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Identification, Characterization and Partial Purification of a Thiol-protease Which Cleaves Specifically the Skeletal Muscle Ryanodine Receptor/Ca^{2+} Release Channel

S. Shevchenko[1], **W. Feng**[1], **M. Varsanyi**[2], **V. Shoshan-Barmatz**[1]

[1]Department of Life Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel
[2]Institute fuer Physiologische Chemie, Ruhr Universitaet, D-44780 Bochum, Germany

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Abstract. A 94 kDa large subunit thiol-protease, as identified by anti-calpain antibodies, has been isolated from skeletal muscle junctional sarcoplasmic reticulum (SR). This protease cleaves specifically the skeletal muscle ryanodine receptor (RyR)/Ca^{2+} release channel at one site resulting in the 375 kDa and 150 kDa fragments. The 94 kDa thiol-protease degrades neither other SR proteins nor the ryanodine receptor of cardiac nor brain membranes. The partially purified 94 kDa protease, like the SR associated protease, had an optimal pH of about 7.0, was absolutely dependent on the presence of thiol reducing reagents, and was completely inhibited by $HgCl_2$, leupeptin and the specific calpain I inhibitor. However, while the SR membrane-associated protease requires Ca^{2+} at a submicromolar concentration, the isolated thiol-protease has lost the Ca^{2+} requirement.

The 94 kDa thiol-protease had no effect on ryanodine binding but modified the channel activity of RyR reconstituted into planar lipid bilayer: in a time-dependent manner, the channel activity decreases and within several minutes the channel is converted into a subconducting state. The protease-modified channel activity is still Ca^{2+}-dependent and ryanodine sensitive.

This 94 kDa thiol-protease cross react with anti-calpain antibodies thus, may represent the novel large subunit of the skeletal muscle specific calpain p94.

Key words: SR — Ryanodine receptor — Calpains — p94

Introduction

Calcium-activated neutral proteases (CANP; EC 3.4.22.17), 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 [29, 38, 46]. There are two homologous isoenzymes with different Ca^{2+} sensitivity: μ-calpain and m-calpain, with low- (μM) and high- (mM) Ca^{2+} concentration requirements, respectively [14, 29]. Calpains (m and μ) have been isolated from many tissues, including brain [12] skeletal [15], cardiac [23], and smooth [33] muscles, and were found to have similar structural and biochemical characteristics. While m- and μ-calpain are expressed ubiquitously, the tissue-specific novel calpains were recently discovered [38, 44–48]. These calpains are expressed specifically in skeletal muscle [43, 47, 48] and stomach [44] and named n-calpain (p94 or nCL-1). Based on their primary amino acid sequence deduced from cDNA the large subunit of n-calpains is expected to have a molecular weight of 94 kDa and to be activated by nano-molar concentrations of Ca^{2+} [38, 46, 48]. Muscle-specific calpain, p94, was identified during fractionation of connectin using specific antibodies and was found to be associated with connectin through a p94-specific sequence [45]. The p94 has a unique feature of rapid autolysis [47].

Limited tissue distribution of n-calpain suggests a restricted range of substrate specificity and may be very

Correspondence to: V. Shoshan-Barmatz

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; HSR, heavy SR; CHAPS; 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane-sulfonate; PMSF; phenylmethylsulfonyl fluoride; RyR, ryanodine receptor, ATPase, Ca^{2+}-ATPase; CS, Calsquestrin.

important for the calpains specificity evaluation in general. There are various hypotheses that attempt to provide a common principle of calpain specificity [37, 50, 52] but none is generally recognized. Calpain activity is also believed to be regulated by both an endogenous inhibitor, calpastatin [30], and by activator proteins [7].

It has been suggested that these Ca^{2+}-activated thiol-proteases function in various Ca^{2+}-mediated cellular processes such as activation of protein kinase C, in turnover of myofibrillar proteins, and of receptors for hormone and growth factors [24, 38, 50]. Calpain catalyzes specific and limited cleavage of substrates including enzymes, myofibrillar proteins, membrane proteins, cytoskeletal proteins and receptor proteins [6, 24, 33]. It has been suggested that calmodulin binding proteins are substrates for calpain and that these proteins are recognized by calpain through their PEST sequence [37]. It was suggested that PEST regions confer the property of rapid degradation to the protein containing them [53].

Calpains first attracted attention as a protease which catalyzes the degradation of muscle proteins [6, 17, 35]. Calpain activity is regulated by the intracellular Ca^{2+} concentration. As such, there is a tight coupling between Ca^{2+} mobilization and modulation of the calpains' activities. It has been suggested that 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 [17].

It is generally accepted that these enzymes are predominantly cytosolic. However, association of calpain and its specific inhibitor protein with cellular membranes has been demonstrated [2, 11, 24, 25, 40, 42]. The association of both calpain and its inhibitor calpastatin with isolated junctional sarcoplasmic reticulum (JSR) has been reported [2, 42]. It has also been shown that ryanodine receptor RyR/Ca^{2+} release channel is specifically cleaved by calpain [2, 10, 34, 40, 42].

Ryanodine receptor [RyR]/Ca^{2+} release channel is a key protein involved in excitation-contraction coupling. It represents one of a new class of channels that is characterized by its large size (homotetramer of 560 kDa for each subunit), high conductance, and by the binding of the toxic alkaloid ryanodine [4, 8, 19]. This channel releases Ca^{2+} in response to depolarization of the surface membrane. The control mechanism(s) of its activation/inactivation and regulation are not as yet understood.

In our recent studies [42] we have characterized an endogenous Ca^{2+}-dependent cysteine-protease of the junctional SR membranes which specifically cleaves one site on the ryanodine receptor/Ca^{2+} release channel. This cleavage results in enhancement of Ca^{2+} efflux from SR vesicles.

In this communication we have identified, characterized and partially purified from skeletal muscle sarcoplasmic reticulum a 94 kDa protein that possesses thiol-dependent proteolytic activity specific towards the skeletal muscle ryanodine receptor.

Materials and Methods

Materials

ATP, EGTA, Tris, Mops, Tricine, CsCl, n-decane, phosphorylase kinase, myelin basic protein, histone IIB, phosphofructokinase, adenylate cyclase, histone III-S, dithiothreitol, CHAPS, and spermine-agarose were obtained from Sigma Chemical. [^{3}H]ryanodine (60 Ci/mmol) was purchased from New England Nuclear. Unlabeled ryanodine and calpain inhibitor were obtained from Calbiochem. Sephadex G-50 (fine) was obtained from Pharmacia. Phosphatidylethanolamine and phosphatidylserine were purchased from Avanti Polar Lipids.

Membrane Preparations

Junctional SR membranes were prepared from rabbit fast twitch skeletal muscle as described by Lai et al. [20]. In every SR preparation, the protease inhibitors PMSF (0.2 mM), and benzamidine (0.8 mM), were included in all solutions. The membranes were suspended at a final concentration of about 25 mg protein/ml in a buffer containing 0.2 M sucrose, 10 mM Tricine, pH 8.0 and 1 mM histidine and stored at −70°C. Cardiac SR [3] and brain microsomes [51] were isolated as described previously. Ryanodine receptor was purified from skeletal muscle SR by using the spermine-agarose column as described previously [41]. The purified protein was stored at −20°C in the presence of 20 mM Mops, pH 7.4, 0.5 mg/ml phosphatidylcholine, 0.5 M NaCl, 1 mM DTT and 20% (W/V) sucrose. Protein concentration was determined by the method of Lowry et al. [21], for membrane preparations and by the method of Kaplan and Pedersen [16] for soluble purified RyR and the 94 kDa containing fractions.

