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Polarized distribution of Na^+, K^+-ATPase α-subunit isoforms in electrocyte membranes

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Abstract

We have previously demonstrated that Na^+, K^+-ATPase activity is present in both differentiated plasma membranes from *Electrophorus electricus* (L.) electrocyte. Considering that the α subunit is responsible for the catalytic properties of the enzyme, the aim of this work was to study the presence and localization of α isoforms (α1 and α2) in the electrocyte. Dose–response curves showed that non-innervated membranes present a Na^+, K^+-ATPase activity 2.6-fold more sensitive to ouabain ($I_{50} = 1.0 \pm 0.1$ μM) than the activity of innervated membranes ($I_{50} = 2.6 \pm 0.2$ μM). As depicted in [^{3}H]ouabain binding experiments, when the [^{3}H]ouabain–enzyme complex was incubated in a medium containing unlabeled ouabain, reversal of binding occurred differently: the bound inhibitor dissociated 32% from Na^+, K^+-ATPase in non-innervated membrane fractions within 1 h, while about 50% of the ouabain bound to the enzyme in innervated membrane fractions was released in the same time. These data are consistent with the distribution of α1 and α2 isoforms, restricted to the innervated and non-innervated membrane faces, respectively, as demonstrated by Western blotting from membrane fractions and immunohistochemical analysis of the main electric organ. The results provide direct evidence for a distinct distribution of Na^+, K^+-ATPase α-subunit isoforms in the differentiated membrane faces of the electrocyte, a characteristic not yet described for any polarized cell.

Keywords: Na^+, K^+-ATPase; α-Subunit isoform; Ouabain binding; Immunohistochemistry; *Electrophorus electricus* (L.)

1. Introduction

Na^+, K^+-ATPase, discovered by Skou in 1957 [1], is an integral membrane protein that maintains transmembrane gradients of Na^+ and K^+ in almost all animal cells. This pump exchanges intracellular Na^+ for extracellular K^+, a process that is coupled to ATP hydrolysis and inhibited by cardiotonic drugs such as ouabain and digitalis [2,3]. Structurally, the Na^+, K^+-ATPase complex consists of equimolar amounts of two subunits, α and β, and in some species a third polypeptide, more recently characterized as γ-subunit [4]. The α-subunit contains Na^+ and K^+ binding sites, the catalytic site for ATP hydrolysis, and it is the cellular receptor for ouabain, as shown by affinity labeling approaches [3,5]. Previous studies have demonstrated that multiple isoforms of both α and β subunits are expressed in different tissues [6–8]. Na^+, K^+-ATPase α isoforms have been described as presenting distinct affinities to ouabain [9–11], different structures that are distinguished by antigenic determinants [7] and different binding site affinities to substrates and metal inhibitors [12–14]. Four α isoforms (α1, α2, α3 and α4) and three β isoforms (β1, β2 and β3) have been identified until now [5,15,16] and at least three of the α isoforms are well conserved even among very distant species of vertebrates [17].

The large heterogeneity of α and β subunits isoforms is highlighted by different kinetics properties, distinct tissue distribution as well as plasma membrane localization, i.e. apical or basolateral membrane. Thus, distinct physiological functions and cellular adaptation to variable ionic and osmotic environments may be explained by this variability. Most epithelial cells, for example, often present Na^+, K^+-ATPase in basolateral membrane; however, it is also found on the apical membrane elsewhere [18,19]. The α1 isoform is believed to be the dominant isoform among all epithelial tissues, but the absence of an uniform

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localization indicates that there should exist different functions attributed to this isoform depending on cellular demands.

The *Electrophorus* electrocyte is an excitable cell with a polarized structure distinguished by an innervated and a non-innervated plasma membrane [20,21], both of them presenting similar Na^+, K^+-ATPase activities [22]. Acetylcholine (ACh) receptors, acetylcholinesterase (AChE) activity and Na^+ channels are preferentially found on the innervated face (IM), giving origin to an electric and chemical excitability [20,23,24]. The non-innervated face (NIM) has been described with a higher cation flux, being however unable to produce action potentials [20,24,25].

