Stull, J. T., & Buss, J. E. (1977) *J. Biol. Chem. 252*, 851–857.
Syska, H., Perry, S. V., & Trayer, I. P. (1974) *FEBS Lett. 40*, 253–257.
Syska, H., Wilkinson, J. M., Grand, R. J. A., & Perry, S. V. (1976) *Biochem. J. 153*, 375–387.
Talbot, J. A., & Hodges, R. S. (1979) *J. Biol. Chem. 254*, 3720–3723.
Talbot, J. A., & Hodges, R. S. (1981a) *J. Biol. Chem. 256*, 2798–2802.
Talbot, J. A., & Hodges, R. S. (1981b) *J. Biol. Chem. 256*, 12374–12379.
Toyo-Oka, T. (1982) *Biochem. Biophys. Res. Commun 107*, 44–50.
Van Eerd, J. P., & Takahashi, K. (1975) *Biochem. Biophys. Res. Commun. 64*, 122–127.
Villar-Palasi, C., & Kumon, A. (1981) *J. Biol. Chem. 256*, 7409–7415.
Wang, C.-L. A., & Gergely, J. (1986) *Eur. J. Biochem. 154*, 225–228.
Wilkinson, J. M. (1980) *Eur. J. Biochem. 103*, 179–188.
Wilkinson, J. M., & Grand, R. J. A. (1975) *Biochem. J. 149*, 493–496.
Wilkinson, J. M., & Grand, R. J. A. (1978a) *Nature (London) 271*, 31–35.
Wilkinson, J. M., & Grand, R. J. A. (1978b) *Eur. J. Biochem. 82*, 493–501.
Yamamoto, K., & Ohtsuki, I. (1982) *J. Biochem. (Tokyo) 91*, 1669–1677.
Zot, A., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535–559.

Trypsin Digestion of Junctional Sarcoplasmic Reticulum Vesicles†

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ABSTRACT: A putative constituent of the junctional processes, connecting the terminal cisternae of sarcoplasmic reticulum and the transverse tubules of skeletal muscle fibers, is a ≥350 000-dalton (Da) protein that displays ryanodine binding and Ca^{2+} channel properties. Ryanodine modulation of Ca^{2+} fluxes suggests that the ryanodine receptor and calcium channel are integral parts of one functional unit corresponding to the ≥350 000-Da protein [Inui, M., Saito, E., & Fleischer, S. (1987) *J. Biol. Chem. 262*, 1740–1747; Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. L., Sutko, J. L., Kahl, S. D., Raab, C. R., & Madson, L. (1987) *J. Biol. Chem. 262*, 6460–6463]. We subjected vesicular fragments of junctional–cisternal membrane to stepwise trypsin digestion. The ≥350 000-Da protein is selectively cleaved in the early stage of digestion, with consequent disappearance of the corresponding band in electrophoretic gels. The Ca^{2+}-ATPase is cleaved at a later stage, while calsequestrin is not digested under the same experimental conditions. While the Ca^{2+}-ATPase yields two complementary fragments that are relatively resistant to further digestion, the ≥350 000-Da protein yields fragments that are rapidly broken down to small peptides. Under conditions producing extensive digestion of the ≥350 000-Da protein, the junctional processes are still visualized by electron microscopy, with no discernible alterations of their ultrastructure. The functional properties of the Ca^{2+} release channel are also maintained following trypsin digestion, including blockage by Mg^{2+} and ruthenium red and activation by Ca^{2+} and nucleotides. Prolonged incubations with and after trypsin produce functional alterations. Ryanodine, at relatively high (micromolar) concentrations, partitions into a nonsaturable compartment of the membrane and inhibits Ca^{2+} efflux through the release channel. On the other hand, high-affinity ryanodine binding ($K_d = 3 \times 10^{-7}$ M) occurs with a stoichiometry approximating that of the ≥350 000-Da protein and produces a reduction of net Ca^{2+} uptake by the vesicles, due to increased Ca^{2+} efflux through the release channel. The bound ryanodine does not interfere with digestion of the ≥350 000-Da protein and is still bound following digestion. We suggest that the ≥350 000-Da protein permits entrance of trypsin into a large crevice (likely at the opening of the channel) where multiple cleavage sites are readily available. The resulting proteolytic fragments remain stabilized by multiple noncovalent interactions and are only dissociated by strong detergents. Additional protein components may contribute to structural stabilization of the junctional processes. Ryanodine binds to a protein domain where it does not interfere with trypsin binding but can regulate the channel through allosteric mechanisms.

Clarification of the mechanism of Ca^{2+} release from sarcoplasmic reticulum (SR) is a missing link for a molecular understanding of excitation–contraction coupling in muscle. Recent progress has been made in this regard by a series of studies demonstrating that high-density SR vesicles ("heavy" SR) are able to release Ca^{2+} rapidly, through a Ca^{2+}- and nucleotide-dependent channel which is absent in light SR (Nagasaki & Kasai, 1983; Meissner, 1984; Ikemoto et al., 1985; Chu et al., 1986). Furthermore, membrane material from heavy SR can be incorporated into model bilayers, forming a high-conductance channel which is modulated by Ca^{2+} and nucleotides (Smith et al., 1985, 1986; Rousseau et al., 1986). The heavy SR has been identified with vesicular

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fragments of terminal cisternae due to its high content in calsequestrin (Meissner, 1975) and the presence of junctional membrane areas (Saito et al., 1984) with processes ("feet") serving as links between the cisternal membrane and the transverse tubules. The junctional processes are organized as tetrads on the cisternal membrane (Franzini-Armstrong & Nunzi, 1983) and contain a high molecular weight protein migrating in electrophoretic gels with a ≥350000-dalton (Da) relative mobility (Cadwell & Caswell, 1982; Seiler et al., 1984; Costello et al., 1986).

Recently, a plant alkaloid insecticide, ryanodine, has been found to bind with high affinity to cisternal vesicles (Pessah et al., 1985; Fleischer et al., 1985) and to the large junctional membrane proteins (Pessah et al., 1986; Inui et al., 1987; Campbell et al., 1987). Ryanodine was also reported to either inhibit or stimulate Ca^{2+} release from cisternal vesicles, depending on the experimental conditions (Fairhurst & Hasselbach, 1970; Seiler et al., 1984; Fleischer et al., 1985; Meissner, 1986; Lattanzio et al., 1987). These findings suggest that the ryanodine receptor and the Ca^{2+} channel are parts of the same functional unit within the junctional processes.

