Identification of 30 kDa calsequestrin-binding protein, which regulates calcium release from sarcoplasmic reticulum of rabbit skeletal muscle

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In a previous study [Yamaguchi, Kawasaki and Kasai (1995) Biochem. Biophys. Res. Commun. **210**, 648–653], we showed that the stilbene derivative 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid activates the Ca^{2+} channel in the sarcoplasmic reticulum (SR) in rabbit skeletal muscle, and it does not bind to the channel protein itself but to the SR 30 kDa protein. Furthermore, the 30 kDa protein was shown to bind to calsequestrin (CSQ), which is one of the regulators of the Ca^{2+} release channel in the SR. In the present study, we determined the partial amino acid sequence of the CSQ-binding 30 kDa protein and, consequently, this protein was proved to be highly similar to ADP/ATP translocase (AAT) expressed in the mitochondria in a variety of cells. By Western-blotting analysis, the CSQ-binding 30 kDa protein was recognized by the antibody raised against bovine cardiac AAT and, furthermore, depolarization-induced Ca^{2+} release monitored in the rabbit skeletal muscle triads was significantly activated by the antibody. As a result of cloning and sequencing of the cDNA encoding AAT of the rabbit skeletal muscle, the amino acid sequence was found to be the same as that of the CSQ-binding 30 kDa protein determined above. Furthermore, the expressed product of the cDNA encoding AAT in *Escherichia coli* was proved to bind to CSQ. These results suggest that AAT itself is expressed in the rabbit skeletal muscle SR and regulates the Ca^{2+} release from the SR; that is, excitation–contraction coupling of the skeletal muscle cell.

INTRODUCTION

In the skeletal muscle cell, Ca^{2+} stored in the sarcoplasmic reticulum (SR) is released depending on the depolarization of the transverse tubular membrane (TTM) and the released Ca^{2+} leads to muscle contraction. This signal transduction is termed excitation–contraction (E–C) coupling, and it is thought that the allosteric coupling between the TTM voltage sensor (dihydropyridine receptor) and the SR Ca^{2+}-release channel [ryanodine receptor (RyR)] takes place [1–3]. However, the mechanisms of signal transduction between dihydropyridine receptors and RyRs, and of Ca^{2+} release regulation, are not known. To understand these mechanisms as chain reactions among proteins, many investigators have suggested the existence of intrinsic SR proteins, such as triadin, calsequestrin (CSQ), junctin and FK-506 binding 12 kDa protein, which regulate E–C coupling [4–18], although the detailed regulation mechanism is still under investigation.

Triadin was identified first as a protein that binds to both RyRs and dihydropyridine receptors and was suggested to mediate the signal transduction between TTM and SR [4–6]. However, as a result of cloning and sequencing of the cDNA encoding triadin by Campbell and his colleagues [7,8], the molecular structure of triadin could not allow it to bind to dihydropyridine receptors, and they suggested that triadin binds to RyRs and CSQ. Now, molecular interactions around triadin are controversial [7–10]. Although CSQ was found to be a Ca^{2+}-storing protein in the lumen side of SR at first, it was shown to regulate the Ca^{2+} release from the SR in a Ca^{2+}-dependent manner [11–13]. However, CSQ had not been shown to bind to

RyRs directly and recently was shown to bind to triadin [8,19]; thus CSQ is thought to regulate RyRs through triadin. Except for triadin, CSQ was shown to bind to junctin, a novel SR 26 kDa protein [14–16] or an SR 30 kDa protein [19,20]. Junctin was first identified in the canine cardiac SR [14] and was subsequently purified, cloned and, furthermore, was confirmed in the skeletal SR [15]. Based on the primary structure, junctin is similar to triadin in its lumen side of the SR; that is, it has many positively charged amino acid residues, which are thought to bind to the carboxyl bases of CSQ, on the lumen side [15]. More recently, junctin was also shown to bind to RyRs and triadin in addition to CSQ; thus, RyR, CSQ, triadin and junctin were thought to form a quaternary complex [16]. As described above, we identified independently the SR 30 kDa protein as a CSQ-binding protein and found that it was a receptor of 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (DIDS) [19]. Because DIDS was known as an activator of RyRs [19,21–23], the 30 kDa protein was thought to possibly regulate RyRs through CSQ. Furthermore, we found that the 30 kDa protein also binds to the CSQ-binding 26 kDa protein, probably junctin [20]; thus we concluded that CSQ, junctin and the 30 kDa protein form a protein complex, which regulates RyRs. However, until now, the primary structure and molecular basis of the 30 kDa protein remained unclear, and evidence for regulation of E–C coupling by it has been somewhat limited.

In the present study, we obtained direct evidence that the 30 kDa protein regulates the Ca^{2+}-release mechanism in the SR using biochemical, immunological and molecular biological strategies.

EXPERIMENTAL

Materials

Antiserum raised against ADP/ATP translocase (AAT) in the bovine cardiac mitochondria was kindly given by Prof. H. Terada of the Faculty of Pharmaceutical Science, University of Tokushima, Japan. Other chemicals were of analytical grade.