Because of its peculiar biochemical and structural characteristics, the electrocyte has been used for many years in our laboratory as a model system to study the Na^+, K^+-ATPase. These studies not only have evidenced its presence in both faces, but also established different biochemical properties of this enzyme. We have demonstrated, by distribution through sucrose gradients [26] and by the effects of different cations on the Na^+, K^+-ATPase activity in both membranes [12–14], that different molecular forms of these enzyme exist in the electrocyte. The variable kinetic behavior of Na^+, K^+-ATPase was attributed to electrocyte distinct isoenzymes which occur in different proportions in both faces. According to these statements, the aim of the present work was to identify the different α subunit isoforms of the Na^+, K^+-ATPase in the innervated and non-innervated plasma membrane of the electrocyte through ouabain binding and specific immunostaining identification. The results presented here provide, for the first time, enough evidence for a polarized distribution of α isoforms in a single cell.

2. Materials and methods

2.1. Reagents

ATP, ouabain, ACh, NaOH, CY3 anti-mouse IgG and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, USA). β-Mercaptoethanol was obtained from Merck (Darmstadt, Germany) and [^{3}H]ouabain was obtained from Amersham Pharmacia Biotech (Cardiff, UK). All other reagents were of analytical grade.

2.2. Animals

All experiments were performed with adult specimens of *Electrophorus electricus* (L.) supplied by Museu Paraense Emílio Goeldi (Belém do Pará, Brazil) and kept in an aquarium filled with fresh filtered water. Each animal was anesthetized in ice-cold water containing 2.0% urethane before being killed by decapitation.

2.3. Isolation procedure

Membrane fractions enriched in Na^+, K^+-ATPase activity were obtained from the *E. electricus* (L.) main electric organ by differential centrifugation according to the method of Somló et al. [12]. These fractions sedimented at $10{,}000 \times g$ (non-innervated membranes-rich fractions) and at $100{,}000 \times g$ (innervated membranes-rich fractions) and were stored at -196 °C and thawed for each experiment.

2.4. Protein assays

Proteins assays were performed according to the Folin-phenol method described by Lowry et al. [27], employing bovine serum albumin as standard.

2.5. Enzyme assays

(a) Na^+, K^+-ATPase assay was performed as previously described [12]. Samples of non-innervated and innervated membrane fractions were added in concentrations of 20 μg protein ml^{-1} to standard assay mediums containing 3.25 mM $MgCl_2$, 0.2 mM EDTA, 14 mM KCl, 72 mM NaCl, 160 mM Tris–HCl pH 7.4 in a final volume of 1.0 ml. Reaction was initiated by adding 2.5 mM ATP and incubations were carried out for 15 min at 37 °C. The reaction was stopped by addition of 5% trichloroacetic acid. Inorganic phosphate (Pi) was determined by the method of Fiske and Subbarow [28]. Na^+, K^+-ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of the specific inhibitor ouabain (0.4 mM). Specific activities were expressed as μmol Pi min^{-1} mg $protein^{-1}$.

(b) AChE activity was assayed in both non-innervated and innervated fractions according to Hassón and Liepin [29] for the characterization of the electrocyte anterior and posterior membranes faces [22]. The enzyme activity was measured by the acetic acid released from a substrate medium containing 10 mM sodium acetate, 20 mM $MgCl_2$ and 2.5 mM ACh at pH 8.0–8.3, titrated with 0.2 N NaOH using a Beckman Century SS-1 pH-meter. The NaOH addition was accomplished by an AGLA micrometer-syringe outfit, with continuous stirring, at 25 °C. Measurements were obtained at 1-min intervals over a 4-min period. The specific activity was defined as the amount of ACh hydrolyzed min^{-1} mg $protein^{-1}$.

2.6. Binding assay

Ouabain binding was studied with a conventional filtration method consisting of:

(a) Ouabain equilibrium binding was carried out by incubating the enzyme with different concentrations of [^{3}H]ouabain (36 μCi/mmol) using the incubation medium described above. In the presence of electrocyte membrane fractions (100 μg protein/tube), increasing concentrations of

unlabeled ouabain from 5 to 1000 μM and a fixed concentration of [^{3}H] ouabain (64 nM) were added to a final volume of 0.5 ml. The mixture was incubated for 15 min, at 37 °C, and the reaction was stopped by filtering it through a 0.45-μm membrane (Millipore, HAWP) and washing in 10 ml of incubation medium. The radioactivity retained by the filter was measured on a liquid scintillation counter with 5 ml of scintillating mixture (670 mM Toluene, 25 mM PPO and 0.5 mM POPOP). Specific binding was determined as the difference in total binding and non-specific binding. Blanks for specific ouabain binding were obtained by preincubation of the enzyme with 1 mM unlabeled ouabain before the addition of labeled ouabain. The blanks corresponded to approximately 5% of total binding.

