Isoform Specific Reductions in Na$^+$,K$^+$-ATPase Catalytic (α) Subunits in the Nerve of Rats with Streptozotocin-Induced Diabetes

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Abstract: Na$^+$,K$^+$-ATPase activity in nerve is reduced in rats with streptozotocin-induced diabetes; three different isoforms of the α (catalytic) subunit of the enzyme are present in nerve. Using western blot to determine subunit isoform polypeptide levels in sciatic nerve, we found a substantial reduction in α1-isoform polypeptide (88% at 3 weeks, 94% at 8 weeks) after induction of diabetes by streptozotocin. Reductions in α2 and α3 polypeptide were smaller and not statistically significant. The reduction in amount of all three isoform polypeptides in the nerve of 3-week diabetic animals was corrected by administration of insulin. Accumulation of α1 polypeptide at a nerve ligature indicated that rapid transport of that polypeptide in nerve occurs with normal kinetics. The results implicate a specific marked deficit in α1, much more than α2 or α3, catalytic subunit isoform of Na$^+$,K$^+$-ATPase in the pathogenesis of diabetic neuropathy. **Key Words:** Na,K-transporting ATPase—Diabetes—Axonal transport—Nerve.
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Neuropathy is a common and often disabling complication of diabetes (Greene et al., 1988). In rat and rabbit models of acute diabetes, activity of Na$^+$,K$^+$-ATPase measured in the nerve is substantially reduced (Das et al., 1976; Greene and Lattimer, 1983, 1984; Greene et al., 1984). The kinetic defect in enzyme activity can be reversed in part by ganglioside treatment in vivo (Bianchi et al., 1988) or by protein kinase C agonists in vitro (Greene and Lattimer, 1986), but the pathogenetic mechanism underlying the defect in Na$^+$,K$^+$-ATPase activity remains obscure.

The functional Na$^+$,K$^+$-ATPase enzyme is a heterodimer composed of an α (M$_r$ 112,000) and a β (M$_r$ 35,000) subunit in 1:1 stoichiometry (Cantley, 1981). The α subunit contains the catalytic site for the exchange of three Na$^+$ for two K$^+$, in addition to the ATP and ouabain binding sites (Shull et al., 1986; Lingrel et al., 1990). In the nervous system, three different isoforms of the α subunit, termed α1, α2, and α3, are expressed as the products of three distinct

members of a multigene family (Shull et al., 1986; Herrera et al., 1987). These isoforms can be characterized by isoform-specific pharmacologic properties (Sweadner, 1985, 1989; Shyjan et al., 1990; Jewell and Lingrel, 1991), suggesting that there may be isoform-specific functions in cells of the nervous system. The β subunit has not been shown to participate in the catalytic function of the sodium pump but may be required for the transport and insertion of functional $\alpha\beta$ heterodimers into the membrane (McDonough et al., 1990; Geering, 1991). Two isoforms of the β subunit have been identified in mammals.

The development of isoform-specific RNA and polypeptide probes has allowed investigation of the distribution of individual isoforms in the nervous system. Each of the α isoform mRNAs has a specific regional and cellular distribution (Filuk et al., 1989; Hieber et al., 1991; Mata et al., 1991c; Watts et al., 1991), and a corresponding distribution of isoform polypeptides can be defined by western blot (Shyjan and Levenson, 1989) and by immunocytochemistry (McGrail et al., 1991). mRNAs coding for the α isoforms are found in motor neuron cell bodies in the spinal cord and in the dorsal root ganglia (DRG) (Mata et al., 1991c). α1 and α2 mRNAs are found in Schwann cells of the peripheral nerve (Mata et al., 1991b,c). All three isoform polypeptides are present in the nerve by western blot (Mata et al., 1993).

