Identification of a Constituent of the Junctional Feet Linking Terminal Cisternae to Transverse Tubules in Skeletal Muscle

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ABSTRACT This study describes the biochemical composition of junctional feet in skeletal muscle utilizing a fraction of isolated triad junctions. [^{3}H]Ouabain entrapment was employed as a specific marker for T-tubules. The integrity of the triad junction was assayed by the isopycnic density of [^{3}H]ouabain activity (24–30% sucrose for free T-tubules, 38–42% sucrose for intact triads). Trypsin, chymotrypsin, and pronase all caused separation of T-tubules from terminal cisternae, indicating that the junction is composed as least in part of protein. Trypsin and chymotrypsin hydrolyzed four proteins: the Ca^{2+} pump, a doublet 325,000, 300,000, and an 80,000 M_r protein. T-tubules which had been labeled covalently with ^{125}I were joined to unlabeled terminal cisternae by treatment with K cacodylate. The reformed triads were separated from free T-tubules and then severed by passage through a French press. When terminal cisternae were separated from T-tubules, some ^{125}I label was transferred from the labeled T-tubules to the unlabeled terminal cisternae. Gel electrophoresis showed that, although T-tubules were originally labeled in a large number of different proteins, only a single protein doublet was significantly labeled in the originally unlabeled terminal cisternae. This protein pair had molecular weights of 325,000 and 300,000 daltons. Transfer of label did not occur to a substantial degree without K cacodylate treatment. We propose that the transfer of ^{125}I label from T-tubules to terminal cisternae during reformation and breakage of the triad junction is a property of the protein which spans the gap between T-tubules and terminal cisternae.

Membrane-to-membrane communication occurs both between cells and within cells. Examples of the former include the gap junction and the chemical synapses of the central nervous system; an example of the latter is the triad junction of mammalian skeletal muscle. The triad junction is formed where transverse tubules (T-tubules) come into close apposition with the terminal cisternae (TC) of sarcoplasmic reticulum (SR). The SR is an anatomically distinct membrane-bounded organelle that releases Ca^{2+} during contraction and sequesters Ca^{2+} during relaxation. The membranes of the T-tubule and TC are separated by a gap of 90–120 Å, and interposed between them are arrays of electron-dense projections called SR feet (8, 9). The membrane potential of the T-tubules directly regulates SR Ca^{2+} permeability and thus the contractile state of the muscle fiber (5). Depolarization of the T-tubule serves as the primary signal for Ca^{2+} release from the SR, initiating tension development (6, 10). The nature of this signal and how it controls Ca^{2+} permeability in SR remain unknown.

The present paucity of biochemical knowledge concerning the junction is due, in part, to the lack of an adequate assay for the presence of junctional feet. For some time, it could not be determined whether the feet were in actual physical contact with the two membranes that form the junctional gap. Caswell et al. (3) demonstrated this physical continuity by the isolation of a fraction of intact triad junctions from a muscle homogenate. The tannic acid mordant-staining technique was used by Saito et al. (15) and Somlyo (17) to enhance the resolution of membrane structure and confirm these findings morphologically. However, the physicochemical composition of the junction remains unknown. Somlyo (17) provided electron microscope evidence that the junctional feet were composed of confluent lipid bilayers that traversed the junctional gap. Campbell et al. (2) observed structures protruding from the surface of isolated SR vesicles and concluded that these were junctional feet. After a KCl wash, the protruding structures were no longer visible in thin-section electron micrographs. This KCl wash removed two proteins (34,000 and 38,000 daltons) from the TC membrane. On this basis the proteins removed by the wash were identified as portions of the junctional feet. However, Caswell et al. (4) have found that the same treatment does not separate triads nor does it preclude the rejoining of T-tubules to TC.

In this paper we seek identification of the molecular component or components that constitute the connection between T-tubules and SR. We have demonstrated earlier that intact triad junctions may be mechanically dissociated into their component organelles, T-tubules and TC (12). The individual organelles can be induced spontaneously to rejoin to form reconstituted triadic junctions if they are incubated in the presence of the salt K cacodylate (4). These reformed junctions bear a close resemblance to the native junctions and exhibit similar properties (see preceding article). We have employed this ability to sever the junction mechanically and to reform it by chemical manipulation as a means to identify the specific component at the interface between the T-tubule and TC.

