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Immunodetection of Na,K-ATPase α3-isoform in renal and nerve tissues

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At least three types of mRNA of the catalytic subunit of Na,K-ATPase namely α-,α+- and α3-isoforms are identified in different tissues. Only two of them α and α+ have well known structural and catalytic properties. Here we present immunochemical data indicating that the α3 protein really exists in pig and human kidney, and human brain. Crude membrane fractions and purified membrane-bound Na,K-ATPases were immunoblotted with α3-specific antibodies raised against the synthetic peptide corresponding to the unique sequence of this isoform. The mature α3-subunit is shown to include the sequence GDKKDDKSSPK followed by the initiating methionine residue. Nephron collecting tubules are proposed to specifically contain Na,K-ATPase α3-isoform.

Catalytic subunit isoform; Antipeptide specific antibody; Western-blot analysis; ATPase, (Na+K)-

1. INTRODUCTION

The Na,K-activated adenosine triphosphatase of animal plasma membranes is a universal system for the active transport of monovalent cations. An enzyme effects vector transmembrane transfer of Na^+ and K^+ against their electrochemical potential gradients due to the energy of ATP hydrolysis. The enzyme molecule consists of two polypeptides, catalytic α-subunit and glycoprotein β of unknown function (for review see [1]). To date, the number of known genes for the Na,K-ATPase catalytic subunit [2] exceeds that of identified protein isoforms (only α and $\alpha+$ subunits have been characterized by biochemical methods [3]). A complete nucleotide sequence of the gene for the α3-isoform of the catalytic subunit is established [4], the gene expression in various organs and tissues is studied (α3 mRNA is detected in rat brain [5], stomach, lung, liver and fetal heart [6], human kidney [7]). All these underlay the search for a protein product of the α3 gene expression. This report presents immunobiochemical data indicating that the α3-isoform of the catalytic subunit of Na,K-ATPase really exists in renal and nerve tissues.

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2. MATERIALS AND METHODS

Kidney microsomes and Na,K-ATPase were prepared as in [8]. Partial purification of Na,K-ATPase from human brain by extraction with SDS was performed as in [3] except that the detergent/protein ratio was decreased to 0.3.

Two peptides corresponding to the α3-subunit regions were synthesized following the solid-phase technique [9]. Peptide I was coupled to Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) using glutaraldehyde and peptide II, coupled to KLH with *N*-succinimidyl 3-(2-pyridyldithio)propionate as described in [10].

Rabbits were immunized by two subcutaneous injections of peptide II (1 mg) or peptide conjugates (500 μg) with a 2 week interval. Antipeptide antibodies (aBSA-I, aKLH-I) were purified by affinity chromatography on Sepharose 4B with the immobilized peptide I. The thiol-Sepharose 4B coupled with peptide II, by specially adding an N-terminal cysteine residue, was used to purify antibodies a-II and aKLH-II.

Antibody binding to the peptides (1–2 μg/ml) or Na,K-ATPase (5–10 μg/ml) was detected by an indirect solid-phase ELISA [9]. SDS-PAGE (4–15%) and immunoblotting was carried out as in [9]. The blots were treated with biotinylated goat anti-rabbit IgG (1:1000) followed by incubation with streptavidin (1:1000) and horseradish-peroxidase (HRP)-conjugated

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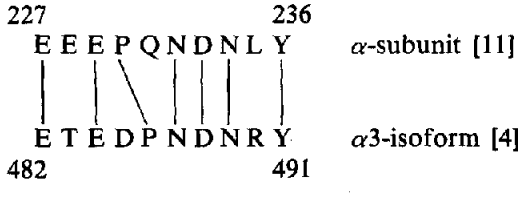
biotin (1:500). An alternative procedure included staining of the specific antigen-antibody complex by goat anti-rabbit IgG conjugated to HRP (1:1000).

3. RESULTS AND DISCUSSION

To elucidate the distribution of the protein product of Na,K-ATPase α3-isoform gene expression, the antibodies obtained to the unique segment of the protein structure proved to be a very useful tool. The comparison of the primary structures derived from the nucleotide sequences of α [11], $\alpha+$ [12] and α3 [4] revealed the notable difference in two regions (fig.1). Both the peptides are located in the regions of the molecule involved directly or indirectly in functioning of the protein. The first peptide adjoins the ATP-binding site, the second is in the N-terminal part of the molecule significant for cation transport [1]. Such disposition implies location of these regions on the molecule surface that is naturally essential for further epitope recognition with antibodies.

