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Regulation of the ryanodine receptor calcium release channel: a molecular complex system

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Abstract

Skeletal muscle contraction is regulated by Ca^{2+} released from the sarcoplasmic reticulum (SR). The Ca^{2+} release channel in the SR has been identified as the ryanodine receptor (RyR). Recently, it was found that the RyR is a large transmembrane protein that is regulated by many intrinsic factors. In this review, we mainly summarize our experimental results. We will first show that calsequestrin and the DIDS-binding 30-kDa protein work as intrinsic factors and regulate the RyR Ca^{2+} release channel. Next, the DIDS-binding 30-kDa protein was identified as the ADT/ATP translocase (AAT) present in mitochondria, based on a cDNA analysis. This result shows that AAT is bifunctional and works as a transporter protein in mitochondria and as a regulator of Ca^{2+} release in the SR. From these results, we propose a model in which calsequestrin, the DIDS-binding 30-kDa protein, and junctin form a ternary complex that regulates the RyR Ca^{2+} release channel through interactions with triadin.

Keywords: Ryanodine receptor; Sarcoplasmic reticulum; DIDS; Calsequestrin; ADP/ATP translocase

1. Introduction

The contraction of skeletal muscle is regulated as follows: An electrical signal comes from the motor nerve and stimulates the plasma membrane of the muscle. When the muscle membrane is excited, a voltage sensor of the T-tubule membrane is activated and the signal is transmitted to the sarcoplasmic reticulum (SR). Ca^{2+} is released from the SR and the muscle contracts. This process is called excitation–contraction coupling. However, the mechanism underlying Ca^{2+} release from the SR has remained unsolved [1,2]. Recently, the Ca^{2+}-induced Ca^{2+} release (CICR) channel was isolated as the ryanodine receptor (RyR) [3,4], and its primary structure was deduced by a cDNA analysis [5]. This channel is

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characteristically activated by micromolar concentrations of Ca^{2+}, caffeine, and ATP, and is blocked by Mg^{2+}, procaine, and ruthenium red [1]. However, how the information is transmitted from the voltage sensor (dihydropyridine [DHP] receptor, L-type Ca^{2+} channel) in the T-tubule to the RyR in the SR is unknown [1,2]. In addition to direct coupling between these two proteins, the contributions of many regulatory proteins, such as triadin, junctin, and calsequestrin, were suggested [6–13]. Triadin was first isolated as a 95-kDa protein that binds to the RyR [13–15], and the importance of this protein in excitation–contraction (E–C) coupling was suggested from an experiment in which a monoclonal antibody against triadin inhibited depolarization-induced calcium release (DICR) [16]. However, the molecular mechanism that controls the E–C coupling has not been clarified. Junctin was first found in the cardiac muscle as a CSQ binding protein with a molecular weight of 26 kDa [17], and was later found in skeletal muscle as well [11]. Recently, junctin was shown to form a protein complex with the RyR and triadin in addition to CSQ [12].

In this review, we mainly summarize our experimental results. First, calsequestrin (CSQ) regulates the RyR Ca^{2+} release channel [6]. Second, 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid (DIDS) opens the RyR, but does not bind it [18]. The 30-kDa protein was shown to bind DIDS [7,8]. Third, it was found that the molecular properties of the DIDS-binding 30-kDa protein were very similar to those of the ADP/ATP translocase (AAT) expressed in the mitochondria of a variety of cells [9]. Furthermore, it was shown that CSQ, the DIDS-binding 30-kDa protein, and junctin form a ternary complex and interact with triadin [8]. Finally, from these results, we proposed a model in which the RyR Ca^{2+} channel in the SR is regulated though a molecular complex of intrinsic factors. From another point of view, the protein–protein interactions within the intracellular Ca^{2+} release channel have recently been reviewed [10]