We report here a series of experiments on characterization of ryanodine binding and its effect on transmembrane Ca^{2+} fluxes. We also report experiments on stepwise proteolysis of junctional membranes, producing selective digestion of the ≥350000-Da protein, with little change in the electron microscopic appearance of the junctional processes, ryanodine binding, or functional properties of the Ca^{2+} channel.

Materials and Methods

SR Preparation. Vesicular fragments of junctional terminal cisternae were prepared from white hind leg muscles of New Zealand white rabbits as described by Saito et al. (1984), with some modification: the homogenates obtained from the Waring blender were centrifuged for 20 min at $7000g_{av}$ (8000 rpm) in a JA-10 rotor (Beckman Instruments, Fullerton, CA). Junctional terminal cisternae were collected at the 38–45% (w/w) sucrose interface and a corresponding light SR fraction at the 32–34% (w/w) sucrose interface.

Purified junctional face membranes were prepared by partial solubilization of junctional terminal cisternae with Triton X-100, according to Costello et al. (1986).

Ca^{2+}-ATPase Assay. The oxidation of NADH was monitored by using the ATP-regenerating system of pyruvate kinase–lactate dehydrogenase as previously described (Chu et al., 1986). The basal ATPase activity [assayed in the absence of Ca^{2+} with 4 mM tris(hydroxymethyl)aminomethane–ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (Tris–EGTA)] was subtracted from the total ATPase rate to obtain the Ca^{2+}-ATPase activity. The reaction mixture contained 20 mM Tris–3-(*N*-morpholino)-propanesulfonic acid (MOPS) (pH 7), 100 μM $CaCl_2$, 5 mM $MgCl_2$, 1 mM Na_2ATP, 2 mM phosphoenolpyruvate, 0.4 mM NADH, 80 mM KCl, 10 μg of SR protein/mL, 8 units/mL pyruvate kinase, and 12 units/mL lactate dehydrogenase. The Ca^{2+} ionophore A23187 (CalBiochem, La Jolla, CA) was used in some cases (see Table I) at a concentration of 2 μg/mL. The reaction was carried out at 25 °C.

Ca^{2+} uptake was measured by following the distribution of $^{45}Ca^{2+}$ tracer (specific activity ~7000 cpm/nmol; New England Nuclear, Boston, MA). The reaction mixture contained 100 mM phosphoric acid neutralized (pH 7) with KOH, 1 mM $MgCl_2$, 50 μM $^{45}CaCl_2$, 50 μg of protein/mL, 1 mM Na_2ATP, and 5 μM ruthenium red when indicated. The reaction was carried out at 25 °C. Aliquots (200 μL) of the reaction mixture were filtered through Millipore filters (0.45 μm; Bedford, MA), which were then washed with 2 mL of ice-cold 10 mM $LaCl_3$/80 mM KCl/20 mM Tris–MOPS (pH 7) and processed for liquid scintillation spectrometry.

Ca^{2+} Release. The SR vesicles (50 μg of protein/mL) were actively loaded in a medium containing 100 μM $^{45}CaCl_2$ (specific activity ~7500 cpm/nmol), 80 mM KCl, 10 mM $MgCl_2$, 20 mM Tris–MOPS (pH 7), and 2 mM acetyl phosphate at 25 °C. After a 2-min incubation, 1-mL aliquots were transferred directly onto Millipore filters (0.65 μm) in a BioLogic rapid filtration apparatus (Pullman, WA), and the loading medium was filtered through (Sumbilla & Inesi, 1987). The vesicles were then rinsed with 2 mL of a nonrelease medium [10 mM $MgCl_2$, 80 mM KCl, 20 mM Tris–MOPS (pH 7), and 1 mM Tris–EGTA] to remove any residual loading mixture. The filters retaining the loaded vesicles (approximately 50 μg of protein per filter) were then flushed with release [80 mM KCl, 20 mM Tris–MOPS (pH 7), 50 μM Tris–EGTA, and either 51.5 μM $CaCl_2$ or 1 mM Na_2ATP and 69.7 μM $CaCl_2$ to yield 5 μM free Ca^{2+}] or nonrelease media for various time intervals (22–25 °C). Velocity and time of flushing through the filters were electronically controlled. The filters were subsequently processed for liquid scintillation spectrometry, without any further washing.

[^3H]Ryanodine Binding. This assay was carried out by a slight modification of the method described by Fleischer et al. (1985). The reaction medium contained 29 nM [^{3}H]ryanodine (specific activity ~30000 cpm/pmol; New England Nuclear), various concentrations (0–1 mM) of nonradioactive ryanodine (Progressive Agri-Systems, Wind Gap, PA), 20 mM Tris–MOPS (pH 7), 150 mM KCl, 20–100 μM $CaCl_2$, 100 μg of protein/mL, and other components as described in the legends to the figures. The reaction, in a 500-μL volume, was allowed to proceed for 30 min (37 °C) in duplicates prior to filtration through Millipore filters (0.45 μm).

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis. Mini-slab gel electrophoresis (gel size, 95 × 55 × 1.5 mm) was carried out at pH 8.8, with a 5–15% linear polyacrylamide gradient and a 4% stacking gel at pH 6.8 (Laemmli, 1970). The gels containing 20 μg of SR protein were stained with 0.125% Coomassie blue R-250 in 50% methanol/10% acetic acid and destained in 5% methanol/7% acetic acid. Molecular weight standards were myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, and α-lactalbumin, with their respective molecular weights 223000, 116000, 97400, 66000, 45000, 36000, 29000, 24000, 20100, and 14200 (Sigma Chemical Co., St. Louis, MO).