Membrane preparations

The heavy fraction of the SR (HSR) was prepared from rabbit skeletal muscle under 4 °C according to the method described previously in [24] with slight modifications. Dorsal and hind-leg muscles were homogenized with 4 volumes of 0.1 M NaCl/1 mM EGTA/5 mM Tris/maleate (pH 7.0)/protease inhibitors (0.5 μg/ml aprotinin/1 μg/ml antipain/1 μg/ml leupeptin/1 μg/ml pepstatin/1 mM PMSF), using a mixer (Toshiba MX A30G) for a total of 2 min with one interval of 30 s. The homogenate was centrifuged at 4000 g (average) for 20 min, and the resultant supernatant was centrifuged at 10000 g (average) for 40 min. The precipitate was suspended in 100 ml of 0.6 M KCl/5 mM Tris/maleate (pH 7.0)/protease inhibitor (0.25 μg/ ml aprotinin/0.5 μg/ml antipain/0.5 μg/ml leupeptin/0.5 μg/ml pepstatin/0.1 mM PMSF) and homogenized in a Teflon-glass homogenizer. The homogenate on further dilution to 200 ml with a solution comprising 0.6 M KCl/5 mM Tris/maleate (pH 7.0) was centrifuged at 16000 g (average) for 40 min. For the resultant precipitate, the same KCl treatment was performed again, and the precipitate was suspended and homogenized with 10 mM Tris/maleate (pH 7.0). The homogenate was centrifuged at 16000 g (average) for 30 min, and the precipitate was suspended and homogenized in a solution comprising 10 % sucrose and 10 mM Tris/maleate (pH 7.0) to dilute to 25–30 mg of protein/ ml. For most experiments, the isolated HSR was frozen in liquid nitrogen and stored at −80 °C.

Junctional face membrane was prepared from HSR according to the method of Costello et al. [25]. The final preparation was suspended in a solution comprising 10 % (w/v) sucrose and 5 mM Hepes-Na (pH 7.4) and then stored at −80 °C.

A terminal cisternae (TC) and triad mixture (TC/triads) was prepared from rabbit skeletal muscle according to the method described in [26]. The final preparation was suspended in 10 mM Tris/maleate (pH 7.0) and then stored at 0 °C. The isolated TC/triads were used within 10 days. In some experiments, the suspended TC/triads were mixed with 10 % (w/v) sucrose (final concentration) and stored at −80 °C.

Mitochondria-rich membrane fraction (MRMF) was prepared from the first precipitate of the HSR preparation as described above according to the method described previously in [27], with slight modifications. The precipitate was homogenized with 0.1 M NaCl/1 mM EGTA/5 mM Tris/maleate (pH 7.0)/protease in-hibitors (0.25 μg/ml aprotinin/0.5 μg/ml antipain/0.5 μg/ml leupeptin/0.5 μg/ml pepstatin/0.1 mM PMSF) and was centri-fuged at 650 g (average) for 10 min. The supernatant was recentrifuged as before, and then the resultant supernatant was centrifuged at 8000 g (average) for 10 min, and the precipitate was diluted with 10 mM Tris/maleate (pH 7.0) and was homo-genized in the same manner as the HSR preparation. The homogenate was centrifuged at 8000 g (average) for 10 min, and the final precipitate was suspended and homogenized in a solution comprising 10 % sucrose and 10 mM Tris/maleate (pH 7.0) and was stored at −80 °C.

The protein concentration was determined by the biuret reaction, calibrated by nitrogen determination or by the Bradford method, calibrated against BSA concentration.

Purification of the 30 kDa protein

Purification of the 30 kDa protein from the junctional face membrane of the rabbit skeletal muscle was performed using hydroxyapatite column chromatography and an electroelution system according to the method described in [20].

Analysis of amino acid sequence

After SDS/PAGE analysis was performed according to the method of Laemmli [28], the proteins in the gel were transferred on to a PVDF membrane (Bio-Rad Co., Hercules, CA, U.S.A.) using a semi-dry transfer system. After staining of the membrane by Ponceau S, the stained band was cut and the amino acid sequence of the peptide in the band was determined from the amino terminus using a protein sequencer (Applied Biosystems model 473A) according to the manufacturer's protocol.

Measurements of enzymic activities

Succinate-cytochrome c reductase and cytochrome c oxidase activities were measured according to methods described pre-viously [29,30] with slight modifications. For succinate-cyto-chrome c reductase, a 1 ml solution comprising 100 mM potassium phosphate/0.3 mM EDTA/KOH/20 mM succinate/ NaOH/2mM NaN_3/HCl/0.1 % (w/v) oxidized cytochrome c (pH 7.4) was mixed with 20 μl of 2 mg/ml membrane fraction (MRMF or HSR) solubilized in 0.1 M potassium phosphate (pH 7.4) in the cuvette. For cytochrome c oxidase, 0.9 ml of 0.244 % (w/v) Lubrol PX, 0.1 ml of 2.7 mg/ml reduced cytochrome c and 0.1 ml of 2 mg/ml membrane fraction were mixed [all solutions were prepared in 40 mM potassium phosphate (pH 6.2)]. In both cases, the measurements of absorbance at 550 nm were started 10 s after the addition of membrane fraction, and the increase or the decrease of the absorbance at the initial 60 s were regarded as the activity of succinate-cytochrome c reductase or cytochrome c oxidase, respectively. The activities were corrected for the spontaneous reduction or oxidation by subtracting the back-ground value, which was determined by substituting potassium phosphate buffer for the membrane fraction.

