Na$^+$–K$^+$ pump location and translocation during muscle contraction in rat skeletal muscle

Michael Kristensen · Martin Krøyer Rasmussen · Carsten Juel

Abstract Muscle contraction may up-regulate the number of Na$^+$–K$^+$ pumps in the plasma membrane by translocation of subunits. Since there is still controversy about where this translocation takes place from and if it takes place at all, the present study used different techniques to characterize the translocation. Electrical stimulation and biotin labeling of rat muscle revealed a 40% and 18% increase in the amounts of the Na$^+$–K$^+$ pump α_2 subunit and caveolin-3 (Cav-3), respectively, in the sarcolemma. Exercise induced a 36% and 19% increase in the relative amounts of the α_2 subunit and Cav-3, respectively, in an outer-membrane-enriched fraction and a 41% and 17% increase, respectively, in sarcolemma giant vesicles. The Na$^+$–K$^+$ pump activity measured with the 3-O-MFPase assay was increased by 37% in giant vesicles from exercised rats. Immunoprecipitation with Cav-3 antibody showed that 17%, 11% and 14% of the α_1 subunits were associated with Cav-3 in soleus, extensor digitorum longus, and mixed muscles, respectively. For the α_2, the corresponding values were 17%, 5% and 16%. In conclusion; muscle contraction induces translocation of the α subunits, which is suggested to be caused partly by structural changes in caveolae and partly by translocation from an intracellular pool.

Keywords Na$^+$–K$^+$ pump translocation · Caveolae · Caveolin-3 · Intracellular pool · Acute exercise

M. Kristensen · M. K. Rasmussen · C. Juel (✉)
Copenhagen Muscle Research Centre,
Department of Molecular Biology, University of Copenhagen,
August Krogh building, Universitetsparken 13,
2100 Copenhagen Ø, Denmark
e-mail: cjuel@aki.ku.dk

Introduction

When muscles are activated during exercise or electrical stimulation, their force gradually declines (muscle fatigue). The ability to generate action potentials and thus muscle contractions depends on the maintenance of concentration gradients for Na$^+$ and K$^+$ across the sarcolemma. These ion gradients, and thus the membrane excitability of muscle fibers, are mainly maintained by the Na$^+$–K$^+$ pump [2, 29]. Functional Na$^+$–K$^+$ pumps in skeletal muscle consist of a catalytic α subunit (α_1–α_2) and a regulatory β subunit (β_1–β_2; for review, see [2]). Long-term exercise training increases Na$^+$–K$^+$ pump density in skeletal muscles [7, 15, 26, 28, 31], but regulation of the Na$^+$–K$^+$ pump during acute exercise has not been fully elucidated. It has been suggested that acute upregulation of Na$^+$–K$^+$ pump transport capacity could be mediated by increases in the number of pump subunits in the plasma membrane (translocation). Several studies using different cellular fractionation techniques have shown that both exercise [12, 14, 36, 37] and insulin [1, 10, 20] induce translocation of α subunits. However, McKenna et al. [24] failed to detect translocation of the α_2 subunit in both electrically stimulated and insulin-treated muscles using ouabain labeling of intact muscles. Another difficulty in the translocation model is the location of the α_1 subunit. This subunit has been shown to be located in or in close proximity to the sarcolemmal membrane which excludes a translocation from intracellular pools or the T tubules. Therefore, although a few studies have shown corresponding reductions in the amount of Na$^+$–K$^+$ pump subunits in the endosomal fraction [1, 36], Sandiford et al. [36] concluded that, approximately 10 years after the first report of translocation of Na$^+$–K$^+$ pumps [37], the origin of the translocated pumps was still unknown.

As mentioned, the Na^+–K^+ pump α_1 subunit seems to be located in or in close proximity to the sarcolemmal membrane, whereas the α_2 subunit is widely distributed throughout the muscle fiber [40], including T tubules, perhaps intracellular pools, and probably caveolae (for review, see [2]). Caveolae are small invaginations of the cell membrane and are normally located in or near the sarcolemmal membrane, meaning that the cell surface membrane can be divided into a caveolae and a non-caveolae sarcolemma membrane. However, a pool of sub-sarcolemmal caveolae that do not connect directly to the interstitium does also exist (for review, see [32]). The caveolae are coated with the protein caveolin and are believed to play several different roles in the cell including mechanosensing, lipid regulation, and as carriers in endo- and exocytosic pathways (for reviews, see [8, 32]). Caveolin (Cav) has been shown to exist in at least three different isoforms (Cav1–3), of which caveolin-3 (Cav-3) has been shown to be muscle specific [38]. In the heart muscle, approximately 20–30% of the Na^+–K^+-pump α_1 and α_2 subunits are present in caveolae, where they co-localize with caveolin-3 [21, 22]. Furthermore, in the heart, the α_2 subunits [22] as well as several other proteins have been shown to relocate from surface membrane caveolae to cell membranes when subjected to different stimuli [19, 27]. In skeletal muscle cells, it has been shown that insulin stimulation changes the amount of Cav-3 in the sarcolemmal membrane, probably mediated by movements from sub-sarcolemmal caveolae [4], confirming that structural changes of the caveolae and a following movement of caveolin can take place in skeletal muscle.

These results suggest that structural changes in the sarcolemmal membrane and caveolae may also occur in skeletal muscle. Thus, our hypothesis is that contraction-induced changes in caveolae containing Na^+–K^+ pumps are involved in pump translocation.

The aims of this paper were therefore to investigate translocation and location of the Na^+–K^+ pump and Cav-3 in skeletal muscle and thereby to elucidate the translocation mechanism, i.e., whether it takes place from intracellular pools (vesicular trafficking) or from other subcellular compartments such as the caveolae, or from both.

