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RELATION BETWEEN α-ISOFORM AND PHOSPHATASE ACTIVITY OF Na^+,K^+-ATPase IN RAT SKELETAL MUSCLE FIBER TYPES

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Abstract

In skeletal muscle the relationship between Na^+,K^+-ATPase activity and isoform content remains controversial (9,6). It could be due to the fiber-type content, membrane isolation and analytical methods. We investigated the distribution of subunit α1 and α2 Na^+,K^+-ATPase catalytic isoforms and the Na^+,K^+-ATPase activity in isolated membranes from white (type I and glycolitic fibers) and red (type II and oxidative fibers) skeletal muscles. Red Gastrocnemius and White Gastrocnemius muscles were sampled from 8 week-old female Wistar rats and crude membranes were performed. The Na^+,K^+-ATPase activity and membrane distribution of Na^+,K^+-ATPase α1 and α2 isoforms were assessed by ouabain sensitive K-phosphatase (Kpase) measurements and Western Blot respectively. The Na^+,K^+-ATPase activity was 6 fold lower in White Gastrocnemius membranes than in Red Gastrocnemius membranes. The α1 and α2-isoform levels are higher in RG than in White Gastrocnemius. The α1 and α2-subunit Red Gastrocnemius content was significantly higher than in WG. The correlation between crude membrane Kpase activity and both catalytic α-subunit of the Na^+,K^+-ATPase exist.These data suggest that the Na^+,K^+-ATPase phosphatase activity correlates with the α1 and α2 isoforms levels in Red Gastrocnemius and White Gastrocnemius and confirms the fiber-specific Na^+,K^+-ATPase catalytic α-subunits and α2-isoform as the major catalytic isoform in rat skeletal muscle.

Key words: Kpase, turnover, Na^+,K^+-ATPase, ouabain.

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Abbreviations: RG : red gastrocnemius muscle; **WG** : white gastrocnemius muscle; **Kpase** : ouabain sensitive potassium phosphatase; **SR** : sarcoplasmic reticulum ; **pNPP**: paranitrophenyl phosphatase; **pNP**: paranitrophenyl ; **LDL** : low density lipoprotein: **3-OMFP** : 3-O-methylfluorescein phosphate hydrolysis by the Na^+,K^+)-ATPase.

INTRODUCTION

Skeletal muscles are composed of different types of fiber types. These fibers are classified by their properties : 1/ phenotypes (red vs white), 2/ Contractility (fast twich (type II) vs slow twich (type I)) and 3/ metabolism (oxidative vs glycolytic). Their physiological roles are for red muscle the gravity, bearing weight and sustained movements and for white the concentrated bursts of power (18). Two examples of skeletal muscles have been used for their phenotypes : soleus with 87 % fast oxidative red fibers (red gastrocnemmius are also used) and the white gastrocnemius with 84 % slow glycolytic white fibers (1). Furthermore, type II fibers possess a specific profile of myosin heavy chains and an extensive sarcoplasmic reticulum (SR) with T tubules which permits the rapid regulation of the cytosolic free Ca^{2+} -transient and rapid activation

of the myofibrillar proteins. This confers to such fiber higher frequency of sarcolemmal action potential (24). As a consequence, these fibers in the rat skeletal muscles are characterized by a high level of P-type ATPases especially the Ca^{2+}-ATPase. Na^+,K^+-ATPase α1 and α2 are major isofoms in adult skeletal muscles whereas the α3 and α4 are not expressed (18). However it was difficult in many studies (*1*-6) to assess the Na^+,K^+-ATPase activity and the α1 and α2 Na^+/K^+-ATPase isoform content in crude homogenates. This discrepancy may be attributed to the different methods used to homogenize and purify membrane from skeletal muscle. However, the yield in enzyme activity was less than 10 % of the whole enzyme with 0.08 % in recovery as outlined by Clausen (6). On the other hand, homogenates seem less appropriated to characterize the protein content with accuracy although Thompson and McDonough (29) illustrate very accurate detection of Na^+,K^+-ATPase in skeletal muscle homogenates in a fiber specific manner. However Fowles et al. (9) recently addressed this issue with extending characterization of Na^+,K^+-ATPase in many conditions and failed to indicate a clear relationship between activity and total pump content and isoform content of Na^+,K^+-ATPase. In the past, there was a consensus that measurements of K^+-activated phosphatase are more accurate for purified plasma membranes from skeletal muscles (7). However these previous measurements are limited in use within the rat since the resistant rat α1 isoform of Na^+,K^+-ATPase is partially inhibited by ouabain, the Na^+,K^+-ATPase specific inhibitor , and do not allow the measure of the whole enzyme activity with accuracy.