Western-blot and ligand-blot analyses

In the Western-blot analysis, the proteins in the gel after SDS/PAGE were transferred electrically on to a nitrocellulose membrane using a semi-dry transfer system. The membrane was incubated with 5 % (w/v) skimmed milk and 5 % (v/v) calf serum in Tris/NaCl buffer [150 mM NaCl/50 mM Tris/HCl (pH 7.6)] for more than 1 h to block non-specific binding sites and then washed with Tris/NaCl buffer. After the membrane had been incubated with the anti-AAT antiserum or the anti-glutathione S-transferase (GST) antibody (Amersham Pharmacia Biotech Ltd., Bucks, U.K.) in Tris/NaCl buffer for more than 3 h at room temperature, it was washed with Tris/NaCl buffer 5–6 times and reincubated with alkaline phosphatase-conjugated anti-rabbit (for anti-AAT antiserum) or anti-goat IgG antibodies (for anti-GST antibody). After washing with Tris/NaCl buffer 5–6 times, the protein that reacted with antibodies was visualized by incubation with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Analysis of the CSQ-binding protein was performed by ligand-blot analysis using biotinylated CSQ according to the method described previously [19].

Measurement of depolarization- or caffeine-induced Ca²⁺ release from skeletal muscle triads

The Ca²⁺ release from TC/triads triggered by TTM-depolarization or caffeine was measured using the membrane-impermeable Ca²⁺ indicator fura-2 (Dojin Co., Kumamoto, Japan) and a stopped-flow spectrofluorimeter (Applied Physics Model SX.17MW) according to the methods described in [26,31].

TC/triads were incubated in the solution comprising 100 mM potassium propionate, 2 mM MgCl₂ and 20 mM Tris/maleate (pH 7.0) for 3 h on ice with normal rabbit serum (Rockland, Inc., Gilbertsville, PA, U.S.A.), anti-bovine albumin antiserum (ICN Pharmaceuticals, Inc., Costa Mesa, CA, U.S.A.), or anti-AAT antiserum (concentrations were each 96.8 μg/ml at the time of incubation). After incubation, an ATP regeneration system (15 mM phosphocreatine disodium salt/15 units/ml creatine phosphokinase/2 mM Na₂-ATP) and 50 μM CaCl₂ were added to load Ca²⁺ into the SR and to polarize the TTM at the same time. After the addition (10 min), the measurements of depolarization-induced Ca²⁺ release (DICR) were performed by mixing TC/triads with 10 volumes of depolarization solution [8.3 mM K⁺-propionate/26 mM TEA (tetraethylammonium)/HCl/69.7 mM TEA/propionate/20 mM Tris/maleate (pH 7.0)/ATP regeneration system/11 μM fura-2 (membrane impermeable)]. For assaying caffeine-induced Ca²⁺ release, TC/triads were mixed with 10 volumes of caffeine-containing solution [100 mM potassium propionate/4 mM TEA/HCl/20 mM Tris/maleate (pH 7.0)/5.5 mM caffeine/ATP regeneration system/11 μM fura-2]. The concentrations of all reagents except serum and antiserum were the final concentrations before mixing. According to the Nernst equation, the magnitudes of depolarization of TTM were +45.1 and +0 mV for DICR and caffeine-induced Ca²⁺ release, respectively. The change in fura-2 fluorescence intensity depending on the Ca²⁺ release was analysed according to the method described previously [26,31].

Isolation and sequencing of cDNA encoding AAT

Total RNA was prepared from the hind-leg muscle of a male rabbit aged 2 weeks according to the acid guanidinium thiocyanate-phenol-chloroform method described previously [32], poly(A⁺) RNA was purified from total RNA using an oligo-dT–cellulose column (Clontech Co., Palo Alto, CA, U.S.A.), and cDNA was synthesized by reverse transcription (RT) of the poly(A⁺) RNA with an oligo-dT primer. cDNA encoding rabbit skeletal muscle AAT was amplified specifically by the RT-PCR method using two forward primers (F1, 5′-GCCAGCAAACAGATCAGTGC-3′; and F2, 5′-CACCCAAGCTCTCAACTTCGC-3′) and two reverse primers (R1, 5′-GCCTCTCAGTACATTGGACC-3′; and R2, 5′-GTCACACTCTGGGCAATCATCC-3′), synthesized according to the cDNA sequence of bovine cardiac AAT T1 [33]. The entire open reading frame was obtained by rapid amplification of the cDNA 5′ and 3′ ends (5′ RACE and 3′ RACE) methods [34], using primers based on the determined sequence.

Determination of the sequence of cDNA encoding rabbit skeletal muscle AAT was performed using a *Taq* polymerase and terminator cycle sequencing ready-reaction kit with a model 310 DNA sequencer (Applied Biosystems).