To investigate contraction-induced translocation of both the Na^+–K^+ pump and Cav-3, we used both cell-surface biotinylation of intact muscles as well as two different fractionation methods based on different centrifugation procedures. The 3-O-methylfluorescein phosphatase (3-O-MFPase) assay was used to investigate if there was a functional translocation of the pump. For Na^+–K^+ pump translocation, we focused on the α_2 subunit because it is by far the most abundant [2] and widely distributed [40] α subunit in skeletal muscle and because the greatest and most consistent translocation has been reported for the α_2

subunit [10, 12, 14, 36, 37]. We used adenosine mono-phosphate (AMP)-activated protein kinase (AMPK) for "noncontractile" stimulation. This kinase is activated during exercise and electrical stimulation [9, 34] and induces translocation of GLUT4 to the sarcolemmal membrane [17], thus mimicking exercise without inducing contraction. Immunoprecipitation with a Cav-3 antibody was used to investigate whether Na^+–K^+ pumps are present in caveolae. Immunofluorescence microscopy and muscle-specific differences were used to further characterize translocation and location of Cav-3 in various subcellular compartments of the muscle.

Materials and methods

Experimental animals

All experiments were done using 4–6 week-old male Wistar rats (Taconic Europe). The animals had unlimited access to food and water and were exposed to a 12:12 h light/dark cycle. The animals were allocated at random to the various experiments and were anesthetized with a blow to the head, immediately followed by cervical dislocation. The animals were treated in accordance with the Danish Animal Welfare Regulations with permission according to Consolidated Act no. 726 of September, 1993.

Contraction-induced changes in the concentrations of membrane proteins

Changes in the amounts of Na^+–K^+ pump α_2 subunits were investigated in the sarcolemmal membrane after acute exercise using sarcolemmal giant vesicles or an outer-membrane-enriched fraction and after in vitro electrical stimulation using biotinylation of the sarcolemmal membrane in intact muscles.

Exercise Rats (100–105 g) were subjected to seven bouts of 3 min treadmill running (20 m/min, slope=10°; Columbus Instruments, Columbus, OH, USA) separated by 1-min rest periods. In some cases, the speed of the treadmill was increased during the last bout to ensure exhaustion. In humans, it has previously been demonstrated that short, exhaustive exercise lasting 3–8 min is sufficient to induce translocation [14]. This high intensity cannot be obtained in rats, but intermittent exercise including short periods of rest has been shown to induce translocation [12]; we have therefore chosen a similar exercise pattern. An air-puff system was used as a motivator. The rats were sacrificed immediately after the final bout of exercise. Weight-matched rats served as controls. Muscle tissue was removed from both hind legs and prepared for production of

sarcolemma giant vesicles or an outer-membrane enriched fraction.

Sarcolemmal giant vesicles In this study, vesicles with a well-known protein profile (no T-tubule membrane and low sarcoplasmatic reticulum content) [13, 16, 30] were purified according to the method of Juel [11] and used as the sarcolemmal fraction. Briefly, after dissection of all rat hind-limb muscles, they were incubated in a KCl–MOPS buffer (140 mM KCl, 5 mM 3-morpholinopropanesulfonic acid, pH 7.4) containing collagenase for approximately 45 min (34°C). The samples were washed, and the supernatants were exposed to 45 min of gradient centrifugation ($50 \times g$). After centrifugation, the giant vesicles were harvested, diluted in a collagenase-free KCl–MOPS buffer, and centrifuged at $830 \times g$ for 30 min. The supernatants were removed, and the pellets (vesicles) were either resuspended in Tris–SDS [4% sodium dodecyl sulfate (SDS), 10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), 2 mM phenyl methyl sulfonyl fluoride (PMSF), pH 7.4] for Western blotting or frozen until use for the 3-O-MFPase assay or immunoprecipitation (see later). The protein recovery with this method was 0.2–0.3% (vesicular protein compared to total protein in homogenate including soluble protein).

Outer-membrane-enriched fraction The outer-membrane-enriched (OME) fraction was made from approximately 450 mg mixed muscle tissue (vastus lateralis, vastus medialis, and rectus femoris), which was minced and incubated for 30 min in buffer A [2 M NaCl, 20 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 0.2 mM PMSF, pH 7.4]. After incubation, the tissue was recovered by centrifugation ($1,000 \times g$), diluted in buffer B (250 mM sucrose, 1 mM EDTA, 20 HEPES, 0.2 mM PMSF, pH 7.4), and homogenized using a motor-driven plastic–glass homogenizer and centrifuged at $1,000 \times g$ for 5 min. The supernatant was harvested and centrifuged at $20,000 \times g$ for 30 min; the resulting supernatant was centrifuged at $190,000 \times g$ for 90 min. The pellet, representing the OME fraction, was resuspended in Tris–SDS and used for Western blotting. The protein recovery with this method was 2.1–2.4%.

Electrical stimulation The rats (80–90 g) were killed, and intact soleus muscles were carefully removed and placed randomly in two different baths containing Krebs–Ringer solution (122 mM NaCl, 25 mM NaHCO$_3$, 2.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 1.3 mM CaCl$_2$, 5.0 mM D-glucose) at 30°C. The buffer was continuously gassed with a mixture of 95% O$_2$ and 5% CO$_2$ (pH 7.4). One soleus muscle was stimulated for 1 s at 3-s intervals (pulse duration=2 ms, electrical field ≈ 100 V/cm) using Ag/AgCl electrodes separated by about 3 mm and a frequency of 33 Hz for 10 min; the contralateral muscle served as the control. The resulting contractile force was recorded by an analog-to-digital converter (Duo-18™ version 1.1). After stimulation, the muscles were exposed to cell-surface biotinylation.

