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Endogenous, Ca^{2+}-dependent Cysteine-Protease Cleaves Specifically the Ryanodine Receptor/Ca^{2+} Release Channel in Skeletal Muscle

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Abstract. The association of an endogenous, Ca^{2+}-dependent cysteine-protease with the junctional sarcoplasmic reticulum (SR) is demonstrated. The activity of this protease is strongly stimulated by dithiothreitol (DTT), cysteine and β-mercaptoethanol, and is inhibited by iodoacetamide, mercuric chloride and leupeptin, but not by PMSF. The activity of this thiol-protease is dependent on Ca^{2+} with half-maximal activity obtained at 0.1 μM and maximal activity at 10 μM. Mg^{2+} is also an activator of this enzyme (CI_{50} = 22 μM). These observations, together with the neutral pH optima and inhibition by the calpain I inhibitor, suggest that this enzyme is of calpain I type.

This protease specifically cleaves the ryanodine receptor monomer (510 kD) at one site to produce two fragments with apparent molecular masses of 375 and 150 kD. The proteolytic fragments remain associated as shown by purification of the cleaved ryanodine receptor. The calpain binding site is identified as a PEST (proline, glutamic acid, serine, threonine-rich) region in the amino acid sequence GTPGGTPQPGVE, at positions 1356–1367 of the RyR and the cleavage site, the calmodulin binding site, at residues 1383–1400. The RyR cleavage by the Ca^{2+}-dependent thiol-protease is prevented in the presence of ATP (1–5 mM) and by high NaCl concentrations. This cleavage of the RyR has no effect on ryanodine binding activity but stimulates Ca^{2+} efflux. A possible involvement of this specific cleavage of the RyR/Ca^{2+} release channel in the control of calpain activity is discussed.

Key words: Ryanodine receptor — Ca^{2+} release channel — Calpain — Junctional sarcoplasmic reticulum — Ca^{2+} release

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Introduction

Calcium-activated neutral proteases (CANP), also known as calpain (calcium-dependent papain-like), are a group of cysteine endopeptidases that have neutral pH optima and are absolutely dependent on Ca^{2+} for catalytic activity [23, 26, 34]. There are two homologous isoenzymes with different Ca^{2+} sensitivity; calpain I and calpain II, with low (μM) and high (mM) Ca^{2+} concentration requirement, respectively [23]. The activity of calpain is also believed to be regulated by an endogenous inhibitor, calpastatin [24] and activator [7] proteins.

These Ca^{2+}-activated thiol-proteases have been suggested to function in various Ca^{2+}-mediated cellular processes such as in activation of protein kinase C, in turnover of myofibrillar proteins and that of receptors for hormone and growth factors [6, 21, 26, 34, 37].

CANP have been isolated from many tissues including brain [10] skeletal [12] cardiac [20] and smooth [27] muscles.

It is generally accepted that these enzymes are predominantly cytosolic. However, association of the CANP and its specific inhibitor protein with cellular membranes has been suggested [2, 9, 18, 21, 22, 32]. The association of both calpain and its inhibitor calpastatin with isolated junctional sarcoplasmic reticulum (JSR) has been reported [2]. Further localization indicates that calpain activity is associated with the transverse tubule membranes [2]. It has also been shown that the SR Ca^{2+} release channel, the receptor for the toxic alkaloid ryanodine, is specifically cleaved by calpain [2, 28, 32].

In this study, we have characterized an endogenous

Ca^{2+}-dependent cysteine-protease of the JSR membranes that specifically cleaves one PEST site on the ryanodine receptor/Ca^{2+} release channel.

Materials and Methods

Materials

ATP, EGTA, Tris, Tricine, MOPS, PMSF, CHAPS, PIPES, polyethylene glycol, iodoacetamide and DTT were obtained from Sigma. [^{3}H]ryanodine (60 Ci/mmol) and $^{45}CaCl_2$ were purchased from New England Nuclear. Unlabeled ryanodine and Calpain I inhibitor were obtained from Calbiochem.

Membrane Preparations

JSR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Lai et al. [17], or according to Saito et al. [31]. Triads were prepared as described by Caswell et al. [4]. The membranes were suspended to a final concentration of about 20 mg protein/ml in a buffer containing 0.3 M sucrose and 10 mM PIPES, pH 7.4 and stored at −70°C. Protein concentration was determined by the method of Lowry et al. [19].