Purification of a 94 kDa Thiol-Protease

The 94 kDa protein was partially purified by a new method using one-step chromatography on spermine-agarose column. HSR membranes (60 mg) were sedimented at 40,000 × *g* for 30 min. The supernatant was collected and the pellet was resuspended to a final protein concentration of about 5 mg/ml in a solution containing 10 mM Tris/HCl, pH 7.5, 0.1 M NaCl and 1 mM EGTA. The vesicles were slowly stirred at 4°C for 20 min, and then centrifuged at 40,000 × *g* for 30 min at 4°C. The supernatant (EGTA-extract) was collected and combined with the first supernatant and EGTA and NaCl were added to final concentrations of 1 mM and 65 mM, respectively. This extract was loaded onto a small spermine-agarose column (0.8/3 cm) pre-equilibrated with 10 mM Tris/HCl, pH 7.5, 65 mM NaCl and 1 mM EGTA (buffer A), and the eluent was reloaded to the same column. The column was washed with 8-ml cold (~8°C) buffer A followed by washing with buffer A containing 0.125 M NaCl. By increasing NaCl concentration to 0.23 M, a 94 kDa protein was eluted. The rate of loading and washing was about 1 ml per 3 min. Fractions (0.6 ml) were collected and 80 μl samples were subjected to SDS-PAGE followed by Coomassie blue or silver staining [26]. The purified protein was stored at 0°C or −20°C after addition of EGTA to a final concentration of 10 mM.

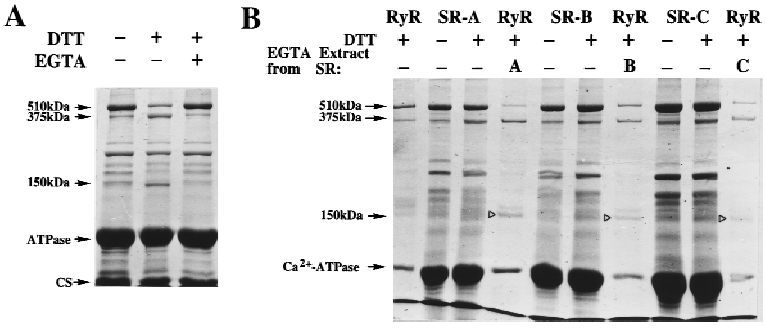

Fig. 1. Endogenous thiol-protease of skeletal muscle junctional SR which specifically cleaves the ryanodine receptor. In *A*, HSR membranes (1 mg/ml) were incubated without (control), with DTT (5 mM) or with DTT and EGTA (1 mM) in 50 mM Tris, pH 7.5. After 30 min at 30°C, aliquots of the treated membranes (50 μg) were solubilized in SDS-buffer and subjected to SDS-PAGE (4.5% acrylamide) as described under Materials and Methods. In *B*, EGTA extracts (A, B and C) obtained from protease nonactive different HSR preparations (SR-A, SR-B and SR-C, isolated according to Refs. 20, 22, and 39, respectively) were added to purified RyR and the samples were incubated as in *A*, in the absence or the presence of 2 mM DTT.

Protease Activity

Aliquots of the EGTA extract of HSR or purified 94 kDa (15–25 μl) were assayed for protease activity by incubation for 30 min at 37°C with purified RyR (5–7 μg) or other substrates (2–6 μg) in a solution containing 50 mM Tricine, pH 7.5 and DTT at the concentrations indicated in the figure legends. Before the assay of protease activity, both purified RyR and the protease containing fractions were passed through Sephadex G-50 columns [32] which were pre-equilibrated with 10 mM Tris/HCl, pH 7.5, and 0.2 mM EGTA [for protease containing fractions] and with 20 mM Mops, pH 7.4, 0.1% CHAPS, 0.5 mg/ml soybean phospholipids and 1 mM DTT (for purified RyR). Endogenous calpain activity of HSR membranes (1 mg/ml) was analyzed under the same conditions, except that DTT concentration was 5–20 mM.

Reconstitution of Single-Channel Activity of RyR in Planar Lipid Bilayers

Single Ca^{2+} release channel/RyR was incorporated by fusion of SR vesicles into planar lipid bilayer as described previously [55]. The bilayers were formed with a 5:3 mixture of phosphatidylethanolamine and phosphatidylserine at 50 mg/ml in n-decane across a 0.2 mm hole drilled into a polystyrene cup which separated two 0.7 ml chambers. The *cis* was the side to which voltage was applied and SR vesicles, 94 kDa protein and other reagents were added. All current recordings were measured at +30 mV holding potential with respect to the *trans* side which was held at ground. For all reconstitution experiments the solutions used contained 50 μM $CaCl_2$ 10 mM Hepes, pH 7.4, and 500 mM CsCl or 100 mM CsCl in *cis* and *trans*, respectively. Picoampere currents were amplified by a Warner Instruments bilayer clamper amplifier (Model BC-525B). The data were filtered through a low-pass Bessel filter at 1 kHz, processed with a VR-10*B* digital data recorder (Instrutech), stored on VCR tape, transferred to a 386 PC computer with VR-111 Interface (Instrutech) and subsequently analyzed with pClamp (Version 5.5.1; Axon Instruments).

Ryanodine binding

Ryanodine binding assay was carried out as described previously [41].

Gel electrophoresis and immunoblot

Analysis of the protein profile and by SDS-PAGE was performed with the discontinuous buffer system of Laemmli [18] in 1.5 mM thick slab gels with 4.5%, 7%, 6–12% or 3–13% acrylamide, using a 3% stacking gel. Gels were stained with Coomassie Brilliant blue or silver ions [26]. Molecular weight standards were: myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine serum albumin, 66,200; and ovalbumin, 42,700 (BioRad). Quantitative analysis of the protein bands was performed by densitometric scanning of the gels with a computing densitometer (Molecular Dynamics) using Image Quant software provided by the manufacturer. Immunoblot analysis has been kindly carried out by Dr. Spencer (University of California) as described previously [49] using polyclonal antibodies recognizing the m-, μ-, and n- (p94) calpain and alkaline phosphatase conjugated to anti rabbit IgG as secondary antibodies.

Results

In our previous study [42], we characterized an endogenous Ca^{2+}-dependent cysteine-protease of the junctional SR membranes which specifically cleaves the RyR/Ca^{2+} release channel in one site to produce 375 kDa and 150 kDa fragments (Fig. 1*A*). This endogenous calpain activity was not obtained in all HSR preparations tested. However, EGTA extract of the protease nonactive HSR preparations was found to be active in the specific cleavage of the purified RyR (Fig. 1*B*). The Ca^{2+} dependency of the RyR cleavage suggests that this protease is of a n-calpain type.

Identification and Partial Purification of a 94 kDa Thiol-Protease

In general, calpains are heterodimers, composed of a large (80 kDa) catalytic and a small (30 kDa) regulatory subunit [29]. In the n-calpain, however, the large subunit is 94 kDa [38, 46]. Both subunits contain calmodulin-like EF hand, Ca^{2+} binding domains, which confer the Ca^{2+} sensitivity to calpain. In a previous study, p94 was not detected in fractionated rat muscle extracts. This was interpreted to suggest that p94 was very short-lived [44]. It has been shown that p94 is predominately expressed in skeletal muscle [48].