2. Regulation of the RyR Ca^{2+} channel by calsequestrin

In order to find intrinsic molecules that regu-

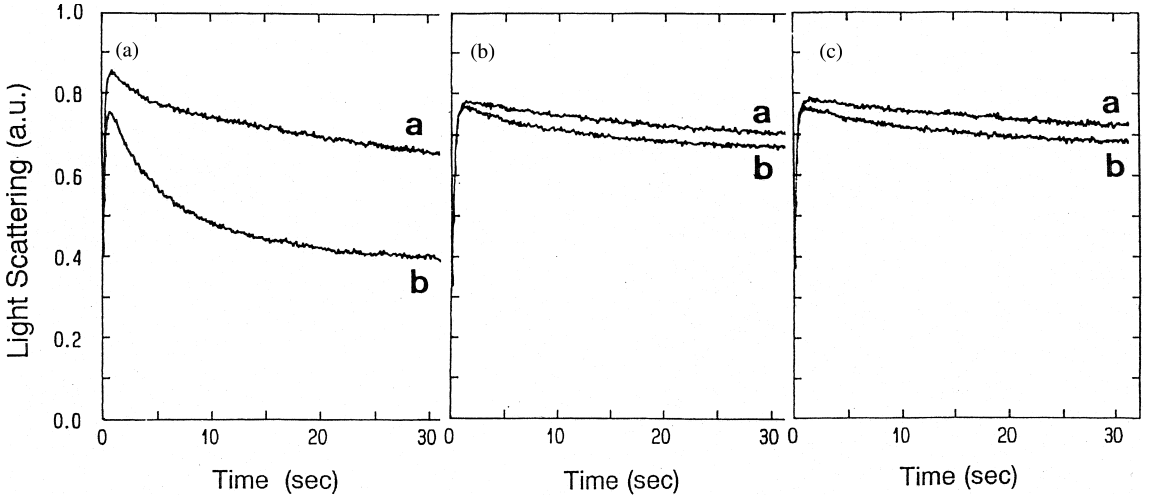


Fig. 1. Decrease in Ca^{2+} response by EDTA treatment. HSR vesicles (10 mg protein ml^{-1}) were incubated overnight at 0°C in 10 mM Tris-maleate (pH 7.0) in the presence and absence of EDTA or EGTA. The conditions were: (a) no addition, (b) 1 mM EDTA, (c) 1 mM EGTA. After the incubation, the HSR vesicles were diluted 20-fold in 10 mM Tris-maleate (pH 7.0) and the $choline^{+}$ influx was measured by the light scattering method, as described [6]. Briefly, the diluted vesicles were mixed in a stopped flow apparatus with a mixing solution comprising 500 mM choline–Cl, 10 mM Tris-maleate (pH 7.0), and Ca^{2+} buffer with different free Ca^{2+} concentrations prepared with 2.5 mM EGTA. The change in the scattered light intensity was then recorded. Ca^{2+} concentrations were: (a) pCa 9, (b) pCa 5. Modified from Kawasaki and Kasai [6].

late Ca^{2+} release from the heavy fraction of SR (HSR) vesicles, the permeation of choline$^+$ through the Ca^{2+} channel was studied using the light scattering method. It is well known that choline$^+$ permeates through the Ca^{2+} channel, like Ca^{2+}, and this method is convenient for studying the gating properties of the Ca^{2+} channel [19–21]. The HSR vesicles showed high permeability to choline$^+$ at pCa 4–6 [21]. Since this property was established as the CICR, the Ca^{2+} response could be estimated from the difference between the choline$^+$ permeabilities at pCa 5 and pCa 9 (Fig. 1a) [6]. Fig. 1b,c show that the Ca^{2+} response was lost when the HSR vesicles were incubated overnight at 0°C in the presence of 1 mM EDTA or EGTA. In this state, caffeine and ATP could not activate the Ca^{2+} channel. The loss of the Ca^{2+} response was attributed to the decrease in the free Ca^{2+} concentration. This loss of the Ca^{2+} response was partially recovered when the HSR vesicles were incubated with 1 mM Ca^{2+} for a few hours (data not shown) [6]. These data suggested that some important factors might be lost during the incubation with EDTA.

In order to determine the relationship between the loss of the Ca^{2+} response and the released proteins, the Ca^{2+} response and the protein profile of the supernatant solution were compared after the HSR preparations were incubated with various Ca^{2+} concentrations and then subjected to ultracentrifugation. At a Ca^{2+} concentration lower than pCa 7, the Ca^{2+} response was practically lost. At a Ca^{2+} concentration higher than pCa 6, the Ca^{2+} response increased with an increase in the Ca^{2+} concentration. When compared with the protein profile, as monitored by SDS-PAGE, a large amount of CSQ was found in the supernatant when the Ca^{2+} response was lost, among other proteins within the supernatant (data not shown) [6]. However, significant amounts of CSQ remained in the membrane fraction after the Ca^{2+} response was completely lost. The remaining CSQ might contribute to the recovery of the Ca^{2+} response after the addition of Ca^{2+}. CSQ seems to be released from intact SR vesicles, but the mechanism by which such large molecules pass through the membrane is not clear. However, this release occurred only at a low ionic strength in the absence of Ca^{2+}, and the release was inhibited in the presence of a physiological concentration of ions, such as KCl or NaCl [6]. Instability of the membrane structure at low ionic strength in the absence of Ca^{2+} might contribute to this phenomenon.

