Structural and Functional Correlation of the Trypsin-digested Ca\(^{2+}\) Release Channel of Skeletal Muscle Sarcoplasmic Reticulum*

Gerhard Meissner†, Eric Rousseau, and F. Anthony Lai

From the Departments of Biochemistry and Physiology, University of North Carolina, Chapel Hill, North Carolina 27599-7260

(Received for publication, August 22, 1988)

The effect of trypsin digestion on the (i) fragmentation pattern, (ii) activity, (iii) \[^{3}H\]ryanodine binding, and (iv) sedimentation behavior of the skeletal sarcoplasmic reticulum (SR) ryanodine receptor-Ca\(^{2+}\) release channel complex has been examined. Mild trypsic digestion of heavy, junctional-derived SR vesicles resulted in the rapid disappearance of the high molecular weight (M\(_{\text{r}}\) > 400,000) Ca\(^{2+}\) release channel protein (to sodium dodecyl sulfate gels and appearance of bands of lower \(M_{r}\), upon immunoblot analysis, without an appreciable effect on \[^{3}H\]ryanodine binding or the apparent S value (30 S) of the 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (Chaps)-solubilized channel complex. Further degradation to bands of \(M_{r}\) > 70,000 on immunoblot correlated with a reduction of channel size from 30 S to 10-15 S and loss of high affinity \[^{3}H\]ryanodine binding to the trypsinized receptor, while low affinity \[^{3}H\]ryanodine binding and \[^{3}H\]ryanodine bound prior to digestion were retained. Parallel \(^{40}\)Ca\(^{2+}\) efflux measurements also indicated retention of the \(M_{r}\) of the \(^{40}\)Ca\(^{2+}\) and ATP regulatory sites, although Ca\(^{2+}\)-induced \(^{40}\)Ca\(^{2+}\) release rates were changed. In planar lipid bilayer-single channel measurements, addition of trypsin to the cytoplasmic side of the high conductance (100 pS in 50 mM Ca\(^{2+}\)), Ca\(^{2+}\)-activated SR Ca\(^{2+}\) channel initially increased the fraction of channel open time and was followed by a complete and irreversible loss of channel activity. Trypsin did not change the unitary conductance, and was without effect on single channel activity when added to the luminal side of the channel.

Rapid release of Ca\(^{2+}\) from skeletal muscle sarcoplasmic reticulum (SR)\(^{1}\) is triggered by a surface membrane activation potential that is thought to be communicated to SR via the transverse (T-) tubule system at specialized areas where the two membrane systems come in close contact (for reviews see Refs. 1-4). Ultrastructural studies have revealed distinct protein bridging structures ("feet") (5) which span the gap between T-tubule and SR and have been immunologically related to high molecular mass proteins of \(\geq\) 300,000 daltons (6). Although the mechanism of signal transmission across the junctional gap remains to be determined, recent single channel recording measurements using isolated membrane fractions have suggested that SR Ca\(^{2+}\) release is mediated by a high conductance, Ca\(^{2+}\)- and ATP-activated Ca\(^{2+}\) release channel (7, 8). Using the Ca\(^{2+}\) release channel-specific probe \[^{3}H\]ryanodine, a 30 S ryanodine receptor complex has been isolated (9) which is composed of polypeptides of apparent molecular mass ~400,000 (9, 11). Upon reconstitution of the 30 S complex into planar lipid bilayers, Ca\(^{2+}\) conducting channels were evident with a conductance and pharmacological behavior similar to those observed when SR Ca\(^{2+}\) conducting vesicles were fused with the bilayers (9, 12, 13). Negative-stain electron microscopy further revealed the four-leaf clover structure described for the feet that span the T-tubule-SR junction (9, 14). These findings have suggested that the feet are synonymous with the high conductance, ligand-gated Ca\(^{2+}\) release channel of sarcoplasmic reticulum. Furthermore, they support the hypothesis that the channel directly senses the T-tubule potential with one end, while the other end regulates a Ca\(^{2+}\) conducting pore in SR (15).

The high molecular weight Ca\(^{2+}\) release channel proteins are highly susceptible to proteolysis (9, 11, 16). Mild digestion of "heavy" SR vesicles with trypsin resulted in partial loss of ATP-dependent Ca\(^{2+}\) accumulation (17) and increased ruthenium red-sensitive Ca\(^{2+}\) efflux from the vesicles (18, 19). Ca\(^{2+}\) efflux was potentiated by Ca\(^{2+}\) and ATP and inhibited by Mg\(^{2+}\) as observed similarly in undigested vesicles. Furthermore, \[^{3}H\]ryanodine binding to trypsin-digested vesicles was reduced (18). These observations have suggested that limited trypsic digestion of heavy SR vesicles activates the SR Ca\(^{2+}\) release channel.

In this paper, the effects of controlled trypsin proteolysis on the SR Ca\(^{2+}\) release channel have been studied using a heavy SR vesicle fraction isolated from rabbit skeletal muscle. We have determined the size and proteolytic fragmentation pattern of the digested channel complex by sedimentation analysis and immunoblot staining using an rabbit anti-rat polyclonal antibody, respectively. In parallel experiments, the Ca\(^{2+}\) release behavior of, and \[^{3}H\]ryanodine binding to, the digested vesicles was determined. In addition, we report the first direct observations of the effects of trypsin on single channels incorporated into planar lipid bilayers. Some of these results have been communicated in abstract form (20).

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin (Type III) from bovine pancreas, soybean trypsin inhibitor protein, disopropyl fluorophosphate (DIFP), and the...
effects of trypsin on Sr ca^2+ release channel

ATP analog AMP-PCP were obtained from Sigma, ruthein red from Fluka, [46Ca^2+] from ICN Pharmaceuticals, [3H]ryanodine (54.7 Ci/mmol) from Du Pont-New England Nuclear, and unlabeled ryanodine from AgriSystems International (Wind Gap, PA). Phospholipids were purchased from Avanti Polar Lipids. All other reagents were of reagent grade.