Expression of cDNA encoding AAT

The cDNA, including the open reading frame of rabbit skeletal muscle AAT, was generated using PCR primers that encoded flanking restriction enzyme sequences (*Eco*RI, forward primer; *Xho*I, reverse primer). Amplified PCR products were cloned into

pGEX-4T-1 (Amersham Pharmacia Biotech). AAT was expressed as a GST fusion protein in *Escherichia coli* BL21 cells.

RESULTS

Determination of the amino acid sequence of CSQ-binding 30 kDa protein

In a previous paper [20], we purified the 30 kDa protein from the SR of the rabbit skeletal muscle and confirmed by ligand-blot analysis that CSQ bound to it. At first, we tried to determine the partial amino acid sequence of the purified natural 30 kDa protein according to the method described under the Experimental section. However, automated Edman degradation of the natural 30 kDa protein failed; thus its N-terminus was thought to be modified. Therefore, we digested the natural 30 kDa protein by protease V8 (Seikagaku Co., Tokyo, Japan), and the amino acid sequence of the digested fragments was determined. Figure 1 (top panel) shows the result of SDS/PAGE of the natural 30 kDa protein (lane 1) and the digested fragments (lane 2). Obviously, the 30 kDa protein was digested into two fragments whose molecular masses were about 15 and 16 kDa. Because the
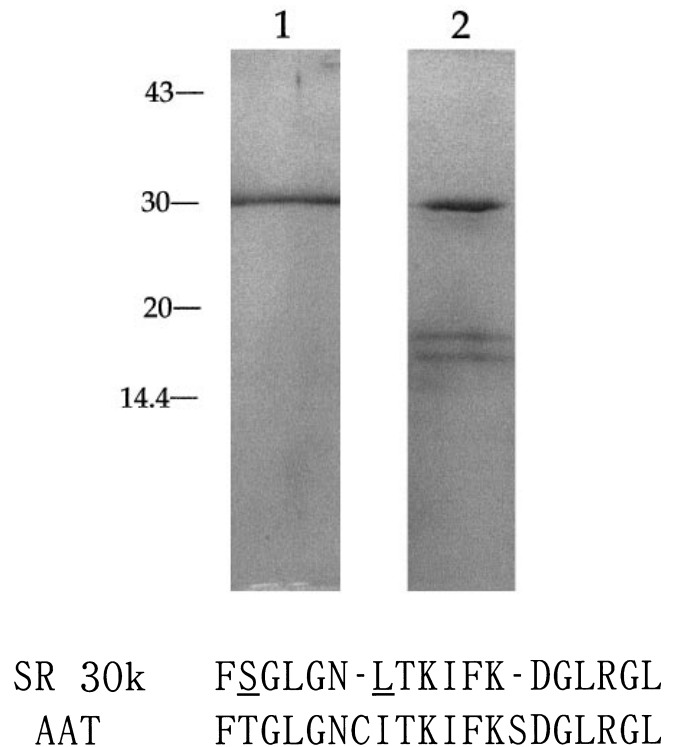

SR 30k   FSGLGN - LTKIFK - DGLRGL
AAT   FTGLGNCITKIFKSDGLRGL

Figure 1   Determination of partial amino acid sequence of the SR 30 kDa protein

(Top panel) Purified SR 30 kDa protein was digested by 2.5–3.0 μg of protease V8 in the gel of SDS/PAGE completed on a 15% polyacrylamide gel, and the gels were stained with Coomassie Brilliant Blue R-250. The results of SDS/PAGE of the purified 30 kDa protein and digested fragments are shown in lanes 1 and 2, respectively. The molecular masses are indicated on the left in kDa. (Lower panel) The partial amino acid sequence of the 15 kDa fragment digested from SR 30 kDa protein (30k) is shown; the seventh and the fourteenth residues (—) could not be determined. The amino acid sequence at positions 154–173 of AAT from bovine cardiac mitochondria is also shown. The underlined amino acid residues of SR 30k are not identical to those of AAT.
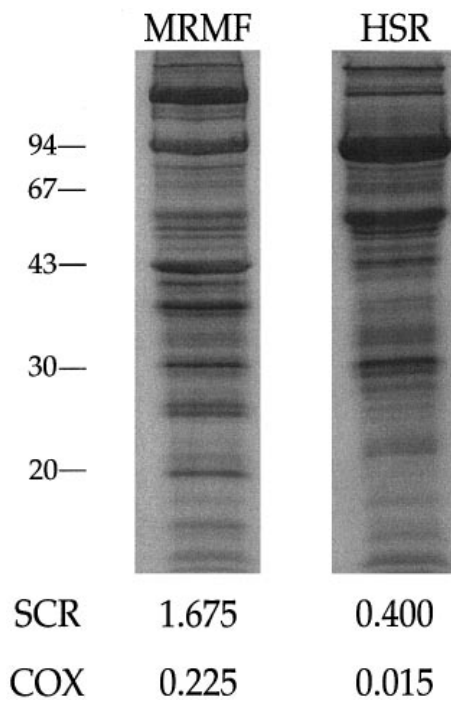
30 kDa protein was also detected in the digested sample (lane 2), the 30 kDa protein was thought to be partially digested under these conditions. We only succeeded in the determination of the amino acid sequence of the 15 kDa fragment; thus the 16 kDa fragment was thought to be half of an N-terminus that was modified, and the 15 kDa fragment was half of a C-terminus. The partial amino acid sequence of the 15 kDa fragment is shown in Figure 1 (lower panel). As a result of the homology search, the 30 kDa protein was found to be highly similar to mitochondrial AATs in a variety of cells; in particular, the sequence was similar to the region between 154 and 173 amino acids of the bovine AAT T1, expressed mainly in the cardiac cell [33] (about 89% identity in 18 amino acid residues).