Purification of the Ryanodine Receptor

HSR membranes were incubated without (control) or with 20 mM DTT for 60 min in 50 mM Tris, pH 7.5, then the membranes were collected by centrifugation (40,000 × *g*, 30 min). The pellets were resuspended to 2 mg/ml in a medium containing 0.5 M NaCl, 20 mM MOPS, pH 7.4, 1.3% CHAPS and the following protease inhibitors; 0.2 mM PMSF, 0.8 mM benzamidine, 0.5 μg/ml leupeptin and 0.2 mM EGTA. Ryanodine receptor was purified from these membranes by the spermine-agarose method [33]. The purified receptor protein (30 to 70 μg/ml) was assayed for [^{3}H]ryanodine binding (in 0.1 ml) as described below, except that soybean lecithin (0.5 mg/ml) was present in the assay medium. After 2 hr at 30°C, the bound ryanodine was assayed by polyethylene glycol 600 (PEG) precipitation in the presence of carrier protein (1.4 mg/ml BSA), followed by filtration through Whatmann GF/B filters and 3 × 4 ml washes with 10% PEG solution [33]. Protein concentration of the purified ryanodine receptor was determined according to Kaplan and Pedersen [13].

Ca^{2+} Efflux from Passively Loaded Vesicles

HSR vesicles were incubated with or without 20 mM DTT as described above, then the membranes were collected by centrifugation (30,000 × *g*, 20 min). The pellets were resuspended to about 3 mg/ml in a medium containing 0.3 M KCl, 20 mM MOPS, pH 6.8 and 0.4 mM $CaCl_2$ (containing $^{45}CaCl_2$, 5×10^4 cpm/nmol) and incubated for 90 min at 24°C. For Ca^{2+}efflux assay the loaded vesicles (25 μl) were placed on 0.45 μm nitrocellulose filters and rinsed with different volumes of 0.3 M KCl and 20 mM MOPS, pH 6.8 and 1 mM EGTA solution for the indicated time. The flow rate was about 1 ml/sec.

Gel Electrophoresis

The membranes were diluted 1:3 with buffer containing: 260 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 4% (v/v) β-mercaptoethanol and incubated for 3 min at 100°C. The analysis of protein profile by SDS-polyacrylamide slab gel electrophoresis was performed using Laemmli's [16] discontinuous buffer system in 1.5 mm thick slab gels with 5, 2.6–6 or 3–13 acrylamide, using 3% stacking gel. Gels were stained with Coomassie Brilliant Blue. Molecular weight standards were: myosin, 200,000, β-galactosidase, 116,000; phosphorylase b, 97,400; bovine serum albumin, 66,200 and ovalbumin, 42,700 (Bio Rad) and the phosphorylase b crosslinked 97 to 873 kD, monomer to octamer (Sigma). Quantitative analysis of the protein bands was performed by densitometric scanning of the Coomassie stained gels, using a Molecular Dynamics computing densitometer. Analysis was performed using ImageQuant software provided by the manufacturer.

Internal Amino Acid Sequence Analysis

This was carried out essentially as described previously [1]. Briefly, the ryanodine receptor was purified from DTT-treated HSR in which the receptor was cleaved to two fragments (375 and 150 kD). This purified receptor or HSR treated with DTT was separated by SDS-PAGE. The 150 kD protein bands obtained only in the DTT-treated membranes or in the RyR purified from them were cut from stained gels, and four to six bands were concentrated in each well of a second gel. The protein bands in this gel were electroblotted in a Hoefer transblot system onto PVDF membranes as described previously [36]. After transfer, the protein bands were stained with Ponceau S, cut out and transferred to an Eppendorf tube. The four cut bands were treated with polyvinylpyrrolidone (PVP-40), to prevent protein absorption to the nitrocellulose and subjected to *in situ* cleavage of the electroblotted protein, by specific grade of trypsin (Promega) or of *Staphylococcus aureus* protease (V8) as described previously [1]. The tryptic fragments were separated on a C18 reverse-phase column and eluted with a linear gradient of 0–84% acetonitrile in 0.1% trifluoroacetic acid at 80 μl/min as described [11]. Several HPLC-separated peptides were subjected to amino acid sequence using amino acid sequencer (Applied Biosystem, model 476) as also described in [11].

Abbreviations

EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tricine, *N*-[2-hydroxy-1, 1-bis (hydroxymethyl)-ethyl]-glycine, MOPS, 3-(*N*-morpholino) propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; PMSF, phenylmethylsulfonyl-fluoride; DTT, dithiothreitol; CHAPS, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HSR, heavy SR; PEST, proline, glutamic acid, serine, threonine-rich region; RyR, ryanodine receptor.