MATERIALS AND METHODS

Preparation of Organelles

TC/triads from rabbit sacrospinalis muscle were prepared by differential centrifugation according to the protocol described by Brandt et al. (1). Free T-tubules and heavy TC were isolated after disruption of TC/Triads followed by density gradient centrifugation as described by Lau et al. (13) and Caswell et al. (3).

Assay for Junctional Intactness

Junctional integrity was determined initially by centrifugation in a solution of uniform (30% sucrose) density. T-tubules (isopycnic point 24–30% sucrose) do not sediment in 30% sucrose whereas TC/triads do. [³H]Ouabain activity was assayed in the pellet. A decrease in the activity found in the pellet indicates that T-tubules have been separated from TC. Analysis of [³H]ouabain activity profiles and protein distribution was carried out in continuous sucrose density gradients. Protein was assayed by the method of Lowry et al. (14) using bovine serum albumin as the standard.

Proteolytic Digestions

All digestions were performed at 37°C for the times and enzyme concentrations indicated in the figure legends. SR protein concentrations were 2–4 mg/ml. Trypsin-treated samples were prepared for electrophoresis by addition of soybean trypsin inhibitor and cooling on ice for 5 min. Sample buffer was added (1:1 by volume) and the sample immediately was placed in boiling water for 1 min. Proteolysis with enzymes other than trypsin was carried out in a similar manner but with the omission of trypsin inhibitor. Soybean trypsin inhibitor, trypsin, chymotrypsin, pronase, and neuraminidase were purchased from Sigma Chemical Co., St. Louis, MO. Gel electrophoresis was performed by the method of Laemmli (11) in 10 or 4.5% acrylamide slab gels.

Iodination of SR

Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; Pierce Chemical Co., Rockford, IL) was employed as a water-insoluble activator of I⁻. A mixture of 15 μg of Iodogen and 200 μl of chloroform was placed in a large (20-ml) test tube. The tube was rotated while the chloroform evaporated, leaving an even film of Iodogen coated on the sides. SR vesicles suspended in 250 mM sucrose, 2 mM histidine (5 ml, 10–20 mg protein) were placed in the tube, and 50 μCi of ^{125}I (carrier-free; New England Nuclear, Boston, MA) per mg of SR protein was added to start the reaction. Incubation was carried out at 0°C for 20 min with frequent stirring. The reaction was terminated by decanting the liquid into another test tube. Unreacted iodine was removed by passage of the suspension through a 4 × 30-cm column containing Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) followed by dialysis for 16 h against two changes of 1,000 vol of 250 mM sucrose, 2 mM histidine at 4°C.

Label Transfer Experiments

The protocol for the label transfer experiments is illustrated in Fig. 1. A preparation of TC/triads was divided into equal parts. One portion was labeled with ^{125}I as indicated by the star in Fig. 1. The second portion remained unlabeled. Both were passed through a French press at 6,000 psi, and the free T-tubules (illustrated by bands in the gradient tube of Fig. 1) were separated from TC on sucrose density gradients as described previously (12). Labeled T-tubules (star) were mixed with unlabeled TC and in a separate experiment unlabeled T-tubules were mixed with labeled TC (star). In both experiments K cacodylate, pH 7.2,

was added to a final concentration of 0.3 M in order to effect rejoining of the organelles as described by Caswell et al. (4). They were concentrated by centrifugation at 125,000 g for 1 h, resuspended in 250 mM sucrose and 2 mM histidine, and placed on sucrose density gradients. Centrifugation was carried out at 150,000 g for 1.5 h in a Sorvall TV-850 vertical rotor (DuPont Instruments, Sorvall Biomedical Div., Newtown, CT), separating rejoined triads from any non-rejoined T-tubules. Generally, only a single condensed band with an isopycnic point of 38–42% sucrose was visible (indicated in Fig. 1). This band was removed from the gradient, concentrated by centrifugation at 125,000 g for 1 h, resuspended in 250 mM sucrose and 2 mM histidine, and passed once more through the French press at a pressure of 6,000 psi. The samples were then layered onto sucrose density gradients and centrifuged in a Sorvall TV 850 rotor at 150,000 g for 1.5 h to separate TC from free T-tubules (indicated by two bands in gradient tube in Fig. 1). Fractions of 1.25 ml were collected and counted in a gamma counter for 10 min per sample.

