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The Na, K-ATPase alpha3-isoform specifically localizes in the Schmidt-Lanterman incisures of human nerve

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Abstract – *Introduction*: To our knowledge, there is little reference in literature with regards to α3-isoform of Na^+,K^+-ATPase in human peripheral nerves. The aim of this study was to determine the expression of the neuronal α3-isoform of Na^+,K^+-ATPase in human sural nerves from patients with a permanent medullary central nervous system injury. *Materials and Methods*: We studied the immunolocalization of α3-isoform of Na^+,K^+-ATPase using a polyclonal antibody against the amino sequence near the phosphorylation site of the α3-isoforms of Na^+,K^+-ATPase using immunohistochemistry and confocal laser scanning microscopy. An antibody specific for α2-isoform of Na^+,K^+-ATPase was used to label the Schwann cells. *Results*: Morphometric analysis of longitudinal section of human sural nerves showed that the α3-isoform of Na^+,K^+-ATPase was distributed along the length of axolemma. The myelin sheath of the Schwann cells showed clearly a distribution of α3- but not α2-isoforms of Na^+,K^+-ATPase at the level of Schmidt-Lanterman incisures. *Conclusion*: The human sural nerve shows a specific localization of the Na^+,K^+-ATPase α3-isoform in the Schmidt-Lanterman incisures of Schwann cells in addition to its localization in axonal membranes.

Key words: Na^+,K^+-ATPase, Isoforms, Immunohistochemistry, Confocal laser scanning microscopy, Central nervous system injury, Peripheral nerve, Schmidt-Lanterman clefts

INTRODUCTION

The Na^+,K^+-ATPase is the ubiquitous membrane protein complex responsible for the active transport of Na^+ and K^+ across the plasma membrane (Skou, 1998). The Na^+,K^+-ATPase also acts as a receptor complex capable of transmitting digitalis signal to various intracellular compartments (Xie, 2001 ; Liu & Askari 2006). The physiological role of the Na^+,K^+-ATPase is particularly critical in excitable cells like neurons where the enzyme insures the maintenance of the Na^+ gradient, required for the propagation of action potentials and the K^+ gradient that sets the cell membrane potential (Levenson et al., 1994). Throughout the peripheral nervous system, axons and Schwann cells the Na^+,K^+-ATPase is responsible for the re-uptake of extracellular K^+. Failure to control extracellular K^+ has been associated with neuronal alterations (D'Ambrioso et al., 2002). Four isoforms of the catalytic subunit of the Na^+,K^+-ATPase have been identified, three of which (α1, α2 and α3) are expressed in the peripheral nervous system (Mata et al., 1994; Kawai et al., 1997; Gerbi et al., 1999). With the exception of the ubiquitous α1-isoform, the expression of Na^+,K^+-ATPase isoforms differs from one cell type to the other (Dobretsov & Stimmers, 2005; Alberti et al., 2007; Arteaga et al., 2004). Remarkably, in the nervous system, α3 isoform is abundantly (Pierre et al., 1999) and selectively expressed in neurons, whereas α2 isoform is the main isoform expressed in glial cells (Schwann cells). Also important to mention, is that there are very few sites of expression of

α3 isoform outside the nervous system. One of these few exceptions is the cardiac tissue (Maixent and Berrebi, 1993; Zahler et al. 1996; Wang et al., 2001). Although such a specific pattern of expression suggests that α3 isoform may serve specific functions in the nervous system, the physiological role of the neuronal α3-Na^+,K^+-ATPase isoform remains unclear and the expression of α2- and α3-Na^+,K^+-ATPase isoforms has not been studied in human sural nerves. Accordingly, the present study investigated the pattern of expression of α2- and α3-Na^+,K^+-ATPase isoforms in human sural nerves from patients with lesions of the central nervous system.

