelin sheath of oligodendrocytes. These data provide additional, new evidence implicating astrocytes in the maintenance of a homeostatic environment in regions of large ionic flux. A functionally equivalent role may be performed by Schwann cells in the PNS. Our observations of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\)-like immunoreactivity in the terminal loops and microvilli processes overlying the node of Ranvier suggest a similar role for Schwann cells in the homeostasis of the extracellular fluid in this region. It is interesting to note along these lines that an abnormality in glial \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) has been established in epileptogenic tissue (reviewed by Grisar, 1984).

Comparison of the different techniques. Cytochemical techniques and immunocytochemical methods for localizing \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) have, on occasion, given different results when compared. As an example, differences in detecting \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) in astrocytes have already been noted. Another example is the epithelial cells of the choroid plexus (see “Results”). The reasons for such a disparity have not been determined. These discrepancies may arise because the immunocytochemical technique requires conservation of enzyme conformation, while the cytochemical method further necessitates preservation of enzymatic activity. Mayahara and Ogewa (1980) have reported in a detailed study in rat kidney that, under their experimental conditions, 45% of the enzymatic activity remains. The immunocytochemical technique may offer greater sensitivity although it could detect nonenzymatically active forms of the antigen.

Some differences have been noted when comparing results of the distribution of \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) from this study with data from other reports using a variety of techniques. However, our polyclonal results did agree where staining has been reported (at the node of Ranvier and plasmalemma) using all of these techniques. Polyclonal and monoclonal data agreed with other immunocytochemical results reported in astrocytes. Thus, the biochemical and immunocytochemical results demonstrate that these monoclonal and polyclonal antibodies are highly specific for \((\text{Na}^+ + \text{K}^+)-\text{ATPase}\) and that the observations reported have been achieved with accurate probes for the enzyme.

Note added in proof. Additional data supporting this interpretation was acquired recently in collaboration with Dr. A. McDonough. It
was determined that the polyclonal antibodies described in this report bind primarily to the α form and less to the α+ form in immunoblot of the enzyme from brain. In contrast, polyclonal antibodies raised to the enzyme from guinea pig kidney (McDonough, 1985) react most strongly with α+ form from rat brain. When these "anti α+" antibodies were used to probe the enzyme’s distribution in peripheral nerve, staining of the Schwann cell plasmalemma was absent. The axolemma of the nodal membrane, however, stained heavily. This indicates that the (Na+ + K+)−ATPase of the nodal membrane is primarily of the α+ variety while that of the Schwann cell plasmalemma is of the α form.

References

Ariyasu, R. G., M. H. Ellisman, J. A. Nichol, and T. J. DeCenfro (1982) Immunocytochemical localization of sodium-potassium adenosine triphosphatase in the rat central nervous system. Soc. Neurosci. Abstr. 8: 415.

Burling, S. J. (1970) Sodium-potassium activated adenosine triphosphatase in cation transport. In Membranes and Ion Transport, E. E. Bittar, ed., pp. 256-363, Wiley-Interscience, London.

Bird, S. H., U. L. Patton, and W. L. Stahl (1979) In vitro localization of potassium-stimulated p-nitrophenylphosphatase activity in dendrites of the cerebral cortex. J. Cell Biol. 77: R13-R17.

Burnette, W. N. (1981) "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195-203.

Craig, W. S., and J. Kyte (1980) Stoichiometry and molecular weight of the minimum asymmetric unit of coninu roanal sodium and potassium ion activated adenosine triphosphatase. J. Biol. Chem. 255: 6262-6269.

Dibona, D. R., and J. W. Mills (1979) Distribution of Na+−pump sites in transporting epithelia. Fed. Proc. 38: 104-145.

Dixon, J. F., and L. E. Hokin (1978) A simple procedure for one preparation of highly purified (sodium and potassium) adenosine triphosphatase from the rectal salt gland of Squalus acanthias and the electric organ of Electrophorus electris. Anal. Biochem. 86: 378-385.