The extent of junction separation produced by the French press was measured by use of a preparation of TC/triads from muscle which had been injected with [³H]ouabain to label T-tubules as described previously (3, 12). The TC/triads were treated in the same way as those used for the label-transfer experiments, except for the omission of the iodine label. This was necessary in order to prevent interference of the ^{125}I with counting of the [³H]ouabain in the scintillation counter.

RESULTS

Separation of TC and T-tubules by Enzymatic Digestion

The effect of proteolysis on junctional integrity was tested by treating intact TC/triads with trypsin and subsequently fractionating the organelles on sucrose density gradients. The measurement of junction breakage is based on the difference in isopycnic densities of free T-tubules and T-tubules joined to TC. The results are shown in Fig. 2. When TC/triads were not treated with trypsin (Fig. 2 C), the major peak of [³H]-ouabain activity appeared in the 38–42% sucrose region of the gradient. Little or no [³H]ouabain activity corresponded to the isopycnic density of free T-tubules (24–30% sucrose wt/wt), indicating that the T-tubules are still associated with the TC. When
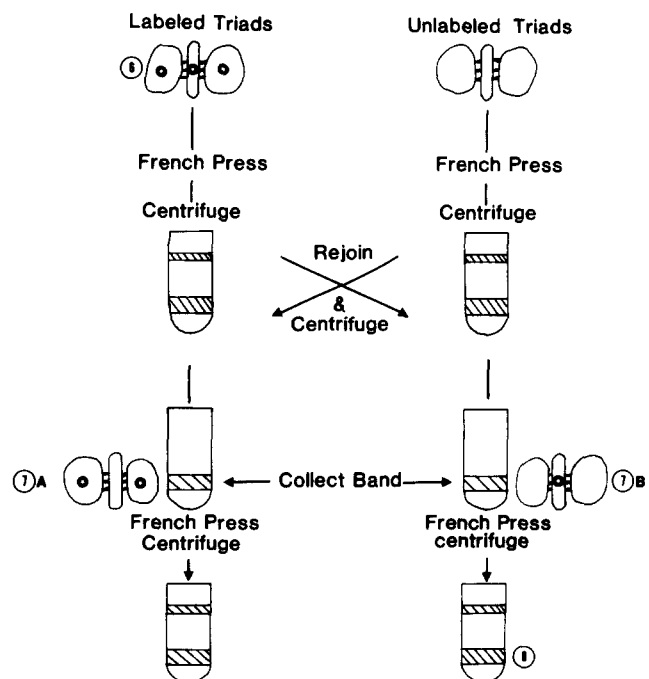