Therefore, our goal in this study is to understand the relation between enzyme activity and isoform content in type I and type II skeletal muscles. To address this, we determined the K^+-dependent phosphatase activity of the Na^+,K^+-ATPase (K-pNPPase activity as sensitive to Na^+) and the immunological characterization of catalytic α-subunit from rat red and white gastrocnemius skeletal muscles crude membrane fractions in term of acceptable enzyme recovery (50 % of the whole Na^+,K^+-ATPase activity) (2).

MATERIALS AND METHODS

Animals

8 weeks female wistar rats were purchased from R. Janvier (Le Genest-St-Isle, France). All experiments were performed according to Institutional Animal Care and Use Committee guidelines (Ministère de l'Agriculture, Article R214-87 and R215-10 from « code rural »). Animals were anesthetized with a mix of Ketamine (3mg/kg ; Jansen Pharmaceutics, Paris, France) and chlorpromazine (1.5ml/kg ; Roche Pharmaceutioicals, Paris, France). Muscles were removed, frozen in liquid nitrogen, and stored at -80 °C.

Muscle Plasma Membrane Isolation

Samples of frozen skeletal muscle (2-12 mg) were homogenized directly in ice-cold buffer containing 250 mM sucrose, 0.1 mM phenylmethane sulfonyl fluoride, 1 mM EDTA, and 20 mM imidazol-HCl, pH 7.4, with a polytron PT 10 (5 sec, setting 3) as previously described in cardiac tissues (2). The homogenate was subfractionated by two sequential differential centrifugations at 12,000 x g for 5 min and 540,000 x g for 5 min, using a TLA100.3 rotor in the Beckman TL 100 centrifuge (Beckman Instruments; Gagny, France). The final pellet was resuspended in 250 mM sucrose and 16 mM HEPES-HCl, pH 7.4 and stored at -80C until use. These preparations consisted of a membrane fraction highly enriched in Na^+,K^+-ATPase.

Since Zhang and Ng showed that the Na^+,K^+-ATPase α-1 isoforms undergo dephosphorylation in rat skeletal muscle cryosections, we have chosen to store muscle membrane preparations in liquid nitrogen after preparation to avoid dephosphorylation (30).

The protein yields for the crude membrane preparations calculated as the amount of sarcolemmal protein available at the end of isolation relative to the known amount of wet weight tissue at the beginning of the isolation (mg protein/g wet wt) were the same as muscle membrane preparations previously used – state the recovery since this was criticized in the introduction (7).

K^+ -stimulated paranitrophenyl phosphatase activity

Na^+,K^+-ATPase activity was measured as K^+-stimulated paranitrophenyl phosphatase (pNPPase) activity using a modified method from Maixent *et al.*(15). The activity was assessed in 96-wells plates using a reaction mixture with KCl or KCl-ouabain-NaCl during 2 h at 37°C. Each well contained (final volume 300 µl) 6 mM $MgCl_2$, 30 mM Imidazole, 250 mM sucrose, 8 mM pNPP (Sigma) with 20 mM KCl or 20 m KCl and 10^{-3} M ouabain. The complete (almost) ouabain inhibition of K^+ -stimulated paranitrophenyl phosphatase (pNPPase) activity was tested after 30 min, 60 min, 90 min and 120 min. This last time of incubation was used to calculate the enzyme activity and ouabain inhibition. The enzymatic reaction was initiated by the addition of protein (5 µg). As similar inhibition of the enzyme activity was obtained between Na^+ (20-100 mM) and ouabain, we did not choose to preequilibrate the membranes and ouabain before addition of K^+. Optical density was measured at 405 nm using the Wallac 1420 VICTOR^{3TM} a multilabel, multitask plate reader. K^+-stimulated paranitrophenyl phosphatase (pNPPase) activity was calculated as the difference in paranitrophenyl (pNP) production with KCl or KCl-ouabain-NaCl. Enzyme activities are expressed as optical density/hr/mg of protein. Protein content was determined by the method of Lowry et al. (13), using bovine serum albumin (Sigma) as a standard.