The HSR vesicles were incorporated into the lipid bilayer membrane, and the effect of CSQ was studied [6]. As shown in Fig. 2, when the vesicles were incorporated into the lipid bilayer, a slight opening of the Ca^{2+} channel was observed. In this experiment, the opening event was measured by the K^+ current, and the *trans* side corresponded to the lumenal side of the SR. When 1 mM EGTA was added to the *trans* side, the probability of opening did not change. After 2 mM $CaCl_2$ was added to the *trans* side, the probability of opening slightly increased. When 500 μg CSQ was added, the channel began to open just after the initiation of stirring. Without the addition of Ca^{2+} to the *trans* side, no activation was observed. The channel was blocked by ruthenium red, indicating that this current passed through the Ca^{2+} channel. These results show that CSQ activated the Ca^{2+} release channel from the lumenal side, and that Ca^{2+} was required for this interaction. This result is consistent with that of the vesicles, as shown in Fig. 1.

CSQ is considered to be a Ca^{2+}-storing protein within the lumen, since it has a large number of carboxyl groups and can bind many Ca^{2+} ions [22]. The present results suggest that CSQ acts as a regulator of the RyR Ca^{2+} channel in the SR membrane, in addition to the Ca^{2+} storing function. The regulatory role of CSQ was also suggested by other researchers [23,24].

3. Effects of DIDS on the RyR Ca^{2+} channel

4,4′-Diisothiocyano-stilbene-2,2′-disulfonic acid (DIDS) is a stilbene derivative, which is known as an inhibitor of the band 3 protein in red blood cells; that is, it inhibits the exchange of hydrogencarbonate and chloride [25]. It was found that the choline$^+$ permeation, as monitored by the light scattering method, was increased by the treat-

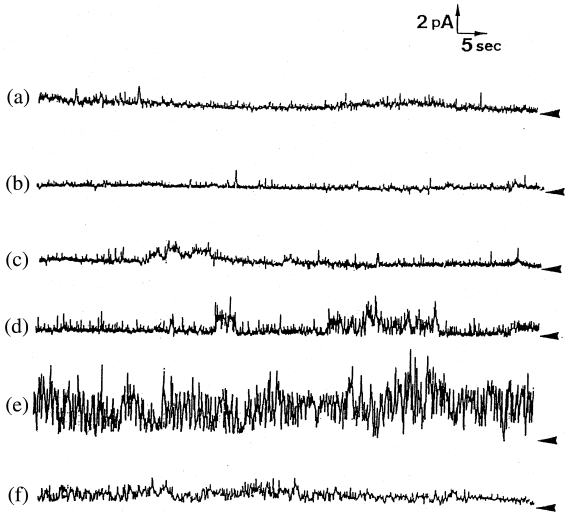


Fig. 2. Effects of CSQ on the Ca^{2+} channel activity. HSR vesicles suspended in 1.5 M KCl were incorporated into the lipid bilayer as indicated [6]. (a) Just after the incorporation, (b) 1 mM EGTA was added to the *trans* side, (c) 2 mM $CaCl_2$ was added to (b), (d) 500 μg CSQ was added to the *trans* side of (c), (e) stirring was started 5 min after the recording of (d), (f) 20 μM ruthenium red was added to the *cis* side. The holding potential was 20 mV in all cases. Arrowheads indicate the closed state of the channel. Modified from Kawasaki and Kasai [6].