FIGURE 1 Protocol in which the transfer of ^{125}I activity from labeled T-tubules to unlabeled TC is observed. Stars denote organelles containing ^{125}I label. The numbers (6, 7A, 7B, 8) indicate points of the protocol at which samples were collected for gel electrophoresis and refer to the figure numbers in which these data are presented.
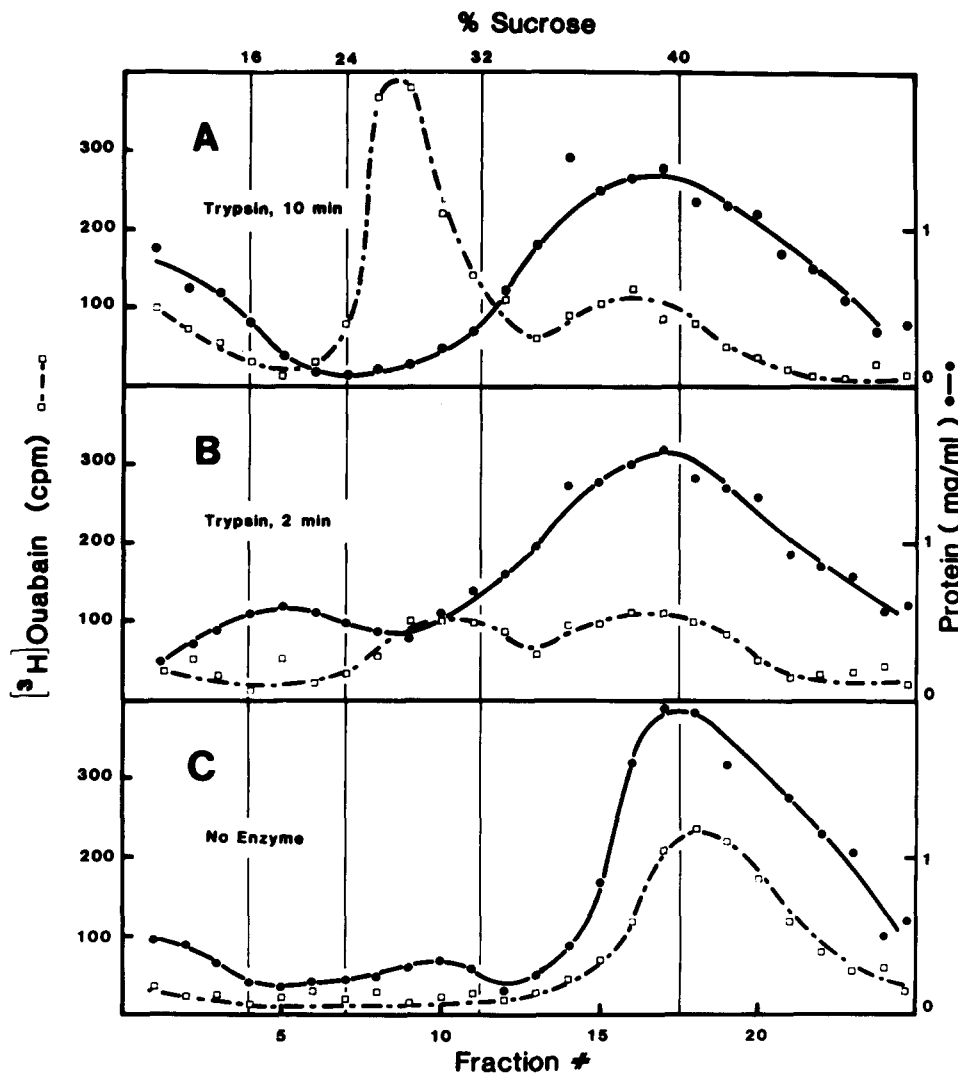
% Sucrose

FIGURE 2 [³H]Ouabain activity (open squares) and protein concentration (solid circles) of [³H]-ouabain-labeled TC/triads in sucrose density gradients (wt/wt) with and without tryptic digestion (0.01 mg/ml) for the times specified. The protocol for this experiment is described in Materials and Methods.

trypsin was added (Fig. 2A and B), [³H]ouabain activity was found in the 24–30% sucrose (wt/wt) region of the gradient indicative of the presence of free T-tubules. No corresponding shift in the protein distribution was seen as the protein content of T-tubules is negligible relative to the TC. Increased time of digestion or enzyme concentration (data not shown) increased the amount of free T-tubules. A trypsin concentration of 0.01 mg/ml for 10 min was sufficient to cause nearly all of the [³H]ouabain activity to appear in the 24–30% (wt/wt) sucrose region of the gradient (Fig. 2A). Very little [³H] activity was found at the top of the gradient, showing that the [³H]ouabain has not been released from the T-tubule vesicles as a result of proteolysis.

Trypsin, chymotrypsin, pronase, and neuraminidase were tested for their ability to separate TC from T-tubules. The amount of separation was assayed by the amount of [³H]-ouabain activity recovered from the pellet after centrifugation through 30% sucrose. Fig. 3 shows that trypsin, chymotrypsin, and pronase all reduced the [³H]ouabain activity in the pellet. All of the proteases caused separation, but pronase was least potent on a milligram basis. Neuraminidase, which enzymatically removes sialic acid residues from glycoproteins, was not effective in reducing the amount of [³H]ouabain activity in the pellet after centrifugation.

Gel Electrophoresis of Digested Triads

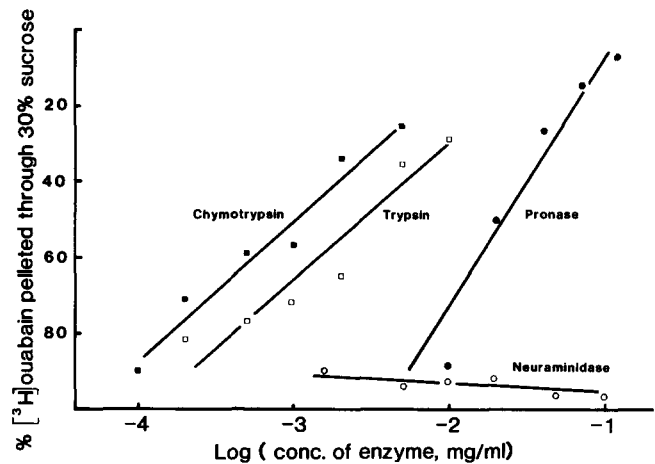The effect of proteolysis on the protein composition of intact