(b) Ouabain dissociation kinetics—For these kinetics studies, 5 ml of the binding medium, containing 200 μM unlabeled ouabain, was added to the same volume of incubation medium containing 100 nM labeled ouabain, at 37 °C for 10 min. At different times, aliquots of 0.9 ml were removed and filtered immediately. All aliquots were filtered under vacuum on Millipore filters (0.45 μm), washed in 10 ml of the reaction mixture and the specific binding was determined as described above.

2.7. Analysis of binding data

(a) The data are presented in dose–response curve as the percentage of remaining activity at the indicated ouabain concentrations, relative to the total Na^+, K^+-ATPase activity in the absence of inhibitor. The data were fit to the equation,

$$V = \frac{(100 - v)}{\left(1 + \frac{[\text{ouabain}]}{IC_{50}}\right)} + v$$

where V is the equivalent percentage of Na^+, K^+-ATPase activity; v is the percentage of Na^+, K^+-ATPase activity inhibited by ouabain, and IC_{50} is the ouabain concentration which inhibits 50% of the enzyme activity.

(b) Dissociation binding—Data are expressed as means of three or more experiments in triplicate and were analyzed by single exponential decay fitting using a nonlinear regression computing software (Sigma Plot v3.0-Jandel Scientific, San Rafael, CA, USA) and Sigma Stat v1.0 (Jandel Scientific) for statistical analysis.

Table 1
Distribution of Na^+, K^+-ATPase and AChE activities in membrane fractions from *E. electricus* (L.) electric organ

Fractions	Total protein[a]	Na^+, K^+-ATPase activity[b]	Total Na^+, K^+-ATPase (U)	AChE activity[c]	Total AChE activity (U)
P_2	131 ± 51	3.93 ± 0.86	514.83	27.0 ± 7.7	3537
P_3	169 ± 69	3.52 ± 0.53	594.88	327 ± 18	55,263

The electric organ membrane fractions were obtained as described in Materials and methods.

[a] Total protein is expressed in mg.

[b] Specific Na^+, K^+-ATPase activity is given in μmoles Pi mg^{-1} min^{-1}.

[c] Specific AChE activity in μmol of ACh hydrolyzed mg^{-1} min^{-1}.

Results are expressed as means ± S.E. of different membrane preparations in triplicate ($n = 4$).

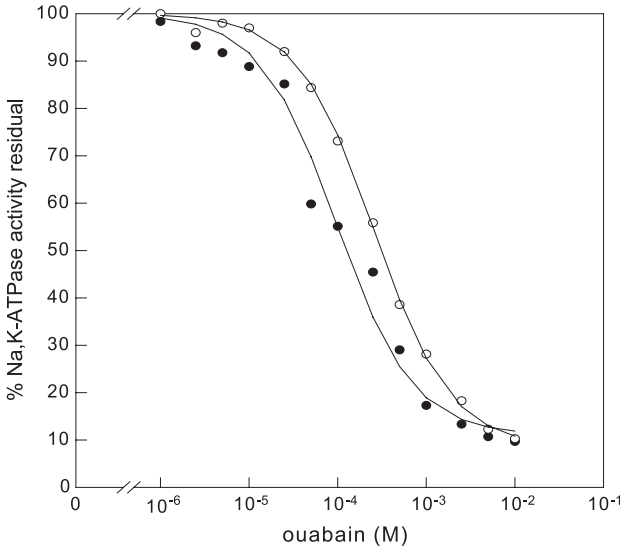

Fig. 1. Inhibition of the Na^+, K^+-ATPase activity by ouabain. Standard assay conditions were used (see Materials and methods) with ouabain concentration varying from 10^{-6} to 10^{-2} M. Values are expressed as percentage of maximal activity without the inhibitor. P_2 (non-innervated membrane), in filled circle; P_3 (innervated membrane), open circle.