Electron Microscopy. Samples of membrane suspensions (approximately 0.3 mg of protein) were diluted to 1-mL volume with 0.1 M sodium cacodylate buffer (pH 7.4) and fixed by adding 50% glutaraldehyde to a final concentration of 2.5%. The samples were incubated overnight at 4 °C and then centrifuged at ~$25250g_{av}$ in a Sorvall centrifuge for 1 h. The resulting pellets were rinsed 3 times with fresh buffer. The samples were then postfixed in 1% osmium tetraoxide overnight, block-stained with 0.5% uranyl acetate at room temperature for 2 h, dehydrated, and embedded in Epon–Araldite. Ultrathin sections were cut on a Porter–Blum MT-2 microtome and poststained with lead citrate for 8 min and 2% uranyl acetate for 30 min. Sections were examined with a Philip EM200 microscope operating at 80 kV.

Proteolytic Digestion. Bovine pancreatic trypsin (specific activity 10000–13000 units/mL, Sigma) concentrations were adjusted to give the desired SR protein:trypsin (w/w) ratios in a reaction mixture containing 0.1–0.2 mg of SR protein/mL

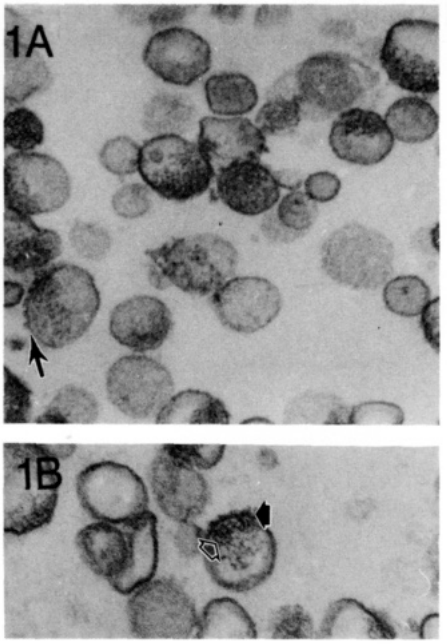

FIGURE 1: Electron microscopic views of junctional SR vesicles. The vesicles were fixed, sectioned, and stained as described under Materials and Methods. The arrow in (A) (76300×) shows a side view of junctional processes ("feet"). Top views of junctional processes are visible in several vesicle sections. A row of junctional processes in side view (filled arrow) and a parallel row in oblique view (open arrow) are shown in (B) (81900×).

in 20 mM Tris–MOPS (pH 7). The digestion was for 2 min at 25 °C. A concentrated soybean trypsin inhibitor (Sigma) stock was then added to the sample, 0.16–1 mg of inhibitor/mL (about 3–5 times the concentration of trypsin), to stop the digestion. The sample was then placed on ice and used for all the above studies on the same day. Samples for electron microscopy were stored frozen at −70 °C and processed at a later date.

Results

Electron Microscopy. A most specific ultrastructural feature of junctional sarcoplasmic reticulum, in thin sections of intact muscle, is the presence of junctional processes that appear as darkly stained, repeating rectangular-shaped entities and connect transverse tubules with SR membranes (Eisenberg & Eisenberg, 1982; Franzini-Armstrong, 1975; Johnson & Sommer, 1967; Kelly, 1969; Somlyo, 1979; Walker & Schrodt, 1968). The presence of these processes on purified membrane subfractions was then used as evidence for their identification as vesicular fragments of junctional SR (Campbell et al., 1980; Cadwell & Caswell, 1982; Saito et al., 1984; Ferguson et al., 1984).

The electron microscopic appearance of the junctional SR vesicles used in our experiments is shown in Figure 1. In thin sections, the vesicles present an asymmetrical profile which is due to the presence of surface structures identified as junctional processes, and diffuse staining of part of the lumen which is attributed to calsequestrin.

The junctional processes are not uniformly distributed over the vesicles. In some cases (Figure 1A), the processes appear in side views as densely stained, repeating structures along the perimeter of the membrane profile. The size and spacing of these structures match those of junctional processes observed in intact muscle. In other cases, top views of the junctional processes show a more complex staining pattern. Due to the high curvature of the vesicles, the processes appear to overlap from top views.

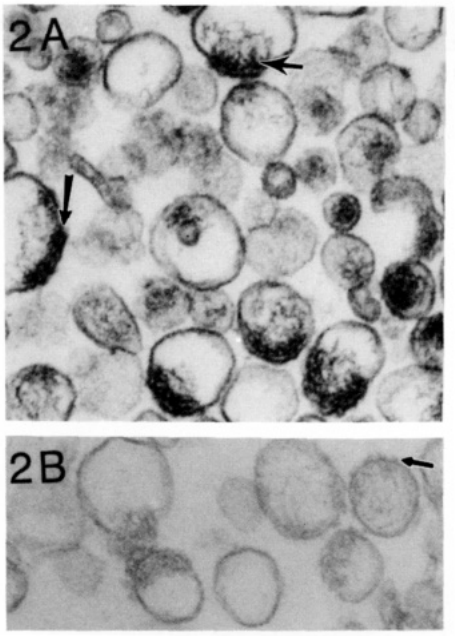

FIGURE 2: Electron microscopic view of junctional SR vesicles digested with trypsin. The vesicles were incubated with trypsin (SR protein:trypsin ratio = 50; corresponding to track 7 in Figure 3) for 2 min at 25 °C in the presence of 20 mM Tris–MOPS (pH 7). The digestion was interrupted by addition of trypsin inhibitor in amounts 5 times higher than trypsin. The samples were then processed for electron microscopy. Rows of junctional processes are shown by the arrows in (A) and (B) (76300×). Figure 2B shows side views of junctional processes as in Figure 1A.

We can interpret our top views of the junctional processes in light of the micrographs of freeze-dried, rotary-shadowed junctional SR membranes published by Ferguson et al. [1984; see also Saito et al. (1984)]. These authors showed that the junctional processes exhibit tetragonal symmetry and resemble a four-leaf clover from the top. In our plastic-embedded membranes, we can identify similar structures also arranged in parallel strings [Figure 1; compare to Figures 1, 4, and 6 in Ferguson et al. (1984)]. Figure 1B shows one row of junctional processes in side view (filled arrow) and parallel rows in oblique view (open arrow). Each process is delineated by stain, and there is a visible black dot in the center of the process (see also Figure 2).