Isolation of SR Vesicles—Heavy SR vesicles enriched in Ca^2+ release activity were isolated from rabbit skeletal muscle as a 2,600-35,000 x g pellet (21) in the presence of 2.5 mm EGTA and 1 mm DPPF. The pellets were resuspended in 0.3 M sucrose, 0.6 M KCl, 0.1 mm EGTA, 0.1 mm DPPF, and 10 mm KPiPipes, pH 7.0, incubated for 1 h at 4°C and centrifuged by centrifugation at 100,000 x g for 30 min. After washing and resuspension in 0.3 M sucrose, 0.1 M NaCl, 100 mm EGTA, 100 mm Ca^2+, and 20 mm NaPiPipes, pH 7.0, membranes were rapidly frozen and stored at -65°C before use. "Light" SR vesicles lacking the Ca^2+ release channel were prepared as described previously (21).

Trypsin Digestion of SR Vesicles—For all experiments, unless otherwise indicated, SR vesicles (2-10 mg of protein/ml) were treated with trypsin at 22°C in 0.1 M NaCl, 0.1 mm EGTA, 1.1 mm Ca^2+, and 20 mm NaPiPipes, pH 7.0. The reaction was quenched with a 20-fold weight excess of bovine trypsin inhibitor protein and 1 mm DPPF and centrifuged at a concentration of 100,000 x g in 0.1 M methanol, 15% acetic acid. In control experiments trypsin was omitted, or the two protease inhibitors were added to the vesicles before the addition of trypsin.

Preparation of Ca^2+ Release Channel Protein Antiserum—a rat and rabbit Ca^2+ release channel protein antiserum were prepared by intradermal injection of an emulsion of 1 ml of Freund's adjuvant (complete) and 1 ml of a saline solution containing a small amount of Chaps and 250 pg of purified Ca^2+ release channel-ryanodine receptor protein (9) prepared from rat skeletal muscle. The [3H]ryanodine-labeled receptor sedimented as a 30 S complex and contained one major high molecular weight band of Mr, ~460,000, as has been previously reported for the rabbit muscle ryanodine receptor (9). After two booster injections at 4-week intervals, rabbit serum was collected. Immunoblot analysis using the complete serum indicated strong immunoreactivity with the rat and rabbit heavy SR Mr, ~460,000 band. A faint band of Mr, ~100,000 was also recognized in the immunoblot. Immunostaining of this latter band was removed by prior incubation of the complete serum for 3 h at 22°C with SR protein coupled to cyanogen bromide-activated Sepharose 4B (1 ml of serum/ml gel containing 1 mg of SR protein). The SR protein fraction used for coupling was obtained by centrifuging Chaps-solubilized heavy SR vesicles from rabbit muscle through a linear sucrose gradient and collecting the top region of the gradient which was devoid of the 30 S channel complex (9).

SDS Gel Electrophoresis and Immunoblot Staining—SDS-PAGE was performed using 4-10% (w/v) acrylamide gels (22) using a 1.5 mm thick, 5-12% linear polyacrylamide gradient gel and 3% stacking gel. Samples were denatured for 3 min at 95°C in 0.1 M Tris-HCl, pH 6.8, containing 2% SDS, 3% β-mercaptoethanol, and 10% glycerol. Electrophoresis was at 15°C and constant current (30 ma/gel) and was monitored with 0.004% bromphenol blue as tracking dye. Gels were stained with 1% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid and destained with 10% methanol, 15% acetic acid.

For immunoblots, the separated proteins from SDS-PAGE were electrophoretically transferred onto Immobilon PVDF membranes (Millipore Corp.) for 1 h at 400 mA then 12 h at 1500 mA and 15°C (23). Transfer membranes were washed in PBS (15 min), blocked with PBS/5% non-fat dried milk for 1 h at 37°C (24), and twice washed for 20 min in PBS/0.5% Tween 20 (PBS-T), before overnight incubation at 4°C with rabbit and anti-rat ryanodine receptor antisem (1:30,000 dilution in blocking buffer) preabsorbed with rabbit SR protein devoid of the ryanodine receptor as described above. After 4 washes in PBS-T as above, membranes were incubated for 1 h at 23°C with peroxidase-conjugated goat anti-rabbit IgG antisem (Caltbiochem), washed 3 times in PBS-T as above, and then the color developed using 2,4-diaminobenzidinide underconstant.

[46Ca^2+] Flux Measurements—[46Ca^2+] efflux rates from vesicles passively loaded with [46Ca^2+] were determined with the use of an Update System 1000 Chemical Quench apparatus (Madison, WI) and by filtration (25). Vesicles (2-10 mg of protein/ml) were passively loaded by incubation for 30 min at 23°C with [46Ca^2+] in a medium containing 50 mm sucrose, 100 mm NaCl, 20 mm NaPiPipes, pH 7.0, 100 mm EGTA, and 1.1 mm Ca^2+. The [46Ca^2+] efflux behavior of the vesicles was measured by 5-300-fold dilution into isosmolar unlabeled release media containing varying concentrations of Ca^2+, Mg^2+, and adenine nucleotide. In the rapid quench experiments, [46Ca^2+] efflux was inhibited at time intervals ranging from 25 to 2000 ms by the addition of 10 mM Mg^2+, 5 mm EGTA, and 10 μm ruthenium red. Untrapped as well as released [46Ca^2+] was separated away from filtration using Millipore Type HA filters. Radioactivity retained by the vesicles on the filters was determined as described previously (25). [46Ca^2+] efflux was inhibited at time intervals ranging from 25 to 2000 ms by the addition of 10 mm EGTA, 5 mm Mg^2+, and 10 μM ruthenium red. Untrapped as well as released [46Ca^2+] was separated away from filtration using Millipore Type HA filters. Radioactivity retained by the vesicles on the filters was determined as described previously (25).

[3H]Ryanodine Binding—SR vesicles (0.5-2 mg of protein/ml) were incubated at 22°C in a medium containing 20 mm NaPiPipes, pH 7.0, 1 mm EGTA, 1 mm DPPF, 0.15 mm Ca^2+, 2.5 mm AMP-PCP, and 2 nm to 1 mm [3H]ryanodine. [3H]Ryanodine (54.7 Ci/mmol) was added to a concentration up to 25 nm; greater concentrations were prepared as mixtures of labeled and unlabeled ryanodine. After 15 h, aliquots of the vesicle suspensions were (i) placed into a scintillation vial to determine total radioactivity, (ii) centrifuged for 30 min at 50,000 x g, in a Beckman Airfuge to remove [3H]ryanodine, and (iii) placed after dilution with 25 volumes of ice-cold water, on a Whatman GF/B filter soaked in 1% polyethylenimine. After rinsing with three 5-ml volumes of ice-cold H2O, radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [3H]ryanodine. [3H]Ryanodine binding experiments were carried out in triplicate. The standard errors were ±10% or less.