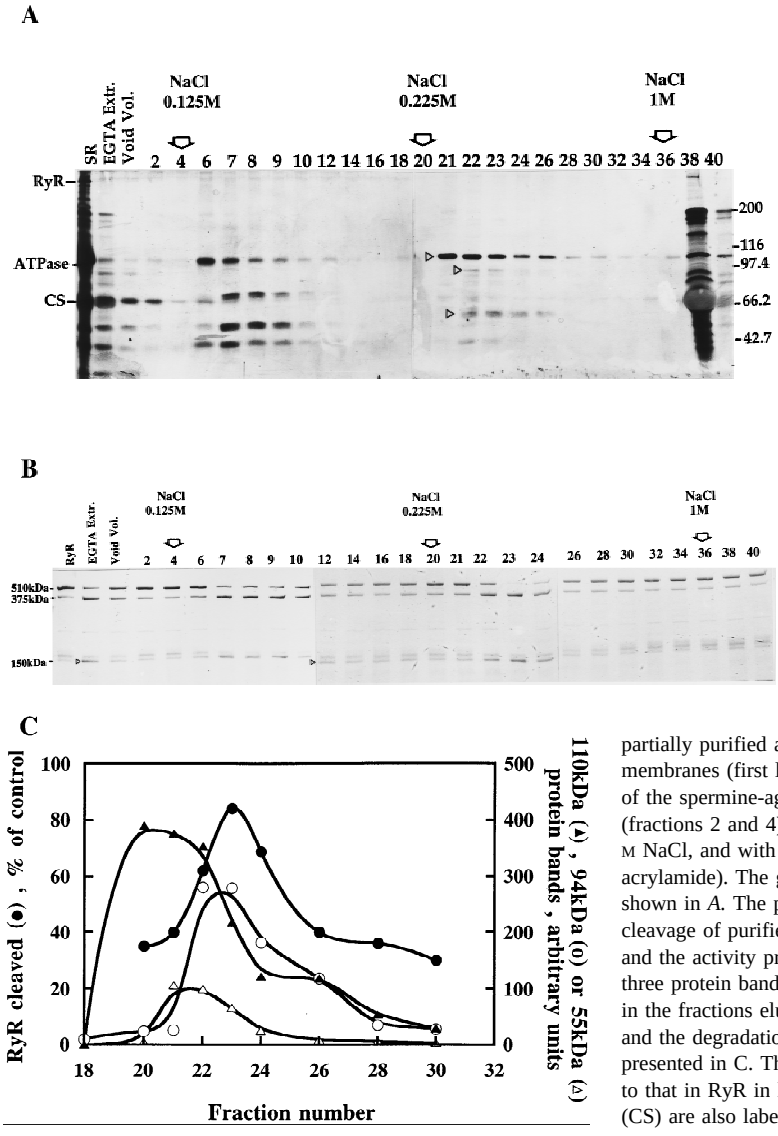

Fig. 2. A simple, one-step method for purification of 94 kDa thiol-protease using a spermine-agarose column. EGTA extract of HSR was obtained and 94 kDa thiol-protease was partially purified as described in Experimental Procedures. The HSR membranes (first lane) EGTA extract (second lane), the void fraction of the spermine-agarose column (third lane), the washed fractions (fractions 2 and 4) and the proteins eluted with 0.125 M NaCl, 0.225 M NaCl, and with 1 M NaCl were subjected to SDS-PAGE (6–12% acrylamide). The gels were stained with silver ions [26] and are shown in *A.* The protease activity was assayed by following the cleavage of purified RyR as described under Materials and Methods and the activity profile is presented in *B.* Quantitative analysis of three protein bands: 110 kDa, 94 kDa and 55 kDa which are present in the fractions eluted by 0.225 M NaCl labeled by arrows (from A) and the degradation of the RyR by these fractions (from B) are presented in C. The amount of 375 kDa fragment formed in relation to that in RyR in lane 1. The RyR, Ca^{2+} ATPase and Calsquestrin (CS) are also labeled.

Partial purification of a 94 kDa protein thiol-protease using a simple one-step method (*see* Materials and Methods) is demonstrated in Fig. 2. On the spermine-agarose column, under the ionic strength and pH used (0.065 M NaCl, pH 7.5), the 94 kDa protein was bound to the column and eluted with 0.225 M NaCl. By increasing NaCl to 1 M, all other bound proteins were effectively washed off from the column. The protein profile of the different eluted fractions is shown in Fig. 2*A.* The thiol-protease activity of the different fractions, using the purified RyR as a substrate is shown in Fig. 2*B.* The elution profiles of 110 kDa, 55 kDa and 94 kDa protein bands as well as the thiol-protease activity distribution in the various fractions eluted from the column were quantitated and are presented in Fig. 2*C.* As shown, the activity peak corresponded to the 94 kDa protein peak. The identity of the 94 kDa protein band as the calpain large subunit is demonstrated in Fig. 3 using specific anti-calpain antibodies.

Characterization of the 94 kDa Thiol-Protease

Stimulation of the protease activity by DTT is shown in Fig. 4*A.* Half-maximal activity was obtained in the presence of 0.1 mM DTT, and maximal activity with 0.5 mM DTT. This DTT-activated protease showed no Ca^{2+} requirement, since EGTA up to 1 mM had no inhibitory effect (Fig. 4*B*). The effect of the incubation pH on the protease activity is shown in Fig. 4*C*, and demonstrates that the optimal pH was in the neutral range as expected for calpains [29].

The effects of different protease inhibitors on the cleavage of the purified RyR by the 94 kDa thiol-

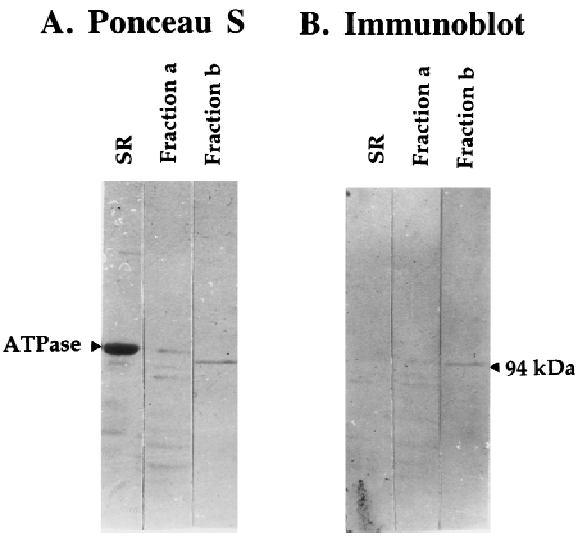

Fig. 3. Immunoenzymatic staining of 94 kDa protein band with anti-calpain antibodies. Immunoblot staining of calpain was carried out as described under Materials and Methods. (*A*) and (*B*) show the Ponceau S staining of the blot and its immunoblot staining, respectively. Fractions (a) and (b) represent fractions obtained in other 94 kDa purification experiment carried out as described in Fig. 2. These fractions cleaved specifically the skeletal muscle RyR.

protease is presented in Fig. 5. The cleavage of RyR (510 kDa) was strongly inhibited by the thiol protease inhibitor mercuric chloride, and also sensitive to leupeptin, as shown previously for other thiol proteases [23]. The specific calpain I inhibitor, at relatively low concentrations, inhibited the cleavage of the RyR by the 94 kDa protease. Lack of inhibition of PMSF shows that the enzyme is not a serine protease. Inhibition of protease activity by the calpain specific inhibitor, the requirement for thiol reagent and the neutral pH optimum suggest that this 94 kDa protease is of a calpain type.

Substrate Specificity of the 94 kDa Thiol-Protease

It has been suggested that calmodulin binding proteins are substrates for calpain [53]. The cleavage of different known calpain substrates by the 94 kDa thiol-protease was examined. Figure 6 shows that among the seven chosen calpain substrates [53] in addition to RyR, histone III-S was the only substrate for this thiol-protease.

