Protein kinase Cα activity is important for contraction-induced FXYD1 phosphorylation in skeletal muscle

Martin Thomassen,1 Adam J. Rose,1,3 Thomas E. Jensen,1 Stine J. Maarbjerg,1 Laurids Bune,2 Michael Leitges,4 Erik A. Richter,1 Jens Bangsbo,1 and Nikolai B. Nordsborg1

1Department of Exercise and Sport Sciences, University of Copenhagen, Copenhagen, Denmark; 2Copenhagen Muscle Research Centre, Rigshospitalet, Denmark; 3Division Molecular Metabolic Control, German Cancer Research Center, Heidelberg, Germany; and 4The Biotechnology Centre of Oslo, University of Oslo, Blindern, Oslo, Norway

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The overall aim of the present study was to investigate whether muscle contractions cause increased FXYD1 phosphorylation, which could be one molecular mechanism of importance for regulation of Na+-K+ pump activity during exercise.

FXYD1 phosphorylation is primarily caused by PKA and PKC (3, 32). PKA-induced phosphorylation of FXYD1 ser68 increases the apparent Na+ affinity of Na+-K+ pump α1β and α2β isozymes, but it has no effect on the maximal Na+-K+ pump activity. In contrast, PKC phosphorylation of FXYD1 ser63 and ser68 increases the maximal activity of the Na+-K+ ATPase α2β isozyme but does not affect Na+ affinity (3). In addition to FXYD1 ser63 and ser68, FXYD1 thr69 has recently been reported as another potential phosphorylation site also regulated by PKC (8). As PKC isoforms, in general, are activated by exercise (1, 27, 30), a second aim of the present study was to investigate whether muscle contractions cause FXYD1 phosphorylation via PKC-dependent pathways. Specifically, the role of the dominating Ca2+-activated (18, 31) conventional PKC isoform in mouse skeletal muscle, PKCα (11), was investigated.

Na+-K+ pump activity in rat muscle is increased after only 2 s of electrical stimulation and is greater after 30 s than 10 s, probably caused by an increase in intracellular Na+ concentration, as well as alterations in pump transport characteristics (19). During moderate-intensity exercise in humans the Na+-K+ pump activity, determined by the K+ reuptake rate, is highest after 20 min (36). Thus, in the present study, a combination of intense brief exercise and moderate prolonged exercise was used to investigate alterations in FXYD1 phosphorylation.

The steep Na+ and K+ gradients across cell membranes are a prerequisite for normal function and viability of excitable tissues, such as skeletal muscle. Skeletal muscle contains more than 90% of the total body K+ (17) and upon transition from rest to exercise, the contraction-induced K+ loss from skeletal muscle is counteracted by an up to a 20-fold increase in Na+-K+ pump activity (5). The increase in skeletal muscle Na+-K+ pump activity is primarily caused by an increased Na+ affinity and an exercise-induced increase in the intracellular Na+ concentration (5, 33). At the onset of exercise, a large K+ efflux from the activated muscle is apparent and indicates that Na+-K+ pump activation lags behind the initiation of muscle contractions (36). This inadequate pump activity may be related to the mechanisms involved in increasing Na+-K+ pump Na+ affinity. However, the molecular mechanisms responsible for the exercise-induced regulation of skeletal muscle Na+-K+ pump activity are only starting to be elucidated.

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MATERIALS AND METHODS

Human exercise protocol. Ten healthy and recreationally active male subjects with an age of 27.4 ± 4.2 years and a $V_{O_2\max}$ of 3.9 ± 0.5 l O$_2$ min$^{-1}$ (means ± SD) participated in the study after giving written informed consent. The study conformed to the code of Ethics of the World Medical Association (Declaration of Helsinki), and the protocol was approved by the Ethics Committee of Copenhagen and Frederiksborg communities. On the day before the experiment, subjects consumed a standardized meal in the evening and refrained from physical activity. On the experimental day, subjects were instructed to consume a standardized breakfast (3.5 MJ, energy distribution: 13, 11, and 76% from proteins, fat, and carbohydrates, respectively) at least 1.5 h before reporting to the laboratory between 0800 and 1000 in the morning. After resting for 30 min, biopsies were obtained from musculus vastus lateralis under local anesthesia (Xylocain 20 mg/ml; AstraZeneca) using a Bergström needle with suction (2) before and after 30 s of high-intensity cycling (447 W; 79% $V_{O_2\max}$) and suspended at resting tension (4–5 mN) in incubation chambers. Following 1 h of preincubation, muscle contractions were elicited by electrical stimulation with 1-s trains (30 V, 0.2 ms, 100 Hz) every 15 s for 10 min. The resting and stimulated mouse muscles were quick-frozen in liquid nitrogen.