Cell-surface biotinylation The cell-surface biotinylation technique was modified from [1]. Muscles were incubated at 4°C with gentle shaking for 1 h in phosphate-buffered saline (PBS1; 137 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4) containing 1.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce). Biotinyl residues attach to protein molecules on the cell surface and were therefore used as a method to label cell-surface proteins from an intact muscle. After the incubation period, muscles were washed for 30 min with PBS1 containing 0.5% bovine serum albumin (BSA) followed by three washes with cold PBS1. Muscles were placed on a towel to remove excess PBS1 and then homogenized in lysis buffer (20 mM Tris base, 135 mM NaCl, 1 mM MgCl$_2$, 2.7 mM KCl, 10 mM Na$_4$P$_2$O$_7$, 10 mM NaF, 1 mM Na$_3$VO$_4$, 1% Triton X-100, 10% v/v glycerol, 0.2 mM PMSF, and 10 μg/ml aprotinin and leupeptin, pH 8.0). The muscles were then rotated gently at 4°C for 1 h, followed by centrifugation ($14,500 \times g$ for 10 min) to remove insoluble material. Protein concentrations were measured using a BSA standard (DC protein assay, Bio-Rad) and a ThermoSpectronis Genesys 10vis (VWR) spectrophotometer. Muscle homogenates containing 1 mg protein were then added to 100 μl immobilized streptavidin–agarose beads (Pierce) and diluted to a volume of 1 ml with lysis buffer. The samples were incubated overnight at 4°C with gentle rotation. Samples were then centrifuged for 1 min at $1,000 \times g$. The supernatant was discarded, and the remaining pellet containing the beads was washed three times with PBS1 containing 0.1% Triton X-100 and three times with cold PBS1. Finally, Laemmli buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue, pH 6.8) was added to the beads, and the samples were heated (20 min, 56°C), to separate the streptavidin–agarose beads from the protein–biotin complex. After mixing, samples were centrifuged at $14,500 \times g$ for 1 min, and the supernatant was used for Western blotting. Due to the methodological approach, it was not possible to measure the protein recovery directly in the samples. However, to ensure that any observed differences were not due to a methodological error, extensor digitorum longus (EDL) muscles were removed as well and subjected to the biotinylation procedure but without being subjected to electrical stimulation. There were no differences in the Cav-3 or α_2 subunit contents of unstimulated EDL muscles ($P > 0.05$, $n=6$, data

not shown). It should be noted that the biotinylation method is probably not able to biotinylate Cav-3 directly [39]. However, Cav-3 is expected to be coprecipitated with other proteins located in the caveolae [e.g., the Na$^+$–K$^+$ pump α subunits (see later) or the dystrophin complex].

3-*O*-methylfluorescein phosphatase assay

Before use, the sarcolemmal giant vesicles were washed in sucrose buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris base, pH 7.4) containing 0.08% deoxycholate to remove K$^+$ and to unmask the Na$^+$–K$^+$ pumps. The solutions were then ultracentrifuged for 1 h at 190,000$\times$g and 4°C and resuspended in sucrose buffer (final K$^+$ concentration <0.1 mM). For the homogenate, 40–50 mg muscle samples were homogenized in sucrose buffer followed by four freeze–thaw cycles. The following procedure was done according to Fraser and McKenna [6]. Briefly, samples were incubated in assay medium [5 mM MgCl$_2$, 1.12 mM EDTA, 100 mM Tris base, 1.25 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 7.4], for 1 min before adding 3-*O*-methylfluorescein phosphate to a final concentration of 160 µM. After 1 min, various concentrations of KCl were added (final K$^+$ concentration 1–15 mM, see Fig. 2) to stimulate the 3-*O*-MFPase reaction, which was monitored for the next 2 min. All measurements were done with stirring at 37°C using a spectrofluorometer with a continuous recording facility.

Immunofluorescence microscopy

Immunofluorescence microscopy was used to investigate the subcellular localization of Cav-3. Rat soleus muscles were embedded in Tissue-Tek (Sakura Finetek) and frozen in liquid nitrogen. Cross sections (8 µm) were cut at −20°C using a cryostat. The sections were placed on glass slides and fixed in acetone at −20°C followed by a 2-min incubation in 4% formaldehyde. The samples were blocked with PBS2 buffer (137 mM NaCl, 1.34 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.2) containing 1% BSA for 1 h. Primary antibody (anti-Cav-3, Abcam) and secondary antibody (Alexa Fluor 488 anti-rabbit, Molecular Probes) were diluted in similar BSA-containing buffers and incubated for 1 h. After repeated washing in PBS2 buffer without BSA, the sections were mounted with DakoCytomation fluorescent mounting medium (DAKO) and stored at −20°C until microscopy.

Immunoprecipitation

For immunoprecipitation, we used sarcolemmal giant vesicles from mixed muscles as well as homogenates of soleus or EDL muscles (rat weight 100–110 g). The soleus and EDL muscles were immediately frozen in liquid nitrogen and then minced and homogenized in ice-cold sucrose buffer (250 mM sucrose, 30 mM HEPES, 2 mM EGTA, 40 mM NaCl, 2 mM PMSF, pH 7.4), followed by centrifugation at 1,000$\times$g at 4°C for 5 min. The supernatant was collected, and the pellet was resuspended in sucrose buffer and homogenized and centrifuged once more. The second supernatant was collected and added to the first supernatant. The pooled supernatant solutions were ultracentrifuged for 1.5 h at 190,000$\times$g at 4°C. The pellets were homogenized in ice-cold lysis buffer (100 mM NaCl, 20 mM Tris base, 10 mM NaF, 1 mM PMSF, 1 mg/ml C$_{12}$E$_8$, pH 7.4) and incubated for 2.5 h (0.5 h for the giant vesicles) at 4°C. The protein concentration of the supernatant was measured after centrifugation at 20,000$\times$g for 15 min. Protein A sepharose beads (Amersham Biosciences) were added to samples containing equal amounts of protein. Lysis buffer was added to equal sample volumes. The relevant antibody was added, and the samples were incubated overnight at 4°C. Negative samples were treated in the same way except that the relevant antibody was substituted with an irrelevant IgG antibody obtained from a similar species. After incubation, the supernatants [IP-lysate (+) (with Cav-3 antibody) or lysate (−) (without Cav-3 antibody)] were removed. The remaining pellets [beads with (IP+) or without (IP−) the immune complexes] were washed three times in ice-cold lysis buffer and three times in ice-cold lysis buffer with only 0.5 mg/ml C$_{12}$E$_8$. Finally, the samples were subjected to Western blotting.

The control homogenate from soleus and EDL muscles were also used to investigate if there were any muscle-specific differences for Cav-3 when subjected to Western blotting.

Activation of AMP-kinase by AICAR injection

Rats (80–90 g) were allocated to an experimental group or a control (placebo) group. A treated rat was always paired with a control rat during the experimental sessions. The experimental group received a 1-ml intraperitoneal injection of 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR; Toronto Research Chemicals) solution (0.7 mg/g body weight), and the control group received an injection of an equal volume of saline. After 1 h, the rats were killed, and a mixture of muscle tissue from their hind legs was removed and prepared for sarcolemmal giant-vesicle production.