In the trypsin digestion studies with prelabeled membranes, vesicles were incubated in the above medium with 2 nm [3H]ryanodine for 2 h at 22°C and then centrifuged for 45 min at 4°C and 100,000 x g in a Beckman ultracentrifuge in a medium of 0.5 mm sucrose to remove unbound [3H]ryanodine and remaining unhydrolyzed DIFP.

Sucrose Gradient Analysis—[3H]Ryanodine-labeled membranes were solubilized at 1.5 mg of protein/ml in buffer A (1 M NaCl, 0.1 mm EGTA, 0.15 mm Ca^2+, 3 mm AMP, 1 mm DPPF, 0.5% soybean phosphatidylcholine, 20 mm NaPiPipes, pH 7.0) containing 1.5% Chaps (9). After 1 h incubation at 22°C and centrifugation at 4°C for 30 min at 100,000 x g in a Beckman ultracentrifuge, the supernatant (1 ml) was loaded onto 5-20% linear sucrose gradients in buffer A containing 1% Chaps and then centrifuged at 26,000 rpm at 2°C for 16 h in a Beckman SW-41 rotor. Gradient fractions were collected from the top and analyzed for radioactivity by liquid scintillation counting. Bound [3H]ryanodine was determined by filtration (26) after dilution of samples with 25 volumes of ice-cold water. Apparent sedimentation coefficients (uncorrected for bound detergent and lipid) of the [3H]ryanodine receptor were determined by extrapolation of an enzyme calibration curve obtained with Escherichia coli β-galactosidase (18 S), bovine catalase (11.2 S), and yeast alcohol dehydrogenase (7.6 S). Biochemical Assays—Protein was determined by the Lowry method using bovine serum albumin as a standard (27). Free Ca^2+ concentrations were calculated by a computer program using binding constants published by Fabiato (28).

RESULTS

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—SDS-polyacrylamide gradient gel electrophoresis of rabbit muscle heavy SR membranes indicated the presence of several major protein bands including two bands of apparent Mr, (relative molecular mass) of 110,000 (110 kDa) and ~400,000 (~400 kDa) (Fig. 1A, lane 1). The band of 110 kDa is the Ca^2+ pump or (Ca^2+ + Mg^2+)-ATPase of sarcoplasmic reticulum. The ~400-kDa polypeptide forms, in Chaps solution, a tetramericy 30 S complex which is identical with the SR Ca^2+ release channel and the foot structures that span the T-tubule-SR junction (9). Proteolytic digestion of heavy SR vesicles at the high protein:trypsin ratio of 10,000:1 for 1 min resulted in the disappearance of the ~400-kDa band (Fig. 1A, lane 3). Under our experimental conditions, the 110-kDa Ca^2+ pump protein was less sensitive to proteolytic digestion.
Treatment at a SR protein:trypsin ratio of 10,000:1 for 1 min caused cleavage of a portion of the Ca\textsuperscript{2+} pump protein (Fig. 1A, lane 3), as indicated by the appearance of a major polypeptide band of \( M_c \sim 55,000 \) (29, 30); however, a longer incubation time of 5 min at a 10 times higher trypsin concentration (SR protein:trypsin ratio of 1000:1) was required to effect disappearance of the 110-kDa band.

The proteolytic fragments of the Ca\textsuperscript{2+} release channel which retained immunoreactivity following degradation were identified by immunoblot staining (Fig. 1B), using a rabbit antiserum raised against the purified rat skeletal muscle \( \sim 400\)-kDa Ca\textsuperscript{2+} release channel protein. Comparison of the minus trypsin control lanes (lane 1 of Fig. 1, A and B) shows that the antibody specifically recognized the \( \sim 400\)-kDa polypeptide among the major protein bands. The antibody also visualized an additional minor band which migrated just below the \( \sim 400\)-kDa protein and whose intensity varied with different membrane preparations. Most likely its appearance is due to partial degradation of the \( \sim 400\)-kDa polypeptide (9). The plus trypsin control lane (lane 2 of Fig. 1, A and B) indicates minimal degradation of the \( \sim 400\)-kDa band when soybean trypsin inhibitor protein and DIFP were added to the vesicles before the addition of trypsin. This suggested rapid and effective inactivation of trypsin in the vesicle suspension under our experimental conditions.

Immunoblots of membranes digested with trypsin are shown in the last five lanes of Fig. 1B. Mild proteolysis (at a SR protein:trypsin ratio of 10,000:1 for 1 min) resulted in the loss of the \( \sim 400\)-kDa protein and a concomitant appearance of six major immunoreactive polypeptides of \( M_c \sim 250,000, \sim 230,000, \sim 220,000, 205,000, 160,000, \) and 65,000 and four minor, with respect to quantity and/or immunoreactivity, bands of \( M_c \) 190,000, 125,000, 55,000, and 50,000 (Fig. 1B, lane 3). More severe proteolytic conditions (SR protein:trypsin ratio of 1000:1) caused the immediate appearance of three major polypeptides of \( M_c \) 160,000, 65,000, and 27,000, and minor bands of \( M_c \sim 220,000, 190,000, 125,000, 55,000, \) and 50,000 after 1 min of trypsin exposure (Fig. 1B, lane 4). Faint bands of \( M_c \) 110,000 and 90,000 also present after 1 min were more pronounced after a 5-min exposure (Fig. 1B, lane 5) as the higher molecular weight bands were substantially decreased. No major immunoreactive bands were observed after 60 min of trypsin exposure (Fig. 1B, lane 7).