FIGURE 3 [³H]Ouabain present in the pellet after treatment of intact TC/triads with proteolytic enzyme and centrifugation through 30% (wt/wt) sucrose as described in Materials and Methods. Percent [³H]ouabain in the pellet is expressed relative to a control experiment in which no enzyme was added.

triads was observed by SDS-PAGE. Triads were digested with 0.01 mg/ml of trypsin or chymotrypsin for 1, 2, 5, and 10 min. Photographs of the resulting gel patterns are presented in Fig. 4. Both proteases hydrolyzed few proteins. Most notably, the Ca²⁺ ATPase (105,000 daltons) and a protein of ~80,000 M_r
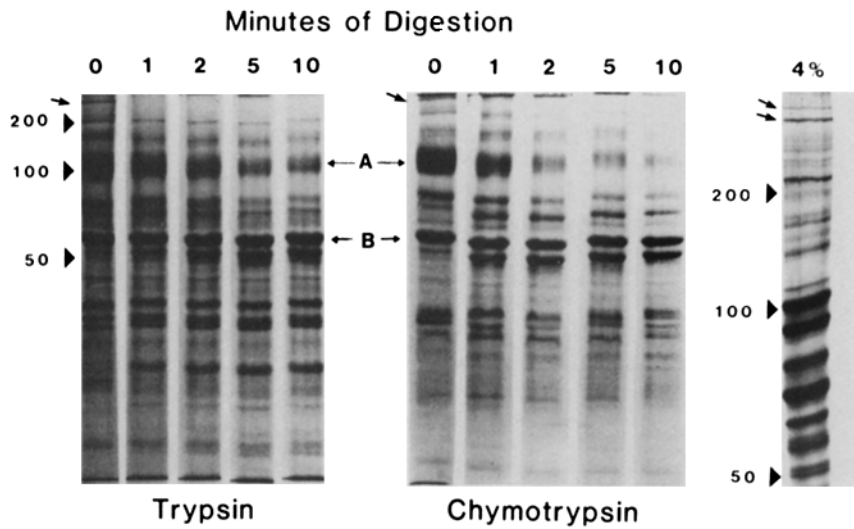
FIGURE 4 Laemmli SDS PAGE of TC/triads digested with trypsin or chymotrypsin (0.01 mg/ml). 0-min digestion represents a control to which no enzyme was added. The Ca^{2+}-ATPase is indicated by A and calsequestrin by B. The high-M_r band is indicated by arrows. Triads from a separate preparation were run on a 4.5% acrylamide gel which further resolved the high molecular weight components into two separate protein bands indicated by arrows. Molecular weights given $\times$ 10^3.
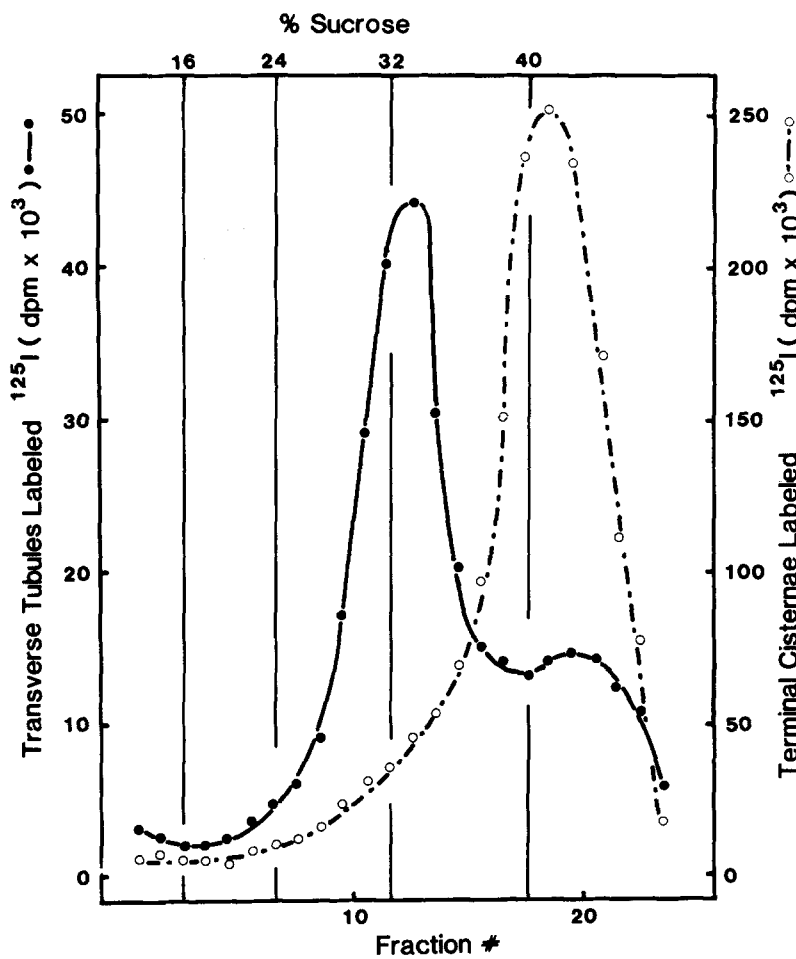