Specificity of the 94 kDa thiol-protease was also demonstrated by testing whether it could cleave the cardiac or brain ryanodine receptors (Fig. 7). Neither the cardiac SR nor brain microsomes showed any endogenous protease activity upon addition of DTT and Ca^{2+} (*data not shown*). These membranes were extracted with EGTA and then exposed to the skeletal muscle 94 kDa thiol-protease. No cleavage of RyR in both membranes was obtained by the 94 kDa thiol-protease in the presence of 2 or 15 mM of DTT (Fig. 7). No cleavage was obtained under the different conditions used such as various Ca^{2+} and DTT concentrations, and time of incubation. These results may indicate the high specificity of this protease towards the skeletal muscle isoform (RyR_1).

The Relationship Between the Ryanodine Receptor Conformational State and its Cleavage by the 94 kDa Thiol-Protease

The functional state of the ryanodine receptor/Ca^{2+} release channel is strongly affected by various ligands such as ATP, ruthenium red, Ca^{2+} and Mg^{2+} and by conditions such as ionic strength and pH [4, 8, 19]. The presence of NaCl almost prevents completely the cleavage of RyR by the 94 kDa thiol-protease. The NaCl concentration dependence of this effect and of the stimulation of ryanodine binding are very similar (Fig. 8). The presence of ATP (1 to 10 mM) during the protease activity assay decreased RyR cleavage by 10 to 50% (*data not shown*). This suggests that high NaCl concentrations or ATP stabilized RyR in a conformation in which the cleavage site is not exposed or unidentified by the protease. The polycationic dye ruthenium red, a known inhibitor of Ca^{2+} release and ryanodine binding [4, 8, 19] that stabilizes the protein in the nonactive conformation, did not interfere with the RyR cleavage by the 94 kDa thiol-protease (*data not shown*). High NaCl concentrations, however, affected the cleavage of histone III-S by this protease (Fig. 8*B*).

Effect of 94 kDa Thiol-Protease on RyR/Ca^{2+} Release Channel Activity

The effect of the isolated 94 kDa thiol-protease on the RyR/Ca^{2+} release channel activity was tested on a single channel incorporated into planar lipid bilayer (Fig. 9). DTT by itself stimulated channel activity by increasing the open probability (P_o from 0.52 to 0.89). The stimulatory effect of DTT on the single channel activity was not observed with all reconstituted channels. Addition of 94 kDa thiol-protease to the *cis* chamber of the bilayer modified the channel activity in a time-dependent manner. Within 1.5 min of incubation, the channel was inactivated as reflected in the decrease in P_o (from 0.89 to 0.62). This was followed by a further decrease in the channel activity (after 3 min, $P_o = 0.48$). Thereafter (after 9 min) the channel was converted into a subconducting state. As expected, addition of 150 μM ryanodine completely blocked the protease-modified channel.

Figure 10 shows an experiment in which the addition of DTT and the 94 kDa thiol-protease together immediately increased the channel activity, most probably

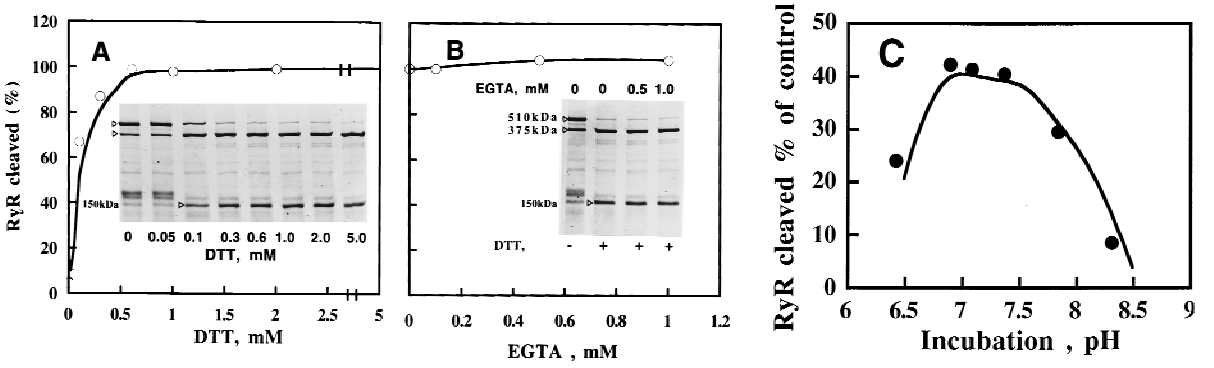

Fig. 4. Effect of DTT, EGTA and pH on the ryanodine receptor cleavage by the 94 kDa thiol-protease. RyR (96 μg/ml) was incubated with the 94 kDa containing fraction in the absence and presence of the indicated DTT (A) or EGTA (B) concentrations as described in Materials and Methods. Aliquots were subjected to SDS-PAGE followed by quantitative analysis of the RyR-proteolytic product 375 kDa (●) as described in Materials and Methods. 100% represents the maximal amount of 375 kDa produced by the protease.
In C, RyR was incubated with the 94 kDa containing fraction in the presence of 2 mM DTT and at different pH. The buffers used were: 50 mM Mops for pH 6.5 and 50 mM Tris for pH 7.0, 7.2, 7.5, 8.0 and 8.5.

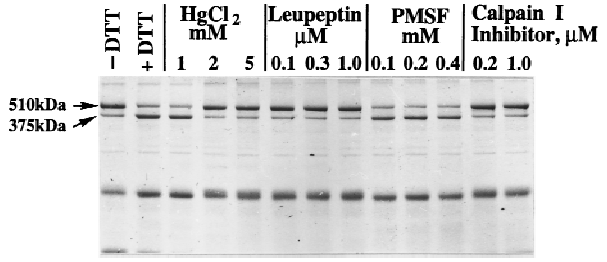

Fig. 5. Effects of antiproteases on the cleavage of the ryanodine receptor by the 94 kDa thiol-protease. Purified RyR (5 μg/ml) was incubated for 30 min, with 20 μl of the 94 kDa containing fraction and 2 mM DTT, and in the absence or presence of the indicated concentrations of the protease inhibitors and then subjected to SDS-PAGE as described in Materials and Methods.

due to the DTT effect on the channel. The channel, however, with time became less active and the subconducting state became apparent. EGTA (2 mM) added to the *cis* chamber (free Ca^{2+} was 0.8 nM) completely closed the channel (trace C), demonstrating that the 94 kDa protease-modified channel still required Ca^{2+} to remain open. Addition of $CaCl_2$ to a final free concentration of 26 μM reactivated the channel to its subconductance state as it was prior to EGTA addition (trace D).

Cleavage of the purified RyR by the 94 kDa thiol-protease had no effect on its ryanodine binding activity (*data not shown*). Similar results were obtained for the degradation of RyR by m-calpain [10, 34].

Discussion

In a previous study [42] we demonstrated the association of an endogenous Ca^{2+}-dependent thiol-protease with the junctional SR membranes. This protease is activated by *submicromolar* concentrations of Ca^{2+}, is absolutely dependent on the presence of thiol reducing reagent, and is inhibited by the specific calpain I inhibitor. These findings may suggest that this protease is of a μ-calpain type, except the difference in the Ca^{2+} sensitivity. This endogenous, Ca^{2+}-dependent, thiol-protease cleaved specifically the RyR/Ca^{2+} release channel at one site regardless of the Ca^{2+} or reducing reagent concentration, the incubation time or temperature. The high sensitivity to Ca^{2+} and high specificity to RyR may suggest that this protease represents the skeletal muscle specific calpain p94 [47]. Using specific antibody, the presence of the skeletal muscle specific calpain p94 protein in muscle tissues was recently demonstrated [45, 49]. The presented work is the first study to partially purify, characterize and demonstrate its high specificity toward the skeletal muscle RyR_1/Ca^{2+} release channel.