\(^{45}\text{Ca}\textsuperscript{2+} \text{Release}\) —Fig. 2 compares the \(^{45}\text{Ca}\textsuperscript{2+} \text{release behavior of undigested and trypsin-digested heavy SR vesicles. Vesicles were passively loaded with 1 mM \(^{45}\text{Ca}\textsuperscript{2+}\) and then diluted into a medium which either inhibited or activated the SR Ca\textsuperscript{2+} release channel. \(^{45}\text{Ca}\textsuperscript{2+} \text{release was slow in the Ca}\textsuperscript{2+} \text{release channel inhibiting medium which contained 10 mM Mg}\textsuperscript{2+} \text{and 10 mM ruthenium red. This allowed determination of the amounts of \(^{45}\text{Ca}\textsuperscript{2+}\) (23 nmol/ml protein) trapped by the vesicles. About 75\% of the trapped \(^{45}\text{Ca}\textsuperscript{2+}\) was released in less than 30 s on dilution of the vesicles into the Ca\textsuperscript{2+} release channel activating medium which contained 10 mM free Ca\textsuperscript{2+}. Some of the \(^{45}\text{Ca}\textsuperscript{2+}\) (\( \sim 5 \) nmol/mg protein) remained with the vesicles for longer times because not all of them contained the Ca\textsuperscript{2+} release channel (21).}

Proteolytic digestion of heavy SR vesicles for 1 and 20 min at a SR protein:trypsin ratio of 25:1 did not appreciably change the amounts of \(^{45}\text{Ca}\textsuperscript{2+}\) trapped by the vesicles in the
Figure 2. Effect of trypsin digestion on \(^{45}\text{Ca}^{2+}\) release behavior of heavy SR vesicles. A heavy SR \(\text{Ca}^{2+}\) release vesicle fraction (10 mg of protein/ml) was passively loaded for 2 h at 22 °C with 1 mM \(^{45}\text{Ca}^{2+}\). Vesicles were then digested with 400 μg of trypsin/ml for the indicated times as described under "Experimental Procedures." The \(^{45}\text{Ca}^{2+}\) efflux behavior of control (minus trypsin, trypsin inhibitor protein, and DIFF) added before addition of trypsin and trypsin-digested vesicles was measured by 200-fold dilution in release media containing 10 mM Mg\(^{2+}\) and 10 μM ruthenium red (solid symbols) or 100 μM EGTA plus 105 μM Ca\(^{2+}\) (10 μM free Ca\(^{2+}\)) (open symbols).

Channel inhibiting medium. At longer time intervals, 60 min, a partial breakdown of the \(^{45}\text{Ca}^{2+}\) permeability barrier was observed for vesicles diluted into the medium containing 10 mM Mg\(^{2+}\) and 10 μM ruthenium red. Inability of the vesicles to trap \(^{45}\text{Ca}^{2+}\) did not appear to be due to the presence of a \(\text{Ca}^{2+}\) release channel which could not be closed by Mg\(^{2+}\) and ruthenium red, based on the following two observations. First, prolonged trypsin digestion similarly decreased the amounts of \(^{45}\text{Ca}^{2+}\) trapped by light SR vesicles lacking the channel (not shown). Second, control experiments with the channel-impermeable molecule \([^{14}\text{C}]\)sucrose showed that trypsin digestion decreased the amounts of \([^{14}\text{C}]\)sucrose trapped by the vesicles to an extent similarly as observed for \(^{45}\text{Ca}^{2+}\) in the channel inhibiting medium in Fig. 2.

There occurred several noticeable changes in the initial rate of \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release after trypsin digestion of the vesicles. Fig. 2 shows that trypsin digestion for 20 min caused a decrease in the rate of \(^{45}\text{Ca}^{2+}\) efflux without appreciably affecting the total amount of \(^{45}\text{Ca}^{2+}\) that was released by the vesicles in 150 s after dilution into the 10 μM \(\text{Ca}^{2+}\) release medium. The effects of more limited trypsin digestion on the initial rates of \(^{45}\text{Ca}^{2+}\) efflux were determined with the use of a rapid mixing and quench apparatus (Fig. 3). Treatment for 1 min at a SR protein:trypsin ratio of 1000:1 resulted in a small decrease of the rate of \(\text{Ca}^{2+}\)-induced \(^{45}\text{Ca}^{2+}\) release. More extensive treatment for 1 and 15 min at a SR protein:trypsin ratio of 100:1 had an opposite effect in that the vesicles lost their \(^{45}\text{Ca}^{2+}\) stores with a faster rate (1.5- and 3.5-fold) than the undigested vesicles. Taken together, the data of Figs. 2 and 3 suggest that mild trypsin digestion is without a marked effect on \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release channel activity. At an intermediate stage of trypsin digestion the channel is activated, whereas extensive proteolysis appeared to result in channel inactivation. This latter stage was difficult to quantitate due to a general breakdown of the permeability barrier of the vesicles.

Figure 3. Effect of trypsin digestion on \(^{45}\text{Ca}^{2+}\) efflux rates from heavy SR vesicles. A heavy SR \(\text{Ca}^{2+}\) release vesicle fraction (2 mg of protein/ml) was digested in the presence of 1 mM \(^{45}\text{Ca}^{2+}\) at a SR protein:trypsin ratio of 1000:1 (○) or 100:1 (□, △), for 1 min (□, △) or 15 min (△), as described under "Experimental Procedures." \(^{45}\text{Ca}^{2+}\) efflux rates from control (minus trypsin, trypsin inhibitor protein, and DIFF) added before the addition of trypsin, and trypsin-digested vesicles were determined in a release medium containing 10 μM free \(\text{Ca}^{2+}\) by a rapid mixing quench protocol as described under "Experimental Procedures." The time course of \(^{45}\text{Ca}^{2+}\) efflux from SR \(\text{Ca}^{2+}\) release vesicles, i.e. from the vesicle population containing the \(\text{Ca}^{2+}\) release channel, was obtained by subtracting the amount not readily released (Ref. 25; see also Fig. 2).