Antibodies

The monoclonal antibody 6F specific for the Na^+,K^+-ATPase α1 isoform was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). The polyclonal antibody HERED specific for α2 isoform was obtained from Thomas A.Pressley (Department of Cell Physiology & Molecular Biophysic, Texas Tech

University Health Sciences Center,Lubbock, Texas) (26)(Tab. 1).

SDS/PAGE and Western Blots

Expression of the α1 and α2 isoforms of the Na^+,K^+-ATPase was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel) and immunoblotting. In a preliminary experiment, we obtained linear calibration curves with the antibodies used in this study between 5 and 20 µg of skeletal membrane proteins loaded on the same gel. A total protein of 10 µg/lane was separated by SDS-PAGE and was transferred onto nitrocellulose membrane (Hybond C^+, Amersham Corp.). Nitrocellulose blots were blocked in 5% BSA in TBS-T for 1 h at room temperature. Primary antibody in 5% BSA TBS-T was incubated overnight at 4°C. Secondary antibody was incubated for 1 hr at room temperature. Table 1 indicates the concentration for each primary antibody and corresponding secondary antibody Detection was performed with the enhanced chemiluminescence (ECL). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. The amount of protein bands was quantitated using imageJ (NIH). All summarized results consist of data from at least four Western blots representing six different membranes preparations of each muscle types.

Table 1. Primary and secondary antibodies used in this study

Antibody	Protein	Caracteristic	Dilution	Secondary Antibody	Dilution
anti 6F[(1)]	α1	Mice monoclonal	1/1000	Anti-mice	1/2000
anti HERED[(2)]	α 2	Rabbit polyclonal	1/1000	Anti-rabbit	1/2000

(1) From Developmental Studies Hybridoma Bank of Iowa University (Iowa City, IA).
(2) Generously provided by Dr. Thomas A. Pressley (Department of Cell Physiology & Molecular Biophysics, Texas Tech University Health Sciences Center,Lubbock, Texas)

Statistical analysis

Results are expressed as means ± SEM of six experiments. Statistical analysis was done using analysis of variance (ANOVA) followed with the Tukey's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered statistically significant.

RESULTS

Na^+,K^+-ATPase activity in crude membrane from frozen skeletal RG and WG

The Na^+,K^+-ATPase activity was determined using Kpase assays in RG and WG muscles crude membrane preparations . The Kpase activity was 6 fold higher ($p<0.01$) in RG than in WG (Fig. 1).

Isoform distribution

The Na^+,K^+-ATPase catalytic subunit isoforms were measured in the same crude membrane preparations. The relative distribution of α1 and α2 isoforms is shown in Fig. 1. Relative density of each isoform was calculated relative to the known standard of rat brain lysate. Rat kidney lysate was used to corrobarate the specificity of α1. The α1- and α2-isoforms distribution is higher in RG than in WG. The α1 and α2-isoforms content was approximately 1.5-fold and 3 fold greater in RG than in WG respectively ($p<0.05$). In conclusion the distribution for α-subunit exhibited a dramatic difference in α2 isoform expression.