Results

Figure 1 shows that in the presence of DTT the 510 ± 20 kD (n = 5) polypeptide band, the ryanodine receptor monomer, is cleaved to two bands with apparent molecular masses of 375 ± 22 kD (n = 5) and 150 ± 7 kD (n = 5). These apparent molecular masses were obtained in the specified electrophoretic conditions and based on the

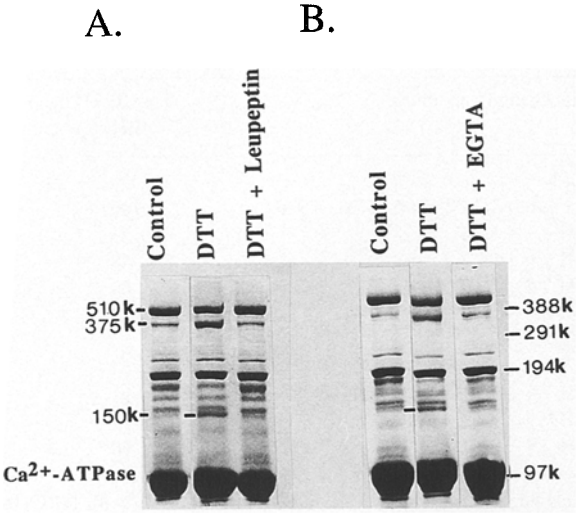

Fig. 1. Specific cleavage of the ryanodine receptor by endogenous, Ca^{2+}-activated thiol-protease. HSR membranes (1 mg/ml) were incubated without (*control*) or with DTT (5 mM) and EGTA (1 mM) or leupeptin (1 μM) in 50 mM Tris, pH 7.5. After 30 min at 30°C, aliquots of the treated membranes (50 μg) were solubilized in SDS-buffer and subjected to SDS-PAGE (5% acrylamide) as described under Materials and Methods. The numbers on the right indicate the molecular masses of phosphorylase b (97 kD) and its oligomeric forms.

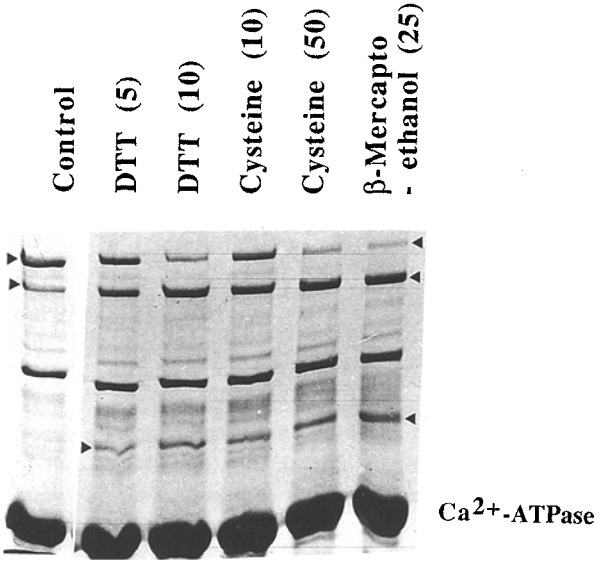

Fig. 2. SDS-PAGE profiles of SR membranes incubated with different reducing agents. HSR membranes (1.0 mg/ml) were incubated without or with the indicated reagents for 30 min at 30°C and analyzed by SDS-PAGE as described in Fig. 1. The numbers in parentheses indicate the concentration of the reagents in millimolar. The arrows indicate the RyR monomer and its proteolytic products.

molecular weight standard used for the proteins. In most of the SR preparations, no other proteins were observed to be cleaved under these conditions (*see* Figs. 3, 5, 7). Stimulation by DTT suggests that cysteine-protease is involved in this specific cleavage of the RyR. This thiol-activated proteolytic activity is also Ca^{2+} dependent. In the presence of DTT, addition of EGTA completely prevented the cleavage of the RyR (Fig. 1*B*). This suggests that the Ca^{2+} contamination in the assay medium is sufficient for the activation of this endogenous thiol-protease. Figure 1 also shows that the Ca^{2+}-activated thiol-protease activity is inhibited by the peptide aldehyde inhibitor leupeptin. Ryanodine binding activity of the DTT-treated membranes was the same as that of untreated vesicles (*data not shown, see* Fig. 8).

Further characterization of this endogenous protease is presented in the following series of experiments. Figure 2 shows that not only DTT, but also other thiol reagents such as β-mercaptoethanol and cysteine, caused almost complete conversion of the 510 kD to 375 and 150 kD polypeptides.