FIGURE 5 ^{125}I activity profile in sucrose density gradients (wt/wt) of TC-labeled triads (open circles) and T-tubule-labeled triads (closed circles) after disruption in the French press and centrifugation to equilibrium. The protocol for this experiment is described in Materials and Methods.

were hydrolyzed by both trypsin and chymotrypsin. A high molecular weight protein band also disappeared after only 2 min of trypsin or chymotrypsin treatment (indicated by arrow). This high molecular weight band is resolved into two discrete components of approximately 300 and 325kdaltons on a 4.5% acrylamide gel (Fig. 4). In contradistinction to these proteins, many of the other bands were largely unaffected by the proteolytic enzymes. For example, trypsin treatment was without effect on calsequestrin and on a number of other lower molecular weight bands.

Label Transfer Experiments

The experimental protocol for this experiment has been described in Fig. 1 and in Materials and Methods. Labeled TC joined to unlabeled T-tubules and subsequently split apart gave the ^{125}I pattern shown by the dashed trace (Fig. 5). Essentially all of the label remained in the 38–42% region of the gradient. Breakage of the junction was seen visually by the appearance of a band in the lighter part of the gradient. When labeled T-tubules were joined to unlabeled TC the ^{125}I activity

distribution was that of the solid trace. It has a well-defined shoulder of ^{125}I activity corresponding in density to TC and a main peak of ^{125}I activity associated with free T-tubules. Therefore these TC, previously unlabeled, now contained ~10% of the total ^{125}I activity. Transfer of labeled material from one organelle to the other had occurred during the reformation and subsequent rupture of the triad junction.