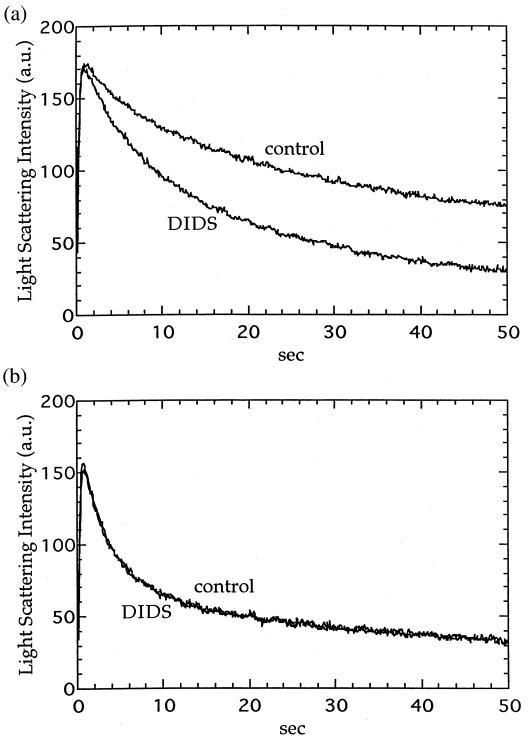


Fig. 3. Activation of choline$^+$ influx through the Ca^{2+} channel by DIDS. HSR vesicles (10 mg protein ml^{-1}) were incubated for 30 min at room temperature in 10 mM bistris-maleate (pH 7.0) in the presence and absence of 100 μM DIDS. After the incubation, the HSR vesicles were diluted 20-fold with 5 mM Tris-maleate (pH 7.0) and the choline$^+$ influx was measured by the light scattering method, as described in Fig. 1. Ca^{2+} concentrations were (a) pCa7, (b) pCa5. Modified from Yamaguchi et al. [7].

ment of the HSR vesicles with DIDS (Fig. 3) [7,18]. When the pCa dependence on the effect of DIDS was measured, it was found that DIDS opened the channel from the closed state. Next, the effect of DIDS was studied using the lipid bilayer method [18]. As shown in Fig. 4, DIDS locked the Ca^{2+} channel in the open state, but the effect is different from that of ryanodine, because DIDS fixed the channel in the completely open state, whereas ryanodine fixed it in a modified state.

Next, we tried to identify the proteins that bind to DIDS. The junctional face membrane (JFM) was incubated with $^3H_2$-DIDS and after SDS-PAGE, the gel was sliced and the radioactivity in each portion was determined [7]. As shown in Fig. 5, a peak of radioactivity was found at the 30-kDa position. The same result was obtained by an autoradiography measurement of the gel [7]. It is clear that DIDS bound to a different protein than the RyR (approx. 500 kDa) and opened the RyR channel. This result shows that this 30-kDa protein regulates the RyR.

4. Identification of the DIDS-binding 30-kDa protein

The above result indicates that the 30-kDa protein has an important function in regulating the RyR Ca^{2+} channel, but the molecular identity of the protein was not clear at that time. At first, we sought to determine the amino acid sequence of this protein. The N-terminus of the intact protein could not be sequenced. After proteolysis by V8 protease, two bands were obtained. Only

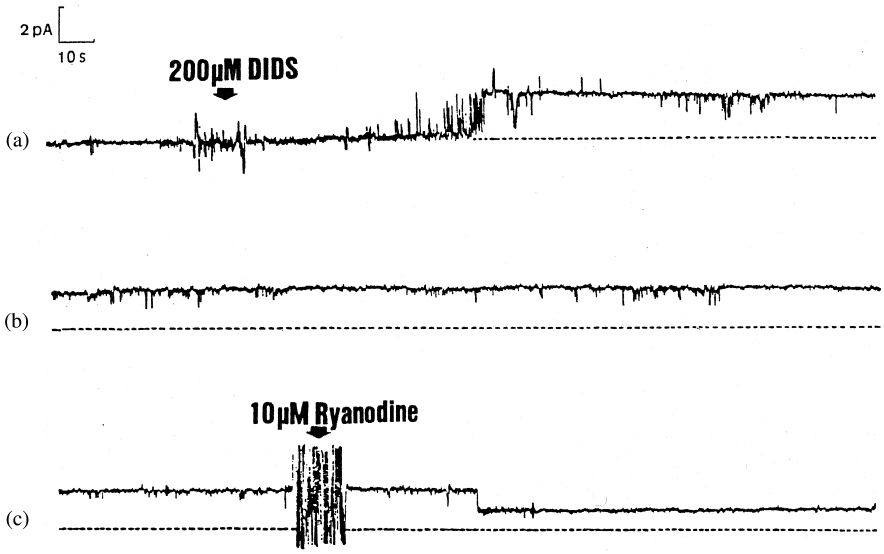


Fig. 4. Effects of DIDS and ryanodine on the Ca^{2+} channel. Experiments similar to those of Fig. 2 were carried out. (a) 100 μM DIDS was added to the *cis* solution containing 2 μM free Ca^{2+} at the point indicated by the arrow. (b) Recording after perfusion of the *cis* side with a solution containing 2 μM free Ca^{2+}, indicating that the activation by DIDS could not be reversed by perfusion. (c) 10 μM ryanodine was added to the *cis* solution of b. The single channel conductance decreased approximately 30 s after the addition of ryanodine. The dashed lines indicate the zero current levels. Modified from Kawasaki and Kasai [18].