The \(\text{Ca}^{2+}\) release channel of skeletal SR contains regulatory binding sites for \(\text{Ca}^{2+}\), Mg\(^{2+}\), and adenine nucleotides (25, 31). The effects of trypsin digestion on these sites was tested by determining the \(\text{Ca}^{2+}\) release behavior of the vesicles in nanomolar and micromolar \(\text{Ca}^{2+}\) media without or with added Mg\(^{2+}\) and adenine nucleotide (Fig. 4). Digestion of the vesicles for 5 and 30 min at a SR protein:trypsin ratio of 100:1
increased and subsequently decreased, respectively, the rate of $^{45}$Ca$^{2+}$ efflux in the nanomolar Ca$^{2+}$ medium. In the three vesicle preparations, $^{45}$Ca$^{2+}$ release was accelerated by the addition of 5 mM AMP to the nanomolar Ca$^{2+}$ medium or by increasing the free Ca$^{2+}$ concentration from $<10^{-6}$ M to $6 \times 10^{-4}$ M. In the undigested vesicles and after digestion of 5 min, a majority of the vesicles released their $^{45}$Ca$^{2+}$ stores in less than 30 s. Digestion for 30 min resulted in a small decrease of the amounts of $^{45}$Ca$^{2+}$ released after 30 s in the micromolar Ca$^{2+}$ and nucleotide containing media. Addition of 1 mM Mg$^{2+}$ to the micromolar Ca$^{2+}$ release medium reduced the rate of $^{45}$Ca$^{2+}$ released in all three preparations. The data of Fig. 4 suggest that trypsin digestion modifies SR $^{45}$Ca$^{2+}$ release activity without causing a loss of regulation of the channel by Ca$^{2+}$, Mg$^{2+}$, and adenine nucleotide.

**Single Channel Measurements**—Another more direct way of measuring Ca$^{2+}$ release channel activity involves the incorporation of single channels into planar lipid bilayers (7, 8). Due to the low frequency of incorporation of only one or two Ca$^{2+}$ release channels into the bilayer, the effects of trypsin were observed in only five separate recordings, two of which are shown in Fig. 5. The current fluctuations of a single skeletal Ca$^{2+}$ release channel which was recorded with 50 mM Ca$^{2+}$ trans as the current carrier are shown in Fig. 5A. In the upper trace, the channel was partially activated by 2.5 $\mu$M free Ca$^{2+}$ in the cis chamber (the SR cytoplasmic side of the channel). In the second trace of Fig. 5A, an increase in channel activity was observed after trypsin (10 $\mu$g/ml) was added to the cis chamber. Channel open time ($P_o$) increased from 0.07 (A, upper trace) to 0.80 (A, second trace) 6 min after the addition of trypsin without a change of unit conductance (100 pS in 50 mM Ca$^{2+}$). The last trace of Fig. 5A shows that after 9 min of trypsin exposure channel activity ceased. This abrupt inactivation was preceded by a short period of reduced channel activity. Channel inactivation was considered to be irreversible since an increase in cis Ca$^{2+}$ or the addition of ATP were ineffective in reactivating the channel (not shown). This behavior contrasted with that observed during the trypsin-induced activation phase where the channel remained sensitive to further activation by ATP and inhibition by Mg$^{2+}$ (not shown). In two recordings, trypsin did not appreciably activate the SR Ca$^{2+}$ release channel when added to the trans chamber (the SR luminal side of the channel) for 30 min. Fig. 5B shows the effects of trypsin on another single Ca$^{2+}$ release channel which was activated ($P_o = 0.69$) by 2.5 $\mu$M Ca$^{2+}$ and 1 mM ATP cis. In this situation, trypsin did not significantly affect channel activity until about 10 min after the addition of trypsin, when the channel abruptly, and irreversibly, ceased to conduct Ca$^{2+}$.

**[H]Ryanodine Binding**—Ryanodine is a neutral plant alkaloid which binds with high affinity and specificity to the SR Ca$^{2+}$ release channel (9-11). The drug has a dual effect in that nanomolar concentrations open the channel, whereas at concentrations above 10 $\mu$M, ryanodine completely closes the skeletal channel (32, 33). [H]Ryanodine binding was measured in the presence of micromolar free Ca$^{2+}$ and 2.5 mM AMP-PCP, a nonhydrolyzable ATP analog (Fig. 6). The two ligands nearly optimally open the skeletal SR Ca$^{2+}$ release channel (8) and thereby favor use-dependent activation and inactivation of the channel by ryanodine (32). Figure 6A shows that trypsin digestion decreased $[^{3}H]$ryanodine binding to a greater extent at nanomolar concentrations than in the micromolar concentration range. Scatchard plot analysis of the $[^{3}H]$ryanodine binding data indicated the presence of a high affinity binding site with a $B_{max}$ value of about 13.5 pmol/mg of protein and a $K_d$ of about 4 nM for undigested vesicles (inset, Fig. 6A). In trypsin-treated vesicles (at a SR protein:trypsin ratio of 400:1) the number of high affinity binding sites was appreciably reduced after a digestion time of 3 min. No high affinity binding was detected in vesicles treated with trypsin for 30 min. The data of Fig. 6A also indicate the presence of additional low affinity $[^{3}H]$ryanodine binding sites which appeared to be less affected by trypsin digestion. In undigested vesicles, the total number of high and low affinity binding sites was 50 ± 15 pmol/mg protein, suggesting that for each high affinity binding site there are approximately three low affinity binding sites. Re-arrangement of the binding data in the form of a Hill plot yielded a non-linear curve which at free ryanodine concentrations in the range of 0.3-3 and 50-1000 nM could be approx-
Effects of Trypsin on SR Ca\(^{2+}\) Release Channel

**Fig. 6. Effect of trypsin digestion on \[^{3}H\]ryanodine binding to SR vesicles.** A heavy SR vesicle fraction (6 mg of protein/ml) was treated with 15 µg of trypsin/ml for 3 min (■) and 30 min (▲). \[^{3}H\]Ryanodine binding to both control (minus trypsin) and trypsin-digested vesicles was determined as described under “Experimental Procedures.” In parallel experiments, it was established that in each condition, equilibrium of \[^{3}H\]ryanodine binding was reached. Specific binding of \[^{3}H\]ryanodine is shown in A and in the form of a Scatchard plot (inset of A) and Hill plots (B), and was obtained by assuming that nonspecific binding is linear with ryanodine concentration. Nonspecific binding was defined as the difference between total and specific binding and amounted to 8 nmol/mg protein at 1 mM \[^{3}H\]ryanodine. Hill plots were obtained assuming that specific binding of \[^{3}H\]ryanodine amounted to 54 pmol/mg protein (one high and three low affinity binding sites in control vesicles). Data shown are from one representative set of results chosen from three separate experiments performed with vesicles digested to slightly different extents by using varying SR protein:trypsin ratios and times.