2.8. α Antibodies

Primary antibodies against α1 and α2-subunits isoforms of Na^+, K^+-ATPase were the generous gift of Dr. K. Sweadner (Massachusetts, USA) to Dr. F. Noel from this University. Horseradish-peroxidase-conjugated goat anti-mouse secondary antibody was purchased from Amersham Bioscience UK Limited (England). Rhodamine (TRITC)-

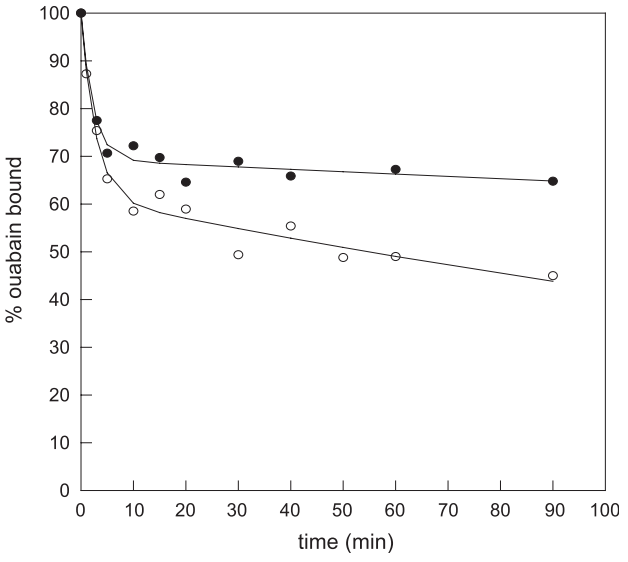

Fig. 2. Dissociation of [^{3}H] ouabain from the enzyme–ouabain complex plotted versus time. Filled circle, P_2 fractions; open circle, P_3 fractions.

conjugated donkey anti-rabbit IgG (H1L) was a product of Jackson Immuno-Research Labs. (West Grove, PA, USA).

2.9. Western blotting

Proteins from non-innervated and innervated membrane fractions were separated using SDS-PAGE (10%). Proteins from gel were transferred to nitrocellulose membrane presoaked in transfer buffer (38.6 mM glycine, 48 mM Tris base, 1.28 mM SDS and 20% methanol, pH 8.3) using BioRad Transfer Cell apparatus. The blotted paper was removed from the gel, notched to provide directional orientation and blocked at room temperature in 5% nonfat dried milk in TBST solution [150 mM NaCl, 5 mM Tris (pH 7.4) and 0.05% Tween-20]. The prehybridized blots were washed with TBST and then incubated for 60 min at room temperature with the appropriate dilution of the primary antibody (both anti-α1 and α2 were diluted 1:1000). The blots were then washed three times with TBST and incubated in a similar manner to that described for the primary antibody with horseradish-peroxidase-conjugated goat anti-mouse secondary antibody, diluted 1:5000 in TBST.

Detection of the immobilized antigens complexed with the sandwich of antibodies was accomplished using chemiluminescence analysis according to the directions supplied by the manufacturer (Amersham Bioscience, UK). For membrane protein size fractionation, standards were used to enable estimation of approximate molecular weight.

2.10. Na^+, K^+-ATPase immunohistochemical analysis

Fragments of the main electric organ were prepared for immunohistochemistry as previously described [30]. Ten-micrometer-thin sections were preincubated with 50 mM