the amino acid sequence of the small peptide could be determined, as shown in Fig. 6 [9]. This result is consistent with the assumption that the larger peptide came from the N-terminal side. The amino acid sequence was subjected to a homology search, and consequently, this protein was found to be very homologous to the ADP/ATP translocase (AAT) expressed in the mitochondria of a variety of cells [26,27].

Next, in order to eliminate the possibility of contamination of the mitochondria in our preparation, a mitochondria rich membrane fraction (MRMF) was purified, and the marker enzymes of the mitochondria were compared between the MRMF and HSR preparations [9]. The amount of the 30-kDa protein band was approximately equal in both preparations (data not shown). The activities of the mitochondrial marker enzymes, succinate cytochrome *c* reductase, were 1.675 and 0.400 units mg^{-1} protein min^{-1} in the MRMF and HSR preparations, respectively, corresponding to a fourfold enrichment in the MRMF. The activities of another marker enzyme, cytochrome *c* oxidase, were 0.225 and 0.015 units mg^{-1} protein min^{-1} in the MRMF and HSR preparations, respectively, which is more than 15 times greater in the MRMF. These results show that the contamination by the mitochondria membrane is small in our HSR preparation. Thus, we concluded that the 30-kDa protein found in the SR preparation originated from the HSR, and that it is either the same as AAT or a very similar protein [27]. To confirm this, an immunoblot analysis was carried out on the various SR membranes: HSR, JFM, and triad. The anti-AAT antibody recognized the 30-kDa protein in all of the membranes (data not shown) [9].

In addition, the effect of the antibody on Ca^{2+} release was studied. Depolarization-induced Ca^{2+} release, monitored in the rabbit skeletal muscle triads, was significantly activated by the antibody [9]. Next, since atractyroside is known as an inhibitor of AAT in mitochondria [27], its effect on the Ca^{2+} release from the SR was studied [28]. This compound inhibited the Ca^{2+} release, as measured by the light scattering method [28]. At a 10-μM concentration of cytoplasmic Ca^{2+}, atractyroside decreased the rate constant of choline^{+}

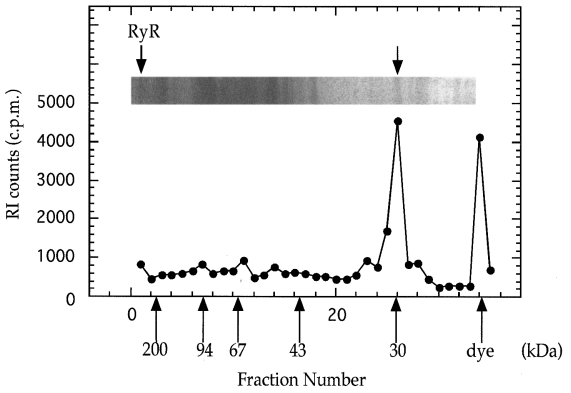
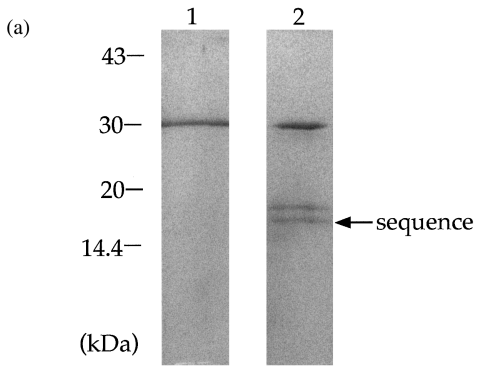