Relationship between Na^+,K^+-ATPase activity and isoforms content

Interrelationships determined from regression correlation between activity and isoform abundance in crude membrane preparations are shown in Fig 2. A trend for a correlation could be seen between crude membrane Kpase activity and both catalytic subunit of the Na^+,K^+-ATPase from both muscle type (Fig. 2 A&B). The correlation was similar for the 2 α-subunits : α1 ($r=0.5$, NS) and α2 ($r=0.5$, NS).

The flat correlation between crude membrane Kpase activity and both catalytic α-subunit of the Na^+,K^+-ATPase could be due to the important difference in Kpase activity between the two muscles (Fig. 2 A&B).

DISCUSSION

It has been known for long time that red and white skeletal muscles differs by their metabolism (oxidative vs. glycolytic), contractility (fast twitch vs. slow twitch) and their functionality. The activity and content of the Na^+,K^+-ATPase is regulated by various factors in order to satisfy the demand of Na^+,K^+-ATPase

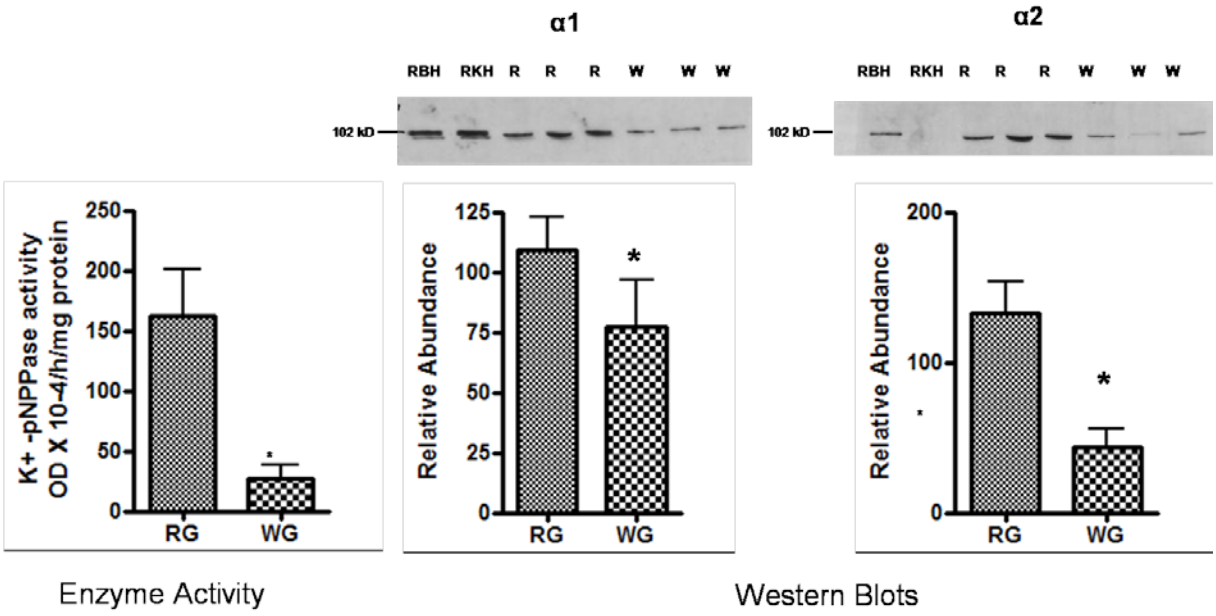

Figure 1. Na^+,K^+-ATPase enzyme activity and α-2 isoform expression are higher in rat red skeletal muscles. RG: Red Gastrocnemius, WG: White Gastrocnemius, relative abundance (in %) was adjusted to 100% for rat brain tissue in α1 and α2 respectively, n=6,* P < 0.05. This graph summarize 4 Western blots representing six different membranes preparations of each muscle types