A further illustration of the ultrastructural features of junctional SR vesicles is given in Figure 2. It is also shown in this figure that no significant change can be shown by electron microscopy, following incubation of the vesicles with trypsin concentrations that produce extensive digestion of the ≥350 000-Da protein and total disappearance of the corresponding electrophoretic band (track 7 in Figure 3A). The arrows in Figure 2 indicate rows of repeating surface structures similar to the junctional feet observed in control preparations (Figure 1).

Electrophoresis. The electrophoretic pattern (Figure 3) of terminal cisternae membrane vesicles includes a large protein component (≥350 000-Da relative mobility) which is known to be an integral structural component of the junctional processes (Cadwell & Caswell, 1982; Saito et al., 1984; Costello et al., 1986; Campbell et al., 1987). It is shown in Figure 2 that trypsin digestion under mild conditions [SR protein:trypsin ratio (w/w) between 2000 and 250] reduces rapidly the intensity of the ≥350 000-Da electrophoretic band (Figure 3). It is noteworthy that under conditions producing digestion and total disappearance of the ≥350 000-Da band, the junctional processes can still be visualized on the surface of the vesicles (Figure 2).

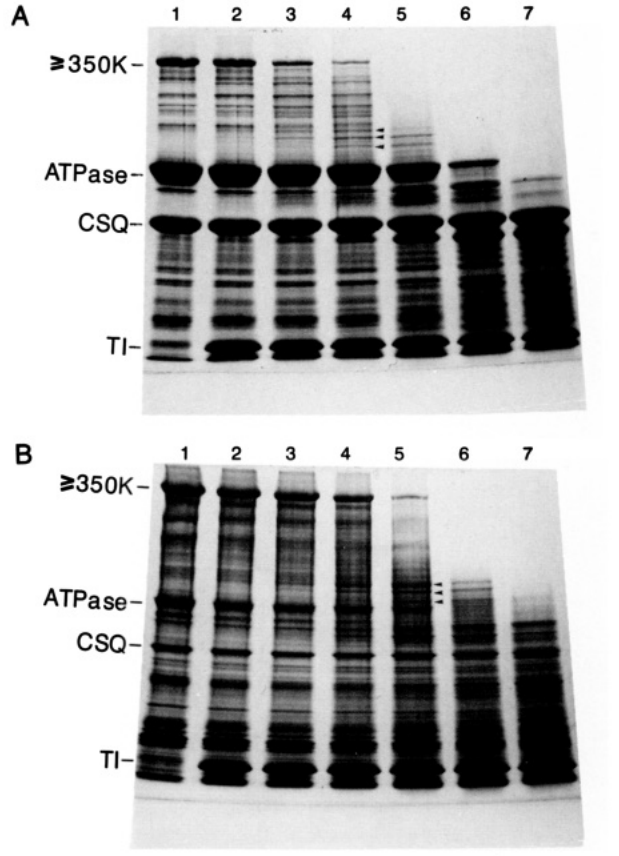

FIGURE 3: Electrophoretic analysis of junctional SR vesicles (A) and junctional face membrane fragments (B) before and after digestion with trypsin. In both experiments, 40 μg of membrane protein was incubated with various concentrations of trypsin in the presence of 10 mM Tris–MOPS (pH 7) for 2 min at 25 °C. The proteolytic reaction was stopped by adding trypsin inhibitor (5 times as much as trypsin), and the samples were solubilized in SDS [1% and 0.312 M Tris-HCl (pH 6.8), 1.25% 2-mercaptoethanol, and 3.75% sucrose] before electrophoresis. Track 1 shows the pattern of control membranes (no trypsin), and tracks 2–7 show samples digested with the following SR protein:trypsin (w/w) ratios: 2000, 1000, 500, 250, 100, and 50. ATPase, CSQ, ≥350K, and TI stand for Ca^{2+}-ATPase, calsequestrin, the ≥350 000-Da protein, and trypsin inhibitor, respectively. Fragments apparently derived from proteolytic digestion of junctional SR vesicles are indicated by small arrowheads. The gels were overloaded to detect proteolytic fragments.

It is shown in Figure 3 that the Ca^{2+}-ATPase is not affected by mild digestion with trypsin, while cleavage into complementary fragments (Migala et al., 1973; Stewart & MacLennan, 1974; Inesi & Scales, 1974) is noted following digestion under more drastic conditions. It is noteworthy that the trypsin digestion patterns of the ≥350 000-Da protein and the Ca^{2+}-ATPase are quite different. ATPase cleavage yields two complementary fragments that resist further digestion and accumulate to produce distinct electrophoretic bands. On the contrary, the primary fragments (115 000–170 000 Da; arrowheads in Figure 3) derived from digestion of junctional SR proteins rapidly undergo further digestion and do not accumulate as heavily stained bands. This pattern of digestion can be visualized more distinctly by the use of partially purified junctional face membranes (Figure 3B) in which the ≥350 000-Da protein is a more prominent component.

Calsequestrin was not affected by trypsin digestion under our experimental conditions.

Functional Characterization of Active Transport. Two important functional features of junctional SR vesicles are the active uptake of Ca^{2+} operated by the Ca^{2+}-ATPase and the passive release of accumulated Ca^{2+} through a separate channel. We first measured ATPase activity and Ca^{2+} transport in our preparation and evaluated the effect of trypsin digestion on these basic functional features. We found that steady-state ATP hydrolysis and Ca^{2+} transport by junctional vesicles were not significantly affected by mild trypsin digestion (Table I). Some inhibition of these parameters was observed following incubation with higher trypsin concentrations [SR protein:trypsin ratio (w/w) between 100 and 50].

Table I: Effect of Trypsin Digestion on ATP Hydrolysis and Ca^{2+} Uptake of Junctional SR Vesicles[a]

	Ca^{2+}-ATPase activity			
protein: trypsin ratio	no A23187		with A23187	
	rate	% control	rate	% control
none	0.73 ± 0.13	100	0.98 ± 0.11	100
500	0.70 ± 0.13	96	0.93 ± 0.12	94
250	0.60 ± 0.09	77	0.86 ± 0.08	90
100	0.68 ± 0.38	73	0.83 ± 0.02	89
50	0.57 ± 0.23	73	0.83 ± 0.02	89
	$^{45}Ca^{2+}$ uptake			
protein: trypsin ratio	no RR		with RR	
	rate	% control	rate	% control
none	88 ± 19	100	673 ± 308	100
500	92 ± 25	103	703 ± 335	102
250	94 ± 20	89	734 ± 495	108
100	85 ± 12	98	525 ± 200	56
50	74 ± 77	77	453 ± 105	45

[a] Junctional SR vesicles were subjected to tryptic digestion and then assayed for Ca^{2+}-ATPase activity and Ca^{2+} uptake in the presence of phosphate (Materials and Methods). The data for Ca^{2+}-ATPase activity (n = 3) and Ca^{2+} uptake (n = 5) are in micromoles per milligram of protein per minute and nanomoles per milligram of protein per minute, respectively. RR, ruthenium red. The percent control is an average of the values of individual rates for trypsinized vs nontrypsinized SR.