MATERIALS AND METHODS

Tissue sampling and processing

Sural nerve surgical biopsies were obtained for diagnostic analysis from three patients with central nervous system lesions (two patients with spinal cord injury and one with stroke) who underwent a selective tibial neurotomy for a spastic equinus foot. Excised tissue samples were rinsed in ice-cold phosphate-buffered saline (PBS) and cut into 1 mm^3. They were then fixed with 3% paraformaldehyde in 0,1 M phosphate buffer (PB) (pH: 7.4) for 2 hours at 4°C. Then, tissues were placed in a graduate sucrose serie (0.5 to 2.1 M) in phosphate-buffered saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl pH: 7.4) and frozen in liquid nitrogen. Longitudinal sections (2 µm) were cut with a cryostat. Sections were mounted on positively- and albumin-coated slides, usually 4 sequential sections per slide.

Ethics

This study has been conducted in agreement with the declaration of Helsinski and with French law relating to the protection of people who consent to biomedical research. Our protocol has been approved by the Ethics Committee (CCPPRB Poitou-Charentes ref. 05.11.21).
Informed consent was obtained, after explaining the protocol to the patient.

Immunohistochemistry

Primary antibodies

Polyclonal anti-Na^+,K^+-ATPase antibodies specific for the various α-isoform subunits were used for immunocytochemical localization in human nerve biopsies. All polyclonal antibodies were provided by Dr. T. A. Pressley (Texas Tech University, Lubbock, Texas, USA; (Pressley 1992). As shown in the Fig. 1, the NASE (anti) polyclonal antibody targets amino acids 489-499 of the α1 polypeptide in the nucleotide-binding domain from the large intracytoplasmic loop ; HERED recognizes amino acids 487-496 of the α2 polypeptide and TED recognizes amino acids 484-494 of the α3 polypetide. These three antibodies, affinity purified from rabbit serum, were used at a 1:100 dilution as previously described (Gerbi et al, 1999). Their specificity was confirmed by using western blot analysis of plasma membrane isolated from human heart (positive control for α1,2,3 isoforms of the Na^+,K^+-ATPase) kidney (positive control for α1 isoform of the Na^+,K^+-ATPase only) and skeletal muscle (positive control for α2 isoform of the Na^+,K^+-ATPase). All antibodies identified only one polypeptide at around 110 kDa in their respective controls. No other immunoreactive was detected between 200 kDa and to 20 kDa, the resolving limit of the gel system (data not shown).

Immunolabeling and image analysis

Human nerves sections were washed for 1 h in a Tris buffer solution containing 2% bovine serum albumin with 0.01% Triton X-100 (TBSC). Then, samples were incubated overnight with primary antibodies at room temperature in wet chamber (anti-HERED α2, anti-TED α3). The primary antibody was omitted in negative control experiments. After washing in TBSC (3 x 10 min), sections were incubated for 1 h in droplets of secondary antibody [1:100; FITC-Phalloïdine (1 µg/ml)]. Following washes in TBS (3 x 5 min), sections were mounted with Vectashield (10 µl) and preserved at 4°C. Slides were then observed with Bio-Rad MRC 1024 confocal laser scanning microscope (Denk & al. 1990). A krypton-argon laser was used to excite FITC at 488 nm and to collect emission at 530 nm. Confocal images (twenty-four-bit RGB, 525x525 pixels) were captured using a 60X oil immersion objective. For illustrations, images (contrast and brightness) were adjusted with ImagePro plus and CorelDraw software.

RESULTS

We used confocal immunofluorescence microscopy to study the α3 isoform of the Na^+,K^+-ATPase expression in human sural nerves. We used antibody specific for α isoform of the Na^+,K^+-ATPase to identify its localization. Morphometric analysis of longitudinal section of human nerve showed that the α3-isoform of Na^+,K^+-ATPase was distributed along the length of axolemma (Fig. 2). The myelin sheath of the Schwann cells was clearly labelled at Schmidt-Lanterman incisures (Fig 2, arrows). The same type of analysis was performed with antibody specific for α2 isoform of the Na^+,K^+-ATPase and revealed the highly compact labelling along the fiber corresponding to the myelin sheath of Schwann cells without any labelling of neither the axonal membrane and axoplasmic profile nor the Schmidt-Lanterman incisures (Fig 3).