To determine whether, in acute diabetes (1) the defect in Na$^+$,K$^+$-ATPase activity in nerve reflects a reduction in the amount of polypeptide and (2) whether such defects are isoform specific, we used a quantitative western blot analysis to determine the levels of each of the three isoform polypeptides in the sciatic
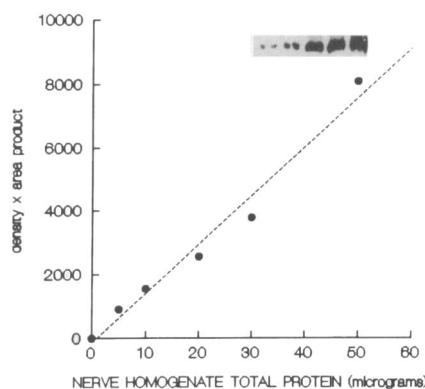
FIG. 1. Quantitative western blot. Western blot with the α1 antibody of total nerve homogenate, detected using Luminol and quantitated by integrated densitometry of the film.

nerve of rats with streptozotocin (STZ)-induced diabetes.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (230–290 g at the time of injection) were rendered diabetic by intraperitoneal injection of STZ. There were three surviving animals in each diabetic and control group, except as indicated. All diabetic rats had randomly obtained blood glucose values in excess of 400 mg/dl measured weekly until the time they were killed. The body weight of the diabetic rats decreased throughout the experiment. The diabetic animals used for studies of Na^+,K^+-ATPase isoform polypeptide levels averaged 205 g at 1 week, 167 g at 3 weeks, and 150 g at 8 weeks of diabetes, compared with simultaneous controls that weighed 285 g at 1 week, 330 g at 3 weeks, and 375 g at 8 weeks. The 3-week diabetic animals used in studies of axonal transport averaged 155 g at 3 weeks compared with simultaneous controls that averaged 380 g at 3 weeks.

To control for direct toxic effects of STZ, one set of animals was treated with NPH (isophane suspension) insulin 5 days of 7 for 3 weeks after induction of diabetes. Treatment was begun with 2 units each morning and 4 units each evening subcutaneously, and the dose increased based on randomly timed blood glucose determinations performed daily; these random glucose measurements were all <210 mg/dl. Two of the five treated animals died during the course of treatment, presumably from hypoglycemia. The average weight of the surviving animals increased from 240 g at induction of diabetes to 320 g at 3 weeks, compared with controls that increased from 250 g to 360 g over a similar time course.

All animal treatments adhered strictly to all institutional and national ethical guidelines.

Ligature experiment

The animals were anesthetized with chloral hydrate, the sciatic nerve exposed in the gluteal region, and a ligature of 4-0 prolene tied tightly around the nerve. Twenty-four hours later the animals were killed by decapitation, the nerve was cut into consecutive 3-mm segments both proximal and distal to the ligature, and was homogenized in 50 mM Tris, 5 mM

NaCl, pH 7, and 25 mg of homogenate protein analyzed for α1 polypeptide by western blot as described below.

Protein preparation

The rats were killed by decapitation. Rapidly dissected sciatic nerves were homogenized in 50 mM Tris, 5 mM NaCl, and centrifuged at 10,000 rpm for 15 min to remove debris. A crude membrane fraction was separated from soluble proteins by centrifugation at 100,000 g for 1 h in a Beckman Airfuge. We have shown previously that all of the immunoreactive Na^+,K^+-ATPase polypeptide in nerve is found in this crude membrane fraction (Mata et al., 1991a).

Western blot

Ten micrograms of nerve membrane protein was separated by 6% polyacylamide gel electrophoresis (Laemmli, 1970) and the proteins transferred to a nitrocellulose membrane (Hybond-ECL) as described (Towbin et al., 1979). The blots were blocked in 5% dried milk in Tris-buffered saline-Tween for 2 h and incubated overnight with commercially obtained isoform specific anti–α-isoform antibodies (Upstate Biotechnologies, Inc.) raised against fusion proteins at a dilution of 1:1,000. The specificities of these antibodies in western blot have previously been demonstrated (Shyjan and Levenson, 1989). After washing, the blots were incubated with peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Cappel) at 1:3,000 for 1 h, followed by reaction with Luminol (Amersham) for 1 min. The reacted blots were exposed to Hyperfilm-ECL for times ranging from 1 to 10 min. The samples to be compared quantitatively were all placed on a single gel and reacted together.