Enzymic activities of membrane fractions

Since the possibility that the 30 kDa protein is derived from contaminating mitochondria could not be neglected, we measured the activities of succinate-cytochrome c reductase and cytochrome c oxidase, major mitochondrial marker enzymes. As shown in Figure 2, the amounts of 30 kDa protein of MRMF and HSR were nearly equal, judging from the bands of the SDS/PAGE gel; however, the activities of succinate-cytochrome c reductase in MRMF and HSR were 1.675 and 0.4 absorbance units/mg of protein per min, respectively, and those of cytochrome c oxidase were 0.225 and 0.015 absorbance units/mg of protein per min, respectively. Therefore, we concluded that the 30 kDa proteins in the HSR preparation used in the present study were derived from SR.
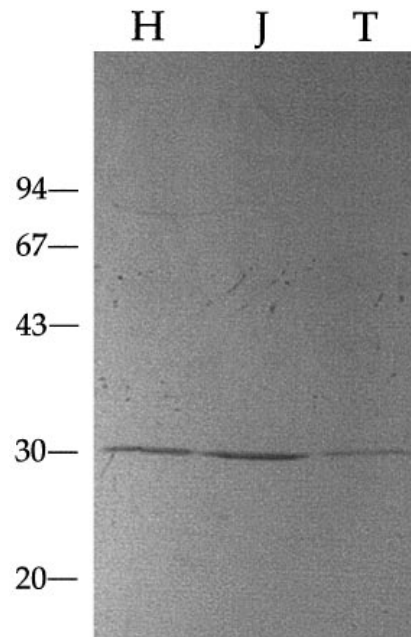
Immunological identification of the 30 kDa protein as a Ca^{2+}-channel regulator

To obtain direct evidence that the SR 30 kDa protein was similar to AAT, we performed Western-blot analysis of the skeletal muscle membrane fractions by the antibody raised against AAT expressed in the bovine cardiac mitochondria. Figure 3 shows the results of Western blot. Only one 30 kDa band was recognized by the antibody in all membrane fractions. A large amount of this protein was detected in the junctional face membrane fraction; on the other hand, in the TC/triads fraction, only a small amount was found. This 30 kDa protein is certainly identical to the CSQ-binding 30 kDa protein reported in previous studies [19,20].

Next, in order to know whether the CSQ-binding 30 kDa protein recognized by the anti-AAT antibody regulated the Ca^{2+} release from the SR, we investigated the effect of the antibody on the Ca^{2+} release from skeletal muscle triads triggered by +45.1 mV depolarization of TTM. Figure 4 shows the representative traces of Ca^{2+} release from skeletal muscle triads. The amount of released Ca^{2+} from the triads incubated in the solution containing anti-AAT antiserum was significantly greater than that from triads in the control experiment. These DICR curves could be best-fitted by a double exponential equation as described previously [26,31]. Consequently, the amounts of released Ca^{2+} normalized by the average for control experiments were calculated to be 100.00 ± 1.43 and $134.33 \pm 20.74\%$ (shown as the average of two experiments $\pm$ S.D.) for triads incubated in the normal rabbit serum (control) and in the anti-AAT antiserum, respectively. The amounts of released Ca^{2+} in the fast and slow phases increased almost equally in the antiserum (fast phase, $128.62 \pm 11.14\%$; slow phase, $139.69 \pm 29.75\%$). Regarding the
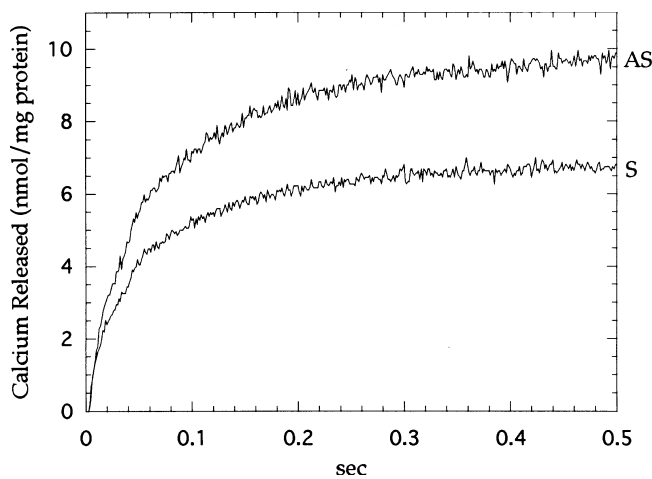
Figure 4   The effect of anti-AAT antibody on DICR from skeletal muscle triads