Western blotting and antibodies

Samples were mixed with sample buffer (10 mM Tris–HCl, 1 mM EDTA, 20 mM DTT, 4% SDS, 10% glycerol, 0.04%

bromophenol blue, pH 8.0), separated by an 8–18% SDS-PAGE (Excell 8–18% Gradient Gel, Amersham) for approximately 80 min at 225–550 mV, and electroblotted to a polyvinylidene-difluoride membrane (Immobilon-P, Millipore). The membrane was blocked for 1 h in Tris buffer (10 mM Tris base, 0.9% NaCl, pH 7.4) containing 2% low-fat milk powder and 0.1% Tween 20 before overnight incubation at 4°C with primary antibody diluted in a similar buffer. After treatment with secondary antibody coupled to horseradish peroxidase (DAKO) for 1.5 h at room temperature, the membrane was repeatedly washed in Tris buffer with and without 0.05% Tween 20 and Millipore H_2O. The membrane was incubated with enhanced chemiluminescence reagent (ECL, Amersham) and visualized on Hyperfilm (ECL, Amersham). Protein content was quantified by scanning the film and analyzing band intensities using UN-SCAN-IT version 5.1 software (Silk Scientific, Orem, UT, USA).

The primary monoclonal antibody (McB2) for detecting the α_2 subunit was kindly provided by Professor Per Amstrup Pedersen (Department of Molecular Biology, University of Copenhagen), and the primary monoclonal α_1 (α6F) antibody was obtained from Hybridoma Bank (IA, USA). A polyclonal α antibody recommended for detection of α_1, α_2, and α_3 isoforms (H-300: sc-28800) was obtained from Santa Cruz Biotechnology. The Cav-3 (ab2912) and GLUT4 polyclonal antibody (AB1340) were obtained from Abcam and Chemicon, respectively.

Statistics

Student's paired and unpaired t tests were used to compare the relative changes of proteins subjected to Western blotting. Two-way analysis of variance was used for comparisons for the 3-O-methylfluorescein phosphatase assay followed by post hoc analysis of mean values using Tukey's test. Hill equation was used to calculate V_{max} and $K_{1/2}$ for K^+ (Sigma Plot). The unpaired t test was used to determine the significance of the differences in V_{max} and $K_{1/2}$. $P<0.05$ was considered significant.

Results

Contraction-induced changes in concentrations of membrane proteins

Exercised muscle showed a 36% and 41% increase in α_2 content of the outer membrane as measured using the OME fraction ($n_{control}=6$, $n_{exercise}=5$) or sarcolemma giant vesicles ($n=8$), respectively ($P<0.05$, Fig. 1). Electrical stimulation followed by biotinylation of the soleus muscle revealed a 40% increase in the α_2 content ($P<0.05$, $n=5$, Fig. 1).

Muscles from exercised rats had a 17% (sarcolemma giant vesicles, $n=8$) and 19% (OME fraction, $n_{control}=6$, $n_{exercise}=5$) greater content of Cav-3 in the outer membrane compared with control rats ($P<0.05$, Fig. 1). Electrical stimulation and biotinylation revealed a similar 18% increase in the Cav-3 content ($P<0.05$, $n=5$; Fig. 1). In the biotinylation experiment, the relative increase in α_2 subunit content on the surface of the soleus muscle was significantly greater than the increase in Cav-3 content ($P<0.05$, $n=5$, Fig. 1), while there was a tendency to a difference for the OME fraction ($P<0.1$, $n=8$, Fig. 1).

3-O-methylfluorescein phosphatase

The V_{max} of the Na^+–K^+ pump measured in sarcolemma giant vesicles increased 37% ($P<0.05$) in exercised rats compared with control rats ($3,393\pm673$ nmol mg protein^{-1} h^{-1} and $2,482\pm648$ nmol mg protein^{-1} h^{-1}, respectively, $n=7$), whereas the $K_{1/2}$ for K^+ remained constant (3.49 ± 0.75 mM K^+ and 3.36 ± 0.97 mM K^+, $n=7$; Fig. 2a). In the homogenate, no differences existed ($P>0.05$) in the V_{max} of exercised rats compared with control rats (183 ± 40 nmol mg protein^{-1} h^{-1} and 176 ± 69 nmol mg protein^{-1} h^{-1}, respectively, $n=8$) or in the $K_{1/2}$ K^+ of exercised rats compared with control rats (2.09 ± 0.56 mM K^+ and 2.10 ± 0.64 mM K^+, respectively, $n=8$; Fig. 2b).

Subcellular localization and muscle-specific differences

Immunohistochemical staining for Cav-3 demonstrated that the protein was located in or near the sarcolemmal membrane as no visible staining existed inside the muscle fiber (Fig. 3a). This pattern was consistent for all fibers and is unlikely to have been an artifact of unspecific binding because Western blotting showed that the antibody had very high specificity (Fig. 3b). The cell membranes of control fibers, which were not incubated with primary antibody, did not exhibit labeling (data not shown). It was not possible to detect any fiber-type differences from the immunofluorescence microscopy. However, comparison in Western blot of the amount of Cav-3 in EDL with that in soleus muscles shows that the soleus muscle contains 40% more Cav-3 protein than the EDL ($P<0.05$, $n=5$; Fig. 3c).