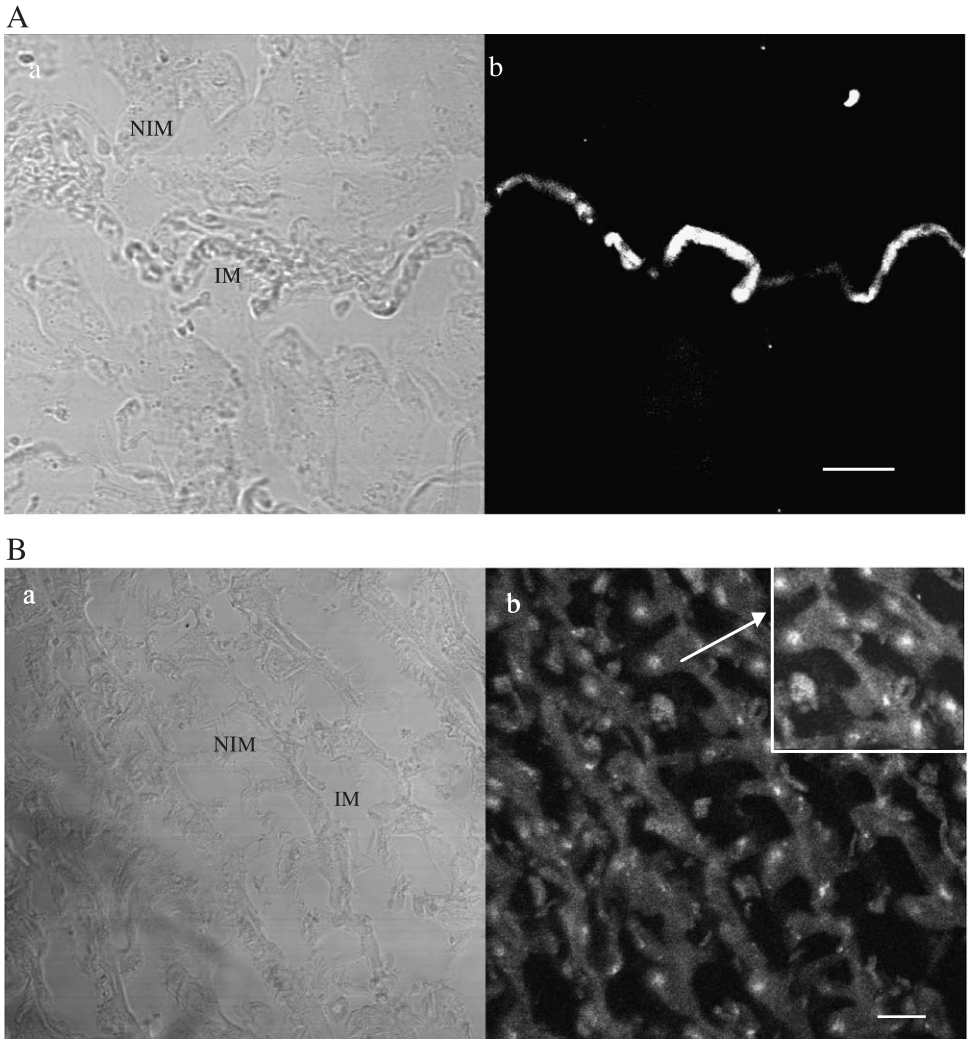

Fig. 3. (A) Confocal microscopy of immunostained electrocyte thin section (10 μm) with Na^+, K^+-ATPase monoclonal α1 antibody. (a) Phase-contrast, non-innervated membrane (NIM); innervated membrane (IM). (b) Immunoreactivity is observed only in the posterior face of the electrocyte, corresponding to the innervated membrane; scale bar, 50 μm. (B) Confocal microscopy of immunostained electrocyte thin section (10 μm) with Na^+, K^+-ATPase monoclonal α2 antibody. (a) Phase-contrast, non-innervated membrane (NIM); innervated membrane (IM). (b) Immunoreactivity is observed only in the anterior face of the electrocyte, corresponding to the non-innervated membrane; inset magnification in b is 10 ×; scale bar, 50 μm.

NH_4Cl for 1 h and washed with PBST solution [10 mM sodium and potassium phosphate buffer (pH 7.4), containing 27 mM KCl, 137 mM NaCl and 0.2% Tween-20]. Sections were then incubated overnight with a monoclonal antibody against α1 and α2-subunits isoforms of Na^+, K^+-ATPase (1:1000 dilution) at 4 °C, washed with PBST containing 10% normal goat serum, and incubated with a CY3 anti-mouse IgG rhodamine conjugated (1:200 dilution) for 2 h at room temperature. Negative controls were obtained by omitting the primary antibody for each experiment. The sections were examined in a Zeiss 310 Confocal Laser Scanning Microscope (CSLM). All images were obtained under the same conditions of brightness and contrast and uniformly processed using Adobe PhotoShop v 7.0.

3. Results

3.1. Na^+, K^+-ATPase and AChE activities

Control values for the Na^+, K^+-ATPase and AChE activities in membrane fractions (non-innervated and innervated faces) of the *E. electricus* (L.) electric organ are shown in Table 1. AChE, which was shown to be localized in the innervated membrane [31], was assayed in order to confirm the identity of innervated membrane-rich fractions and the success of membranes separation. As described by Somló et al. [22], Na^+, K^+-ATPase activity is present in both fractions.