DISCUSSION

Since the α1-isoform of Na^+,K^+-ATPase is an ubiquitous protein for all neuronal cells, we have examined the profile of protein expression for α2 and α3-isoforms of Na^+,K^+-ATPase in the human peripheral nervous system. A non uniform profile of the α2- and α3-isoforms of Na^+,K^+-ATPase was evidenced in this study.

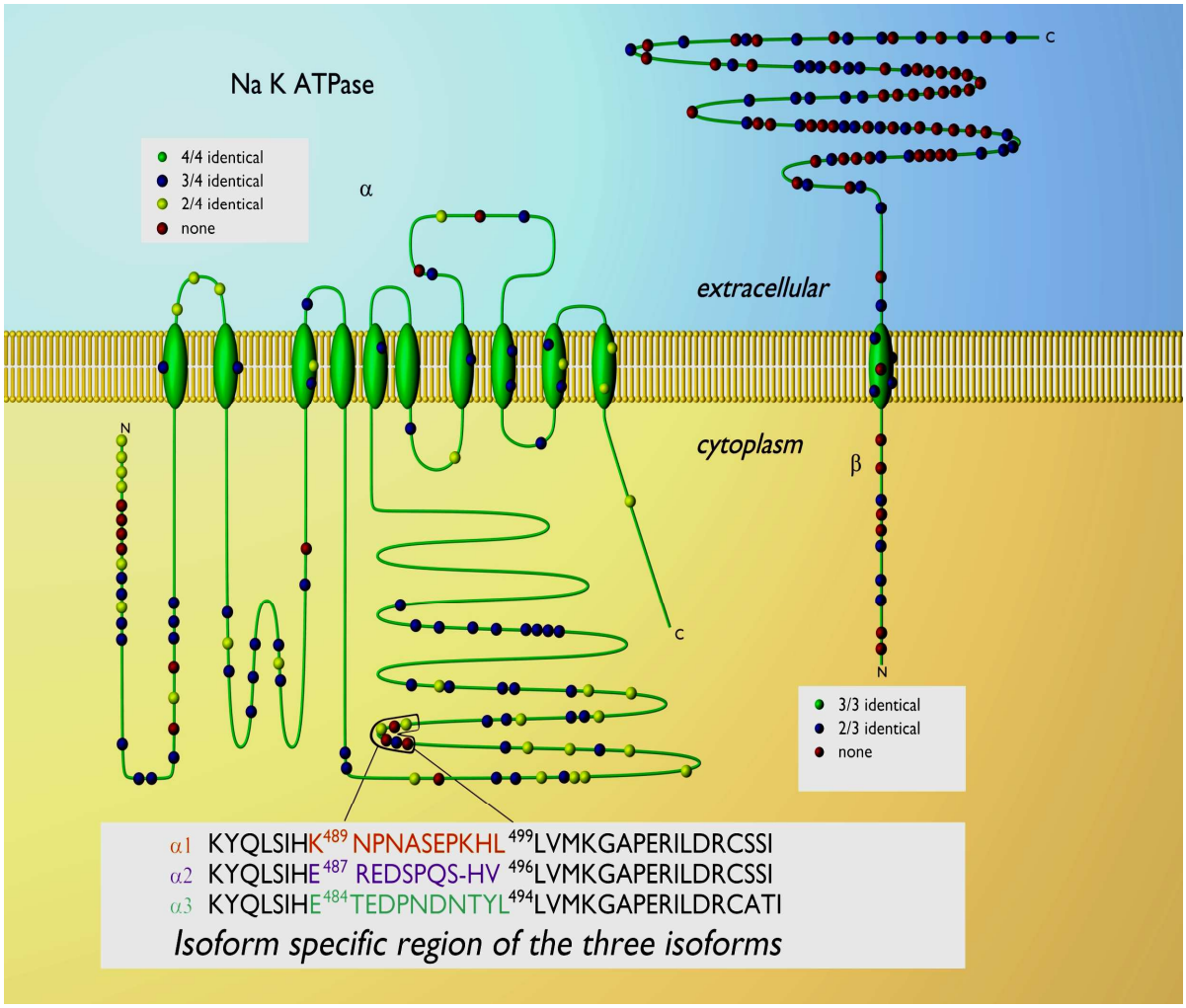

Figure 1. Schematic structure of aminoacid sequences of Na^+,K^+-ATPase subunit isoforms and specific amino acid Na^+,K^+-ATPase subunit isoforms sequences recognized by antibodies(adapted from Blanco & Mercer, 1998; Dobretsov & Stimers, 2005). These polyclonal antibodies were used in this study for the evaluation of the distribution of the Na^+,K^+-ATPase isoforms in human nerve by immunohistochemistry. All antibodies were provided by Dr. T. A. Pressley (Texas Tech