Quantitation

The amount of immunoreactive polypeptide was quantitated by integrated densitometric analysis of the film using a PC-based image analysis system (MCID, Imaging Resources Inc.). The Luminol method allows quantitative determination of immunoreactive polypeptide (Young et al., 1991), and we confirmed that using this method, the measurement of α isoform polypeptide in nerve is linear over the range of concentrations found in a 3-mm nerve segment (Fig. 1). The statistical significance of differences was determined by two-tail t test.

RESULTS

Western blot of membrane fractions isolated from nerve confirmed the presence of all three α-isoform polypeptides in control nerve (Fig. 2, first lane). Previous in situ hybridization studies have shown that α1,
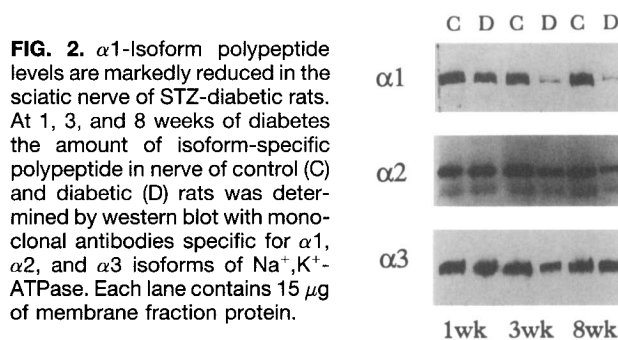

FIG. 2. α1-Isoform polypeptide levels are markedly reduced in the sciatic nerve of STZ-diabetic rats. At 1, 3, and 8 weeks of diabetes the amount of isoform-specific polypeptide in nerve of control (C) and diabetic (D) rats was determined by western blot with monoclonal antibodies specific for α1, α2, and α3 isoforms of Na^+,K^+-ATPase. Each lane contains 15 μg of membrane fraction protein.
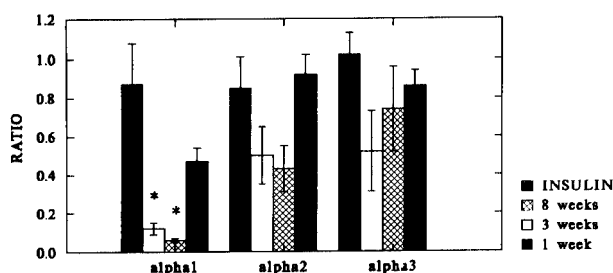
FIG. 3. Quantitative analysis of α-isoform polypeptide levels in nerve of diabetic and treated animals. The amount of polypeptide from each nerve segment was quantified by integrated densitometry of the film. The data are presented as the ratio of diabetic to age-matched controls at each time point. *$p < 0.01$, compared with control animals. The insulin-treated animals were significantly different both from the untreated 3-week diabetic animals and from control animals ($p < 0.01$). None of the other apparent differences are statistically significant.

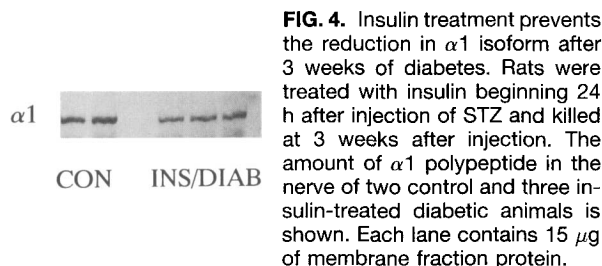
FIG. 4. Insulin treatment prevents the reduction in α1 isoform after 3 weeks of diabetes. Rats were treated with insulin beginning 24 h after injection of STZ and killed at 3 weeks after injection. The amount of α1 polypeptide in the nerve of two control and three insulin-treated diabetic animals is shown. Each lane contains 15 μg of membrane fraction protein.