Mouse muscle electrically stimulated contractions. Generation and characterization of the used male PKCo knockout (KO) mice on a 129S2/Sv background have previously been described (15). Soleus and extensor digitorum longus (EDL) muscles from male wild-type (WT) or PKCo KO mice were electrically stimulated, as described previously (11). In short, soleus and EDL muscles were obtained from fed anesthetized WT or PKCo KO mice (6 mg of pentobarbital sodium per 100 g body wt) and suspended at resting tension (4–5 mN) in incubation chambers. Following 1 h of preincubation, muscle contractions were elicited by electrical stimulation with 1-s trains (30 V, 0.2 ms, 100 Hz) every 15 s for 10 min. The resting and stimulated mouse muscles were quick-frozen in liquid nitrogen.

Muscle lysate preparation. Muscle samples were homogenized with a Polytron 3100, 7-mm diameter (Kinematica) 1.300 rpm in a fresh batch of homogenization buffer [10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na$_2$VO$_4$, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine] for no more than 30 s. After rotation end over end for 1 h, the samples were centrifuged for 30 min at 16,000 g at 4°C, and the clarified lysate was collected as the supernatant. Protein concentrations were determined in the lysates using BSA standards (Pierce). Finally, samples were diluted to the same protein concentration in ddH$_2$O and 6 × Laemmli buffer (7 ml 0.5 M Tris-base, 3 ml glycerol, 0.93 g DTT, 1 g SDS, and 1.2 mg bromophenol blue).

SDS-PAGE and Western blotting. Equal amounts (2–15 μg) of muscle lysate proteins, determined during optimization of the different antibodies, were loaded in each well. Proteins were separated using 16.5% Tris-Tricine gels (Bio-Rad Laboratories) and semi-dry transferred to PVDF membranes (Immobilon Transfer Membrane, Millipore A/S). After blocking (Tris-buffered saline + 0.1% Tween-20 + 2% skimmed milk or 3% BSA), the membranes were incubated with primary antibodies overnight followed by incubation in horseradish peroxidase-conjugated secondary antibody. Quantification was done using a charge-coupled device image sensor and one-dimensional software (Kodak Image Station, 2000MM, Kodak) and determined as the total intensity of the band minus the background intensity.

Immunoprecipitation. FXYD1 proteins were immunoprecipitated (IP) from 200–400 μg of total human muscle lysate proteins using specific antibodies (1.4–6 μl) and Sepharose-coupled protein G overnight in 200 μl of the homogenization buffer. Samples were centrifuged at 6,000 g for 1 min at 4°C, and 170 μl of the supernatants were harvested (Post-IP) and mixed with 30 μl of Laemmli buffer (0.85–1.7 μg/μl). The remaining pellets (IP) were washed once with the homogenization buffer, twice with 10 mM Tris-base and 150 mM NaCl (pH 7.4) and resuspended in 40 μl of Laemmli buffer (5–10 μg/μl if the immunoprecipitation was 100%) and boiled at 96°C for 3 min. To confirm the use of the motif- and phospho-specific antibodies, the effect of the immunoprecipitation was determined by a direct comparison between the Post-IP and Pre-IP (supernatant from an IP without antibody) signal based on the same amount of total protein: IP Effect (%) = (Pre-IP signal – Post-IP signal)/Pre-IP signal × 100%.