Immunoprecipitation

Approximately 90–100% of the Cav-3 protein was immunoprecipitated from the IP-lysate (+) sample (with Cav-3 antibody) when compared with the lysate (−) sample (without Cav-3 antibody) in the soleus (Fig. 4a), EDL (Fig. 4b) and sarcolemma giant-vesicle samples (Fig. 4c; $P<0.05$; $n=5$, $n=7$, and $n=6$, respectively). A corresponding
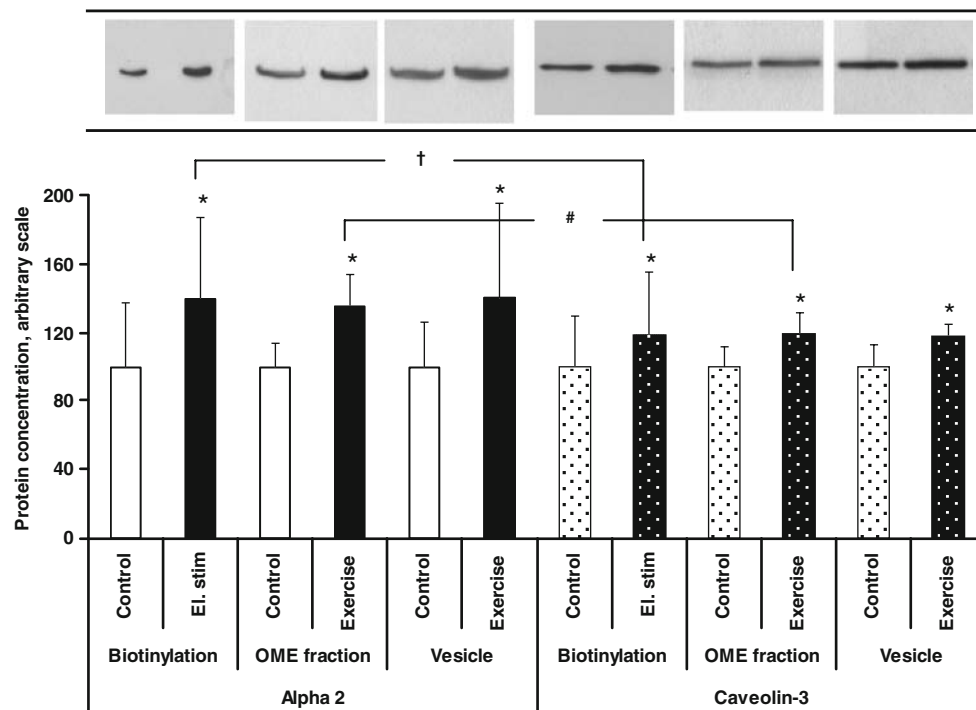
Fig. 1 Contraction-induced changes in muscle-membrane protein distribution. Means (±SD) are expressed as relative amounts of the Na$^+$–K$^+$ pump α_2 subunits and Cav-3 in the sarcolemmal membrane of either soleus muscles subjected to biotinylation or mixed muscle from an outer-membrane-enriched (OME) fraction, or sarcolemmal giant vesicles (vesicle). The mixed muscles (*OME fraction* or *vesicles*) were from animals exposed to treadmill running, and the soleus muscles (*biotinylation*) were exposed to electrical stimulation (*El. Stim*). Representative immunoblots are placed *above the quantified data*. The α_2 and Cav-3 antibodies only labeled one band during Western blotting. *Pure black* and *white bars* of the quantified data represent changes for the α_2 subunits, while *dotted bars* represent changes for the Cav-3 protein. *Asterisk* Significantly different from control; *dagger* significant difference in the relative protein change between two different proteins (α_2 and Cav-3); *pound sign* tendency for a difference in the relative protein change between two different proteins (α_2 and Cav-3)

Cav-3 band was evident in the IP+ fraction, whereas no Cav-3 was detected in the negative (IP–) control experiment (Fig. 4c).

The Na$^+$–K$^+$ pump coimmunoprecipitated with Cav-3 in the soleus muscle homogenate; thus, a 17% reduction was found for both α_1 and α_2 subunits, ($P<0.05$, $n=5$; Fig. 4a) in the IP-lysate (+) sample compared with the lysate (−) sample. For the EDL muscle homogenate, the reductions were 11% ($P<0.05$, $n=7$) and 5% ($P>0.05$, $n=7$) for the α_1 and α_2 subunits, respectively (Fig. 4b), while the corresponding reductions were 15% and 16%, respectively ($P<0.05$, $n=6$; Fig. 4c) for the sarcolemma giant vesicles (mixed muscles). The α_1 and α_2 subunits were only detectable in the IP+ fraction and not in the control IP− fraction (Fig. 4d).

Coimmunoprecipitation of Cav-3 was also evident when immunoprecipitating with an isoform-unspecific Na$^+$–K$^+$ pump α antibody (data not shown).

Activation of AMP-kinase by AICAR injection

An 11% increase occurred in the relative amount of GLUT4 in the mixed muscle sarcolemmal membrane from AICAR-

infused rats compared with that of saline-infused (control) rats ($P<0.05$, $n=10$; Fig. 5). In the same muscles, a 20% decrease in the relative amount of α_2 subunits occurred in sarcolemmal membranes of the AICAR-treated rats ($P<0.05$, $n=8$; Fig. 5), while no differences existed in the contents of α_1 subunits or Cav-3 ($P>0.05$, $n=6$; Fig. 5).

Discussion

Na$^+$–K$^+$ pump translocation

Our studies with electrical stimulation and biotinylation showed that muscle contractions resulted in a 40% increase in the α_2 subunit content of the sarcolemmal membrane compared with control muscles (Fig. 1). Almost identical increases (36% and 41%) were observed after exercise when using the OME fraction or the sarcolemma giant-vesicle technique, respectively. These results are within the range reported previously for α_2 translocation (25–94%; [12, 14, 36, 37]) and strengthens the assumption that translocation of the Na$^+$,K$^+$-ATPase takes place during
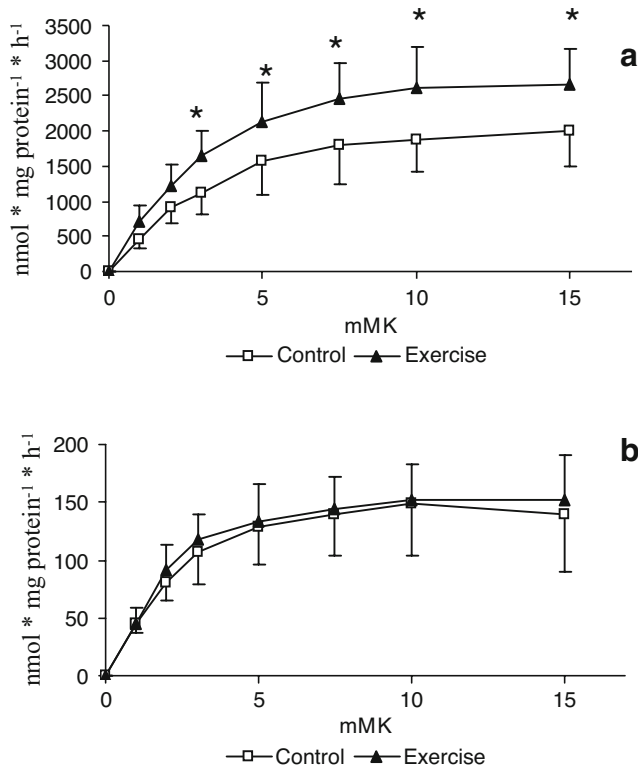
Fig. 2 Effect of exercise on K^+-stimulated Na^+–K^+ pump activity measured by the 3-O-MFPase technique in sarcolemmal giant vesicles (a) and homogenates (b) of skeletal muscles from control and exercised rats. Values are expressed as means±SD. *Asterisk* Significantly different from the control