3.2. Inhibition of Na^+, K^+-ATPase activity by ouabain

Dose–response curves for ouabain inhibition of the non-innervated and innervated membrane fraction Na^+, K^+-ATPase activities (Fig. 1) were performed in order to investigate their sensitivities towards ouabain. Our data indicate that these activities are highly sensitive to ouabain, and exhibit statistically different IC_{50} values ranging over 1.0 ± 0.1 μM (non-innervated fractions) and 2.6 ± 0.2 μM (innervated fractions) ($P < 0.001$, as determined by Student's t test).

3.3. [^{3}H]ouabain binding

The dissociation reactions were performed by incubating both the radioligand and Na^+, K^+-ATPase for a period of time as shown on Fig. 2. These dissociation reactions occurred according to a biphasic component: a rapid initial phase, probably associated with the sites of the lowest affinity, and a second phase corresponding to the sites of highest affinity. At 1-h period of incubation, it was observed that 32% of ouabain dissociated from non-innervated face Na^+, K^+-ATPase. Nevertheless, 50% of the ouabain bound to the enzyme in innervated face was released in the same time. These results confirm the data showed in Fig. 1 and demonstrate that Na^+, K^+-ATPase from non-innervated face has a higher sensitivity to ouabain.

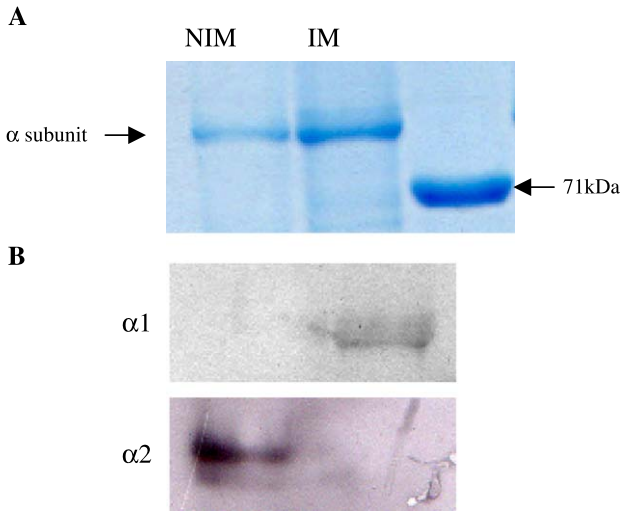

Fig. 4. Identification of α subunit isoforms by Western blotting. (A) Coomassie blue-stained gels of SDS-PAGE. (B) α1 and α2 subunit isoform immunodetection of 40 μg of total protein from membrane fractions of non-innervated (NIM) and innervated membranes (IM).

3.4. Immunodetection

Immunodetection analysis became necessary for identification of different α-subunits isoforms of Na^+, K^+-ATPase from non-innervated and innervated faces, which could definitely explain the results shown above. The employed primary antibodies are described as being specific to Na^+, K^+-ATPase α-subunit isoforms identification [32]. Immunohistochemical analysis was carried out to localize in situ the α1 and α2-subunits isoforms. The presence of Na^+, K^+-ATPase α1 isoform was observed by immunofluorescence only in the innervated electrocyte membrane (IM) from the *E. electricus* main electric organ (Fig. 3A), while α2 isoform was restricted to the non-innervated electrocyte membrane (NIM) (Fig. 3B).

Western blots were assayed to eliminate the possibility of a cross-contamination during the process of membrane fractioning that would interfere with the results obtained with membrane fractions experiments (Figs. 1 and 2), and also to confirm the polarized distribution of α1 and α2-subunits isoforms (Fig. 3). Fig. 4 establishes membrane domain specificity of both isoforms in the electrocyte, demonstrating that α1 isoform is present only in innervated membrane-rich fractions while α2 isoform can be found exclusively in non-innervated membrane-rich fractions.

4. Discussion

Our previous studies showed differences in Na^+, K^+-ATPase activity profiles on innervated and non-innervated electrocyte membrane fractions when submitted to nonlinear sucrose gradients [26]. In addition, we studied the kinetic effects of metal ions on these activities [12–14],

and suggested that the presence of distinct isozymes of Na^+, K^+-ATPase should explain the results obtained in these fractions. It seemed likely that an involvement of separate α-subunit isoforms exist, or at least variable contents of them, in innervated and non-innervated electrocyte faces, although it has been demonstrated early that the non-innervated face presents a high Na^+, K^+-ATPase-like immunoreactivity detected through an antibody against α-subunit [33].