Antibody specificity. Two polyclonal rabbit antibodies were used to determine contraction-induced changes in FXYD1 phosphorylation (kindly provided by Dr. J. Randall Moorman, University of Virginia, and Dr. D. Bers, Loyola University, respectively). These antibodies were developed to recognize either unphosphorylated FXYD1 proteins (AB_FXYD1, originally denoted as C2) or FXYD1 phosphorylated at serine 68 (AB_FXYD1ser68, originally denoted as CP68) (26). To verify that FXYD1 phosphorylation status could be determined in human skeletal muscle, phosphorylation was either reduced by extraction without phosphatase (PPase) inhibitors followed by incubation with PPase (A-PPase; New England BioLabs) or preserved with extraction with PPase inhibitors and afterward not incubated with PPase, as described previously (29). The intensity of the AB_FXYD1 Western blot signal was 2.4 ± 0.1-fold higher (P < 0.001, Fig. 1, A and B) in the dephosphorylated samples compared...
Successful immunoprecipitation with the use of AB_FXYD1 (4 analyses of immunoprecipitates obtained by use of the AB_FXYD1 motif-specific antibodies for FXYD1 phosphorylation was determined by proteins phosphorylated at ser68 were determined with an antibody and RRXS, respectively (AB_ser68/thr69, no. 9621), and FXYD1 phosphorylation were determined with an antibody targeting RXXT sequence: (R/K)XS(hydrophobic)(R/K). FXYD1 ser68 and thr69 determined with an antibody (AB_ser63, no. 2261) targeting the following commercially obtained (no. 2261, no. 9621, and no. 9624; Cell Signaling Technology), FXYD1 ser63 phosphorylation was determined with an antibody (AB_ser63, no. 2261) targeting the following sequence: (R/K)XS(hydrophobic)(R/K). FXYD1 ser68 and thr69 phosphorylation were determined with an antibody targeting RXXT and RRXS, respectively (AB_ser68/thr69, no. 9621), and FXYD1 proteins phosphorylated at ser68 were determined with an antibody (AB_ser68, no. 9624) targeting RRX(S/T). The specificity of the motif-specific antibodies for FXYD1 phosphorylation was determined by analyses of immunoprecipitates obtained by use of the AB_FXYD1. Successful immunoprecipitation with the use of AB_FXYD1 (4 μl AB_FXYD1 and 400 μg total protein from human skeletal muscles) was confirmed by subsequent Western blot analysis of the IP with the AB_FXYD1 antibody, which resulted in a clear ~12-kDa band signal (Fig. 2). Furthermore, the POST-IP obtained subsequent to AB_FXYD1 IP revealed no detectable signal when applied either the AB_FXYD1 or AB_FXYD1ser68, respectively (Fig. 2). Thus, even though AB_FXYD1 was shown to be phospho-sensitive when used for Western blot analysis, it was possible to immunoprecipitate all proteins recognizable by the phospho-specific AB_FXYD1ser68, most likely because of the polyclonal nature of AB_FXYD1 (Fig. 2). The three motif and phospho-specific antibodies yielded signals at the expected ~12 kDa when applied to AB_FXYD1 IPs (Fig. 3). Furthermore, the level of phosphorylated proteins that AB_FXYD1 was able to pull down at ~12 kDa recognized by the motif- and phospho-specific antibodies was calculated (IP effect). Determined in a mixed human skeletal muscle pool consisting of both resting and exercised tissue, the AB_FXYD1 IP effect was 100% (nondetectable signal in the Post-IP), 99.6%, and 100% for AB_ser63, AB_ser68, and AB_ser68/thr69, respectively. Thus, the signal from the motif and phospho-specific antibodies at ~12 kDa is specific to FXYD1 proteins. Because no other signals than that from FXYD1 were detected around 12 kDa, analyses with the motif and phospho-specific antibodies were done using human skeletal muscle lysates.

For further confirmation, the effect of increased PKA activity on the motif- and phospho-specific antibody signals was determined by maximal insulin stimulation. In a single human subject, a basal muscle sample was taken at rest and compared with a biopsy obtained after a 20-min hyperinsulinemic clamp (500 μU/ml). Insulin stimulation increased the Western blot signal by 297%, 184%, and 74% for AB_ser63, AB_ser68, and AB_ser68/thr69, respectively, compared with basal. As well, the Western blot AB_FXYD1 and AB_FXYD1ser68 signal was increased by 15% and 63%, respectively. This confirms that the motif- and phospho-specific antibodies recognize FXYD1 proteins, since PKA has been shown to increase FXYD1 ser63 and ser68 phosphorylation (8). Interestingly, the increase in FXYD1 ser68 phosphorylation was more pronounced as determined by AB_ser68 than by AB_FXYD1ser68.