α2, and α3 mRNAs are present in the sensory neurons of the DRG and the motor neurons of the ventral horn of spinal cord (Mata et al., 1991c, and authors' unpublished data). Both α1 and α2, but not α3, mRNAs are present in the nerve, where virtually all the mRNA is located in Schwann cells (Mata et al., 1991c).

After 1 week of STZ-induced diabetes, there was a slight reduction in the amount of α1-immunoreactive polypeptide in sciatic nerve. By 3 weeks of diabetes, α1 polypeptide was markedly reduced in nerve and was barely detectable at 8 weeks of diabetes (Fig. 2, top row). Levels of α2-polypeptide immunoreactivity in nerve appeared unchanged at 1 week of diabetes, and slightly reduced at 3 and 8 weeks of diabetes (Fig. 2, second row). α3-Polypeptide immunoreactivity in contrast was unchanged at 1 week, mildly reduced at 3 weeks, but appeared normal after 8 weeks of diabetes (Fig. 2, third row). Quantitative analysis by integrated densitometry of the western blot showed that α1-polypeptide levels were reduced to 12% of control after 3 weeks of diabetes and only 6% of control at 8 weeks of diabetes (Fig. 3). These results were statistically significant ($p < 0.01$). α2 was reduced ~50% at 3 and 8 weeks of diabetes, and α3 was reduced ~50% at 3 weeks of diabetes but recovered to ~75% at 8 weeks. These changes at 8 weeks were not statistically significant (Fig. 3).

Treatment of STZ-induced diabetes with insulin, beginning 24 h after STZ injection, corrected the loss of α1-immunoreactive polypeptide in nerve at 3 weeks of diabetes (Fig. 4). In these treated animals with random glucose levels <210 mg/dl, the weight gain was near but not quite normal. Quantitative analysis showed that the amount of α1 polypeptide was corrected to ~50% of normal, which was significantly different both from the 3-week untreated diabetic animals and from the controls (Fig. 3). The amount of α2 and α3 polypeptides in the treated animals was essentially normal.

Because α1 isoform is found in Schwann cells and

fibroblasts in addition to neurons, it was important to determine whether the immunoreactive α1 polypeptide in the STZ-diabetic animals was neuronal or Schwann cell in origin. We have previously shown that α1-isoform polypeptide ATPase is carried into the nerve by rapid axonal transport and consequently accumulates at a ligature placed on the nerve (Mata et al., 1993). When a ligature was placed on the sciatic nerve, and accumulation of transported polypeptide allowed to proceed for 24 h, α1 polypeptide accumulated at the ligature in both normal and diabetic animals (Fig. 5), indicating normal kinetics of axonal transport of the immunoreactive polypeptide. The amount of immunoreactivity was less in the diabetic than in the control animals both in the segments proximal to the ligature as well as in the segment immediately adjacent to the ligature in which the accumulation occurred. A quantitative comparison of this reduction was not obtained, because the samples were run on different gels.

DISCUSSION

This is the first description of a disease process affecting the nervous system in adult animals in which differential effects on isoforms of the catalytic subunit of Na^+,K^+-ATPase α subunit have been found. Thyroid hormone regulates Na^+,K^+-ATPase isoform gene expression in a variety of tissues during development (Gick et al., 1988; McDonough et al., 1988; Orlowski and Lingrel, 1990; Melikian and Ismail Beigi, 1991), and affects the abundance of Na^+,K^+-ATPase during development in neonatal rat brain (Schmitt and McDonough, 1988), but does not appear to affect Na^+,K^+-ATPase abundance or activity in the brain of adult animals (Lin and Akera, 1978; Horowitz et al., 1990).