University, Lubbock, Texas, USA; (Pressley 1992). As shown here, the NASE (anti-α1) polyclonal antibody interacts with amino acids 489-499 in the N-domain. The HERED (anti-α2) polyclonal antibody interacts with amino acids 487-496 in the N-domain. The TED (anti-α3) polyclonal antibody interacts with amino acids 484-494 in the N-domain.

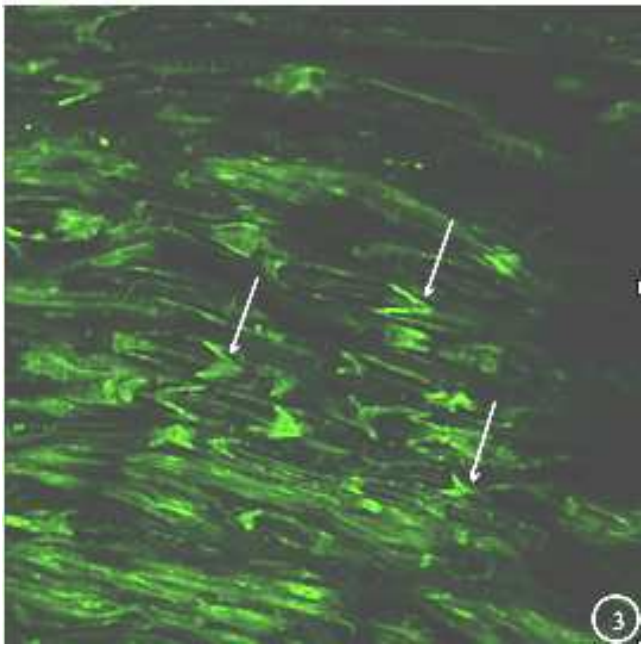

Figure 2. Confocal microscopy images of immunofluorescence in sural human nerve. Myelinated axons are shown cut longitudinally. α3-isoform of Na^+,K^+-ATPase was distributed along the length of axolemma (Axons). The myelin sheath of the Schwann cells was clearly immunolabeled by antiTED (α3-isoform) at Schmidt-Lanterman incisures (Arrows). Note the absence of immunoreactivity in the myelin sheath surrounding the axons. This analysis was done with biopsies from 3 patients.