The Ca^{2+} release from SR triggered by $+45.1$ mV depolarization of TTM was measured using skeletal muscle triads incubated with the normal rabbit serum (S) or the anti-AAT antiserum (AS) according to the method described in the Experimental section. Both traces are the averages of 5–10 traces collected sequentially in a single-sample experiment.

kinetic parameters, the Ca^{2+}-release rates in the fast and slow phases of DICR were found not to be activated by antiserum [fast phase, 39.59 ± 5.93 and 38.51 ± 6.34 (1/s); slow phase, 7.83 ± 0.90 and 7.16 ± 1.18 (1/s) for triads incubated in the normal rabbit serum and in the anti-AAT antiserum, respectively]. Therefore, Ca^{2+} release activated by the antibody is thought to be through the same Ca^{2+} channel as in the control experiments (the channel has already been proved to be RyR [26,31]). These results suggest that the CSQ-binding 30 kDa protein recognized by anti-AAT antibody regulates the number of RyRs in the SR opening in response to a depolarization stimulus. As a negative control experiment, we investigated the effect of anti-bovine albumin antiserum on DICR, and found that it could not increase the amount of released Ca^{2+}; the amounts of released Ca^{2+} normalized by the average for control experiments were 100.00 ± 11.7 and $98.58 \pm 8.97\%$ ($n = 3$) for triads incubated in the normal rabbit serum and in the antiserum, respectively. Therefore, anti-AAT antiserum was concluded to activate DICR specifically.

Cloning, sequencing and expression of cDNA encoding rabbit skeletal muscle AAT

At first, using primers based on the sequence of bovine cardiac AAT (see the Experimental section), a DNA fragment was

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                                                             CCCTGCTGTCATC          -1
ATGAGTGATCAGGCTTTGAGCTTCCTCAAGGACTTCCTGGCAGGTGGCGTCGCCCGCTGCCGTCTCCAAGACTGCG        75
 M  S  D  Q  A  L  S  F  L  K  D  F  L  A  G  G  V  A  A  A  V  S  K  T  A

GTCGCCCCCATCGAGAGGGTCAAACTGCTGCTGCAGGTCCAGCATGCCAGCAAACAGATCAGCGCTGAGAAGCAG       150
 V  A  P  I  E  R  V  K  L  L  L  Q  V  Q  H  A  S  K  Q  I  S  A  E  K  Q

TACAAAGGGATCATTGATTGTGTGGTGAGGATCCCCAAGGAGCAGGGCTTTCTCTCCTTCTGGAGGGGTAACCTG       225
 Y  K  G  I  I  D  C  V  V  R  I  P  K  E  Q  G  F  L  S  F  W  R  G  N  L

GCCAACGTGATCCGGTACTTCCCCACCCAAGCTCTCAACTTCGCCTTCAAGGACAAGTACAAGCAGATCTTCTTG       300
 A  N  V  I  R  Y  F  P  T  Q  A  L  N  F  A  F  K  D  K  Y  K  Q  I  F  L

GGGGGCGTGGATCGGCATAAGCAGTTCTGGCGCTACTTCGCTGGTAACCTGGCCTCCGGTGGGGCAGCTGGGGCC       375
 G  G  V  D  R  H  K  Q  F  W  R  Y  F  A  G  N  L  A  S  G  G  A  A  G  A

ACCTCCCTCTGCTTTGTCTACCCGCTGGACTTTGCCAGGACCAGGTTGGCTGCCGACGTGGGCAAGGGTGCCGCC       450
 T  S  L  C  F  V  Y  P  L  D  F  A  R  T  R  L  A  A  D  V  G  K  G  A  A

CAGCGTGAGTTCAGTGGTCTGGGCAACTGTCTCACCAAGATCTTCAAGTCTGATGGCCTGAGGGGTCTCTACCAG       525
 Q  R  E  F̲  S̲  G̲  L̲  G̲  N̲  C̲  L̲  T̲  K̲  I̲  F̲  K̲  S  D̲  G̲  L̲  R̲  G̲  L̲  Y  Q

GGTTTCAATGTCTCTGTGCAAGGCATCATTATCTACAGAGCTGCCTACTTCGGAGTCTATGATACTGCCAAGGGG       600
 G  F  N  V  S  V  Q  G  I  I  I  Y  R  A  A  Y  F  G  V  Y  D  T  A  K  G

ATGTTGCCTGACCCCAAGAACGTGCACATTATCGTGAGCTGGATGATCGCCCAGACCGTGACGGCAGTGGCCGGG       675
 M  L  P  D  P  K  N  V  H  I  I  V  S  W  M  I  A  Q  T  V  T  A  V  A  G

CTGGTGTCCTACCCCTTTGACACTGTTCGTCGTAGGATGATGATGCAGTCTGGCCGGAAAGGGGCTGACATTATG       750
 L  V  S  Y  P  F  D  T  V  R  R  R  M  M  M  Q  S  G  R  K  G  A  D  I  M