Imitated by two straight lines (Fig. 6B). From the slopes, \(n\) values of 0.95 and 0.35 were obtained, respectively. Hill plots of binding data of vesicles treated with trypsin at a protein ratio of 400:1 for 5 min showed a less non-linear behavior, whereas binding to more extensively digested vesicles (for 30 min) could be reasonably well fitted by a straight line with an \(n\) value of 0.75. These results suggested that in trypsin-digested vesicles there occurred a significant change in the affinity and cooperativity of \[^{3}H\]ryanodine binding.

**Sedimentation Coefficients—**The apparent sedimentation coefficient of the Chaps-solubilized \[^{3}H\]ryanodine receptor-Ca\(^{2+}\) release channel complex was determined by sucrose density gradient centrifugation. In the control condition in the absence of trypsin digestion, a single peak of bound radioactivity was observed in the lower half of the gradient (Fig. 7). Bound radioactivity comigrated with a minor protein peak with an apparent sedimentation coefficient of 30 S and composed of polypeptides of \(M_{r} \approx 400,000\) (9). Trypsin treatment of membranes for 5 min at a protein:trypsin ratio of 1000:1 followed by Chaps solubilization resulted only in a small shift of the S value (Fig. 7). However, a more dramatic decrease of the S value from 30 S to ~10 S was observed after digestion for 120 min. This reduction in sedimentation rate suggested that there had occurred a significant change in size and/or protein conformation of the trypsin-digested, Chaps-solubilized receptor complex.

**Time Course of Trypsin Digestion—**Heavy SR vesicles prelabeled and not labeled with \[^{3}H\]ryanodine were digested for varying times with trypsin in the presence of 1 mM Ca\(^{2+}\). Vesicle suspensions were then divided to compare the time course of the effects of trypsin digestion on (i) \(^{45}Ca\(^{2+}\) release, (ii) \[^{3}H\]ryanodine binding, and (iii) the apparent sedimentation coefficient of the Chaps-solubilized \[^{3}H\]ryanodine receptor (Fig. 8). In control experiments, an essentially identical fragmentation pattern of the \(\sim 400\, \text{KDa}\) band was observed on immunoblots when vesicles were not labeled (Fig. 1B) or were prelabeled with a saturating concentration of ryanodine (20 µM) prior to trypsin digestion, suggesting a similar pattern of degradation for the unlabeled and labeled receptor. Tryptic digestion at a SR protein:trypsin ratio of 500:1 for 1 min resulted in a 1.5-fold decrease of the \(^{45}Ca\(^{2+}\) efflux rate, measured by diluting the vesicles into a nanomolar free Ca\(^{2+}\) release medium, without an appreciable effect on \[^{3}H\]ryanodine binding or the sedimentation behavior of the channel complex. Continued exposure to trypsin resulted in an increase of the \(^{45}Ca\(^{2+}\) release rate, a decrease of the S value of the solubilized complex from 30 S to about 15 S and a loss of high affinity \[^{3}H\]ryanodine binding when assayed after trypsination.
Fig. 8. Time course of the effects of trypsin digestion on 
\(^{45}\text{Ca}^++\) release, \(^{[3]H}\)ryanodine binding, and sedimentation coefficient of the \(^{[3]H}\)ryanodine receptor. Heavy SR vesicles (5 mg of protein/ml) prelabeled and not labeled with 2 nm \(^{[3]H}\)ryanodine were treated for the indicated times with 10 \(\mu\)g (solid symbols) of trypsin or for 30 min with 50 \(\mu\)g (open symbols) of trypsin as described under "Experimental Procedures." Aliquots of the unlabeled vesicles were used to determine (i) \(^{45}\text{Ca}^++\) release rates at 10\(^{-6}\) m free \(\text{Ca}^++\) (cf. Fig. 4) and (ii) the \(B_{\text{max}}\) value of high affinity \(^{[3]H}\)ryanodine binding (cf. Fig. 6A). Aliquots of the \(^{[3]H}\)ryanodine labeled vesicles were used to determine (i) amounts of bound \(^{[3]H}\)ryanodine remaining with the vesicles, and (ii) sedimentation coefficients of the Chaps-solubilized \(^{[3]H}\)ryanodine receptor. The 100% control values were an initial \(^{45}\text{Ca}^++\) release rate of 0.3 \(\pm\) 0.05 nmol of \(^{45}\text{Ca}^++\)/mg protein/s, a \(B_{\text{max}}\) value of 12 \(\pm\) 2 pmol of \(^{[3]H}\)ryanodine/mg protein for the postlabeled samples, 0.4 \(\pm\) 0.1 pmol of \(^{[3]H}\)ryanodine bound per mg protein for the prelabeled samples, and an \(S\) value of 30 S. Data are the average of three determinations.

Vesicles were also first labeled with 2 nm \(^{[3]H}\)ryanodine before being treated with trypsin. In this case, membranes digested at a protein:trypsin ratio of 500:1 for 30 min retained greater than 80% of the bound \(^{[3]H}\)ryanodine (Fig. 8). By comparison, high affinity \(^{[3]H}\)ryanodine binding was reduced to less than 1% when vesicles were labeled after digestion at the same SR protein:trypsin ratio for 30 min.

Fig. 8 further shows that digestion at a lower SR protein:trypsin ratio, 100:1, for 30 min decreased the \(S\) value of the solubilized complex to \(~10\) S and the \(^{45}\text{Ca}^++\) efflux rate to a value close to that of undigested vesicles. The effects of more extensive digestion were not compared because of a loss of the general permeability barrier of the vesicles (see Fig. 2) and a large decrease in the amount of bound \(^{[3]H}\)ryanodine on solubilization with Chaps (not shown).