exercise [12]. Consequently, the translocation measured with vesicles could be underestimated. It is therefore a surprise that the ouabain technique could not confirm translocation. One reason could be the earlier-mentioned differences in recovery obtained for the different techniques. Another reason could be due to back translocation of the pump. There are indications that the ouabain α complex can be internalized [18, 41]. It is therefore a possibility that ouabain is internalized during the 2 h of incubation at 30°C [24], which would blur translocation. Biotin labeling, on the other hand, was performed at 4°C, which is expected to inhibit back translocation.

Functional importance

The increase in Na^+–K^+ pump subunits shown in our study is of functional importance as demonstrated by the 3-O-MFPase experiments (Fig. 2a). The Na^+–K^+ pump V_{max} of the sarcolemmal membrane from exercised muscles was 37% greater than that of control muscle, which also corresponds with the observed increase in the amount of

muscle contraction. One point of criticism for the giant-vesicle experiments has been the low protein recovery (0.2–0.3%). Based on the small recovery, it may be questioned if these proteins are representative for the entire muscle. We therefore supplemented with another membrane-purification method, the OME-fractionation technique (2.1–2.4% protein recovery) and the biotinylation technique. The biotinylation technique used in our study detects membrane proteins thanks to their extracellular domains and therefore measures protein changes in intact muscles. This is methodologically similar to the ouabain technique used by McKenna et al. [24] but differs markedly from vesicles and other subcellular fractionation methods [12, 14, 36, 37]. Based on the similar results obtained by the three different methods, the present findings strengthen the assumption that translocation of the Na^+,K^+-ATPase takes place during muscle contraction and confirm that the methods used are representative for what happens in the intact muscle.

It should also be mentioned that due to the long incubation periods and high temperature, the possibility exists that pumps could be translocated back to their original location during giant-vesicle production. It has been shown that the back translocation of the Na^+,K^+-ATPase subunits is almost completed already 30 min after
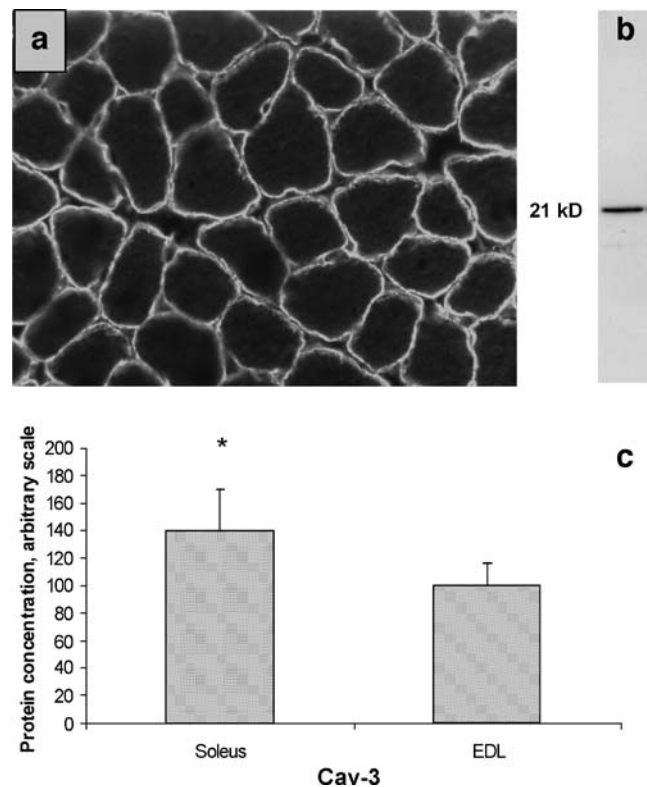

Fig. 3 Immunofluorescence microscopy image of a soleus muscle cross section (a) using Cav-3 antibody. The image shows that Cav-3 is located in (caveolae) or very near to the sarcolemmal membrane. No staining occurred in negative controls obtained by omission of primary antibody (data not shown). Western blotting (b) indicates that the Cav-3 antibody had a very high specificity for Cav-3 in muscle homogenate. c The relative amount (mean±SD) of Cav-3 in oxidative (*Soleus*) compared with glycolytic (*EDL*) muscle homogenates. *Asterisk* Significantly different from the control