Fig. 5. Identification of the $^3H_2$-DIDS-binding protein. The $^3H_2$-DIDS-binding assay was carried out as described [7]. SDS-PAGE was performed on a 10% polyacrylamide gel using JFM (100 μg of protein) treated in 100 μM $^3H_2$-DIDS, 5 mM Hepes–Na (pH 7.4), and 10% sucrose for 1 h at room temperature. After the electrophoresis, the gel was stained with Coomassie brilliant blue R-250 for 1 h and was destained in a solution of 5% methanol and 7.5% acetic acid for approximately 10 h. The destained gel was then sliced into 3-mm sections and each section was incubated in 1 ml H_2O_2 for approximately 3 h at 90°C. After the addition of 10 ml of Scintisol 500, the radioactivity was measured in liquid scintillation counter. RyR refers to the protein, RyR, and the other arrow indicates the 30-kDa protein. Molecular weights are indicated on the bottom in kDa. Modified from Yamaguchi et al. [7].



(b)

SR 30k FSGLGN-LTKIFK-DGLRGL
AAT FTGLGNCITKIFKSDGLRGL

Fig. 6. Determination of the partial amino acid sequence of the SR 30-kDa protein. (a) Purified SR 30-kDa protein was digested by 2.5–3.0 μg protease V8, and after electrophoresis on a 15% polyacrylamide gel, the gels was stained with Coomassie brilliant blue R-250. The purified 30-kDa protein and the digested fragments are shown in lanes 1 and 2, respectively. The molecular weights are indicated on the left side of the panel in kDa. (b) The partial amino acid sequence of the 15-kDa fragment derived from the SR 30-kDa protein (30 k) is shown (SR 30 k); however, the seventh and the fourteenth residues (shown in bars) could not be determined. The amino acid sequence of positions 154 through 173 of AAT from bovine cardiac mitochondria is also shown (AAT). The underlined amino acid residues of SR 30 k are not identical to those of AAT. Modified from Yamaguchi and Kasai [9].

influx through the Ca^{2+} channels up to approximately 60%. A similar experiment was carried out using the lipid bilayer method. Atractyroside completely inhibited the channel, but interestingly, the effect was observed in only half of the cases. This result is consistent with that of the light scattering method. Furthermore, the inhibition of the Ca^{2+} channels by atractyroside was effective at lower Ca^{2+} concentrations. These results suggest that the number of Ca^{2+} channels regulated by AAT depends on the cytoplasmic Ca^{2+} concentration [28], and that a protein similar to AAT functions in the SR membrane.

Next, the cDNA encoding AAT from rabbit skeletal muscle was cloned and sequenced [9]. The deduced amino acid sequence was found to be the same as that of the CSQ-binding 30-kDa protein determined above. Furthermore, the product of the cDNA encoding AAT in *E. coli*. BL21 cells, expressed as a GST fusion protein, was proved to bind to CSQ, as discussed in the next section (Fig. 7). These results suggest that AAT itself is expressed in the rabbit skeletal muscle SR and regulates the Ca^{2+} release from the SR in vivo. These results show that AAT, the same protein expressed in the mitochondria, is also expressed in the SR but exhibits a different function. Judging from these facts, we propose that the SR 30-kDa protein is probably AAT itself; that is, AAT is a dual functional protein, involved in the transport of adenine nucleotides across the mitochondrial inner membrane and the regulation of the Ca^{2+} release from the SR. This is similar to ε-crystallin in the lens, which

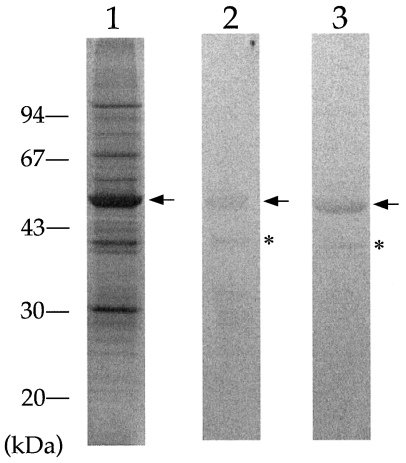


Fig. 7. Expression of the cDNA encoding rabbit skeletal muscle AAT. The plasmid encoding the open reading frame of rabbit skeletal AAT was transfected into *E. coli* BL21 cells, and expression was induced. After the cells were sonicated and solubilized in a solution comprising 1% (v/v) Triton X-100 and PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3)], the insoluble fraction was collected by centrifugation and was analyzed 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 onto a nitrocellulose membrane, and then western blot analysis using the anti-GST antibody (lane 2) or ligand blot analysis using the biotinylated CSQ (lane 3) was performed [9]. The arrows indicate the position of the GST-AAT fusion protein, and the asterisks indicate the position of the presumed digested product of the fusion protein. The molecular weights are indicated on the left side of the panel in kDa. Modified from Yamaguchi and Kasai [9].