Figure 3 shows the dependency of 510 kD cleavage on DTT concentration. Quantitative analysis of the 510 kD and of the proteolytic products 375 and 150 kD shows that treatment of the SR with increasing DTT concentrations leads to a progressive disappearance of the 510 kD, and in parallel, the appearance of the 375 and 150 kD protein bands. The relative proportions of the 375 kD, 150 kD proteins and 510 kD suggest that the two

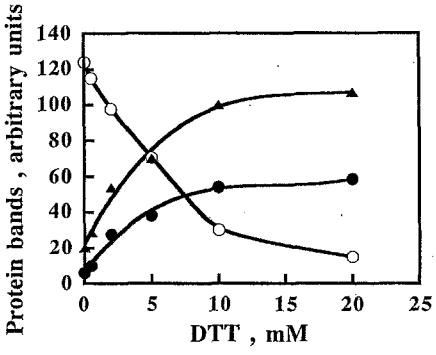

Fig. 3. DTT dependence of the ryanodine receptor cleavage by the endogeneous protease. HSR membranes (1 mg/ml) were incubated in the absence and in the presence of the indicated DTT concentration as described in Fig. 1. Aliquots were subjected to SDS-PAGE followed by quantitative analysis of the ryanodine receptor monomer (○) and of the proteolytic products 375 kD (▲) and 150 kD (●) protein bands, as described in Materials and Methods.

newly formed bands are derived from the 510 kD protein band.

Similar results were obtained when the cleavage of the 510 kD by the Ca^{2+}-activated endogenous protease was analyzed as a function of time (Fig. 4). Quantitative analysis shows that increasing the time of exposure to DTT resulted in the disappearance of the ryanodine receptor monomer and in an accumulation of the cleavage products. This quantitative analysis, as that in Fig. 3,

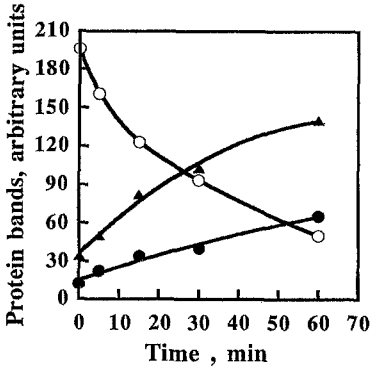

Fig. 4. Cleavage of the ryanodine receptor by Ca^{2+}-activated thiol-protease as a function of incubation time. HSR membranes (1.0 mg/ml) were incubated without or with 10 mM DTT for the indicated time as described in Fig. 1. Aliquots (50 μg) were subjected to SDS-PAGE (3 to 6% acrylamide), and to quantitative analysis of the 510 kD and of the proteolytic products as described under Materials and Methods. The relative amount of the 510 kD (○) and of the 375 (▲) and 150 kD (●) cleavage products are plotted as a function of time.

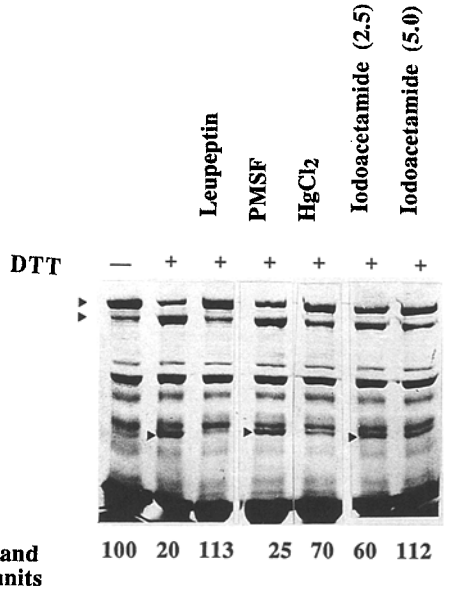

Fig. 5. Effects of antiproteases on the cleavage of the ryanodine receptor by the endogenous, Ca^{2+}-activated thiol-protease. HSR membranes (1 mg/ml) were incubated for 30 min with 10 mM DTT in the absence or presence of the indicated protease inhibitors and then subjected to SDS-PAGE and to quantitative analysis, as described in Materials and Methods. Control (100%) represents the relative amount of the 510 kD protein band (indicated by the first arrowhead) in HSR membranes incubated without DTT. The concentrations of the reagents were; 1 μM, 0.2 mM and 5 mM for leupeptin, PMSF and $HgCl_2$, respectively. The indicated concentrations of iodoacetamide are in mM.

suggests the existence of a direct stoichiometric relationship between the 510 kD and the 375 and 150 kD proteolytic products.