Fig. 4 Immunoprecipitation of Cav-3 from rat soleus muscle (**a**), EDL muscle (**b**), and sarcolemmal giant vesicles (**c**). Cav-3 protein was precipitated [the difference between lysate (−), in which Cav-3 antibody was not used, and IP-lysate (+), in which Cav-3 antibody was used] from the soleus and EDL homogenate and sarcolemma giant vesicles from mixed muscles. Coimmunoprecipitation of the α_1 and α_2 subunits was calculated in the same way. Representative immunoblots are placed *above the quantified data*. Values are expressed as mean±SD. **d** Positive confirmation that the removed protein was present in the immunoprecipitation fraction containing the Cav-3 antibody (IP+) and that no protein was present in the negative control immunoprecipitation fraction (IP−), which did not contain Cav-3 antibody. Results for IP− illustrate that no protein was removed from the lysate (−) shown in **a**, **b** and **c**, while results for IP+ illustrate that some protein was removed from the IP-lysate (+). *Asterisk* IP-lysate (+) is significantly different from lysate (−)
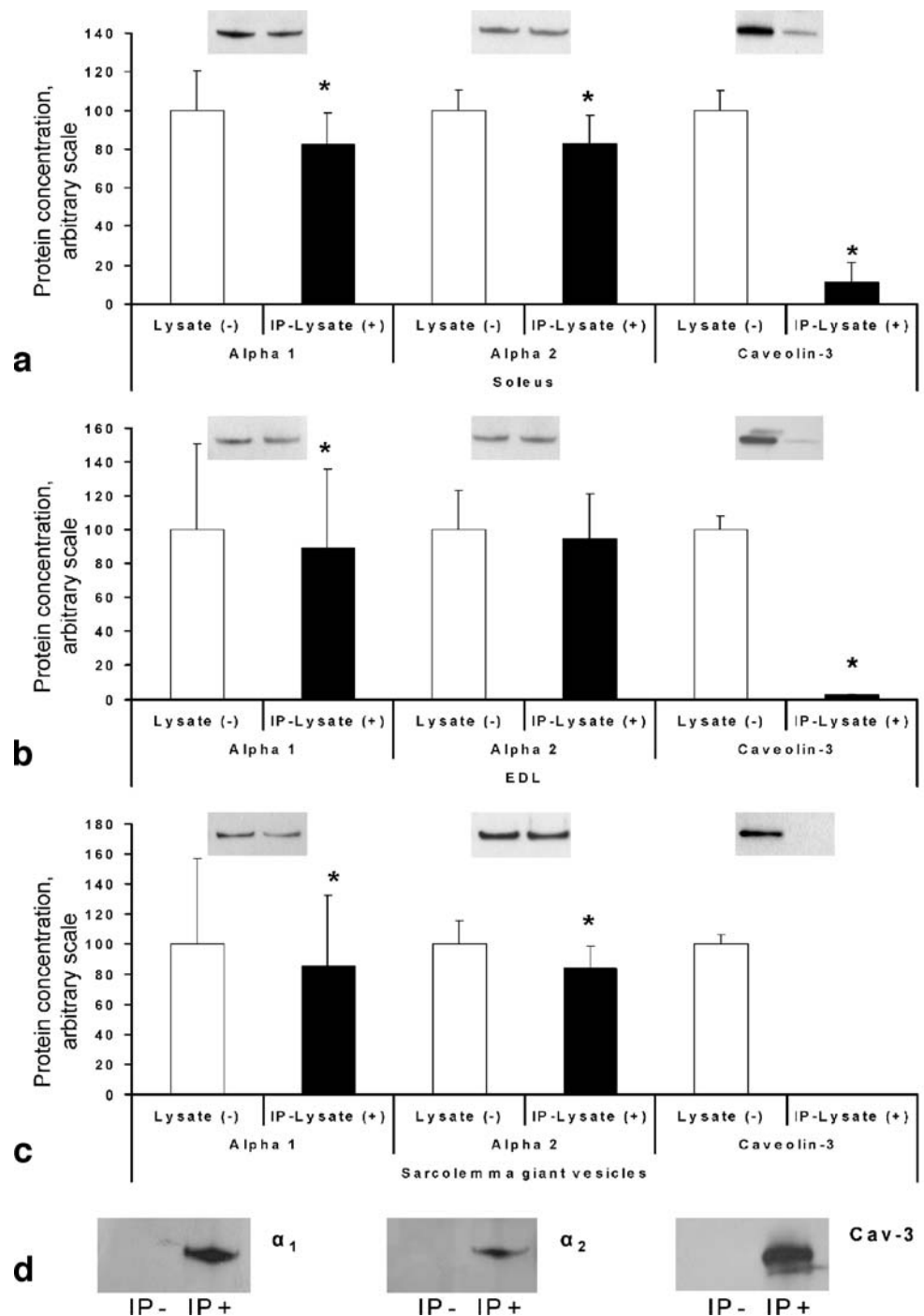
Na^+–K^+ pumps in the sarcolemmal membrane after exercise (Fig. 1). This increase in V_{max} was not caused by an increase in the activities of individual pumps because the V_{max} was unchanged in the homogenates, which contain a constant number of pumps (Fig. 2b). There seems to be some controversy in the literature about exercise-induced changes in pump activity measured with 3-O-MFPase method. Sandiford et al. [36] showed a similar increase (37–40%) in K^+-stimulated Na^+–K^+ pump activity (V_{max}) of a sarcolemma-enriched fraction from electrically stimulated muscles compared with control muscles and a corresponding increase in levels of the α_1 and α_2 subunits. Surprisingly, they also showed a 40–53% increase in pump activity in muscle homogenate, indicating that the increased activity in the sarcolemma-enriched fractions was not necessarily caused by translocation but could also be due to a change in the activities of individual pumps. In contrast, Fraser et al. [5] and McKenna et al. [25] observed an exercise-induced reduction in Na^+–K^+ pump activity (14–20% and 22–24%, respectively) in muscle homogenate.
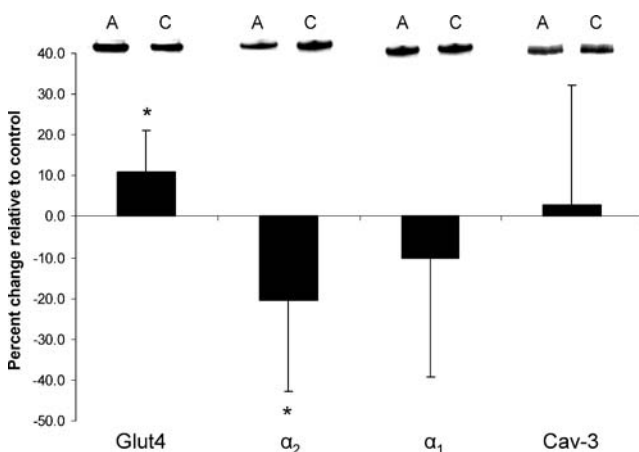
Fig. 5 Relative changes in the amount of proteins for GLUT4, Na$^+$–K$^+$ pump α_1 and α_2 subunits, and Cav-3 in the sarcolemma membrane (sarcolemmal giant vesicles) from mixed muscles from AICAR-treated rats compared with controls. Changes are expressed as percentages of control values. Representative immunoblots are placed *above the quantified data*. *A* AICAR-treated rats, *C* control rats. Values are expressed as means±SD. *Asterisk* Significantly different from the control

Protein location

The term "translocation" has been used in the literature to describe movement of proteins from one pool to another, such as from an intracellular pool to the sarcolemmal membrane, and the transport process involved has been called "vesicular trafficking". Therefore, a pool of non-sarcolemmal proteins (Cav-3 and the α_1 and α_2 subunits) must exist for vesicular trafficking to take place. However, an increase in a subunit observed with a labeling procedure may be caused by increased accessibility of the protein, for instance, as a result of opening of caveolae, restructuring of the membrane, or changes in T tubules.