To confirm that the homogenization procedure, as well as the exercise intervention, did not change the composition of the individual muscle samples in relation to the distribution between membrane and intracellular proteins, we used a polyclonal rabbit antibody (ab2912; Abcam) to detect the membrane marker caveolin-3 (CAV3). When CAV3 expression was the same over time, the same amount of membrane-bound proteins were extracted in the samples. As a marker for total protein amount and equal loading, we used a rabbit actin antibody (A2066; Sigma) developed using a COOH terminus actin fragment and thus recognizing all known isoforms. The secondary antibody used was HRP-conjugated goat-anti-rabbit in a 1:5,000 dilution (P-0448; Dako).

Statistics. To determine changes in FXYD1 phosphorylation, a ratio between the signal intensity for each sample and the mean signal intensity for all resting samples at the same membrane (human experiment) or the mean signal intensity for resting WT samples (mouse experiment) was calculated. Where multiple samples were used, this ratio was averaged as the mean ratio for each time point. The human skeletal muscle exercise-induced changes in Western blot signal intensity were examined by one-factor repeated-measures ANOVA (rest, 30 s, and 20 min). Changes in mouse muscle Western blot signal intensity were determined by two-way repeated-measures ANOVA (one-factor repetition) with rest vs. electrical stimulated contractions as one factor and WT vs. PKCα KO mice as another factor. The level of significance was set at P < 0.05. Statistical analyses were performed in SigmaPlot version 11. All data are expressed as means ± SE, unless otherwise stated.
RESULTS

Human exercise. In human skeletal muscles, the AB_FXYD1 signal intensity relative to rest was decreased to 84 ± 8% (P < 0.05) after 30 s and 68 ± 6% (P < 0.001) after 20 min of exercise, with the decrease at 20 min being larger (P < 0.05) than after 30 s. This demonstrates that exercise gradually increases unspecific FXYD1 phosphorylation (Fig. 4). When comparing samples obtained after 20 min of exercise to samples obtained at rest, FXYD1 ser63 phosphorylation (AB_ser63) was 43 ± 20% higher (P < 0.01), FXYD1 ser68 phosphorylation (AB_ser68) was 26 ± 14% higher (P < 0.05), and combined FXYD1 ser68 and thr69 phosphorylation (AB_ser68/thr69) was 45 ± 14% (P < 0.05) higher (Fig. 5, B–D). However, no change was apparent in FXYD1 ser68 phosphorylation when using the AB_FXYD1ser68 (Fig. 5A). Exercise did not change the expression of the skeletal muscle membrane marker CAV3 and the total protein marker actin.

Electically stimulated mouse muscle. Both at rest and after contractions, no difference was apparent between WT and PKCα KO AB_FXYD1 signal in either soleus or EDL muscle muscles (Fig. 6, A and B). In WT soleus, contractile activity decreased (P < 0.05) AB_FXYD1 signal intensity to 86 ± 12% of the intensity in the resting muscle (Fig. 6A), while no effect was seen in EDL (Fig. 6B). In PKCα KO mice contractions had no effect in soleus (Fig. 6A) and decreased (P < 0.05) the AB_FXYD1 signal intensity in EDL from 124 ± 16% to 89 ± 12% of resting WT intensity (Fig. 6B).

FXYD1 ser68 phosphorylation, as determined by use of AB_FXYD1ser68, was lower (P < 0.001) at rest in PKCα KO relative to WT (20 ± 4% and 12 ± 2% of rest in soleus and EDL, respectively). In WT soleus muscle electrically stimulated contractions increased (P < 0.001) the AB_FXYD1ser68 signal intensity to 159 ± 18% of the intensity in the resting muscle (Fig. 6C), while contractions in EDL decreased (P < 0.001) AB_FXYD1ser68 signal intensity to 38 ± 7%, corresponding to similar level as seen in PKCα KO mice (Fig. 6D). In PKCα KO mice, the electrically stimulated contractions had no effect on the AB_FXYD1ser68 signal in either soleus or EDL (Fig. 6, C and D).