Increase of net Ca^{2+} transport by ruthenium red is a characteristic effect which is observed in experiments with junctional vesicles under appropriate conditions (e.g., low [Mg^{2+}]) and is attributed to inhibition of Ca^{2+} leakage through a passive channel (Chu et al., 1986). We found the rates of net transport to be approximately 7 times higher in the presence, as compared to those in the absence, of ruthenium red, demonstrating that our vesicles contain a prominent pathway for passive release of Ca^{2+}. This large enhancement of net Ca^{2+} transport by ruthenium red was observed even following digestion with trypsin at higher concentrations, indicating that the Ca^{2+} release pathway retains its ruthenium red sensitivity following trypsin digestion.

Functional Characterization of Ca^{2+} Release. Kinetic resolution of Ca^{2+} release from junctional vesicles was first obtained by rapid-quench methods, following passive loading by preincubation with high Ca^{2+} concentrations and sudden dilution to lower the Ca^{2+} concentration in the medium outside the vesicles (Meissner et al., 1986). We have used an alternative (Sumbilla & Inesi, 1987) method based on loading of the vesicles by active transport, placing the loaded vesicles on Millipore filters, and flushing the release medium through the filters for serial time intervals under electronic control. The Ca^{2+} load, obtained by a 2-min incubation with $^{45}Ca^{2+}$ in the presence of acetyl phosphate, was 75 nmol/mg of protein in control vesicles. The load was somewhat lower in the heavily digested junctional SR vesicles (Figure 4). Such a reduction was observed also in longitudinal ("light") SR vesicles and may be related to alteration of Ca^{2+} pump function (Table I).

As previously reported (Meissner et al., 1986), Ca^{2+} release is blocked by Mg^{2+}, activated by Ca^{2+}, and potentiated by nucleotides (Figure 4). The Ca^{2+}- and nucleotide-activated

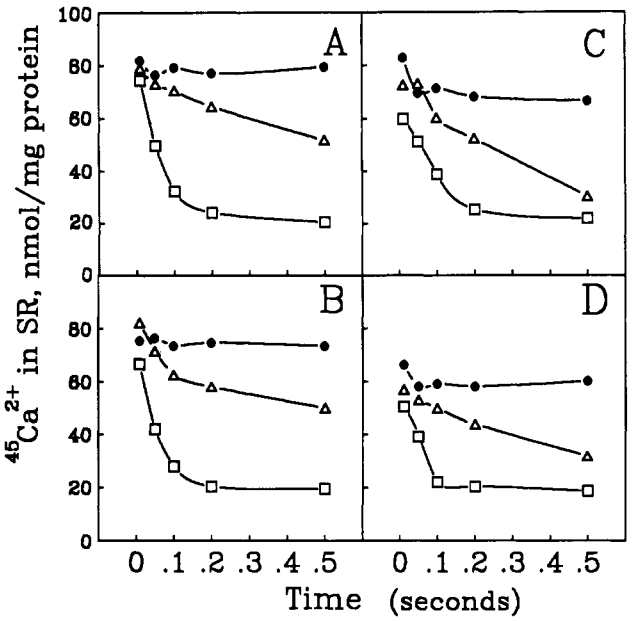

FIGURE 4: Ca^{2+} release from junctional SR vesicles subjected to various degrees of trypsin digestion. The vesicles were first incubated for 2 min in 10 mM Tris–MOPS (pH 7) at 25 °C with no trypsin (A) or with trypsin [SR protein:trypsin ratio = 500 in (B), 100 in (C), and 50 in (D)]. The vesicles were then loaded with $^{45}Ca^{2+}$ by a 2-min incubation (25 °C) in the following reaction mixture: 50 μg of SR protein/mL, 20 mM Tris–MOPS (pH 7), 80 mM KCl, 10 mM $MgCl_2$, 100 μM $^{45}CaCl_2$, and 2 mM acetyl phosphate. For each time point of the release curves, a sample (1 mL) of loaded vesicles was filtered in a rapid filtration apparatus. The loaded vesicles collected on the Millipore filter were washed with 2 mL of nonrelease medium and then flushed either with nonrelease medium (●) or with release media containing 5 μM free Ca^{2+} (▲) or 1 mM ATP and 5 μM free Ca^{2+} (□). The filters were then processed for liquid scintillation spectrometry with no further washing.

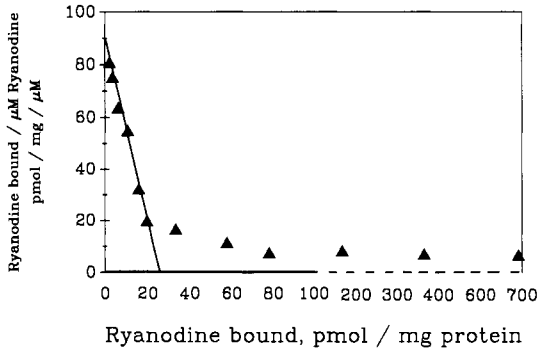

FIGURE 5: Ryanodine binding to junctional SR vesicles. The membrane vesicles (100 μg of protein/mL) were incubated with 29 nM [^{3}H]ryanodine and various concentrations of nonradioactive ryanodine in the presence of 20 mM Tris-MOPS (pH 7), 150 mM KCl, and 0.1 mM $CaCl_2$ at 37 °C for 30 min. The Scatchard plot distinguished a saturable binding component ($K_d = 3 \times 10^{-7}$ M; B_{max} = 24 pmol/mg of protein) from a nonsaturable component.