The presence of a thiol-protease containing a large subunit of 94 kDa in junctional SR membranes is demonstrated using anti-calpain antibodies (Fig. 3). This protease was partially purified from SR (Figs. 2 and 3), and like the endogenous protease, the 94 kDa protease has optimal pH of around 7.0 and its activity is absolutely dependent on the presence of thiol reducing reagents (Fig. 4). Similar to the SR associated protease, the proteolytic activity of the 94 kDa protein is completely inhibited by $HgCl_2$, leupeptin and the specific calpain I inhibitor (Fig. 5). However, the isolated 94 kDa thiol-protease has no requirement for Ca^{2+} and possesses activity in the presence of EGTA (Fig. 4*B*).

The expression of two alternative splicing products with and without Ca^{2+}-binding domain of the novel stomach calpain has been reported [44]. The loss of Ca^{2+} requirement by the EGTA extract and by the 94 kDa protease purified from it could be due to protease activation during extraction from the SR or to dissociation

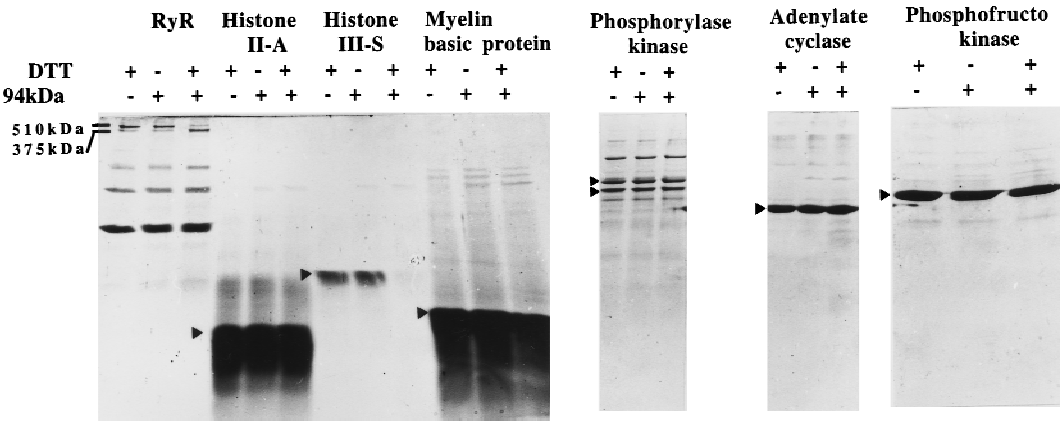

Fig. 6. Specific cleavage of the skeletal muscle RyR by the 94 kDa thiol-protease. The purified RyR and the indicated proteins (4 μg) were exposed to the 94 kDa thiol-protease (150 ng) in the absence and presence of 2 mM DTT, as in Fig. 2. After 30 min at 37°C the samples were subjected to SDS-PAGE (3–15% acrylamide). The arrows point to the indicated protein bands and in phosphorylase kinase lane the different bands represent its subunits.

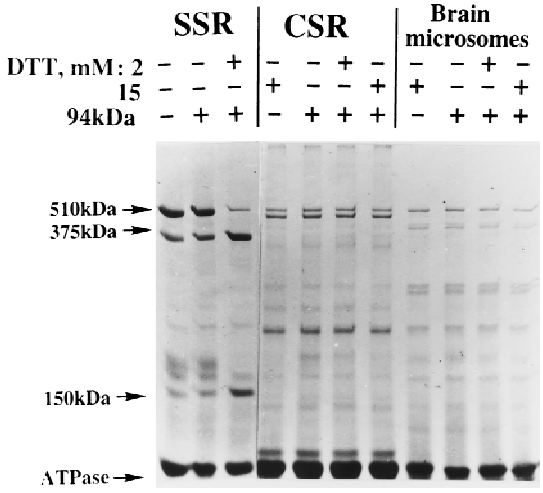

Fig. 7. Cleavage of skeletal muscle but not cardiac or brain RyR by the 94 kDa thiol-protease. Skeletal muscle SR (SSR), cardiac SR (CSR) and brain microsomes were isolated and extracted with EGTA as described under Materials and Methods. The different membrane preparations (after their extraction with EGTA) (1 mg/ml) were exposed to the 94 kDa thiol-protease, isolated from skeletal muscle SR as described in Fig. 2, in the absence and the presence of DTT. After 30 min at 37°C, the membranes (35 μg) were subjected to SDS-PAGE (4.5% acrylamide).

from the membrane. *In vitro* studies have demonstrated that autolysis of amino terminal peptides from each calpain subunit occurs upon activation and results in increased calcium sensitivity [5, 14].

This 94 kDa thiol-protease may represent the novel large subunit of the skeletal muscle specific calpain (p94 or nCL-1; stands for *n*ovel *C*alpain *L*arge subunit). Comparison of the amino acid sequence of p94 with other calpains large subunits has revealed that domain I of the p94 is distinct in both length and primary structure. Since the N-terminal portion of domain I plays an important role in the activation of μ- and m-calpains [7, 13], it was suggested that p94 should be activated by a mechanism different from the activation mechanism of the μ- and m-calpain [14]. Thus, our finding of Ca^{2+} *independent* activity of the soluble 94 kDa thiol-protease is in accord with the above suggestion.

The specificity of the 94 kDa thiol-protease for the skeletal muscle RyR is remarkable. Calpain associated with the SR degraded no other SR proteins, and the 94 kDa thiol-protease isolated from the SR cleaves the skeletal muscle RyR_1 protein but not the brain (RyR_3?) or cardiac (RyR_2) isoforms. Furthermore, the RyR_1 protein is cleaved at only one site and this cleavage is strongly affected by its conformational state. In the presence of high NaCl concentrations or millimolar ATP, which strongly stimulate ryanodine binding and single channel activities [4], cleavage of RyR is inhibited. This may suggest that when RyR is in its active conformation the cleavage site is inaccessible or not recognized by the protease. The effect of the high NaCl concentrations could be also on the protease itself, since its activity on histone III-S was also prevented by high NaCl concentrations (Fig. 8). It has been shown that m-calpain (requiring mM Ca^{2+}) exogenously added to SR membranes degraded the RyR/Ca^{2+} release channel to several fragments and that this cleavage was inhibited by calmodulin [2], a modulator of RyR activity [4, 19].

Our results show that the activity of the RyR single channel incorporated into a planar liquid bilayer modified several minutes after the addition of the 94 kDa thiol-protease (Figs. 9 and 10). The modification was reflected in the appearance of a channel with subconductance and lower open probability (Figs. 9, 10). Thus, cleavage of RyR at a specific site (*see* Figs. 1, 2, 4, 5, 7 and Ref. 42) resulted in a modified channel. This may

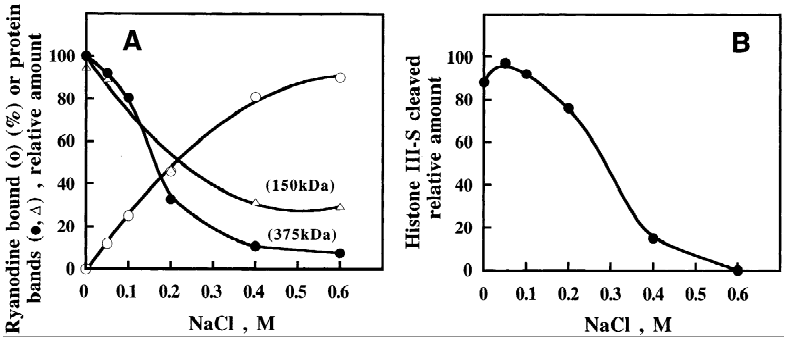

Fig. 8. Effect of NaCl concentration on the RyR activity and cleavage by the 94 kDa thiol-protease. Purified RyR (*A*) or histone III-S (*B*) were incubated for 30 min with the 94 kDa thiol-protease as in Fig. 2, except that NaCl at the indicated concentration was present. The effect of NaCl concentration on the ryanodine binding activity of junctional SR membranes is also presented in *A*. Control activity (100%) was 2.6 pmol ryanodine bound/mg. Ryanodine binding was assayed as described previously [41]. The samples were subjected to SDS-PAGE 4.5% for (*A*) and 3–15% acrylamide for histone III-S (*B*). The Coomassie stained gels were quantitatively analyzed as described in Fig. 2. In *A* the relative amount of 375 kDa and 150 kDa fragments formed by the 94 kDa protease at the indicated NaCl concentration is presented. In *B*, the relative amount of histone III-S cleaved by the protease in the presence of the indicated NaCl concentrations.