DISCUSSION

Heavy junctional-derived SR vesicles contain a ligand-gated \(\text{Ca}^{2+}\) release channel which is composed of polypeptides of \(M_r \sim 400,000\). Earlier studies showing the rapid degradation of high molecular mass SR proteins by \(\text{Ca}^{2+}\)-activated protease (16) have been corroborated by this report and other recent observations that the \(~400\)-kDa channel protein is highly susceptible to proteolysis (9, 18, 20). The appearance of smaller peptides of \(~300\) and 160 kDa upon isolation of the ryanodine receptor, in addition to the \(~400\)-kDa protein, initially suggested them to be distinct subunits of a hetero-

meric complex (34, 35). However, more recent evidence has revealed that, when purified in the presence of protease inhibitors, the \(~400\)-kDa protein is the major constituent of the ryanodine receptor complex (9-11), indicating that the \(~300\) and \(~160\)-kDa proteins were derived from proteolytic fragmentation caused by endogenous proteases (9). In this regard, recent studies suggesting the involvement/association of proteins smaller than the \(~400\)-kDa channel protein with SR \(\text{Ca}^{2+}\) release (36-38), may conceivably be related to its acute lability. These developments in defining the subunit composition of the SR \(\text{Ca}^{2+}\) release channel are the converse of that which occurred for the muscle nicotinic acetylcholine receptor channel complex, where early purification studies identified a single subunit (\(~40\) kDa), which turned out to have derived from proteolysis of a heteropentameric complex (\(\alpha_2\), \(\beta\), \(\gamma\), \(\delta\)) comprising subunits of \(>40\) kDa (39, 40).

Mild trypsin digestion of SR membranes resulted in the transient appearance of protein bands of \(115-170\) kDa which underwent further rapid proteolysis (18). In the present study, the use of a specific anti-30 S complex antiserum has allowed a more definitive determination of the trypsin fragmentation pattern of the \(\text{Ca}^{2+}\) release channel proteins and has enabled its correlation with the \(^{[3]H}\)ryanodine binding, sedimentation behavior, and functional characteristics of the \(\text{Ca}^{2+}\) release channel. Our studies indicate that short term trypsin digestion of ryanodine-labeled and unlabeled vesicles at a high SR protein:trypsin ratio results in the complete disappearance of the \(~400\)-kDa channel band on SDS gels and the corresponding appearance of a series of lower molecular mass bands between \(~250\) and 27 kDa upon immunoblot analysis (Fig. 1B, lanes 3 and 4) without an appreciable effect on \(^{[3]H}\)ryanodine binding or the sedimentation behavior of the Chaps-solubilized channel complex. In addition, the rate of \(^{45}\text{Ca}^{2+}\) release from the vesicles decreased maximally 1.5-fold under conditions of partial activation of the SR \(\text{Ca}^{2+}\) release channel, i.e. when vesicles were diluted into media containing nanomolar (Fig. 8) or micromolar (Fig. 3) free \(\text{Ca}^{2+}\).

More extensive trypsin exposure produced several more marked changes in the structure and function of the channel complex. Disappearance of polypeptides with \(M_r > 70,000\) on immunoblots of ryanodine-labeled (not shown) and unlabeled membranes (Fig. 1B, lanes 6 and 7) correlated with a decrease of the apparent \(S\) value of the solubilized receptor complex from \(30\) S to \(10-15\) S. Although \(^{[3]H}\)ryanodine bound to the receptor prior to digestion remained bound, significant differences in the affinity and interaction of the binding sites were noticed when \(^{[3]H}\)ryanodine binding was studied after trypsin digestion. Since a similar number of total binding sites (high and low affinity) were measured in the postlabeling experiments, trypsin digestion appeared to induce protein conformational changes in the complex which specifically altered the high affinity sites without causing a measurable loss of low affinity \(^{[3]H}\)ryanodine binding sites. This surprising finding may help to elucidate the subunit composition and stoichiometry of the \(\text{Ca}^{2+}\) channel oligomeric complex. An additional observation was that more extensive trypsin digestion resulted in a transient increase of the \(^{45}\text{Ca}^{2+}\) efflux rate (Figs. 3 and 8). That this increase was specifically due to an activation of the high conductance SR \(\text{Ca}^{2+}\) release channel was strongly suggested by the following two observations. First, trypsin digestion did not appreciably increase the \(\text{Ca}^{2+}\) permeability of light SR vesicles which lacked the \(\text{Ca}^{2+}\) release channel. During trypsin digestion the SR \(\text{Ca}^{2+}\) pump in heavy and light SR vesicles can be cleaved into two peptides of \(M_r \sim 55,000\) and 50,000 (29, 30) which appeared as one major broad band in our gel system (Fig. 1A). Although further slow cleavage of the \(\text{Ca}^{2+}\) pump protein has been reported to render SR vesicles "leaky" to \(\text{Ca}^{2+}\) by uncoupling \(\text{Ca}^{2+}\) transport from
ATP hydrolysis (41, 42) and this may have occurred under our experimental conditions, we did not observe a significant increase in the number of leaky vesicles. In this regard, it should also be noted that Shoshan-Barmatz et al. (17) have recently found the loss of Ca$^{2+}$ accumulation by trypsin-digested vesicles is not due to the cleavage of the Ca$^{2+}$ pump protein, but rather to that of some other component of the SR membrane. A second observation supporting a direct action of trypsin on the Ca$^{2+}$ release channel was that in planar bilayers trypsin increased the fraction of open time of the Ca$^{2+}$-activated channel. Subsequent to channel activation, a brief period of reduced activity was noticed which was followed by a complete and irreversible loss of channel activity. Trypsin digestion did not induce sub-levels of conductance, a phenomenon previously observed when the purified skeletal 30 S channel complex was reconstituted into the bilayers (9), and was ineffective in changing channel activity when added to the luminal side of the channel. Our measurements further revealed that the trypsin-digested channel remained sensitive to activation by Ca$^{2+}$ and adenine nucleotide and inhibition by Mg$^{2+}$ and ruthenium red. As a consequence, the extent of channel activation depended on the composition of the release/bilayer media (Figs. 4 and 5).

In a recent study, under conditions of extensive digestion of the ~400-kDa protein, the junctional processes of heavy vesicles were visualized by electron microscopy without discernible alterations in ultrastructure (18). This observation suggested that the proteolytic fragments were stabilized by multiple noncovalent interactions which can only be dissociated by strong detergents. In the present study, a decrease in the apparent sedimentation coefficient of the trypsin-digested, Chaps-solubilized receptor complex from 30 S to 10 S was observed with retention of prelabelled [3H]ryanodine binding (Fig. 8). Since ultrastructural studies with the purified channel complex have revealed the presence of four peripheral protein “loops” which project from an electron-dense core region of the complex (9), a full or partial removal of the protein loops, during detergent solubilization of the membranes would appear to be the most plausible explanation for the decrease in S value. Retention of [3H]ryanodine to the ~10 S complex would therefore suggest that the central portion of the complex comprises the high affinity ryanodine binding site and is held together by noncovalent bonds which are not broken by the mild detergent Chaps.