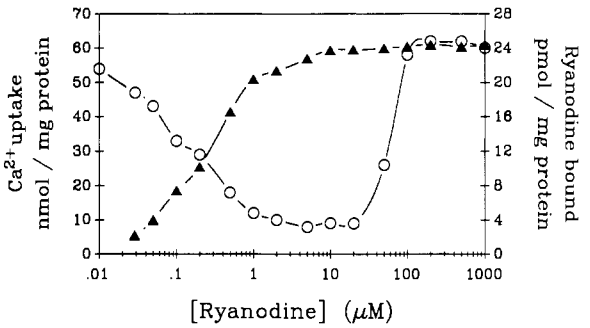

FIGURE 6: Steady-state levels of Ca^{2+} uptake (○) and specific (saturable component) ryanodine (▲) in junction SR vesicles as functions of ryanodine concentration. The membrane vesicles (1 mg of protein/mL) were first preincubated with various concentrations of ryanodine (0–1 mM) in the presence of 20 mM Tris–MOPS (pH 7), 100 μM $CaCl_2$, and 150 mM KCl at 37 °C for 30 min. The preincubated vesicles were then diluted to 50 μg of protein/mL in a medium containing 100 μM $^{45}CaCl_2$ (~7500 cpm/nmol), 10 mM $MgCl_2$, 150 mM KCl, 20 mM MOPS (pH 7), and 2 mM acetyl phosphate. Ca^{2+} uptake was allowed to proceed for 2 min at 25 °C and stopped by filtering 1-mL samples. The vesicles collected on the filter were then washed with nonrelease medium (Materials and Methods) and processed for measurement of radioactivity. Ryanodine binding was measured in parallel runs, utilizing [^{3}H]ryanodine tracer. The figure shows a reduction of *net* Ca^{2+} uptake proportional to specific (saturable) ryanodine binding. *Net* uptake rises again to maximal levels in the presence of concentrations of ryanodine higher than 100 μM, which allow further binding of the drug (Figure 5) and prevent Ca^{2+} release (Figure 8).

release occurs within the millisecond time scale, and a rate constant of approximately 10–20 s^{-1} in this set of experiments (Figure 4). Trypsin digestion, under our experimental conditions, did not alter the blocking effect of Mg^{2+} on Ca^{2+} release, and the digested vesicles retained their load just like control vesicles (Figure 4). It is of interest that trypsin digestion did not interfere with both the activating and potentiating effects of Ca^{2+} and nucleotides on the kinetics of release (Figure 4). In the presence of Ca^{2+} and ATP, the amount of Ca^{2+} released reached a maximum whether the vesicles were digested or not. However, when only Ca^{2+}, and no nucleotides, was present in the release medium, we noted a tendency for more Ca^{2+} to be released faster when the trypsin concentration was increased (Figure 4C).

It is of interest that even under drastic conditions of trypsin digestion, when the ability of the vesicles to be actively loaded was somewhat reduced, the channel retained its Ca^{2+}-dependent release function (Figure 4D).

Ryanodine Binding. A specific feature of junctional SR is its ability to bind ryanodine (Pessah et al., 1985, 1986; Fleischer et al., 1985; Inui et al., 1987; Campbell et al., 1987). The ryanodine binding characteristics of our preparation are shown in Figure 5. A saturable ("specific") binding component can be clearly identified, yielding a maximal binding stoichiometry of 24 pmol/mg of protein and a dissociation constant of 3×10^{-7} M. At higher ryanodine concentrations, a nonsaturable component is observed, likely due to drug partitioning into the membrane (Figure 5). An approximate estimate of the ≥350 000-Da protein stoichiometry, based on its molecular weight and its fractional value with respect to total membrane protein on the electrophoretic gels, suggests that ryanodine binding occurs within a similar stoichiometric range.

The involvement of bound ryanodine in functional regulation of Ca^{2+} fluxes is clearly demonstrated by measuring the steady-state levels of Ca^{2+} uptake (coupled to acetyl phosphate utilization) in the presence of various ryanodine concentrations. It is shown in Figure 6 that within the ryanodine concentration range sustaining specific binding, a large reduction of net Ca^{2+} uptake is observed. We found that this effect is not related to inhibition of the Ca^{2+} pump, as reflected by Ca^{2+}-ATPase activity (not shown), but rather to increased efflux through the release channel [Figure 7; see also Fleischer et al. (1985) and Meissner (1986)]. It is noteworthy that in the experiment shown in Figure 7, significant Ca^{2+} efflux probably occurs during the washing of ryanodine-treated vesicles with a nonrelease medium (as suggested by a dashed line for the first time interval in Figure 7).

At higher ryanodine concentrations, net Ca^{2+} uptake returns to its maximal level, due to an inhibitory effect of low-affinity

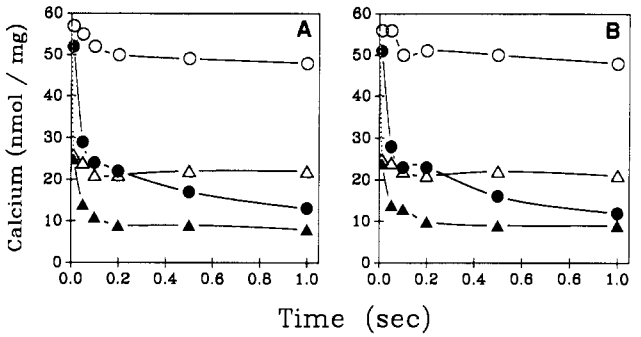

FIGURE 7: Ca^{2+} release from trypsin-digested junctional SR vesicles in the presence of low concentrations of ryanodine. The vesicles (1.0 mg of protein/mL) were passively loaded for 2 h at 25 °C in the presence of 1 mM $^{45}CaCl_2$ (~10000 cpm/nmol), 20 mM Tris–MOPS (pH 7), and 80 mM KCl. They were then further incubated at 25 °C for 1 h in the presence (●, ▲) or absence (○, △) of 1 μM ryanodine. The reaction was allowed to proceed for another 2 min at 25 °C in the absence (A) or presence (B) of trypsin (SR protein:trypsin ratio of 500) and then stopped by the addition of trypsin inhibitor. Aliquots (50 μg) were diluted into 1 mL of nonrelease medium (Materials and Methods). The entire 1 mL was then placed on a filter in the rapid filtration apparatus, washed with 2 mL of nonrelease medium, and finally flushed with either nonrelease (○, ●) or release medium containing 5 μM free Ca^{2+} and 1 mM ATP (△, ▲). The filters were then processed for radioactivity measurement without any further washing.