DISCUSSION

The major findings in the present study are that contractile activity increases FXYD1 phosphorylation in human skeletal muscle at multiple sites. Furthermore, in mouse skeletal muscle, the contraction-induced increases of FXYD1 ser68 phosphorylation in soleus and the reduction of FXYD1 ser68 phosphorylation in EDL is dependent on normal PKCα expression.

The present results indicated that FXYD1 phosphorylation at multiple sites is a potential mechanism causing increased Na⁺-K⁺ pump Na⁺ affinity and turnover number with exercise. This is supported by the fact that FXYD1 phosphorylation increases the apparent Na⁺ affinity of α1/β1 and α2/β1 Na⁺-K⁺-ATPase complexes, as well as α2/β1 Na⁺-K⁺ pump turnover number in a Xenopus oocyte expression system (3) and the notion that α2/β1 and α1/β1 are the major skeletal muscle complexes (9, 12).

With regard to site-specific phosphorylation, the present results show that contraction-induced FXYD1 phosphorylation does occur at multiple sites in human skeletal muscle, including ser68, ser63, and possibly thr69. The increase in human muscle FXYD1 ser68 phosphorylation is detected with the motif- and phospho-specific antibodies (Fig. 5, C and D). In support, contractions induced FXYD1 ser68 phosphorylation in mouse soleus muscles (Fig. 6C). The apparent unchanged phosphorylation of FXYD1ser68 in human skeletal muscles, as indicated by application of the AB_FXYD1ser68 (Fig. 5A), is likely a result of phosphorylation at multiple sites because phosphorylation of FXYD1 at multiple sites induced by incubation with histamine and PKC has been shown to reduce the binding of AB_FXYD1ser68 compared with selective phosphorylation of FXYD1 at ser68 induced by incubation with forskolin and PKA (8, 26). Previous findings in rat soleus and mixed thigh skeletal muscles of unchanged FXYD1 ser68 phosphorylation during contractions using AB_FXYD1ser68 (24) is, therefore, also likely to be a result of FXYD1 phosphorylation at multiple residues.

Increased FXYD1 phosphorylation after 30 s was only detected by one of the applied antibodies (AB_FXYD1), indicating that the degree of phosphorylation was not as pronounced as after the subsequent 20 min of exercise, when phosphorylation was clearly detected by several approaches. The present finding of a submaximal FXYD1 phosphorylation level after 30 s of intense exercise provides a possible explanation for the initial large efflux of K⁺ from contracting muscle at the onset of exercise (10, 21, 36). The more pronounced phosphorylation level after the subsequent 20-min exercise bout could indicate that it takes a while before activated intracellular signaling results in full phosphorylation of protein targets, optimal pump activity, and maximum K⁺ re-uptake (36).
From the present mouse muscle experiments, it is clear that PKCα expression is a prerequisite for contraction-induced regulation of FXYD1 phosphorylation in a fiber type-specific way. Electrically evoked contractions increased FXYD1 ser68 phosphorylation in slow-twitch fiber-dominated soleus muscles of WT mice, but no change in soleus FXYD1 ser68 was observed in PKCα KO mice. Thus, contraction-induced activation of PKCα is required for contraction-induced FXYD1 ser68 phosphorylation in soleus. However, contractile activity in WT fast-twitch EDL muscle resulted in decreased phosphorylation of FXYD1 ser68 but had no effect on the level of phosphorylation in PKCα KO mice. These muscle-specific differences in FXYD1 phosphorylation indicate that exercise induces different regulation of Na⁺-K⁺ pump activity (23) in slow-twitch and fast-twitch fibers. It is noteworthy that the changes observed in soleus resemble the findings in human vastus lateralis muscles, whereas the response in EDL is different. This may be related to the mixed fiber-type distribution in human skeletal muscles (16).