Gel electrophoresis patterns of membranes collected after certain steps of the protocol (Fig. 1) produced the Coomassie Blue staining and ^{125}I-labeling patterns in Figs. 6–8. The numbers beside the bands in Fig. 1 refer to the figures in which the corresponding gel patterns are shown. Some samples were also treated with trypsin (0.01 mg/ml, 10 min). The gel patterns of the initial labeled triads are shown in Fig. 6. A fairly general pattern of labeling is observed and the specific activity of labeling is quite variable. A high molecular weight protein is highly labeled whereas the Ca^{2+} pump was labeled to a lower specific activity. Tryptic digestion removed the ^{125}I label from these regions while an intensely labeled low molecular weight band appeared. Rejoined triads with either labeled TC (A) or labeled T-tubules (B) are shown in Fig. 7. Few bands are intensely labeled in the TC-labeled triads, the high M_r band and the Ca^{2+} ATPase being the most notable. The specific activity of the high M_r protein is considerably higher than that of the Ca^{2+} pump protein and is the most heavily labeled band in the gel. Triads containing labeled T-tubules displayed a more complex pattern. The high M_r band is present in low concentrations but is still the most heavily labeled protein. Considerable labeling of proteins of intermediate (20,000–50,000 M_r) molecular weight is also seen.
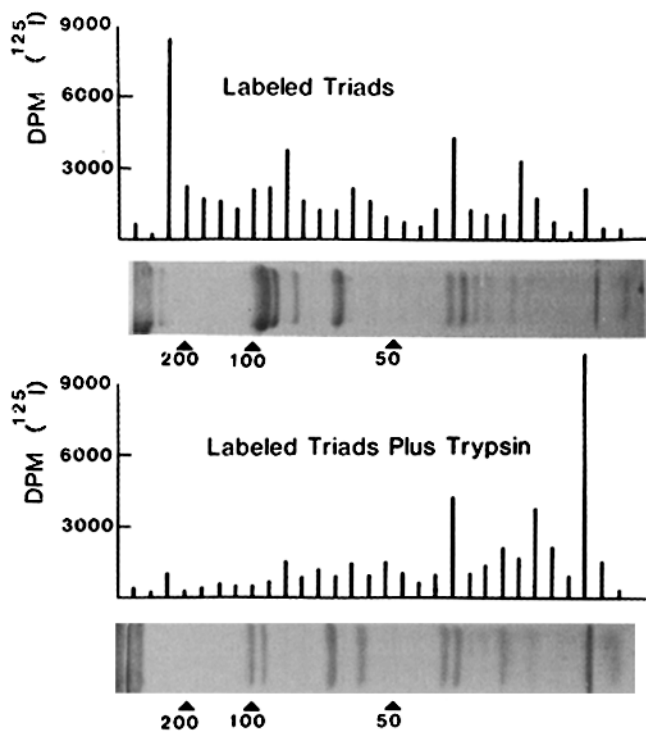
FIGURE 6 Coomassie Blue–staining and ^{125}I-labeling patterns for undigested and digested (trypsin, 0.01 mg/ml, 10 min) ^{125}I-labeled TC/triads. The histogram of ^{125}I activity for this and the following two figures was produced by drying the gel onto filter paper and cutting into 3-mm slices and counting for 10 min in a gamma counter. The stage in the label transfer protocol at which the samples were taken is illustrated by 6 in Fig. 1. Molecular weights given × 10^3.

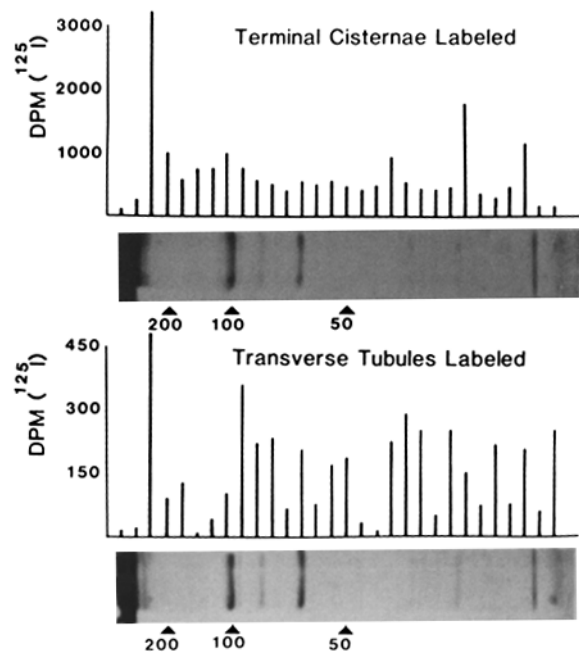FIGURE 7 Coomassie Blue–staining and ^{125}I-labeling patterns for TC/triads containing either labeled TC (top) or labeled T-tubules (bottom). These are referred to in Fig. 1 as 7A and 7B, respectively. Molecular weights given × 10^3.

The previously unlabeled TC were removed from the denser (40–43% sucrose) part of the shoulder (Fig. 5) to avoid contamination with labeled T-tubules. When these TC were run on PAGE, sliced, and counted, a single high M_r band was seen to contain ^{125}I label (Fig. 8). No other band was seen to contain significant label. Some random counts occur owing to the low overall content of label. The distribution of label in Fig. 8A contrasts sharply with the distribution shown in Fig. 7B despite the fact that the T-tubules from Fig. 7B are the source of label for Fig. 8. Thus the latter pattern is dramatically more specific. The material of Fig. 8 was treated with trypsin, 0.01 mg/ml, for 10 min. Fig. 8B shows that the ^{125}I activity was absent from the high M_r region of the gel, and that essentially all the ^{125}I activity had moved very near the dye front confirming that this protein was highly susceptible to tryptic digestion.