All Na^+, K^+-ATPase catalytic subunits (with the exception of α4, for which not enough information is obtained) have shown to differ in some of their properties, with discrepancies in: molecular weight by SDS-PAGE [2], sensitivities to proteolysis and dephosphorylation by K^+ [34], number of reactive sulfhydryl groups [35] and different antigenic determinants [7]. Although Na^+, K^+-ATPase isolated from different sources have similar affinities for most ligands such as Na^+, K^+, Mg^{2+}, and ATP, which are involved in the transport process, the ouabain affinity of distinct α isoforms varies widely [35]. It is also known that endogenous ouabain may regulate cardiovascular tonicity by inhibiting a discrete number of Na^+, K^+-ATPase pumps, to modulate cellular excitability and muscle contractility [8,36], and also the role of Na,K-ATPase as a ouabain receptor and a signal transducer [37]. Consequently, the existence of multiple α isoforms represents important tools in the regulation of cardiac glycosides action. The preliminary molecular identification of the α-isoforms in electrocytes was performed in order to verify Na^+, K^+-ATPase ouabain sensitivity of innervated and non-innervated membrane-rich fractions. The differences observed in inhibition curves (Fig. 1) can be explained by the existence of at least one distinct Na^+, K^+-ATPase isozyme in each membrane fraction. This statement is fully supported by the IC_{50} values obtained, which showed that the pump found in non-innervated membrane fractions is 2.6-fold more sensitive to ouabain than that present in innervated membrane fractions (Fig. 1).

The interaction of cardiac glycosides with Na^+, K^+-ATPase α subunit has been postulated to occur in two steps: an initial, rapidly reversible binding event followed by a conformational change to a more stable form of the enzyme–ouabain complex [38]. Accordingly, when the [^{3}H]ouabain–enzyme complex was incubated in a medium containing unlabeled ouabain, it showed a 1.6-fold higher dissociation in innervated membrane fractions (50%) when compared to that observed in non-innervated membrane fractions (32%) within 1 h (Fig. 2), although the initial phases of both plots were undistinguishable due to the fast decay of the bound complex. Altogether, these data indicate that the Na^+, K^+-ATPase ouabain site sensitivities and interactions differ from one electrocyte membrane face to another and suggest the existence of distinct α subunits in these regions. This hypothesis was confirmed by immunohistochemical and Western blotting analysis of the Na^+, K^+-ATPase α1 and α2 subunits distribution in the electrocyte, which strikingly demonstrated that the former is restricted to the innervated membrane (Fig. 3A), while the latter is localized only in the non-innervated membrane (Fig. 3B), and consequently the same pattern was observed in their respective membrane fractions (Fig. 4).

The immunohistochemical localization was employed by other authors to determine the differential expression in different tissues fragments and polarized cells [19,20] and even in distinct domains in non-polarized cells [39]. Because the multiple α-isoforms exhibit tissue-specific localization among diverse species, their functional and physiological importance has been extensively studied [35]. Variations in α-isoforms composition induced by altering the expression levels of α1 and α2-isoforms demonstrated their different roles in skeletal muscle contractility [40].The work of He and coworkers supported previous studies [41] which showed that α1-isoform was uniformly distributed along the plasma membrane of rat astrocytes and arterial myocytes, whereas α2-isoform was confined in specific regions, co-localized with the Na^+, Ca^{2+} exchanger, suggesting that low and high ouabain affinity isoforms should undergo different regulation mechanisms. This hypothesis is in well agreement with a recent statement demonstrating a coherent link between isoform-specific interactions of the pump and structural characteristics of α1 and α2 isoforms [42].

This paper deals with the identification/distribution analysis of catalytic isoforms of Na^+, K^+-ATPase, but its function–localization relationship in the electrocyte remains yet to be determined. Our study presents evidences that undoubtedly shows, by ouabain sensitivity and employing specific antibodies for immunostaining analysis, that electrocyte Na^+, K^+-ATPase has a polarized α-subunit isoform distribution (at least α1 and α2). These results provide, for the first time, an apical/basal isoforms localization in a single cell; therefore, further studies about their possible functions for a polarized cell must be performed.

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