The effects of different protease inhibitors on the cleavage of the 510 kD by the Ca^{2+}-dependent thiol-protease is presented in Fig. 5. The cleavage of the 510 kD is strongly inhibited by the thiol-protease inhibitors, iodoacetamide and mercuric chloride. As expected, the inhibitory effect of iodoacetamide and mercuric chloride was obtained at lower concentrations of the inhibitors when the membranes were preincubated with the reagents before the addition of DTT (*data not shown*). The cleavage is also sensitive to leupeptin, as shown previously for other thiol-proteases [20, 40]. Lack of inhibition by PMSF shows that the enzyme is not a serine protease. Calpain I inhibitor (*N*-acetyl-Leu-Leu-norleucinal) at 0.2 μM inhibits completely the cleavage of the RyR by the endogenous Ca^{2+}-dependent thiol-protease (*data not shown*). These findings confirmed that the 510 kD cleaving enzyme is a thiol-protease and most probably a calpain I type protease (*see* Note Added in Proof).

Table. Effect of pH, ATP and NaCl on the endogenous Ca^{2+}-activated thiol-protease

Preincubation conditions	510 kD band Relative amount
Exp. I	
Control (-DTT); pH 6.1, 7.0, 7.5, 8.0	100
pH 6.1 + DTT	81
pH 7.0 + DTT	48
pH 7.5 + DTT	60
pH 8.0 + DTT	72
Exp. II	
pH 7.5 + DTT	54
pH 7.5 + DTT + NaCl (0.3 M)	91
pH 7.5 + DTT + ATP (1 mM)	77
pH 7.5 + DTT + ATP (5 mM)	90

SR membranes (1 mg/ml) were incubated for 30 min with DTT (10 mM) at different pH values as in Fig. 1. In Exp. II, NaCl or ATP at the indicated concentration was present. Aliquots were subjected to SDS-PAGE and to quantitative analysis of the 510 kD as described in Materials and Methods. The buffers used were: 50 mM MOPS for pH 6.5 and 50 mM Tris for pH 7.0, 7.5 and 8.0.

The pH dependence of the RyR specific cleavage by the endogenous protease is shown in the Table. Maximum RyR cleavage was observed between pH 7.0 and 7.5. Similar optimal pH was reported for calpain I [40]. The presence of ATP (1–5 mM) and of high NaCl concentrations in the preincubation of the membranes with DTT, strongly decreases the cleavage of the RyR (Table).

The cleavage of the RyR by the endogenous Ca^{2+}-dependent thiol-protease was not affected by the incubation temperature or by preincubation of the membranes with ruthenium red or with ryanodine at low (20 nM) or high (5 μM) concentrations (*data not shown*).

The dependency of the endogenous protease activity, as reflected in the specific cleavage of the 510 kD, on Ca^{2+} concentration is shown in Fig. 6. Half-maximal

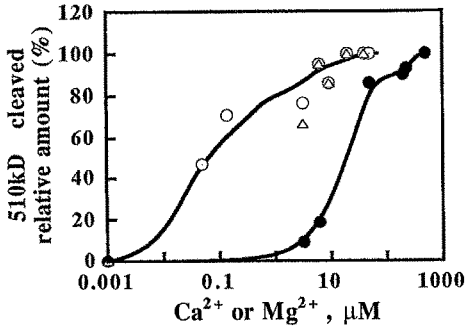

Fig. 6. Ca^{2+} and Mg^{2+} dependency of the thiol-activated endogenous protease. HSR (1 mg/ml) were incubated for 30 min with 20 mM DTT, and 0.5 mM EGTA and in the presence of the indicated free concentrations of Ca^{2+} (○, △) or Mg^{2+} (●). Aliquots (50 μg of protein) were subjected to SDS-PAGE and to quantitative analysis as described in Materials and Methods. Free Ca^{2+} and Mg^{2+} concentrations were calculated with a computer program using the EGTA association constants reported by Fabiato [8]. (○, △) indicate two different experiments with two different HSR preparations.