Two synthetic peptides 10–11 amino acids in length were used as antigens to raise specific antibodies: (I) ETEDPNDNRY; (II) GDKKDDKSSP. As the α3-isoform mRNA content is substantial in renal [7] and nerve tissues [6], the protein is assumed to enter the enzyme isolated from kidney as a minor component. The membrane-bound Na,K-ATPase from pig kidney outer medulla was subjected to ELISA with various dilutions of aKLH-I, aBSA-I and a-αp999 [9]. (Only two amino acid substitutions Ile(α)/Leu(α3) and Arg(α)/Asn(α3) allowed supposition that the antibodies will recognize both enzyme isoforms equally well.) The same height of the upper plateau on titration curves demonstrates the identical antigen quantity in three binding processes. This can be explained by nonspecific interaction of aBSA-I antibodies and the Na,K-ATPase α-subunit region (227–236)



There is no doubt about the exposure of this fragment in membrane-bound ATPase complex, and

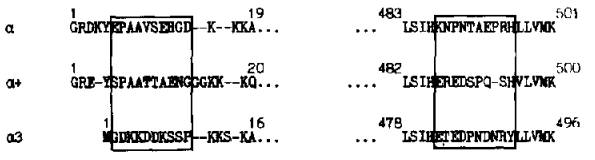


Fig.1. Comparative sequence analysis of the Na,K-ATPase α [11], $\alpha+$ [12] and α3 [4] isoforms. The regions of nonhomology have been boxed.

consequently, about its accessibility, since this region is obviously involved in acception of the enzyme-specific inhibitor, ouabain [13,14].

Hence a series of experiments on obtaining antibodies against synthetic peptide II was performed, the peptide being supposed to include the N-terminal region of the α3-subunit molecule. Since four Lys and three Asp residues enter the peptide, we specially added a cysteine residue to the N-terminus of peptide II for coupling. Simultaneous immunization was performed by two distinct antigens: the peptide conjugated to keyhole hemocyanin (KLH-II) and free peptide II. Then polyclonal sera and affinity purified monospecific antibodies were used in Western-blot analysis to probe the presence and relative quantities of the corresponding α3 antigen. To avoid a possible pitfall of unequal recovery of Na,K-ATPase isozymes during membrane preparation, initial experiments were performed with the most inclusive possible particulate fractions from pig kidney medulla and cortex as well as from human kidney and brain. No positive hybridization signal was observed when an anti-rabbit IgG conjugated to horseradish peroxidase was used to reveal specific complexes. The lack of detectable α3 subunit could indicate that the antigen is absent from the microsomes analyzed. Besides, the lack of a region corresponding to peptide II in the mature protein of the α3-isoform could also be one of the reasons. However, it may be the result of a low level of α3 antigen (below detection level of the ELISA with secondary antibody-HRP conjugate).

Use was made of the avidin-biotin system to amplify a possible positive signal. In this case (fig.2), only a polypeptide with molecular mass of 100 kDa and its dimer were detected in microsomal fractions. Na,K-ATPase purified preparations used as antigens in immunoblotting provided the identical results (fig.3). The specificity of the reac-

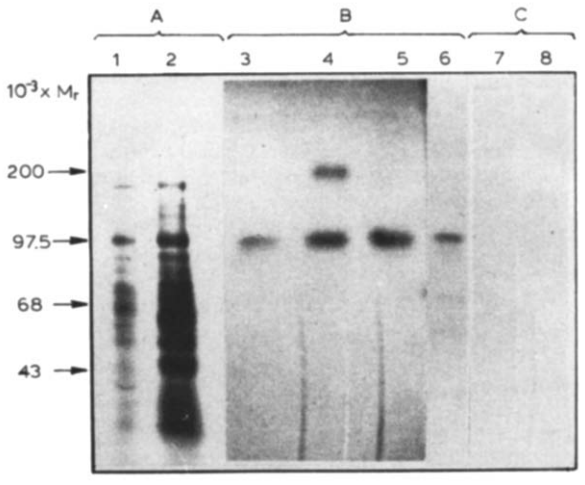


Fig.2. Immunodetection of Na,K-ATPase α3-isoform in kidney and brain. Western type immunoblots were performed on 4–15% gel gradient containing the microsomes from human kidney (100 μg) (lanes 1,5,7), human brain (250 μg) (lanes 2,6,8), pig renal outer medulla (100 μg) (lane 3) and cortex (100 μg) (lane 4). The blots were stained with (A) Coomassie G-250; (B) the antipeptide antiserum a-II diluted 100-fold; (C) the nonimmune serum (1:100).