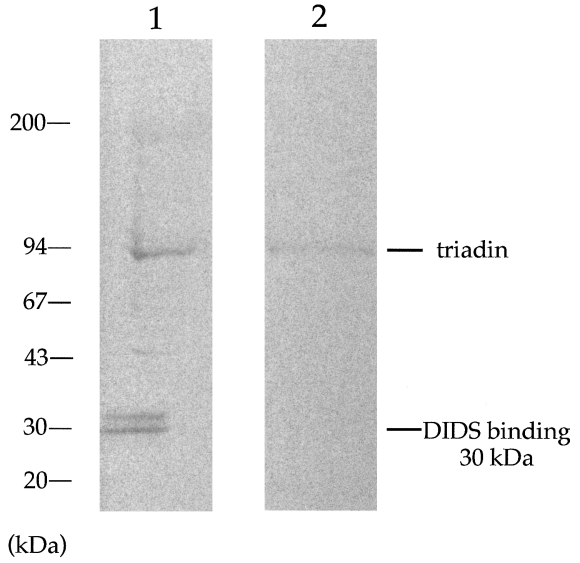


Fig. 8. Identification of CSQ interacting proteins. Ligand blot analysis using biotin labeled CSQ (400 μg) was carried out as described [7]. SDS-PAGE was carried out on discontinuous gels made of equal amounts of 7.5% and 12.5% polyacrylamide. (Lane 1) JFM (30 μg), (lane 2) partially purified triadin (5 μg). Molecular weights are indicated on the left side in kDa. Modified from Yamaguchi et al. [7].

has been shown to be identical to lactate dehydrogenase and prostaglandin F synthase [29,30].

5. Complex formation by regulator proteins

As described above, we found that CSQ and the DIDS-binding 30-kDa protein are regulators of the RyR. Furthermore, triadin and junctin were also reported to have regulatory functions [11–13]. In order to determine the relationship between these regulatory proteins, their interactions were studied. First, biotin-labeled CSQs were prepared, and the CSQ binding proteins were assayed after electrophoresis of the JFM membrane fraction [8]. As shown in Fig. 8, three bands were visible (lane 1). The band near 94 kDa was identified as triadin from comparison with the position of purified triadin (lane 2). Two bands near 30 kDa were also present. The 30-kDa protein was identified as the DIDS-binding protein reported above. Thus, CSQ was shown to bind to the 30-kDa protein and triadin. A protein near 34 kDa was also seen, but it has not yet been identified.

The CSQ binding proteins were fractionated after solubilization by CHAPS. After absorption onto the CSQ column, many proteins were eluted by the KCl gradient. The 30- and 25-kDa proteins were obtained, but triadin was not present, since it cannot be solubilized by CHAPS (data not shown) [8]. By using hydroxyapatite column chromatography and an electroelution system, the 30-kDa protein was purified [8]. The purified 30-kDa protein was confirmed by the overlay assay to bind to CSQ.