Muscle caveolae membrane located in or in very close proximity of the sarcolemmal membrane contains a very high density of Cav-3 (Fig. 3a, [33, 35]). However, because of the very large area of T-tubule membranes compared with that of the sarcolemma, the total content of Cav-3 in the core of the soleus fiber is approximately 40% greater than that of the sarcolemma [35]. Therefore, Cav-3 seems to be located in two main subcellular compartments in skeletal muscle: caveolae and T tubules, although this is not visible in immunohistochemical pictures as the one in Fig. 3.

The α_1 subunit has been found to be located in or very close to the sarcolemmal membrane, while the α_2 subunit is widely distributed throughout the fiber, including T tubules and perhaps intracellular pools [10, 23, 40]. Due to the location of the α_1 subunit, it has been difficult to explain the observed α_1 translocation [12, 14, 36, 37]. In our study, immunoprecipitation with anti-Cav-3 removed 90–100% of Cav-3 from soleus, EDL, and sarcolemma giant-vesicle samples. Corresponding coimmunoprecipitation indicates

that up to approximately 20% of the Na$^+$–K$^+$ pumps are located in caveolae (Fig. 4), which corresponds to what has been observed in heart muscles [21, 22]. Therefore, one fraction of the α_1 (and α_2) subunits is most likely present in the caveolae, and it could be that translocation of the α subunits takes place from the caveolae to the sarcolemma membrane during muscle contraction (see below).

We also found that a greater amount of Cav-3 existed in soleus muscle than in EDL muscle (Fig. 3c). This was unexpected because the amount of T tubules in glycolytic muscles is approximately 50% greater than in oxidative muscles [3]. Consequently, the density of Cav-3 in the caveolae and T-tubule membrane must be approximately twice as high in oxidative muscles compared to glycolytic muscles. This higher amount of Cav-3 in soleus compared to EDL muscles could explain the apparent higher relative amount of α_1 and α_2 subunits coimmunoprecipitated from soleus muscles compared to EDL muscles.

Translocation: changes in caveolae or vesicular trafficking from an intracellular pool?

Translocation has normally been suggested to be mediated by vesicular trafficking from some yet unidentified intracellular pools. However, caveolae are another subcellular membrane compartment from where the translocation could take place. This could be either due to a relocation of the subunits from the caveolae to the sarcolemma membrane as observed for several different proteins [19, 22, 27] or if caveolae make contact with the cell surface during muscle contraction either by subsarcolemmal caveolae "docking" the sarcolemmal membrane or, as suggested by Marette et al, [23], by opening up. These mechanisms could mediate an increase in functional Na$^+$–K$^+$ pumps in the sarcolemmal membrane. Our results show that translocation of Cav-3 proteins takes place during muscle contraction (Fig. 1). This was demonstrated in three independent experiments using three different methods. The amount of Cav-3 in the sarcolemma of stimulated muscles was 17% and 19% greater after exercise (measured using the sarcolemmal giant-vesicle and OME-fraction techniques) and 18% greater after electrical stimulation (measured using the biotinylation technique) compared with control muscles (Fig. 1). Combined with the location of Cav-3, these observations indicate that Cav-3 translocation was probably caused by translocation of Cav-3 from caveolae to the sarcolemma, and not vesicular trafficking from an intracellular pool. Therefore, a potential explanation for the reported increase in the contents of α_1 and α_2 subunits (Fig. 1, [12, 14, 36, 37]) is that it is caused by structural changes in the sarcolemmal membrane and/or caveolae occurring during muscle contractions. For the α_1 subunit, translocation from the caveolae would explain why several

studies show an increase in the amount of this subunit in the sarcolemma despite its location in or very close to the sarcolemmal membrane [40].

If it is assumed that more Cav-3 protein than α_2 subunit protein exist in caveolae, and as the relative increase in α_2 subunits was significantly greater than that of Cav-3 (biotinylation experiment, Fig. 1), it can be suggested that α_2 subunits also translocate from an intracellular pool (vesicular trafficking) different from caveolae. In support of this, a significant change occurred in the α_2 subunit in AICAR-treated rats, whereas no change in the α_1 subunit or Cav-3 was found (Fig. 5). Thus, part of the α_2 subunit, but not the α_1 subunit, could be involved in Cav-3-independent translocation to the sarcolemmal membrane. Yuan et al. [42] showed that subcellular changes in α_1 and α_2 subunits occurred in muscle cells subjected to passive stretching but that vesicular trafficking was only involved in translocation of the α_2 subunit, and they concluded that different mechanisms were involved in the regulation of the two α isoforms. We therefore suggest that both the α_1 and α_2 subunits relocate from caveolae to the sarcolemma during muscle contraction and that the α_2 subunit also translocates via vesicular trafficking. This would explain why contraction-induced translocation reported in the literature is greater for the α_2 subunit than for the α_1 subunit; the reported increase in the content of the α_1 subunit is between 0% and 55% while the increase in the content of the α_2 subunit is between 25% and 94% [12, 14, 36, 37].

Conclusion

Muscle contractions (electrical stimulation or exercise) induce an increase in the amount of Na^+–K^+ pump α_2 subunits and Cav-3 in the sarcolemmal membrane. Coimmunoprecipitation of the Na^+–K^+ pump with Cav-3 indicates that the pump is present in caveolae. On the basis of this evidence, the increase in the Cav-3 level in sarcolemmal membrane during contraction, and the AICAR data, we suggest that part of the observed Na^+–K^+ pump translocation was caused by structural changes in or relocation from caveolae as well as vesicular trafficking from intracellular pools.

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