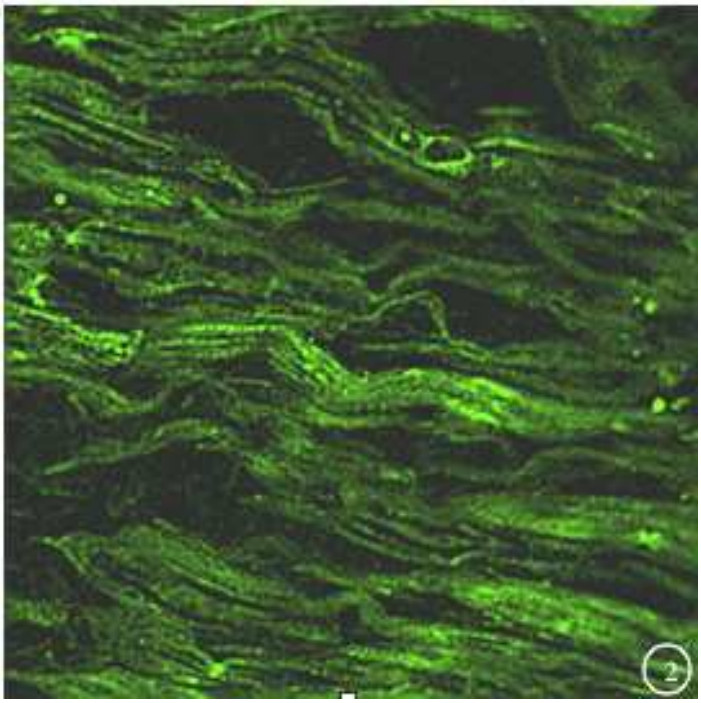

Figure 3. Confocal microscopy images of immunofluorescence in sural human nerves. Myelinated axons are shown cut longitudinally. The myelin sheath of the Schwann cells was clearly immunolabeled by antiHERED (α2-isoform). The α2-isoform of Na^+,K^+-ATPase was distributed along the length of Schwann cells. Note the absence of immunoreactivity in the the axons. The Schmidt-Lanterman incisures of the Schwann cells appear immunolabeled in a similar extent to that of Schwann cell membranes.

One of the most striking findings of the present study was the expression of immunoreactivity corresponding to the α3-isoforms of Na^+,K^+-ATPase in the Schmidt-Lanterman. This issue was studied in sural human nerves from patients with central nervous system lesions. We cannot exclude that such conditions over time would have modified (amplified) the pattern of Na^+,K^+-ATPase expression. Even if we assume that peripheral nerves should remain intact after a central nervous system injury, one limitation of this study is that the α3-isoform localization probably does not reflect the normal isoform distribution from healthy individuals. Nevertheless, our study demonstrates a typical cell type-dependent asymetric distribution in the peripheral nerve and confirms that the α2-isoform of Na^+,K^+-ATPase is specific of Schwann cell membrane whereas the α3-isoform of Na^+,K^+-ATPase specifically located at the axonal membrane (Dobretsov and Stimers 2005).

The Schwann cells present a unique architecture with a myelin sheath around peripheral nerve axons. Furthermore, the structure of myelin includes regions of highly compacted membrane and cytoplasm and noncompacted regions of myelin with the Schmidt-Lanterman incisures (Cooper & Kidman, 1984). They are tube-like cytoplasmic structures that cross the compact myelin and connect the Schwann cell peripheral (abaxonal) cytoplasm to the periaxonal (adaxonal) cytoplasm. The results of the present study indicate that α3-isoform of Na^+,K^+-ATPase was localized and distributed on the axonal membrane, confirming the significance of this enzyme for the transmission of electrical signals (Alberti et al, 2007). However, the intramyelinic localization corresponding to α2- and α3-isoforms of Na^+,K^+-ATPase is specifically related to the compacted structural composition of myelin and non compacted structural composition of Schmidt-Lanterman incisures respectively. The latter suggests an enrichment of α3-isoforms of Na^+,K^+-ATPase in the Schmidt-Lantermann incisures without excluding the presence of α2-isoforms of Na^+,K^+-ATPase in the Schmidt-Lanterman incisures.