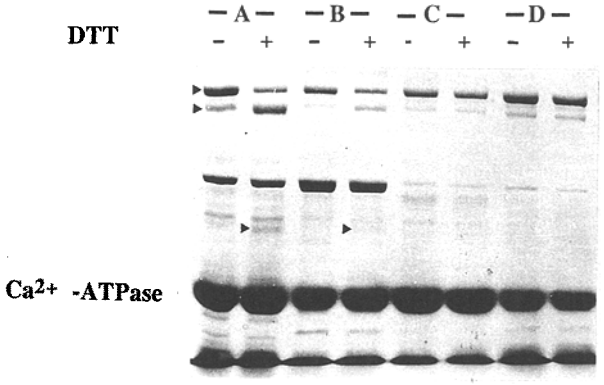

Fig. 7. The presence of endogeneous Ca^{2+}-activated cysteine-protease in different SR membrane preparations. Junctional SR membranes were prepared according to Lai et al. [17] (*A*), Saito et al. [31] (*B*). The HSR in *B* are the unfractionated heavy SR preparation from which R_3 (*C*) and R_4 (*D*) were obtained by sucrose gradient [31].

activity was obtained at below 0.1 μM and maximal activity was observed in the presence of 10 μM of Ca^{2+}. In addition to activation by Ca^{2+}, the endogenous protease is also activated by Mg^{2+} with half-maximal activation at about 22 μM of Mg^{2+}. Activation by Mg^{2+} was previously observed with the Ca^{2+}-activated protease isolated from skeletal muscle [6]. Activation by Mn^{2+}, Ba^{2+} and Sr^{2+} and only slightly by Mg^{2+} was reported for calpain I [26, 40].

This Ca^{2+}-dependent, cysteine-protease activity was not observed in all the different SR preparations tested. As shown in Fig. 7, JSR isolated according to Lai et al. [17] or Saito et al. [31] show the activation of 510 kD cleavage by DTT. However, further fractionation of the HSR isolated according to Saito et al. [31] resulted in R_3 and R_4 fractions without DTT-activated endogeneous protease activity. This could be due to either inactivation or removal of this protease during this fractionation. When the protease-containing triads (terminal cisternae/T-system membranes) [4] were disrupted in a French Press following separation on an isopycnic gradient, the ryanodine receptor containing fractions showed no cleavage of the RyR by endogenous, Ca^{2+}-dependent cysteine-protease (*data not shown*). This observation is in agreement with a previous study [2] showing that calpain activity is associated with the transverse tubule membranes.

To test whether the proteolytic products 375 and 150 kD fragments remain associated, we purified the ryanodine receptor using the spermine-agarose method [33], from SR membrane where the RyR was cleaved by the endogenous Ca^{2+}-dependent, cysteine-protease. Figure 8 shows the SDS-PAGE protein profile of the CHAPS extracts of control and that of DTT-treated membranes applied to the spermine-agarose columns and of two fractions eluted from each column by 2 mM of spermine. The results clearly show that the proteolytic products remain associated after solubilization and purification of the ryanodine receptor. The binding of [^{3}H]ryanodine to the receptor purified from DTT-treated membranes is similar to that of receptor purified from control membranes, indicating that the cleavage of the receptor has no effect on its ryanodine binding activity as has been shown previously [2, 28]. Furthermore, the results demonstrate clearly that the receptor is cleaved at only one site in each monomer to yield the 375 and 150 kD fragments.

To identify the protease cleavage site, we have purified the 150 kD proteolytic fragment from gels in which the RyR purified from DTT-treated membranes was subjected to SDS-PAGE. The 150 kD protein bands were cut, concentrated in a second gel and then electroblotted onto PVDF membranes, and the blots were applied to *in situ* trypsin or *S. aureus* protease (V_8) digestion. The resulting peptide fragments were separated by reverse phase HPLC. Selected peptides were applied to a gas phase sequenator, and their sequences are presented in Fig. 9, together with the corresponding sequences of the ryanodine receptor monomer [35].

Figure 10 shows the effect of cleavage of the ryanodine receptor by the Ca^{2+}-dependent, thiol-endogenous protease on the Ca^{2+} efflux from HSR vesicles passively loaded with $^{45}CaCl_2$. As shown, the cleavage of the RyR enhances Ca^{2+} efflux from the Ca^{2+}-loaded SR vesicles. The extent of Ca^{2+} efflux enhancement is dependent on the degree of cleavage of the RyR as controlled by the DTT concentration (Fig. 10*B*). It is most likely that this enhanced Ca^{2+} efflux is not due to a direct effect of DTT on the Ca^{2+} release system, because the presence of leupeptin during the treatment with DTT prevented both the