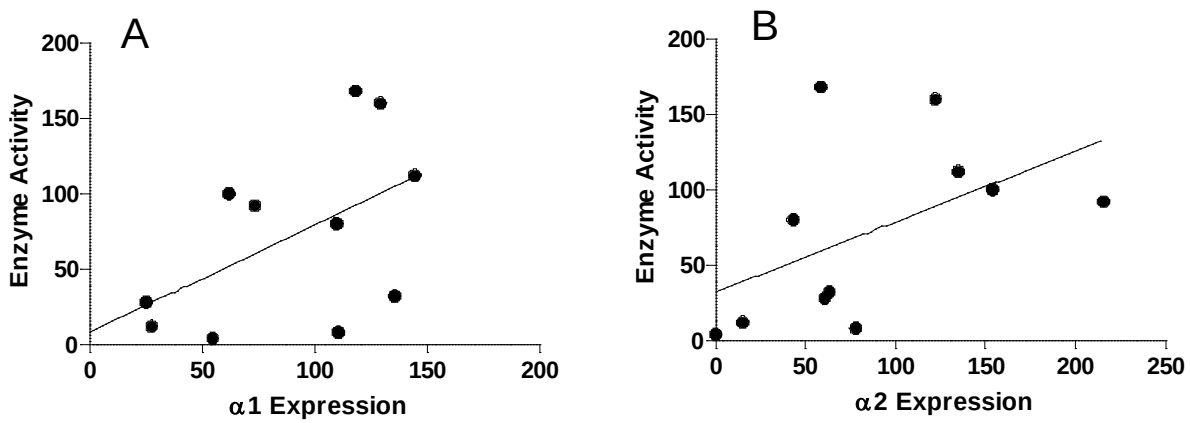

Figure 2. Regression correlation between activity and isoform abundance in crude membrane preparations of red and white skeletal muscles. Linear regression : R=0.5 for α1 and : R=0.5 for α2. Enzyme activity : K^+ pNPPase Activity OD x 10^{-4}/h/mg protein, α1 and α2 expression : relative abundance to rat brain homogenate (%) .

(phosphorylation, signal transduction, etc.) in the skeletal muscle (6,18,21). It has been assumed that the changes in K^+ transport in muscle are dependent of Na^+,K^+-ATPase activity and abundance, especially the α2-isoform which is the predominant isoform in skeletal muscle (19). However, the mechanisms underlying these regulations are still not well understood (6). In skeletal muscle the relationship between Na^+,K^+-ATPase activity and isoform content remains controversial[9]. It could be due to the fiber-type content, membrane isolation and analytical methods. We investigated the distribution of Na^+/K^+ ATPase α1 and α2 isoforms and its activity in isolated membranes from white (type I and glycolytic fibers) and red (type II and oxidative fibers) skeletal muscles. To avoid the dilemma to use homogenate or purified membrane preparation, in this study we isolated a simple crude membrane preparation and revealed that an apparent fiber type of Na^+,K^+-ATPase α1- and α2-subunit abundances in RG and WG respectively.

We found a trend for a regulation between crude membrane Kpase activity and both catalytic subunit of the Na^+,K^+-ATPase from both muscle type (Fig. 2 A&B). The correlation was similar for the 2 α-subunits (r=0.5). Previous studies showed discrepancies between Na^+,K^+-ATPase activity and α-subunit content in the skeletal muscle (9,20,21). Fowles et al.(9) investigated the hypothesis that muscle Na^+,K^+-ATPase activity was directly related to Na^+,K^+-ATPase and to α2-catalytic isoform contents from different fiber-type composition. However Fowles et al. found disparate correlation between content and activity of Na^+,K^+-ATPase in the skeletal muscle among and the methods to measure the enzyme activity ie. Na^+,K^+-ATPase, 3-O-MFP and ^3H ouabain binding (9) and conclude that the best measure of Na^+,K^+-ATPase activity was the ATPase reaction in crude membrane fractions.