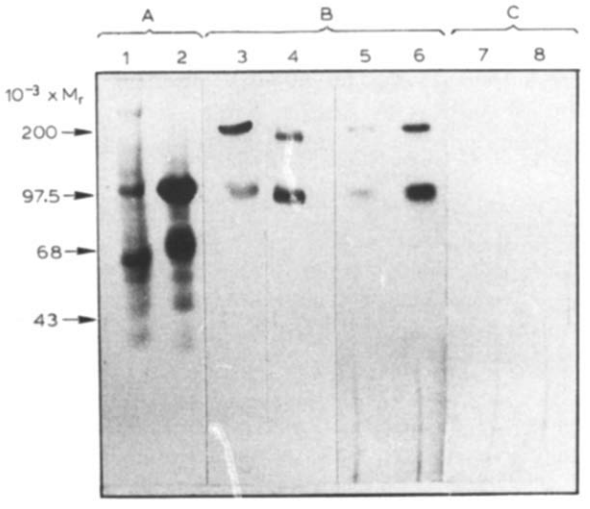


Fig.3. Immunoblot staining of purified Na,K-ATPase from human kidney (lanes 1,6,8), human brain (lanes 2,5,7), pig kidney outer medulla (lane 4) and cortex (lane 3). The blots were treated with (A) Coomassie G-250; (B) the antipeptide antiserum a-II (1:100); (C) the antiserum a-II preliminary depleted with peptide II immobilized on thiol-Sepharose (1:100).

tion was proved by the lack of immunolabelling when electrotransferred proteins were incubated with sera absorbed with peptide II. In preparation, adsorption on thiol-Sepharose immobilized peptide was carried out. Titer decrease with respect to the peptide demonstrated serum depletion. No reactive band was seen on immunoblots when either this preparation or nonimmune IgG (figs 2,3) were used.

Positive hybridization signals detected in immunoblotting after amplification give evidence that first, the α3-isoform of the catalytic subunit of Na,K-ATPase is present as a minor component in mammalian kidney and brain (pig, human), with the content, evidently, being two orders lower of that of the α-subunit. Second, the N-terminus of the mature protein molecule includes peptide II or at least its part, whereupon this region is sufficiently conservative in the mammalian enzyme (rat, pig, human).

The functional properties of the Na,K-ATPase containing the α3 chain remain uncertain. One of them could deal with endogenous regulators. By studying Na,K-ATPase at the level of single well defined nephron segments, the differences in Na,K-ATPase sensitivity to and affinity for ouabain along the nephron were demonstrated [15]. The proximal tubule was found to have a low sensitivity to ouabain whereas the collecting tubule was about 30-fold more sensitive. So there would obviously be a need for more than one form of Na,K-ATPase in kidney. The α3-isoform of the catalytic subunit can be such a protein, its sensitivity to ouabain seems to be two orders of magnitude higher in comparison with Na,K-ATPase α-polypeptide [16]. Nephron collecting tubules are known to span medulla and cortex, so it is logical to speculate that they contain the α3-isoform that may be the target for endogenous ouabain-like factors.

A weaker signal observed when analysing the preparations isolated from human brain, certainly gives no evidence of less protein content in nerve tissues as compared with kidney. Probably, the fine analysis of brain segments and its tissues will reveal a precise localization of the α3-isoform.

The quantity estimation of the α3-isoform distribution in various tissues is difficult in a way and can be caused by insufficient sensitivity of the methods applied, by possible partial protein loss in the course of fraction enrichment and electrophoretic transfer upon immunoblotting. This is

why the application of immunocytochemical methods seems more reasonable in this case. At present we are engaged in works on localization of the α3-isoform by specific antibodies on sections and cell cultures of various animal tissues.

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