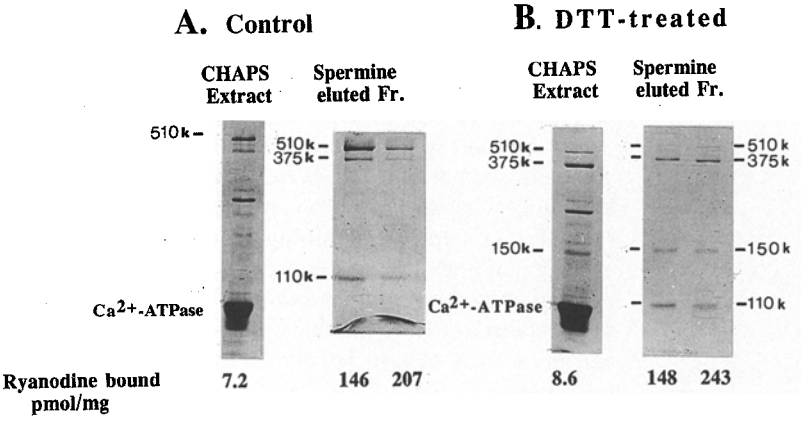

Fig. 8. SDS-PAGE protein profiles of ryanodine receptor purified from control and DTT-treated membranes. Ryanodine receptor was isolated from HSR membranes incubated without (*A*) or with 20 mM DTT (*B*) using the spermine-agarose method, as described in Materials and Methods. The SDS-PAGE patterns of the CHAPS extracts and of two fractions eluted from the column with 2 m*M* spermine are shown. The specific ryanodine binding activity is also presented at the bottom of the gel.

641 VTSIRPNIFVG RAEGSTQYG 660 Trypsin
RPNIFVGR

685 WALTEGYSPYPGGGEGWGGNGVDD 710 Trypsin
GYSPYPXXXEG X X GN XV

1351 GTAKEGTPGGTPQPGVEAQP 1370 V8
GTPGGTPQPGVE

Fig. 9. Deduced amino acid sequence for the tryptic fragments of the 150 kD polypeptide derived from the RyR by the endogenous Ca^{2+}-dependent thiol-protease. The 150 kD fragment derived from the RyR was purified, electroblotted onto PVDF membranes and subjected to trypsin or V8 digestion, as described in Materials and Methods. The reverse-phase HPLC separation of the digest mixture on a C18 column was carried out as described previously [11]. The peptide peaks were sequenced as described in Materials and Methods and the obtained sequences, in the single letter amino acid code, are presented with the corresponding RyR sequences published in [35].

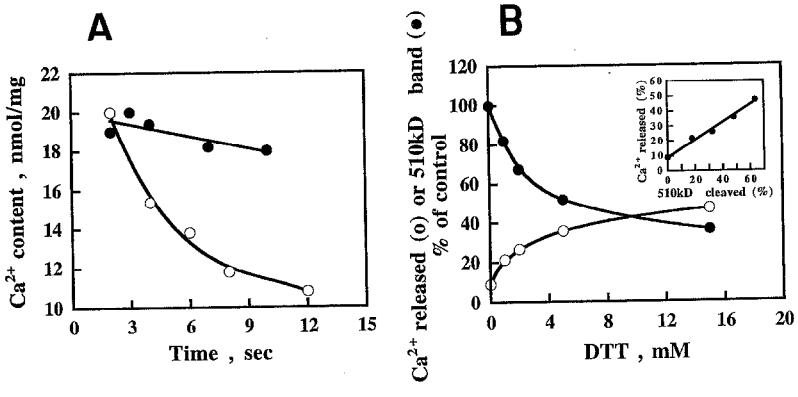

Fig. 10. Ca^{2+} efflux from intact and cleaved ryanodine receptor containing HSR vesicles. In *A*, control (●) and DTT (20 mM)-treated (○) SR vesicles were prepared, loaded with $^{45}CaCl_2$ and assayed for Ca^{2+} efflux, as described in Materials and Methods. In this experiment, only about 50% of the 510 kD protein band was cleaved to the 375 and 150 kD fragments. In *B*, SR membranes were incubated for 30 min with the indicated concentrations of DTT in the absence (treated) or in the presence of 5 μM leupeptin (control). The vesicles were loaded with $^{45}Ca^{2+}$ and assayed for Ca^{2+} efflux, as described in Materials and Methods. The presented amount of the 510 kD protein band is relative to that of corresponded control (treated with DTT and leupeptin). In the inset, the data from *B* have been replotted where the percentage of Ca^{2+} released was plotted as a function of the percentage of the 510 kD (RyR) cleaved at the different DTT concentration.

enhancement of Ca^{2+} efflux and of RyR cleavage (controls in Fig. 10*B*).