Ellisman, M. H., and S. R. Levinson (1982) Immunocytochemical localization of sodium channel distribution in the excitable membrane of Electrophorus electricus. Proc. Natl. Acad. Sci. U. S. A. 79: 6707-6711.

Ellisman, M. H., J. D. Lindsey, C. A. Wiley-Livingston, and S. R. Levinson (1983) Differentiation and maintenance of the membrane cytoskeletal structure in nerve. In Structure and Function in Excitable Cells, D. C. Chang, I. Tasiak, W. J. Adalman, Jr., and H. R. Lauchtad, eds., pp. 3-23. Plenum Publ. Corp., New York.

Eng, L. F., and S. J. DeArmond (1981) Gial fibrillary acidic (GFA) protein immunocytochemistry in development and neuropathology. Prog. Clin. Biol. Res. 59: 49-77.

Eng, L. F., J. J. Vanderhaegen, A. Bignami, and B. Gerstl (1971) An acidic protein from cerebral cortical cytoskeleton that is strongly antigenic in guinea pig kidney. J. Histochem. Cytochem. 19: 1005-1022.

Ernst, S. A., and J. W. Mills (1980) Autoradiographic localization of tritiated ouabain-sensitive sodium pump sites in ion transporting epithelia. J. Histochem. Cytochem. 28: 72-77.

Fambrough, D. M., and E. K. Bayne (1983) Multiple forms of (Na+ + K+)−ATPase in the chicken. J. Biol. Chem. 258: 3926-3935.

Familaro, D. M., and E. K. Bayne (1983) Localization of potassium-stimulated p-nitrophenylphosphatase activity in the chicken. Proc. Natl. Acad. Sci. U. S. A. 77: 6707-6711.

Fitzgerald, B. E., J. H. Jones, Jr., and T. W. Smith (1976) Sodium- and potassium-activated adenosine triphosphatase in the hawk moth, Manduca sexta. Biochim. Biophys. Acta 356: 36-52.

Fouquet, J. P. (1980) Properties of the two polypeptides of sodium-potassium dependent adenosine triphosphatase. J. Biol. Chem. 247: 7642-7649.

Fouquet, J. P. (1976) Localization of Na+− dependent adenosine triphosphatase in the rat kidney. Acta Histochem. Cytochem. 19: 53-65.

Fouquet, J. P. (1976) Immunofluorescence localization of Na+− dependent adenosine triphosphatase on the plasma membranes of renal convoluted tubules. Ann. N.Y. Acad. Sci. 28: 351-354.

Gerstl, A., and S. J. DeArmond (1972) Development of a monoclonal antibody against the sodium pump. Exp. Eye Res. 14: 173-185.

Görgen, P. L., and J. C. Skou (1969) Preparation of highly active (Na+ + K+)−ATPase from the outer medulla of rabbit kidney. Biochem. Biophys. Res. Commun. 37: 39-46.

Görgen, P. L., and J. C. Skou (1969) Mechanism of the Na+−,K+− pump. Biochem. Biophys. Acta 694: 27-68.

Görgen, P. L., and J. C. Skou (1969) Purification and characterization of (Na+ + K+)−ATPase. III. Purification from the outer medulla of mammalian kidney after detergent removal of membrane components by sodium dodecyl sulphate. Biochim. Biophys. Acta 356: 36-52.
Schenk, D. B., and H. L. Leffert (1983) Monoclonal antibodies to rat Na+,K+-ATPase block enzymatic activity. Proc. Natl. Acad. Sci. U. S. A. 80: 5281-5285.

Schenk, D. B., J. J. Hubert, and H. L. Leffert (1984) Use of a monoclonal antibody to quantify (Na+ + K+)-ATPase activity and sites in normal and regenerating rat liver. J. Biol. Chem. 259: 14941-14951.

Schwartz, M., S. A. Ernst, G. J. Siegel, and B. W. Agranoff (1981) Immuno-cytocchemical localization of (Na+K+)-ATPase in the goldfish optic nerve. J. Neurochem. 36: 107-115.