FIG. 5. α1 is carried by rapid axonal transport in normal and diabetic rats. Twenty-four hours after sciatic nerve ligature, the amount of α1-immunoreactive polypeptide in consecutive 3-mm segments of the nerve was determined by western blot. α1 accumulates proximal to the ligature in both the normal and diabetic nerve. Each lane represents the protein from one segment, and the third lane is the segment just proximal to the ligature. The experiment was repeated twice.
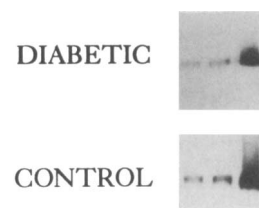
In vitro, Na^+,K^+-ATPase gene expression is also regulated by the ionic composition of the extracellular environment (Pressley et al., 1988; Taormino and Fambrough, 1990) and by glucocorticoids (Pressley, 1988; Bhutada et al., 1991; Celsi et al., 1991).

Substantial evidence implicates deficits in Na^+,K^+-ATPase activity in nerve in the pathogenesis of diabetic neuropathy (Greene and Winegrad, 1981; Greene and Lattimer, 1984; Greene et al., 1988). Whereas the exact role of this metabolic defect in the pathogenesis of the neuropathy is not yet clear, evidence suggests that it is an important step in the development of physiologic and structural correlates of diabetic neuropathy.

Direct determination of Na^+,K^+-ATPase pump concentration in peripheral nerve of STZ-diabetic rats by [^{3}H]ouabain binding showed a 16% reduction after 4 weeks of diabetes. In that study the binding appeared to occur to only a single population of sites, with an apparent K_D of 2.2×10^{-6} M (Kjeldsen et al., 1987). The western blot data of the current study indicate that all three isoform polypeptides are found in the peripheral nerve, but only $\alpha1$ is significantly reduced in the diabetic animals. More recently, a 10–30% decrease in Na^+,K^+-ATPase enzymatic activity in different regions of brain in STZ-diabetic rats has been found (Leong and Leung, 1991), with no alteration in other enzymes of the glucose metabolic pathway.

The ligature experiments further indicate that most of the Na^+,K^+-ATPase $\alpha1$ polypeptide detected in nerve is synthesized at the neuronal cell body and transported into the axon, despite the potentially confounding observation that Schwann cells also express $\alpha1$ mRNA (Hieber et al., 1991) and polypeptide (Shyjan and Levenson, 1989). We have previously shown that all neurons in the spinal cord and in the DRG express $\alpha3$ mRNA, with some of those neurons coexpressing high amounts of $\alpha1$ mRNA (Mata et al., 1991c). Whether neurons that in normal conditions express high amounts of $\alpha1$ become selectively impaired in diabetic neuropathy is not known.

What is the significance of the relatively selective loss of $\alpha1$ isoform from the axolemma? Voltage clamp analysis of the node of Ranvier in diabetic rats has shown an elevation in intraaxonal Na^+ (Sima and Brismar, 1985; Brismar et al., 1987), which correlates with the reduction in nerve Na^+,K^+-ATPase. The results of the current study suggest that the $\alpha1$ isoform may be responsible for Na^+,K^+ exchange at the node of Ranvier. We have recently shown, by quantitative in situ hybridization, that in response to increased electrical activity induced by salt treatment, the neurons of the supraoptic and paraventricular nuclei of the hypothalamus increase the amount of $\alpha1$ but not $\alpha2$ or $\alpha3$ mRNA (Mata et al., 1992). This finding led us to propose that in those cells $\alpha1$ isoform might be specifically involved in exchanging Na^+, which entered through voltage-gated channels during electrical depolarization at the neuron terminal. These two experiments taken together suggest that $\alpha1$ isoform may

function specifically at the node and the terminal where the major Na^+ fluxes occur.

Ongoing studies will define whether the deficit in $\alpha1$ occurs at the level of transcription or translation or represents a posttranslational effect.

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