In conclusion, we have presented evidence that the cytoplasmic transverse tubule-SR junction spanning portion of the SR Ca$^{2+}$ release channel is highly sensitive to proteolysis. Our results indicate that the channel retains its ability to conduct Ca$^{2+}$ after cleavage of the ~400-kDa channel subunits to fragments identifiable by immunoblotting of less than 70,000 daltons. In addition, although measurement of channel activity and [3H]ryanodine binding activity after proteolysis suggested the occurrence of protein structural changes, the channel remained sensitive to activation by Ca$^{2+}$ and adenine nucleotide and inhibition by Mg$^{2+}$ and ruthenium red.

Acknowledgments—We thank Andrea Denk, Julia Henderson, Jeffrey LaDine, and Amy Smith for excellent technical assistance.

Note Added in Proof—The effects of trypsin on SR Ca$^{2+}$ release activity have been also recently reported by U. Shoshan-Barmatz and A., Zarka ([1998] J. Biol. Chem. 263, 16772-16779) and J. L., Trimm, G., Salama and J. L., Abramson ([1988] J. Biol. Chem. 263, 17443-17451).

REFERENCES

1. Endo, M. (1977) Physiol. Rev. 57, 71-108
2. Stephenson, E. W. (1981) Am. J. Physiol. 240, C1-C19
3. Fabiato, A. (1983) Am. J. Physiol. 245, C1-C14
4. Somlyo, A. P. (1985) Nature 316, 298-299
5. Ferguson, D. G., Schwartz, H. W., and Franki-Armon, C. (1984) J. Cell Biol. 99, 1735-1742
6. Kawamoto, R. M., Brunswig, J.-P., Kim, K. C., and Caswell, A. H. (1986) J. Cell Biol. 103, 1405-1414
7. Smith, J. S., Coronado, R., and Meissner, G. (1985) Nature 316, 446-449
8. Smith, J. S., Coronado, R., and Meissner, G. (1986) J. Gen. Physiol. 88, 573-586
9. Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q. Y., and Meissner, G. (1988) Nature 331, 315-319
10. Imagawa, T., Smith, J. S., Coronado, R., and Campbell, K. P. (1987) J. Biol. Chem. 262, 16636-16643
11. Inui, M., Saito, A., and Fleischer, S. (1987) J. Biol. Chem. 262, 15637-15642
12. Hymel, L., Inui, M., Fleischer, S., and Schindler, H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 441-445
13. Smith, J. S., Imagawa, T., Ma, J., Fill, M., Campbell, K. P., and Coronado, R. (1988) J. Gen. Physiol 92, 1-26
14. Saito, A., Inui, M., Rademacher, M., Frank, J., and Fleischer, S. (1988) J. Cell Biol. 107, 211-219
15. Schneider, M. F. (1981) Annu. Rev. Physiol. 43, 507-517
16. Seiler, S., Wegener, A. D., Whang, D. D., Hathaway, D. R., and Jones, L. R. (1984) J. Biol. Chem. 259, 8550-8557
17. Shoshan-Barmatz, V., Ouzil, N., and Chipman, D. M. (1987) J. Biol. Chem. 262, 11559-11564
18. Chu, A., Sumbilla, C., Scales, D., Platta, A., and Inesi, G. (1988) Biochemistry 27, 2827-2833
19. Trimm, J. L., Salama, G., and Abramson, J. L. (1988) Biophys. J. 53, 357 (abstr.)
20. Rousseau, E., Lai, F. A., Henderson, J. S., and Meissner, G. (1988) Biophys. J. 53, 455 (abstr.)
21. Meissner, G. (1984) J. Biol. Chem. 259, 2365-2374
22. Laemmli, U. K. (1970) Nature 227, 680-685
23. Otter, T., King, S. M., and Witman, G. B. (1987) Anal. Biochem. 162, 370-377
24. Johnson, D. A., Gauthcs, J. W., Sportsman, J. R., and Elder, J. M. (1984) Gene Anal. Tech. 1, 3-8
25. Meissner, G., Darling, E., and Eveleth, J. (1986) Biochemistry 25, 236-244
26. Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., and Casida, J. E. (1987) J. Biol. Chem. 261, 8643-8648
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
28. Fabiato, A. (1981) J. Gen. Physiol. 78, 457-497
29. Inesi, G., and Scales, D. (1974) Biochemistry 13, 3286-3290
30. Shamoo, A. E., Ryan, T. E., Stewart, P. S., and MacLennan, D. H. (1976) J. Biol. Chem. 251, 4147-4154
31. Nagasaki, K., and Kashi, M. (1983) J. Biochem. (Tokyo) 94, 1101-1109
32. Meissner, G. (1986) J. Biol. Chem. 261, 6300-6306
33. Lattanzio, F. A., Jr., Schlatterer, R. G., Nicar, M., Campbell, K. P., and Sutko, J. L. (1987) J. Biol. Chem. 262, 2711-2718
34. Lai, F. A., Erickson, H., Block, B. A., and Meissner, G. (1987) Biochem. Biophys. Res. Commun. 143, 704-709
35. Inui, M., Saito, A., and Fleischer, S. (1987) J. Biol. Chem. 262, 1740-1747
36. Morii, M., Danio, S., Kim, D. H., and Ikemoto, N. (1986) J. Biol. Chem. 261, 2534-2548
37. Kim, D. H., and Ikemoto, N. (1986) J. Biol. Chem. 261, 11674-11679
38. Zoratto, F. Margreth, A., and Volpe, P. (1986) J. Biol. Chem. 261, 13252-13257
39. Conti-Tronconi, B. M., and Raftery, M. A. (1982) Annu. Rev. Biochem. 51, 491-530
40. Soma, N., Noda, M., Takahashi, H., Tanabe, T., Toyoshio, M., Furutani, Y., and Kikyotani, S. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 57-60
41. Scott, T. L., and Shimoo, A. E. (1982) J. Membr. Bioi. 64, 137-144
42. Huang, J. L., Topping, T. B., He, Z., Folsom, B., and Dunker, A. K. (1987) J. Membr. Biol. 100, 193-206