TACACGGGGACAGTTGACTGCTGGAAGAAGATTGCAAAAGATGAAGGAGCCAAGGCTTTCTTCAAAGGTGCCTGG       825
 Y  T  G  T  V  D  C  W  K  K  I  A  K  D  E  G  A  K  A  F  F  K  G  A  W

TCCAATGTGTTGAGAGGCATGGGTGGCGCTTTTGTATTGGTGTTGTATGATGAGATCAAAAAATATGTCTAATGC       900
 S  N  V  L  R  G  M  G  G  A  F  V  L  V  L  Y  D  E  I  K  K  Y  V  -

AGTTGAAAATCAAGTTCAT                                                             919
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Figure 5   Nucleotide and deduced amino acid sequences of the rabbit skeletal muscle AAT

The underlined amino acid residues are identical to the residues determined in Figure 1 (lower panel).
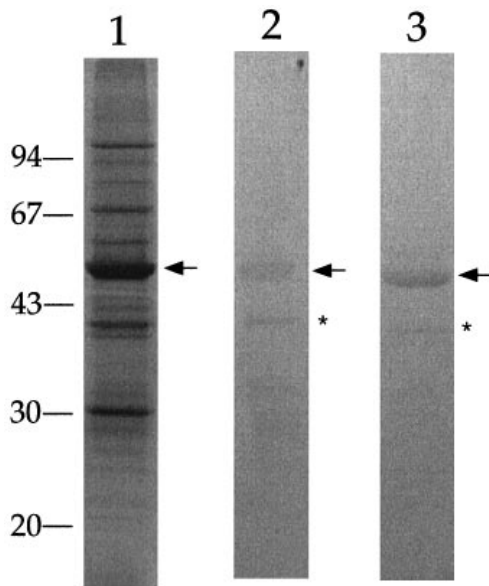
Figure 6   Expression of cDNA encoding rabbit skeletal muscle AAT

The cloned plasmid including the open reading frame of rabbit skeletal AAT was transfected in BL21 cells, and expression was induced. After the cells were sonicated and solubilized in the solution comprising 1% (v/v) Triton X-100/PBS (140 mM NaCl/2.7 mM KCl/10 mM Na$_2$HPO$_4$/1.8 mM KH$_2$PO$_4$, pH 7.3), the insoluble fraction was collected by centrifugation and was analysed by SDS/PAGE (lane 1) on a 10% polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue R-250. The proteins in the gel were transferred on to the nitrocellulose membrane, and then Western-blot analysis using anti-GST antibody (lane 2) or ligand-blot analysis using the biotinylated CSQ (lane 3) was performed according to the method described in the Experimental section. The arrows indicate the position of the GST–AAT fusion protein, and the asterisks indicate the presumed position of the digested fusion-protein product. The molecular masses are indicated on the left in kDa.

amplified from the rabbit skeletal muscle poly(A$^+$) RNA by the RT-PCR method. For example, when using primers F1 and R1, a fragment of about 700 bp was amplified. The amplified fragment was sequenced, and other primers were designed from the determined nucleotide sequence. Finally, the complete nucleotide sequence of the open reading frame of rabbit skeletal muscle AAT was determined by 5′- and 3′-RACE methods. The predicted open reading frame consists of 894 nucleotides. The determined nucleotide and deduced amino acid sequences are shown in Figure 5. As a result of a homology search, the nucleotide sequence is 94% and the amino acid sequence is 98% homologous to those of the bovine AAT T1 [33]. Furthermore, it was confirmed that the deduced amino acid sequence totally included the 18 amino acids of the SR 30 kDa protein (FSGLGN-LTKIFK-DGLRGL) shown in Figure 1 (the underlined letters in Figure 5).

Next, the open reading frame of cDNA encoding the rabbit skeletal muscle AAT was cloned into a pGEX-4T-1 vector in order to obtain the GST fusion protein. After the cloned plasmid was transformed to *E.coli* BL21 cells, expression of AAT as a GST fusion protein was induced. The molecular mass of GST was about 30 kDa; thus the molecular mass of the GST-fusion product of AAT was expected to be about 60 kDa. As shown in Figure 6 (lane 1), a large amount of the product was found in the insoluble fraction at the position of 60 kDa. This 60 kDa product was recognized by the anti-GST antibody (lane 2). These results indicate that the 60 kDa protein was the expressed GST fusion protein. Further, the 40 kDa protein, whose amount was much

smaller than that of the 60 kDa protein, and lower-molecular-mass proteins were also recognized by the anti-GST antibody (lane 2). These proteins were thought to be digested products of the intact 60 kDa protein. In addition, by ligand-blot analysis using biotinylated CSQ, the GST fusion 60 kDa protein of AAT was found to be able to bind to CSQ, and the 40 kDa protein described above was also bound (lane 3). These results strongly suggest that AAT itself is expressed in the skeletal muscle SR as a CSQ-binding protein and plays an important role in intracellular Ca^{2+} mobilization, that is, E–C coupling.