Using western blot analysis, Kritensen and Juel (12) investigated the relative α-isoform distribution in rat skeletal muscle membranes. Interestingly the distribution of RG and WG Na^+,K^+-ATPase in the purified membranes (sarcolemma giant vesicles) was found to be very similar to our results with a crude membrane preparation. However they found an inverse ratio in contrast to our results with a very crude membrane preparation. In this outer membrane fraction, the α1 and α2 were the major protein found in WG. In contrast, Fowles et al. (9) found the same distribution with the homogenate and crude membranes presented: homogenates; α1 RG >>WG & α2 RG >WG and crude membranes; α1 RG >WG and α2 RG>WG. Our results confirm the constant RG>WG for α1 and α2 whatever the fraction (homogenate or crude membranes). The relative isoform distribution in our membrane preparation confirms two facts as previously evidenced in a more complete analysis of muscle types by Thompson and MCDonough (29) : the fiber-specific Na^+,K^+-ATPase α-subunits distribution and the α2-isoform as the major catalytic isoform in rat skeletal muscle (29). All our results are integrated with the existing literature and are schematically represent in Fig. 3 & in Fig. 4 for red skeletal muscle and white skeletal muscle respectively (These figures are adapted from Mcdonough et al., 2002 (18)). It remains to assess the subcellular distribution of α-isoforms in terms of enzyme activity and protein expression in endosomal membranes, caveolae and t-tubules.

Skeletal muscle crude homogenates contain very high concentrations of SR and mitochondrial ATPases, and only a minor fraction can be identified as specifically activated by Na^+, K^+, and Mg^{2+} or suppressed by cardiac glycosides. It is difficult, therefore, to obtain accurate values for the total content of Na^+-K^+-ATPase activity as well as the Na^+-K^+-ATPase protein distribution (6,18). Moreover, Zhang et al. (30) recently showed a differential phosphorylation of the α1-subunit in the RG. Using an elegant experimental design, and two well characterized specific antibodies for the Na^+,K^+-ATPase α1-subunit (McK1 and α6F), these authors demonstrated that in RG skeletal muscle, only a small number of the fibers were stained and that the type IIA, IID and IIB were minimally stained due to a phosphorylation on the Ser 18. This amino acid is specific for the α1 on the rat and can be phosphorylated (5,8). The phosphatase activity during the cryosection and immunostaining could have dephosphorylated the phosphorylation sites in the Na^+,K^+-ATPase and render more homogeneous (in the way of irrespective of the phosphorylation status) the RG and WG crude membrane preparations for western blot and enzyme. Since we used a phosphatase in our assay, this heterogenous phosphorylation could be less present.

Since the challenge is to access Na^+,K^+-ATPase in skeletal muscles, our enzymological procedure coupled to our membrane fractionation appears a good compromise. To understand better the distribution of Na^+,K^+-ATPase coupled to enzyme activity in the high and low-oxidative

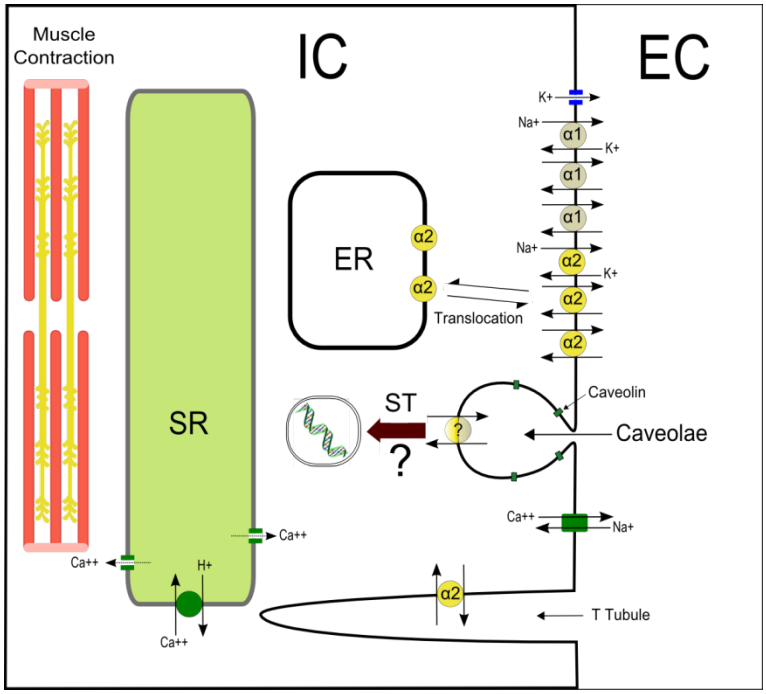

Figure 3. Schematic representation of the Na^+,K^+-ATPase in red skeletal muscle. IC : Intracellular, EC : Extracellular, ST : Signal Transduction, ER : Endoplasmic Reticulum, SR : Sarcoplasmic Reticulum. Adapted from Mcdonough et al., 2002 (18).