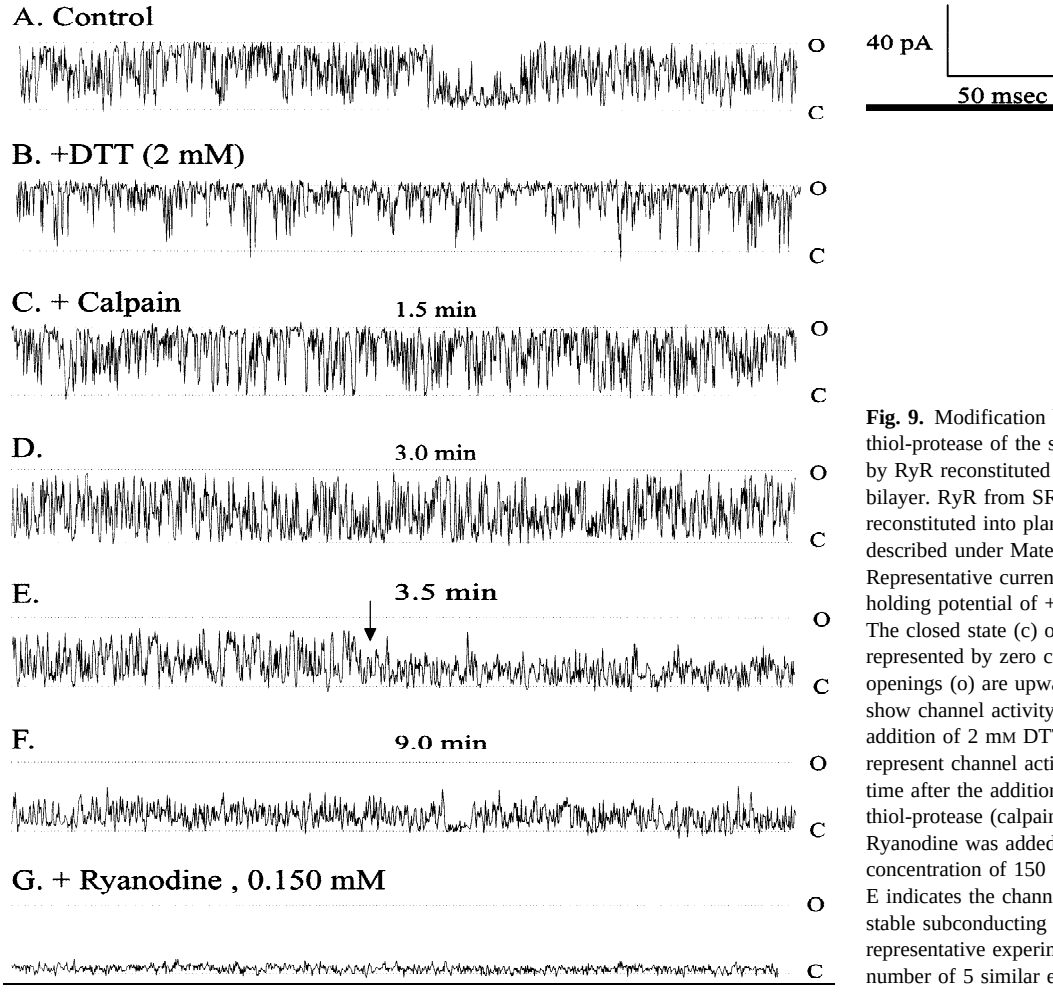

Fig. 9. Modification by the 94 kDa thiol-protease of the single-channel activity by RyR reconstituted into planar lipid bilayer. RyR from SR membranes was reconstituted into planar lipid bilayer as described under Materials and Methods. Representative current tracings recorded at a holding potential of +30 mV are presented. The closed state (c) of the channel is represented by zero current, and channel openings (o) are upward deflections. *A* and *B* show channel activity before and after the addition of 2 mM DTT. Traces C to F represent channel activity at the indicated time after the addition of 94 kDa thiol-protease (calpain) to the *cis* chamber. Ryanodine was added to a final concentration of 150 μM. The arrow in trace E indicates the channel converting into a stable subconducting state. This is a representative experiment from a total number of 5 similar experiments.

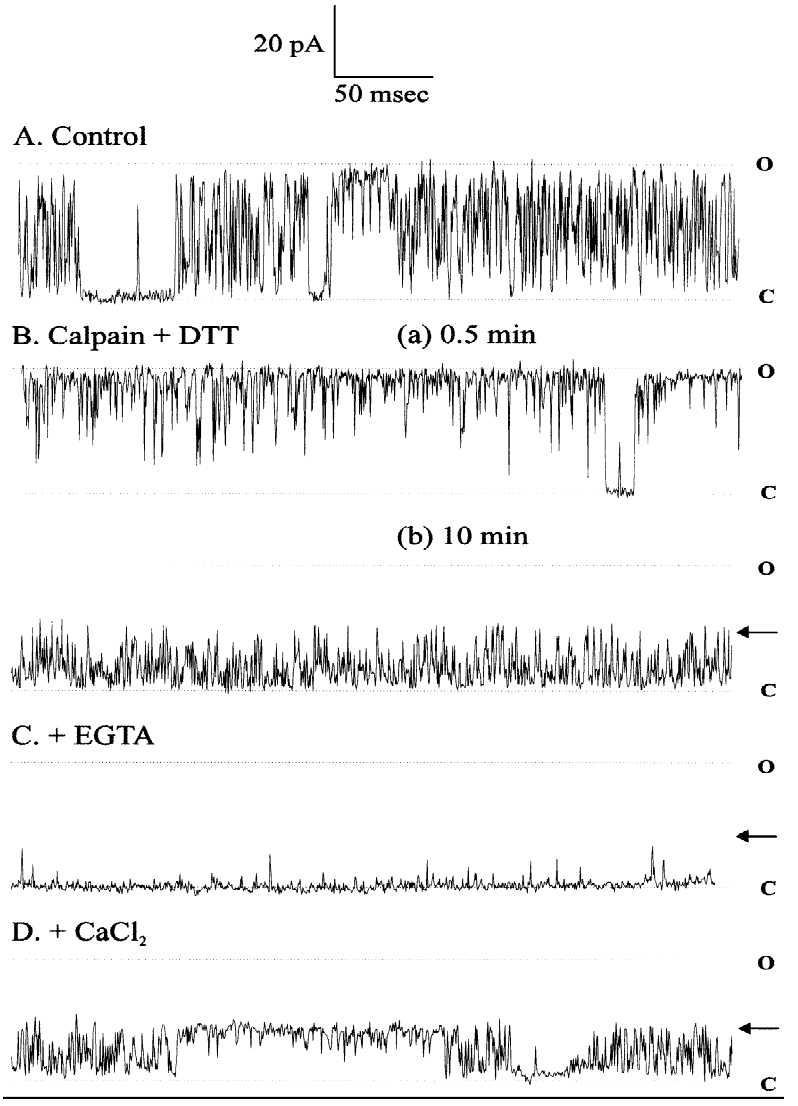

Fig. 10. RyR channel activity modified by 94 kDa thiol-protease remains Ca^{2+} dependent. RyR single channel activity was reconstituted into PLB as described in Fig. 9. The channel current was recorded at +30 mV before (*A*) and 0.5 and 10 min (*B*) after the addition of the 94 kDa thiol-protease (calpain) and DTT to the *cis* chamber. Within several minutes the channel appeared in the subconducting state (b). EGTA was added to the *cis* solution to a final concentration of 2 mM (free Ca^{2+} = 0.8 nM) (*C*), followed by addition of $CaCl_2$ to a free Ca^{2+} concentration of 26 μM (*D*). Arrows on the right side of the traces indicate the maximal opening level of the subconducting channel.