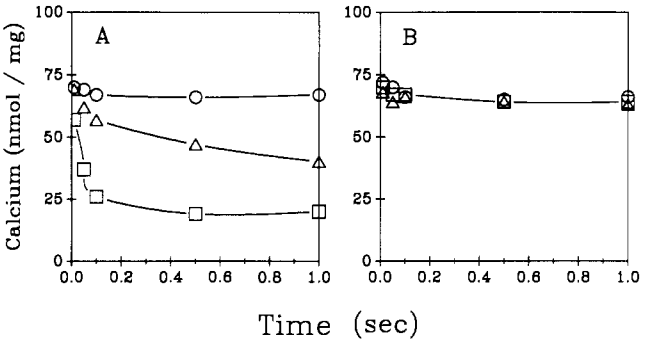

FIGURE 8: Inhibition of Ca^{2+} release from junctional SR vesicles in the presence of high concentrations of ryanodine. The vesicles were preincubated in the absence (A) or in the presence (B) of 100 μM ryanodine, before being actively loaded with $^{45}Ca^{2+}$ in the presence of acetyl phosphate (see legend to Figure 6). The loaded vesicles were then placed in the rapid filtration apparatus, washed with 2 mL of nonrelease medium, and then flushed with nonrelease (○) or release medium containing 5 μM free Ca^{2+} (△) or 5 μM free Ca^{2+} and 1 mM ATP (□). The filters were then processed for radioactivity measurement without any further washing.

ryanodine binding on Ca^{2+} efflux through the release channel (Figure 8). Mild or extensive digestion with trypsin did not alter the effect of either 1 or 100 μM ryanodine on Ca^{2+} release.

We then investigated whether ryanodine binding is altered by digestion of the junctional SR vesicles with trypsin. In one set of experiments, we first preequilibrated the membranes with ryanodine and then added trypsin to start proteolysis. We found that the ≥350 000-Da protein was digested by trypsin equally well in the presence and absence of ryanodine by SDS gel electrophoresis (not shown). Furthermore, ryanodine remained bound to the digested vesicles, with a stoichiometry nearly identical with that of control vesicles (Figure 9).

In another set of experiments, we first digested the vesicles with trypsin and then added ryanodine. In this case, the level of ryanodine binding was reduced. It is possible that ryanodine binding in the previous set of experiments prevented or retarded protein denaturation following the tryptic cut. It should be pointed out that ryanodine binding is rather slow and requires prolonged equilibration at 37 °C. Such a prolonged incubation following trypsin digestion produces alteration of several functional parameters, including active Ca^{2+} transport and regulation of release. This effect is most likely due to thermal unfolding of the tryptic fragments.

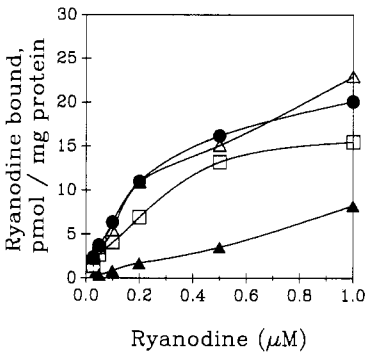

FIGURE 9: Residual ryanodine binding following trypsin digestion of junctional SR vesicles. Incubations for ryanodine binding were carried out as described in the legend to Figure 5. Incubations (2 min at 25 °C) with no trypsin (●) or with trypsin at SR protein:trypsin (w/w) ratios of 500 (△) and 50 (□) were carried out after prior incubation with ryanodine (30 min at 37 °C and then cooled to 25 °C). (▲) represents residual ryanodine binding at SR protein:trypsin ratio of 500 when the SR was first trypsinized for 2 min at 25 °C and then incubated with ryanodine for 30 min at 37 °C.

DISCUSSION

Junctional SR membrane contains a ≥350 000-Da protein which is a putative component of the junctional processes (Cadwell & Caswell, 1982; Seiler et al., 1984; Costello et al., 1986; Inui et al., 1986; Campbell et al., 1987). We find that incubation of junctional SR vesicles with trypsin produces early digestion of the ≥350 000-Da protein, before involving other protein components such as the Ca^{2+}-ATPase and calsequestrin. Primary fragments of 115 000–170 000 Da, produced as a consequence of junctional SR protein cleavage, undergo further digestion quite rapidly and do not reach high steady-state levels. Therefore, the corresponding bands stain lightly on electrophoretic gels. The trypsin digestion pattern of the ≥350 000-Da protein differs from that of the Ca^{2+}-ATPase in that the latter requires a more prolonged digestion and yields two primary fragments that are relatively resistant to further digestion. Calsequestrin is not digested under our experimental conditions.

It is likely that the multiple cuts produced almost simultaneously by trypsin on the ≥350 000-Da protein are due to penetration of the proteolytic enzyme into a large crevice of the protein, where several peptide bonds are susceptible to hydrolysis. In fact, electron microscopy of stained thin sections shows a discrete black spot in the middle of each junctional process, suggesting a polar environment in the center of the membrane-bound protein, which favors binding of electron-dense stain. A similarly heavily stained central area of the acetylcholine receptor has been identified with a large opening of the ion channel (Stroud, 1987). It is possible that the crevice accepting trypsin in the ≥350 000-Da protein of junctional SR is also at the opening of the permeability channel.

If proteolytic digestion is allowed to proceed until total disappearance of the electrophoretic bands corresponding to the ≥350 000-Da protein and high molecular weight fragments, no alteration of the junctional processes ultrastructure is noted by electron microscopy. Assuming that the ≥350 000-Da protein is a structural component of the junctional processes, our findings suggest that the peptides produced by trypsin digestion are held together by the multiple weak bonds that

stabilize protein folding and its interaction within the lipid bilayer; thereby the protein may retain its channel and ligand binding functions. It is also possible that additional proteins contribute to structural stabilization of the junctional processes.