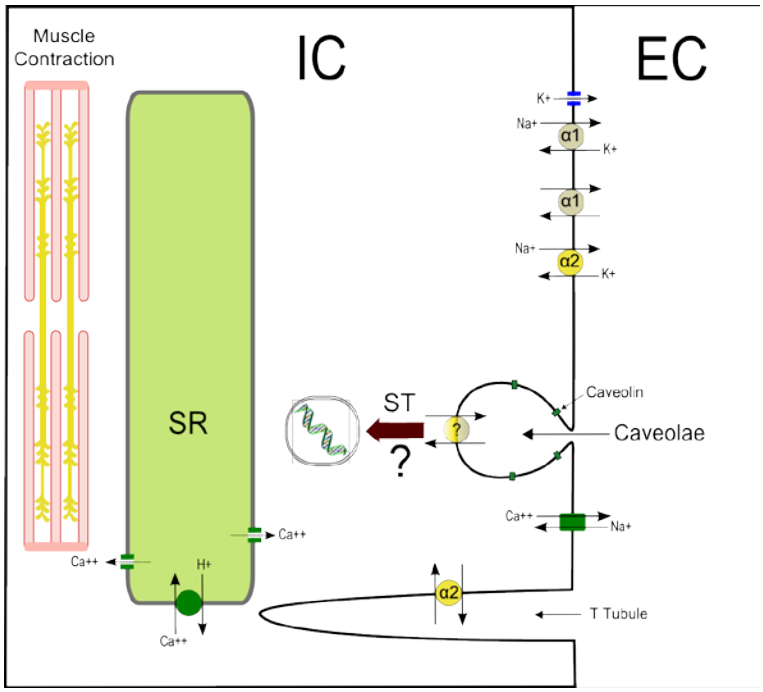

Figure 4. Schematic representation of the Na^+,K^+-ATPase in white skeletal muscle. IC : Intracellular, EC : Extracellular, ST : Signal Transduction, ER : Endoplasmic Reticulum, SR : Sarcoplasmic Reticulum. Adapted from Mcdonough et al., 2002 (18).

muscles (4,16), it will be interesting to study it during physiological and pathophysiologicals conditions such as exercise, muscle fatigue (with a pronounced loss of K^+ from contracting muscles (22), age (23), lipidic modulation by cholesterol, oxidized LDL and fatty acids especially the polyunsaturated fatty acids like the omega-3 (3,17,25)), as well as obesity, diabetes, cardiac hypertrophy, heart failure, peripherical nerve dysfunctions (10,14,27,28).

The role of the Na^+,K^+-ATPase β subunit as well as the Phospholemman (FXYD1) protein associated with its αβ heterodimers within this membrane preparation remains to be elucidated although α2β2 heterodimers is the predominant heterodimer in fast glycolytic muscle (9,11,12,29,30). The extrapolation of the rat to human (7,22) and the control of the dephosphorylation status of Na^+,K^+-ATPase as previously shown by Zhang et al. (30) in plasma membranes should be addressed.

The main finding of the present study is that membrane enzyme activity using the phosphatase activity of the Na^+,K^+-ATPase from crude RG and WG membrane preparations reflects the α1 and α2 Na^+,K^+-ATPase isoform distribution in RG and WG rat skeletal muscles and confirms the fiber-specific Na^+,K^+-ATPase catalytic α-subunits and α2-isoform as the major catalytic isoform in rat skeletal muscle. This procedure could be extended to other characterizations of the Na^+,K^+-ATPase in skeletal muscles.

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