Discussion

In the present study we show the association of an endogenous Ca^{2+}-dependent cysteine-protease with the junctional SR membranes. This protease is activated by submicromolar concentrations of Ca^{2+} ($C_{50} \leq 0.1$ μM), has optimal pH of about 7.0, its activity is absolutely dependent on the presence of thiol-reducing reagents and completely inhibited by calpain I inhibitors. These findings suggest that this is a calpain I type protease (*see* Note Added in Proof). This endogenous, Ca^{2+}-

dependent thiol-protease cleaved specifically the ryanodine receptor/Ca^{2+} release channel at one site (Fig. 8), regardless of the Ca^{2+} or reducing reagent concentration, the time- or temperature-incubation or other incubation conditions such as the presence of ryanodine or ruthenium red—compounds known to interact with the Ca^{2+} release channel and to modify its activities.

These observations suggest that this cleavage site is the only one exposed to the protease and/or recognized by it. The accessibility of this site, however, is modified by high concentrations of NaCl or by the presence of ATP (Table). High NaCl concentrations and ATP stimulate ryanodine binding probably by stabilization of a RyR conformational state with higher binding affinity for ryanodine [41]. As shown in the Table, the protein conformation stabilized by high NaCl concentrations or ATP is less sensitive to the endogenous protease. Exogenously added calpain II (requiring mM Ca^{2+}) to SR membranes degraded the ryanodine receptor/Ca^{2+} release channel to several fragments, and this cleavage is inhibited by calmodulin [2].

It is accepted that calpain catalyzes specific and limited cleavage of substrates including enzymes, myofibrillar proteins, membrane proteins, cytoskeletal proteins, and receptor proteins [5, 21, 27, 37]. It has been suggested that calmodulin binding proteins are substrates for calpain [38] and that these proteins are recognized by calpain through their PEST sequence [38]. PEST regions were suggested to confer the property of rapid degradation to the protein containing them [30]. The RyR/Ca^{2+} release channel has eight PEST sequences [2]. Using such predicated PEST sequences for proteolysis and based on the sequences of several proteolytic fragments of the 150 kD protein band derived from the RyR by the endogenous Ca^{2+}-dependent thiol-protease (Fig. 9), the PEST sequence GTPGGTPQPGVE at the positions 1356–1367 is suggested as the protease binding site. The predicated splitting site is near a calmodulin binding site [38]. Several candidate sites (up to six [39]) for calmodulin binding in RyR were suggested including sites at residues 1383–1400 and 1974–1996 [2, 3, 35, 42]. Thus, based on the apparent molecular mass of the proteolytic fragments 150 k, the cleavage site is suggested to be at residues 1383–1400.

The function of this specific cleavage of the RyR/Ca^{2+} release channel is not clear. Rardon et al. [28] have shown that calpain treatment of junctional SR membranes increased the open probability of the Ca^{2+} release channel. This observation is in agreement with the observed enhanced Ca^{2+} efflux from SR vesicles where the RyR was cleaved at one site to yield the two fragments (Fig. 10). Calpains first attracted attention as a protease that catalyzes the degradation of muscle proteins [3, 5, 6, 14, 15, 29]. Calpain activity is regulated by the intracellular Ca^{2+} concentration. Thus, there is a tight coupling between Ca^{2+} mobilization and modulation of the calpain activities. It has been suggested that the enhanced activity of the Ca^{2+}-dependent protease in muscle is responsible for several structural changes occurring under pathological conditions such as in atrophying muscle tissue and myocardial infection [15, 25, 26]. Thus, for pathophysiological consequences, a hypothesis for sequential events leading to Ca^{2+}-regulated muscle degradation could be postulated. Upon a small increase in intracellular Ca^{2+}, a membrane-associated Ca^{2+}-dependent protease (calpain I) is activated. This would specifically cleave the RyR, thereby activating Ca^{2+} release. These initial events would lead to a further increase in the intracellular Ca^{2+} concentration that would further increase the protease activity (calpain I and II) and lead to muscle degradation. Further pursuit of this hypothesis may reveal a novel role for this Ca^{2+}-dependent thiol-protease in the control of proteolysis of muscle proteins.

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Note Added in Proof

In most of our HSR preparations the $[Ca^{2+}]$ requirement for the specific cleavage of the RyR is in the nM range. Thus, it is possible that the calpain activity demonstrated in this study is of the newly identified skeletal muscle–specific calpain (nCL-1) (Sorimachi, H., Saido, T.C., Suzuki, K. 1994. New era of calpain research, discovery of tissue—specific calpains. *FEBS Lett.* **343:**1–5).