Siegel, G. J., C. Holm, J. H. Schreiker, T. Desmond, and S. A. Ernst (1984) Purification of mouse brain (Na+ + K+)-ATPase catalytic unit, characterization of antisera, and immunocytocchemical localization in cerebellum, choroid plexus and kidney. J. Histochem. Cytochem. 32: 1309-1318.

Specht, S. C., and K. J. Sweadner (1984) Two different Na,K-ATPases in the optic nerve: cells of origin and axonal transport. Proc. Natl. Acad. Sci. U. S. A. 81: 1234-1239.

Stahl, W. L., and D. G. Baskin (1984) Immunocytocchemical localization of Na+K+ adenine triphosphatase in rat retina. J. Histochem. Cytochem. 32: 248-250.

Stahl, W. L., and S. H. Broderson (1976) Localization of Na+,K+-ATPase in brain. Fed. Proc. 36: 1260-1265.

Stemberger, L. A. (1979) Immunocytochemistry, Ed. 2, John Wiley & Sons, Inc., New York.

Sweedner, K. J. (1979) Two molecular forms of (Na+ + K+)-stimulated ATPase in brain. Separation, and difference in affinity for strophantidin. J. Biol. Chem. 254: 6060-6067.

Ternynck, T., and S. Avrameas (1976) Polymerization and immobilization of proteins using ethylchloroformate and glutaraldehyde. Scand. J. Immunol. Suppl. 3: 29-35.

Thomas, R. C. (1975) Electrogenic sodium pump in nerve and muscle cells. Physiol. Rev. 52: 563-594.

Towbin, H., T. Staehelin, and J. Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76: 4350-4364.

Trachtenberg, M. C., D. J. Packey, and T. Sweeney (1981) In vivo functioning of the Na+,K+-activated ATPase. Curr. Top. Cell. Regul. 19: 159-217.

Ueno, S., H. Meyakawa, I. Tsukahara, and K. Oyama (1961) Ultrastructural and cytochemical localization of ouabain-sensitive, potassium-dependent p-nitrophenylphosphatase activity in guinea pig retina. II. Neurons and Muller cells. Acta Histochem. Cytochem. 14: 189-206.

Urayama, O., and M. Nakao (1979) Organ specificity of rat sodium- and potassium-activated adenosine triphosphatase. J. Biochem. 86: 1371-1381.

Uyeda, C. T., L. F. Eng, and A. Bignami (1972) Immunological study of the glial fibrillary acidic protein. Brain Res. 37: R1-R9.

Varon, S. S., and G. G. Somjen (1979) Neuro-Glia. Neurosci. Res. Program Bull. 17: 147-174.

Voller, A., D. E. Bidwell, and A. Bartlett (1979) The Enzyme Linked Immunosorbent Assay (ELISA): A Guide with Abstracts of Microplate Applications, A. Voller, D. E. Bidwell, and A. Bartlett, Publishers.

Vorbrodt, A. W., A. S. Lossinsky, and H. K. Wiener (1982) Cytoskeletal and functional localization of ouabain-sensitive, K+-dependent p-nitrophenylphosphatase (transport ATPase) in the mouse central and peripheral nervous systems. Brain Res. 243: 225-234.

Walz, W., and L. Hertz (1982) Ouabain-sensitive and ouabain-resistant net uptake of potassium into astrocytes and neurons in primary cultures. J. Neurochem. 39: 70-77.

Winston, J. W. (1981) The reaction of sulfhydryl groups of sodium and potassium ion-activated adenosine triphosphatase with N-ethylmaleimide. The relationship between ligand-dependent alterations of nucleophility and enzymatic conformation states. J. Biol. Chem. 256: 9522-9531.

Wood, J. G., D. H. Joan, J. N. Whittaker, B. J. McLaughlin, and R. W. Albers (1977) Immunocytocchemical localization of the sodium, potassium activated ATPase in knife fish brain. J. Neurocytol. 6: 571-581.