The distributions of these α2- and α3-isoforms of Na^+,K^+-ATPase in myelinated nerve highlight the elaborated molecular specializations of these membranes (Dobretsov et al., 2003). Recently a study aiming at understanding the role of proteins in the adherens junctions, a juxtamembrane domain abundantly distributed in the Schmidt-Lanterman incisures, presented evidence for the presence and enrichment of some proteins such as p120 catenin (a cytoplasmic partner) and E-Cadherin (an adhesion partner) in the adherens structure of Schmidt-Lanterman incisures (Tricaud et al 2005). The immunolabeling of these proteins were however restricted to the adherens junctions in constrat with that of connexin 29 that labelled the entire Schmidt-Lanterman incisures. Our study suggests the presence of α3-isoform of Na^+,K^+-ATPase not only in the adherens junctions but in the rest of Schmidt-Lanterman incisures near to the axolemma. A colocalization study of connexin 29 with α3-isoform of Na^+,K^+-ATPase would be of interest in human nerve Schmidt-Lanterman incisures. As cadherin is a calcium-dependent adhesion molecule, the presence of α3-isoforms of Na^+,K^+-ATPase in the adherens junction of Schmidt-Lanterman incisures could be invoved in calcium homeostasis, a role already demonstrated in the axolemme of neurons (Tricaud et al 2005).

Other physiological role for the α3-isoforms of Na^+,K^+-ATPase in the Schmidt-Lanterman incisures could be the transport of metabolic substances across the myelin sheath and the metabolic maintenance and longitudinal growth of the sheath (Ghabriel and Allt, 1980; Arroyo and Scherer, 2000, Alberti et al, 2007).

The location of Na^+,K^+-ATPase in the Schmidt-Lanterman incisures is of importance but Na^+,K^+-ATPase should be active as an enzyme. This has been previously observed by numerous authors in several species but not in human (Ghabriel and allt, 1980; Baumann and Pham-Dinh, 2001; Kanoh and Kumoi, 1994; Kanoh and Sakagami, 1996). All these studies used the immunoenzyme activity (K^+-pase activity) assay to demontrate the location of the Na^+,K^+-ATPase enzyme activity. For histochemical analysis, the localization of ouabain-sensitive, K^+-dependent p-nitrophenylphosphatase (K^+-pase) activity represents the second dephosphorylative property of the Na^+,K^+-ATPase. Facial nerves in normal guinea pigs show a fine-granular reaction product of the K^+-pase activity. Furthermore, after reserpinization of guinea pig facial nerves, a process that inhibits cerebral palsy, the K^+-NPPase activity was absent along the internodal axolemma and the Schmidt-Lanterman incisures. This is an evidence that Na^+,K^+-ATPase activity in the Schmidt-Lanterman incisures could be modulated and associated to physiopathological states (Kanoh & Sakagami, 1996).

Regeneration after axotomy of peripheral nerves has been recently found to influence transiently the expression of the Na^+,K^+-ATPase subunit isoforms (Arteaga et al., 2004). By analyzing all the known isoforms of the Na^+,K^+-ATPase protein complex (which is composed of α, β and γ-subunits), these studies suggested that this adaptation phenomena concerns virtually all Na^+,K^+-ATPase isoforms (Arteaga et al., 2004).

The functional differences observed within the individual isoform in terms of kinetic difference for K^+ and Na^+ could result in ionic homeostasis specific to Schmidt-Lanterman incisures. Indeed Na^+,K^+-ATPase exhibiting α3 isoforms have an approximately 3-fold lower $K_{0.5}$ for extracellular K^+ and an approximately 2-fold higher $K_{0.5}$ for cytoplasmic Na^+ than pumps exhibiting α2 and α1 isoforms (Munzer et al., 1994).

In conclusion, Na^+,K^+-ATPase isoforms are found in adult human nerve with specific localization. α3-isoform of Na^+,K^+-ATPase are expressed in Schwann cells and axon membrane but differ in their distribution. α3-isoform of Na^+,K^+-ATPase is localized in the Schwann cell at the Schmidt-Lanterman incisure membrane. Our results provide evidence that closely related Na^+,K^+-ATPase isoforms can be localized differentially in the same cell and highly concentrated in the axonal membrane and Schmidt-Lanterman incisure regions of human Schwann cells.

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