The plant alkaloid ryanodine has been reported to be a specific ligand for the ≥350 000-Da protein (Pessah et al., 1986; Inui et al., 1987; Campbell et al., 1987). Consistent with these reports, we find that the stoichiometry of high-affinity binding sites is within the range of the ≥350 000-Da protein. A precise relationship can be established (Figure 6) between high-affinity ryanodine binding and the loss of the ability of the junctional SR vesicles to retain Ca^{2+} which is taken up through active transport. This is evidently due to an effect favoring the open state of the release channel (Fleischer et al., 1985; Meissner, 1986).

High-affinity ryanodine binding to the ≥350 000-Da protein does not interfere with its digestion by trypsin, suggesting that the ryanodine and trypsin domains do not overlap. It is likely that ryanodine binds on the crest surrounding the crevice leading to the channel, rather than in the crevice itself, in analogy with ligand binding to the acetylcholine receptor (Stroud, 1987). The gating signals then must be transmitted some distance through an allosteric mechanism.

It is of interest that higher concentrations of ryanodine have an effect opposite to that produced by low concentrations of the drug [Figures 7 and 8; see also Meissner (1986)]. While low concentrations of the drug increase Ca^{2+} release, higher concentrations block Ca^{2+} release. Binding of ryanodine at high concentrations occurs through a nonsaturable mechanism which is likely to be related to partitioning of relatively large amounts of the drug into the membrane. It is possible that in this location, the drug interferes with structural adjustments of transmembrane protein segments, which are required to open the channel.

With respect to the effects of ryanodine, it is worth considering that while electrical measurements of single-channel activity in bilayers provide very important information of the behavior of the channel, kinetic measurements on SR vesicles yield quantitative evaluation of net Ca^{2+} fluxes through the native membrane in the presence of physiological concentrations of Ca^{2+} and other electrolytes. Furthermore, they test whether passive fluxes are functionally meaningful in the presence of coexistent active transport.

REFERENCES

Cadwell, J. J. S., & Caswell, A. H. (1982) *J. Cell Biol. 93*, 543–550.

Campbell, K. P., Franzini-Armstrong, C., & Shamoo, A. E. (1980) *Biochim. Biophys. Acta 602*, 97–116.

Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. L., Sutko, J. L., Kahl, S. D., Raab, C. R., & Madson, L. (1987) *J. Biol. Chem. 262*, 6460–6463.

Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A., & Fleischer, S. (1986) *J. Cell Biol. 103*, 741–753.

Chu, A., Volpe, P., Costello, B., & Fleischer, S. (1986) *Biochemistry 25*, 8315–8324.

Eisenberg, B. R., & Eisenberg, R. S. (1982) *J. Gen. Physiol. 79*, 1–19.

Fairhurst, A. S., & Hasselbach, W. (1970) *Eur. J. Biochem. 13*, 504–509.

Ferguson, D. G., Schwartz, H. W., & Franzini-Armstrong, C. (1984) *J. Cell Biol. 99*, 1735–1742.

Fleischer, S., Ogunbunmi, E. M., Dixon, M. C., & Fleer, E. A. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7256–7259.

Franzini-Armstrong, C. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 1382–1389.

Franzini-Armstrong, C., & Nunzi, G. (1983) *J. Muscle Res. Cell Motil. 4*, 233–252.

Ikemoto, N., Antoniu, B., & Meszaros, L. G. (1985) *J. Biol. Chem. 260*, 14096–14100.

Inesi, G., & Scales, D. (1974) *Biochemistry 13*, 3298–3306.

Inui, M., Saito, A., & Fleischer, S. (1987) *J. Biol. Chem. 262*, 1740–1747.

Johnson, E. A., & Sommer, J. R. (1967) *J. Cell Biol. 33*, 103–129.

Kelly, D. E. (1969) *J. Ultrastruct. Res. 29*, 37–49.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lattanzio, F. A., Jr., Schlatterer, R. G., Nicar, M., Campbell, K. P., & Sutko, J. L. (1987) *J. Biol. Chem. 262*, 2711–2718.

Meissner, G. (1975) *Biochim. Biophys. Acta 389*, 51–68.

Meissner, G. (1984) *J. Biol. Chem. 259*, 2365–2374.

Meissner, G. (1986) *J. Biol. Chem. 261*, 6300–6306.

Meissner, G., Darling, E., & Eveleth, J. (1986) *Biochemistry 25*, 236–244.

Migala, A., Agostini, B., & Hasselbach, W. (1973) *Z. Naturforsch. C: Biochem., Biophys., Biol., Virol. 28C*, 178–182.

Nagasaki, K., & Kasai, M. (1983) *J. Biochem. (Tokyo) 94*, 1101–1109.

Pessah, I. N., Waterhouse, A. L., & Casida, J. E. (1985) *Biochem. Biophys. Res. Commun. 128*, 449–456.

Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., & Casida, J. E. (1986) *J. Biol. Chem. 261*, 8643–8648.

Rousseau, E., Smith, J. S., Henderson, J. S., & Meissner, G. (1986) *Biophys. J. 50*, 1009–1014.

Saito, A., Seiler, S., Chu, A., & Fleischer, S. (1984) *J. Cell Biol. 99*, 875–885.

Seiler, S., Wegener, A. D., Whang, D. D., Hathaway, D. R., & Jones, L. R. (1984) *J. Biol. Chem. 259*, 8550–8557.

Smith, J. S., Coronado, R., & Meissner, G. (1985) *Nature (London) 316*, 446–449.

Smith, J. S., Coronado, R., & Meissner, G. (1986) *Biophys. J. 50*, 921–928.

Somlyo, A. V. (1979) *J. Cell Biol. 80*, 743–750.

Stewart, P. S., & MacLennan, D. H. (1974) *J. Biol. Chem. 249*, 985–993.

Stroud, R. M. (1987) in *Proteins of Excitable Membranes* (Hille, B., & Fambrough, D. M., Eds.) pp 67–76, Wiley-Interscience, New York.

Sumbilla, C., & Inesi, G. (1987) *FEBS Lett. 210*, 31–36.

Walker, S. M., & Schrodt, G. R. (1968) *J. Cell Biol. 37*, 564–569.