Fig. 5. Site-specific phosphorylation of FXYD1 in human m. vastus lateralis lysates at rest, after 30 s of high-intensity exercise (30 s Exercise), and following 20 min of moderate-intensity exercise (20 min Exercise). A: FXYD1 ser68 phosphorylation (n = 8 – 10) obtained using a phospho-specific antibody against FXYD1 phosphorylated at ser68 (AB_FXYD1ser68). B: FXYD1 ser63 phosphorylation (n = 8 or 9) obtained using a motif and phospho-specific antibody that detects FXYD1 phosphorylated at ser63 (AB_ser63). C: FXYD1 ser68 phosphorylation (n = 8 or 9) obtained using a motif and phospho-specific antibody that detects FXYD1 phosphorylated at ser68 (AB_ser68). D: FXYD1 ser68 and thr69 phosphorylation (n = 8 or 9) obtained using a motif and phospho-specific antibody that detects FXYD1 phosphorylated at ser68 and/or thr69 (AB_ser68/thr69). The Western blot data are presented as sample signal intensity vs. mean rest signal intensity. Results are expressed as means ± SE. Significant differences from rest are indicated by * (P < 0.05) and ** (P < 0.01). Significant differences from 30 s Exercise are indicated by: # (P < 0.05).
The greatly reduced FXYD1 ser68 phosphorylation observed in the PKCα KO mice indicate that PKCα is an important regulator of FXYD1 phosphorylation. However, PKCα may not be the only regulator of FXYD1 phosphorylation in skeletal muscles. In example, both PKCα and PKCε induced phosphorylation of FXYD1 at ser63, ser68, and thr69, while PKCδ did not affect the phosphorylation levels in cardiac myocytes (8). Furthermore, PKA is reported to phosphorylate FXYD1 ser68 and, thereby, increase Na\(^+\)/H\(^+\) affinity of the Na\(^+\)/K\(^+\) pump (3).

The present results from the mouse study show that PKCα could be involved in the signaling pathway by which skeletal muscle Na\(^+\)/K\(^+\) pump activity is regulated during exercise. However, the importance of PKCα for regulation of pump activity in human skeletal muscle is still not clear. In skeletal muscle cells, PKCε is activated by contractions (20) and because PKCε increases FXYD1 phosphorylation (8), it may be involved in the exercise-induced increases in FXYD1 phosphorylation. Numerous studies have reported increased activity of other PKC isoforms with exercise in humans. For example, skeletal muscle atypical PKC activity is increased by moderate-intensity exercise (1, 28, 30). Despite this, no increase in PKCα activity appears to exist after 40 min of exercise at ~70% of VO\(_{2\max}\) (30), even though increases in Ca\(^{2+}\) concentrations in L6 myotubes induced increases in conventional PKC (cPKC) activity (13). However, the use of buffers containing the Ca\(^{2+}\) chelators EDTA and EGTA in muscle extractions, which probably reverse the translocation of cPKC to the membrane and, thereby, diminish contraction-induced PKCα activity, could likely explain the observed lack of PKCα activity increase (11, 30). Thus, it is tempting to speculate that the observed exercise-induced increase in FXYD1 phosphorylation in human skeletal muscle could be partly mediated by an exercise protocol-specific increase of PKCα activity.

In summary, exercise increases human skeletal muscle FXYD1 phosphorylation at multiple sites. Using PKCα KO mice, we demonstrated that electrical stimulation-induced FXYD1 ser68 phosphorylation in the soleus muscle is partly caused by PKCα activity.

Fig. 6. FXYD1 expression and phosphorylation in wild-type (WT) and PKCα KO mice at rest and after electrically stimulation are shown. A: unphosphorylated FXYD1 expression obtained with AB_FXYD1 in soleus mouse muscles. B: unphosphorylated FXYD1 expression obtained with AB_FXYD1 in EDL muscles. C: phosphorylation of FXYD1 ser68 determined with AB_FXYD1ser68 in soleus mouse muscles. D: phosphorylation of FXYD1 ser68 determined with AB_FXYD1ser68 in EDL mouse muscles. Data are normalized to the mean signal intensity of WT muscles at rest and expressed as means ± SE. Significant differences between rest and stimulated muscles are indicated by *** (P < 0.001) and * (P < 0.05). Significant differences between WT and PKCα KO mice muscles are indicated by ### (P < 0.001).
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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