suggest the involvement of the cleaved site in the channel control mechanism. The cleavage site of RyR by 94 kDa thiol-protease was localized at the N-terminal part of RyR [42], suggesting that the N-terminal also has a functional role in the control of channel conductance. In cardiac and skeletal SR it has been shown that specific degradation of RyR to two fragments (315 kDa and 150 kDa) by exogenously added μ-calpain resulted in modification of the channel activity as reflected in an increase in the percentage of channel open times with no effect on the unitary channel conductances [34]. The differences between these and our results may be due to the different proteases studied.

RyR/Ca^{2+}channel has been shown to be converted into subconducting states under different conditions. Ryanodine interacts with the channel protein by locking it into a permanently open subconducting state [4, 19]. A similar effect has been obtained with the venom toxin ryanotoxin [28]. Modification of RyR with the lysine specific reagent FITC (fluorescein isothiocyanate) converted the channel into a noisy ~60% subconductance state [31]. Propranolol, a β-blocker, modified the RyR channel into much lower conducting states only in the presence of ATP [55]. In addition, QX-314, a quaternary amine derivative of lidocaine was found in a voltage-dependent manner to reduce RyR channel conductance [54]. All of these observations suggest that the RyR/Ca^{2+} release channel has multiconducting conformations that can be stabilized by different treatments or effectors. The effect of the 94 kDa thiol-protease on the RyR channel conductivity supports this suggestion.

It has been shown that the p94 mRNA is expressed only in skeletal muscle, with no expression in any other organs including heart muscle and smooth muscle [48]. This specific expression of p94 mRNA may suggest a specific function of this protease. The specific cleavage

of the RyR/Ca^{2+} release channel at a specific site and its conversion to a channel in an open sub-conductance state may suggest that the function of this protease is to modify RyR activity under specific conditions. The observation that when the RyR/Ca^{2+} release channel is in the active state, such as in the presence of ATP or a high NaCl concentration, no degradation of the RyR was obtained may suggest that the protease action is possible only under abnormal conditions such as pathological conditions. It has been suggested that degradation of the Ca^{2+} release channel is prevented by the presence of dystrophin [9]. Recently, it has been demonstrated that calpains are the active components in the pathology of dystrophin-deficient muscle [49], and that p94 is responsible for limb Girdle muscular dystrophy type 2A [45]. A hypothesis regarding the involvement of the protease specific cleavage and modification of RyR/Ca^{2+} release channel and pathological effects on muscle proteins was presented in our previous study [42]. Briefly, activation of the skeletal muscle-specific calpain (p94 thiol-protease) would specifically cleave RyR, thereby modifying its Ca^{2+} release activity by stabilizing it in an open subconducting state. These initial events would lead to an increase in the intracellular Ca^{2+} concentration which would activate μ-calpain and m-calpain which could lead to degradation of muscle proteins [7, 17]. A calpain cascade of μ-calpain activating m-calpain was demonstrated recently, and the calpain cascade suggested to play an important role in coordinating the functioning of calpains in living cells [52]. Further pursuit of the suggested hypothesis may reveal a novel pathological mechanism leading to muscular dystrophy in which the condition is caused by mutations affecting calpain rather than a structural component of muscle tissue as recently suggested [36]. Mutations in n-calpain (calpain 3) were shown to cause limb-girdle muscular dystrophy type 2A and it has been hypothesized that n-calpain plays an active role in signal transduction [36]. This is in agreement with our suggestion that n-calpain has a regulatory rather than degradative role, and this is mediated by the specific cleavage of RyR and thus, activation of the RyR/Ca^{2+} release channel. Evidence for a pathological increase in intracellular Ca^{2+} concentration has been obtained in several studies [1, 9, 27].

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References

1. Bakker, A.J., Head, S.I., Williams, D.A., Stephenson, D.G. 1993. Ca^{2+} levels in myotubes grown from the skeletal muscle of dystrophic (mdx) and normal mice. *J. Physiol.* **460:**1–13
2. Brandt, N.R., Caswell, A.H., Brandt, T., Brew, K., Mellgren, R.L. 1992. Mapping of the calpain proteolysis products of the junctional foot protein of the skeletal muscle triad junction. *J. Membrane Biol.* **127:**35–47
3. Chamberlain, B.K., Levitsky, D.O., Fleischer, S. 1983. Isolation and characterization of canine cardiac sarcoplasmic reticulum with improved Ca^{2+} transport properties. *J. Biol. Chem.* **258:**6602–6609
4. Coronado, R., Morrissette, J., Sukhareva, M., Vaughan, D.M. 1994. Structure and function of ryanodine receptors. *Am. J. Phys.* **266:**C1485–C1504
5. Croall, D.E. 1989. Proteolytic modification of calcium-dependent protease in erythrocytes treated with ionomycin and calcium. *Biochemistry* **28:**6882–6888
6. Dayton, W.R., Schollmeyer, J.V., Lepley, R.A., Cortes, L.R. 1981. A calcium-activated protease possibly involved in myofibrillar protein turnover. *Biochim. Biophys. Acta* **659:**48–61
7. De Martino, G.N., Huff, C.A., Croall, D.E. 1986. Autoproteolysis of the small subunit of calcium-dependent protease II activates and regulates protease activity. *J. Biol. Chem.* **261:**12047–12052
8. Fleischer, S., Inui, M. 1989. Biochemistry and biophysics of excitation-contraction coupling. *Ann. Rev. Biophys. Biophys. Chem.* **18:**333–364
9. Franco, A.J. and Lansman, J.B. 1990. Calcium entry through stretch-inactivated ion channels in mdx myotubes. *Nature* **344:** 670–673
10. Gilchrist, J.S.C., Wang, K.K.W., Katz, S., Belcastro, A.N. 1992. Calcium-activated neutral protease effects upon skeletal muscle sarcoplasmic reticulum protein structure and calcium release. *J. Biol. Chem.* **267:**20857–20865
11. Gopalakrishan, R., Barsky, S.H. 1986. Hydrophobic association of calpains with subcellular organs compartmentalization of calpains and the endogenous inhibitor calpastatin in tissues. *J. Biol. Chem.* **261:**13936–13942
12. Guroff, G. 1964. A neutral, calcium-activated protease from soluble fraction of rat brain. *J. Biol. Chem.* **239:**149–155
13. Ilian, M.N., Forsberg, N.E. 1992. Gene expression of calpains and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. *Biochem. J.* **287:**163–171
14. Imajoh, S., Kawasaki, H., Suzuki, K. 1986. Limited autolysis of calcium-activated neutral protease (CANP): reduction of the Ca^{2+} requirement is due to the NH_2-terminal processing of the large subunit. *J. Biochem.* **100:**633–642
15. Inomata, M., Hayashi, M., Nakamura, M., Imahori, K., Kawashima, S. 1983. Purification and characterization of a calcium-activated neutral protease from rabbit skeletal muscle which requires calcium ions of μM order of concentration. *J. Biochem.* **93:**291–294
16. Kaplan, R.S., Pedersen, F.L. 1985. Determination of microgram quantities of protein in the presence of milligrams level of lipid with amido black 10B. *Anal. Biochem.* **150:**94–104
17. Kay, J. 1984. Ca^{2+}-activated proteases, protein degradation and muscular dystrophy in: *Proteases: potential role in health and disease* (W.H. Hörl and A. Heidland editors.) pp. 519–532. Plenum, New York
18. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227:**680–685
19. Lai, F.A., Meissner, G. 1989. The muscle ryanodine receptor and its intrinsic Ca^{2+} channel activity. *Bioenerg. Biomembr.* **21:**227–245
20. Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.Y., Meissner, G. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature* **331:**315–319
21. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1941.