Next, the proteins that bind to the 30-kDa

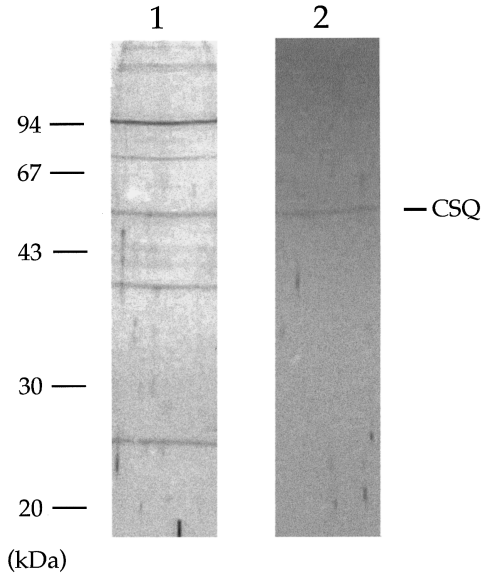


Fig. 9. HSR proteins that were adsorbed on the 30-kDa protein affinity column. The HSR (0.4 mg protein ml^{-1}) was solubilized in a solution (equilibrium solution) comprising 1% (w/v) CHAPS, 150 mM KCl, and 20 mM Hepes–Na (pH 7.4) for 1 h at 4°C and was centrifuged for 1 h at $100\,000 \times g$ (av.). The resultant supernatant was passed through the 30-kDa protein affinity column, which had been pre-equilibrated with the equilibrium solution for more than 5 h. After washing the column with 20 ml of equilibrium solution, the proteins adsorbed on the column were eluted with 200 μl of 1% CHAPS, 1 M KCl, and 20 mM Hepes–Na (pH 7.4). (Lane 1) SDS-PAGE of the eluted proteins was performed on a 12.5% polyacrylamide gel, and the gel was stained with silver. (Lane 2) Immunoblotting of the eluted proteins by anti-CSQ antiserum was performed as described [8]. The molecular weights are indicated on the left side of the panel in kDa. Modified from Kagari et al. [8].

protein were studied by affinity chromatography using the purified 30-kDa protein. As shown in Fig. 9, few proteins were found in the HSR (lane 1), and only the 25-kDa protein was found in the JFM (data not shown) [8]. The band near 57 kDa was recognized by the CSQ antibody (lane 2). From these experiments, CSQ and the 25-kDa protein were identified as proteins binding to the 30-kDa protein. The 25-kDa protein is probably the recently reported 26-kDa CSQ-binding protein (junctin) [11,12].

Previously, we showed that the 30- and 34-kDa proteins can bind to CSQ in the skeletal muscle SR [7], whereas Damiani and Margreth found 30- and 31-kDa proteins bound to [^{125}I]CSQ in skeletal muscle [31]. Furthermore, Jones and his colleagues showed that the 31-kDa protein was a minor CSQ-binding and junctin-like protein in the cardiac SR [11,12]. The 30- and 34-kDa proteins found in our previous study may correspond to the 30- and 31-kDa proteins found by Damiani and Margreth [31], respectively. Since the skeletal muscle AAT does not have a sequence homologous to junctin, the CSQ-binding and junctin-like 31-kDa protein in the cardiac SR [11,12] may correspond to the 34-kDa protein in our previous report [7] or the 31-kDa protein found by Damiani and Margreth [31].

In summary, from these experiments, we concluded that three proteins, CSQ, junctin, and the 30-kDa protein, make a ternary complex in the terminal cisternae of the SR and regulate the RyR Ca^{2+} release channel, probably through an interaction with triadin. The SR 30-kDa protein is probably identical to the mitochondrial AAT, and AAT has a dual function in mitochondria and the SR. We have developed a model of the interactions among these proteins (Fig. 10). However, the molecular mechanism underlying the regulation of the RyR Ca^{2+} release channel remains to be solved.

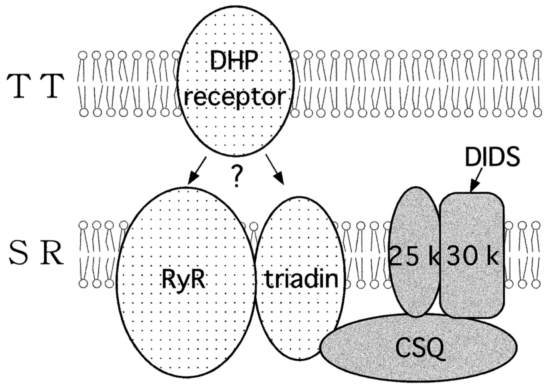


Fig. 10. A schematic model of the molecular complex and the regulation of the RyR Ca^{2+} release channel. The 25 kDa protein (junctin), the 30-kDa protein (DIDS-binding 30-kDa protein), and CSQ form the ternary complex and regulate the RyR through interactions with triadin. TT, T-tubule membrane; SR, sarcoplasmic reticulum.

6. Nomenclature

AAT:	ADP/ATP translocase
CICR:	Ca^{2+}-induced Ca^{2+} release
CSQ:	Calsequestrin
E–C coupling:	Excitation–contraction coupling
DICR:	Depolarization-induced calcium release
DIDS:	4,4′-diisothiocyanostilbene-2,2′-disulfonic acid
HSR:	Heavy fraction of sarcoplasmic reticulum
JFM:	Junctional face membrane
MRMF:	Mitochondria-rich membrane fraction
RyR:	Ryanodine receptor
SR:	Sarcoplasmic reticulum.

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