DISCUSSION

The stilbene derivative DIDS has been shown by planar lipid-bilayer measurement and ion-flux measurement in the skeletal and cardiac muscle SR to activate RyRs [19,21–23], and in a previous paper [19], we found that DIDS bound not to RyRs but to the 30 kDa protein in the skeletal muscle SR. Furthermore, the 30 kDa protein was also found to bind to CSQ [19], which had been already reported to regulate RyRs in the SR [11–13]. Therefore, we have thought that the 30 kDa protein plays an important role through CSQ in E–C coupling of skeletal muscle. In the present study, the 30 kDa protein was found surprisingly to be highly similar to AAT expressed in the mitochondria (Figures 1 and 3), although the SR fraction used in the present study hardly included any mitochondrial contaminants (Figure 2). As shown in Figure 4, DICR from skeletal muscle triads was significantly activated by the anti-AAT antibody; thus the protein recognized by the antibody (CSQ-binding 30 kDa protein) is thought certainly to participate in the regulation of intracellular Ca^{2+} mobilization in skeletal muscle. Furthermore, we found by ion-flux and single-channel measurements that atractyloside, the specific inhibitor of AAT [35], inhibits RyR activity (N. Yamaguchi, T. Kagari and M. Kasai, unpublished work). This result is strong evidence that the AAT-like protein regulates the SR Ca^{2+} channel. Because DIDS which was found to activate RyR was also an AAT inhibitor [36], it is reasonable that RyR was activated by DIDS through an AAT-like protein. Probably, the conformation change in the AAT-like protein caused by atractyloside or DIDS is thought to determine whether RyRs can open or close. We also investigated the effect of anti-AAT antibody on caffeine-induced Ca^{2+} release from triads; however, a significant activation by the antibody was not observed in contrast with the DICR shown in Figure 2 (results not shown). In a previous paper [19], we showed that DIDS, which binds to the 30 kDa (AAT-like) protein, could not activate RyRs that were already activated by Ca^{2+} through the Ca^{2+}-induced Ca^{2+} release mechanism. Because caffeine is thought to be a Ca^{2+}-induced Ca^{2+}-release activator or a substitute for Ca^{2+}, the results described above are consistent with the previous results. At the regulation site of RyRs, the effects of the AAT-like protein may compete with those of caffeine or Ca^{2+}.

The deduced amino acid sequence of rabbit skeletal muscle AAT (the amino acid residues at positions 154–173 in Figure 5) from the cDNA sequence completely included the partial amino acid sequence of the 15 kDa fragment digested from the SR 30 kDa protein by protease V8, shown in Figure 1. The deduced sites digested by protease V8 from Figure 5 include the position between amino acid residues 153 and 154. Therefore the results of Figure 1 are consistent with the results of Figure 5. Judging from these facts, we consider that the SR 30 kDa protein is probably AAT itself; that is, AAT is a dual-function protein (for carrying adenine nucleotides across the mitochondrial inner membrane and for regulating Ca^{2+} release from SR), like ε-crystallin in the lens, which has been shown to be identical to

lactate dehydrogenase and prostaglandin F synthase [37,38]. Also, that expressed AAT in BL21 cells could bind to CSQ (Figure 6) is evidence of this consideration. It is probable that in the skeletal muscle SR, AAT does not function as a carrier of ATP and ADP, because it has already been reported that atractyloside could not inhibit the [^{32}P]ATP flux through the SR membrane [39]. Alternatively, porin, also one of the mitochondrial proteins, was suggested to exist in the skeletal muscle SR as a carrier of ATP and ADP [39]. Carrier activity of AAT might be prohibited by binding of other proteins such as CSQ in the SR.

Previously, we showed that the 30 and 34 kDa proteins can bind to CSQ in the skeletal muscle SR [19], whereas Damiani and Margreth reported that ^{125}I-labelled CSQ could bind to 30 and 31 kDa proteins in the skeletal muscle [40]. Furthermore, Jones and his colleagues reported the 31 kDa protein as a minor CSQ-binding and junctin-like protein in cardiac SR [15,16]. The 30 and 34 kDa proteins in our previous study are thought to correspond to the 30 and 31 kDa proteins, respectively, in the study of Damiani and Margreth [40]. Because the skeletal muscle AAT does not have a homologous sequence of junctin, the CSQ-binding and junctin-like 31 kDa protein in the cardiac SR [15,16] is thought to correspond to the 34 kDa protein in our previous study [19] or the 31 kDa protein in the study of Damiani and Margreth [40].

Triadin and junctin were suggested previously to bind to CSQ through the charge interaction at the C-terminus located in the lumen side of the SR, which contains a high concentration of positively charged amino acids [7,8,15,16]. Regarding AAT, many lysine and arginine residues exist on the N-terminal side (residues at positions 31–72 in Figure 5), thought to be in the matrix side of the mitochondrion [35], which corresponds to the lumen side of SR. Therefore, AAT is also thought to bind to CSQ on the lumen side of SR through charge interaction like triadin and junctin.

In summary, we found that the CSQ-binding 30 kDa protein in the skeletal muscle SR is highly homologous to (and is probably the same as) AAT in mitochondria, and that it regulates RyRs. From now on, the signal transduction around the 30 kDa protein, including triadin, CSQ and junctin, should be studied.

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