Protein measurements with folin phenol reagent. *J. Biol. Chem.* **193:**265–275
22. MacLennan, D.H. 1970. Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* **245:**4508–4518
23. Mellgren, R.L. 1980. Canine cardiac calcium-dependent proteases: Resolution of two forms with different requirements for calcium. *FEBS Lett.* **109:**129–133
24. Mellgren, R.L. 1987. Calcium-dependent protease: an enzyme system active at cellular membranes. *FASEB J.* **1:**110–115
25. Mellgren, R.L., Lane, R.D., Kakar, S.S. 1987. Isolated bovine myocardial sarcolemma and sarcoplasmic reticulum vesicles contain tightly bound calcium dependent protease inhibitor. *Biochem. Biophys. Res. Commun.* **142:**1025–1031
26. Merril, C.R., Goldman, D., Van Keuren, M.L. 1983. Silver staining methods for polyacrylamid gel electrophoresis. *Meth. Enzymol.* **96:**230–239
27. Mongini, T., Ghigo, D., Doriguzzi, C., Bussolino, F., Pescarmona, G., Pollo, B., Schiffer, D., Bosia, A. 1988. Free cytoplasmic Ca^{2+} at rest and after cholinergic stimulus is increased in cultured muscle cells from Dunchenne muscular dystrophy patients. *Neurology* **38:**476–480
28. Morrissette, J., Beurg, M., Sukhareva, M., Coronado, R. 1996. Purification and characterization of ryanotoxin, a peptide with actions similar to those of ryanodine. *Biophysiol. J.* **71:**707–721
29. Murachi, T. 1989. Intracellular regulatory system involving calpain and calpastatin. *Biochem. Int.* **18:**263–294
30. Murachi, T., Tanaka, K., Hatanaka, M., Murakami, T. 1981. Intracellular Ca^{2+}-dependent protease [calpain] and its high-molecular weight endogenous inhibitor [calpastatin]. *Adv. Enzyme Regul.* **19:**407–424
31. Orr, I., Martin, C., Ashley, R., Shoshan-Barmatz, V. 1993. The interaction of fluorescein isothiocyanate with the ryanodine receptor/Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem.* **268:**1376–1382
32. Penefsky, H.S. 1977. Reversible binding of Pi by beef heart mitochondrial. *J. Biol. Chem.* **252:**2891–2899
33. Puca, G.A., Nola, E., Sica, V., Bresciani, F. 1977. Estragon binding proteins of calf uterus; molecular and functional characterization of the receptor transforming factor: A calcium-activated protease. *J. Biol. Chem.* **252:**1358–1370
34. Rardon, D.P., Cefali, D.C., Mitchell, R.D., Seiler, S.M., Hathaway, D.R., Jones, L.R. 1990. Digestion of cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles with calpain II; Effect on the Ca^{2+} release channel. *Circ. Res.* **67:**84–96
35. Reddy, M.K., Rabinovitz, M., Zak, R. 1983. Stringent requirement for Ca^{2+} in the removal of Z-line and α-actinin from isolated myofibrils by Ca^{2+} activated neural proteinase. *Biochem. J.* **209:**635–641
36. Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannikulchai, N., Bourg, N., Brengular, L., Devaud, C. et al. 1994. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* **81:**27–40
37. Rogers, S., Wells, R., Rechsteiner, M. 1986. Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234:**364–368
38. Saido, C.T., Sorimachi, H., Suzuki, K. 1994. Calpain: New perspectives in molecular diversity and physiological-pathological involvement *FASEB J.* **8:**814–822
39. Saito, A., Seiler, S., Chu, A., Fleischer, S. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* **99:**875–885
40. Seiler, S., Wegener, A.D., Whang, D.D., Hathaway, D.R., Jones, L.R. 1984. High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum bind calmodulin are phosphorylated and are degraded by Ca^{2+}-activated protease. *J. Biol. Chem.* **259:**8550–8557
41. Shoshan-Barmatz, V., Zarka, A. 1992. A simple, fast, one-step method for purification of the skeletal muscle ryanodine receptor. *Biochem. J.* **285:**61–64
42. Shoshan-Barmatz, V., Weil, S., Varsanyi, M., Heilmeyer, L.M.G. 1994. Endogenous Ca^{2+}-dependent, cysteine-protease cleaves specifically the ryanodine receptor/Ca^{2+} release channel in skeletal muscle. *J. Membrane Biol.* **142:**281–288
43. Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., Suzuki, K. 1989. Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ- types. *J. Biol. Chem.* **264:**20106–20111
44. Sorimachi, H., Ishiura, S., Suzuki, K. 1993. A novel tissue specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. *J. Biol. Chem.* **268:**19476–19482
45. Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Sorimachi, N., Shimada, H., Tagawa, K., Maruyama, K., Suzuki, K. 1995. Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, association with connectin through IS2, a p94-specific sequence. *J. Biol. Chem.* **270:**31158–31162
46. Sorimachi, H., Saido, T.C., Suzuku, K. 1994. New era of calpain research, Discovery of tissue-specific calpains. *FEBS Lett.* **343:**1–5
47. Sorimachi, H., Toyama-Sorimachi, N., Saido, T.C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S., Suzuki, K. 1993. Muscle specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. *J. Biol. Chem.* **268:** 10593–10605
48. Sorimachi, H., Tsukahara, T., Okada-Ben, M., Sugita, H., Ishiura, S., Suzuki, K. 1995. Identification of a third ubiquitous calpain species-chicken muscle expresses four distinct calpains. *Biochim. Biophys. Acta* **1262:**381–393
49. Spencer, M.J., Croall, D.E., Tidball, J.G. 1994. Calpains are activated in narcotic fibers from mdx dystrophic mice. *J. Biol. Chem.* **270:**10909–10914
50. Suzuki, K. 1987. Calcium activated neutral protease: domain structure and activity regulation. *Trends Biochem. Sci.* **12:**103–105
51. Tamkun, M.M., Catterall, W.A. 1981. Ion flux studies of voltage-sensitive sodium channel in synaptic nerve-ending particles. *Molec. Pharmacol.* **19:**78–86
52. Tompa, P., Baki, A., Schad, E., Friedrich, P. 1996. The calpain cascade: μ calpain activates m calpain. *J. Biol. Chem.* **271:**33161–33164
53. Wang, K.K.W., Villalobo, A., Roufogalis, B.D. 1989. Calmodulin-binding proteins as calpain substrates. *Biochem. J.* **262:**693–706
54. Xu, L., Jones, R., Meissner, G. 1993. Effects of local anesthetics on single channel behavior of skeletal muscle calcium channel. *J. Gen. Physiol.* **101:**207–233
55. Zchut, S., Feng, W., Shoshan-Barmatz, V. 1996. Ryanodine receptor/Ca^{2+} release channel conformations as reflected in the different effects of propranolol on its ryanodine binding and channel activity. *Biochem. J.* **315:**377–383