To establish the authenticity of the transfer of ^{125}I label, control experiments were performed to determine the amount of T-tubules remaining attached to TC after passage through the French press. Fig. 9 shows the distribution of [3H]ouabain in a sucrose density gradient after passage through the French press. [3H]Ouabain has been employed previously as a specific marker for T-tubules in density gradients (3, 12). The top portion of this figure shows the [3H] activity after intact TC/triads were passed through the French press. A peak of [3H]-activity is present in the region corresponding to free T-tubules whereas [3H] activity is low in the denser regions of the gradient corresponding to TC/triads, demonstrating essentially complete separation of T-tubules from TC. The lower portion of Fig. 9 shows the [3H] activity profile in a sucrose density gradient of rejoined triads after passage through the French press. Once again, the [3H] activity is associated with free T-tubules (24–30% sucrose) whereas little activity is associated with the TC (38–42% sucrose). Breakage of the junction is essentially complete under conditions in which the transfer of label is observed. This result has been found consistently in our laboratory (1, 4).
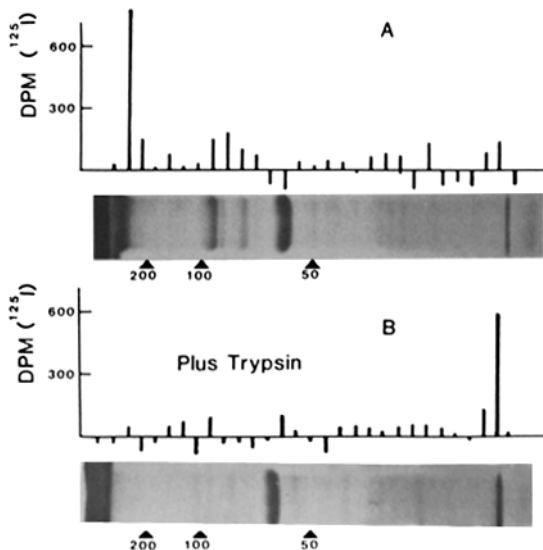
FIGURE 8 Coomassie Blue–staining and ^{125}I-labeling patterns for material taken from the TC region after ^{125}I T-tubule triads were passed through the French press and centrifuged on sucrose density gradients. Fractions 20–23 were taken from the gradient illustrated in Fig. 5 and concentrated by centrifugation at 125,000 g for 45 min (A). (B) Material from A after treatment with trypsin (0.01 mg/ml, 10 min). Molecular weights given 10^3.
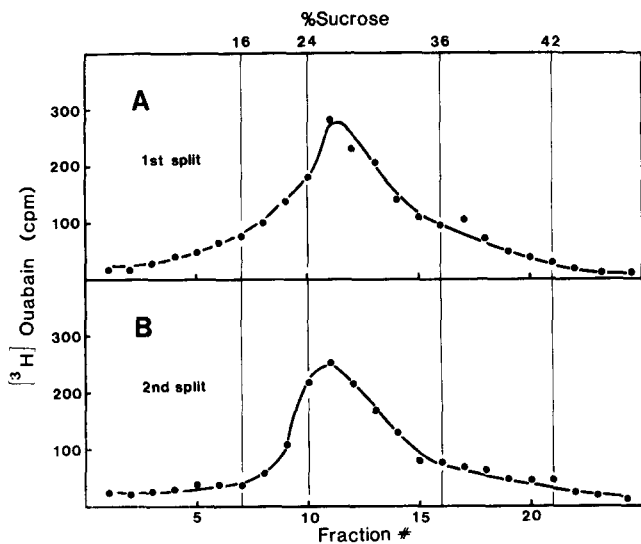

FIGURE 9 [^{3}H]Ouabain distribution in sucrose density gradients (wt/wt) of triads after passage through the French press. (A) Intact triads, (B) rejoined triads. This is representative of the data from three separate experiments.

Fig. 10 shows the effect of K-cacodylate treatment on the transfer of ^{125}I label from labeled T-tubules to unlabeled TC. In the upper part of the figure, ^{125}I-labeled T-tubules have been mixed with unlabeled TC in the presence (solid trace) and absence (dashed trace) of K cacodylate. K cacodylate caused the majority of the ^{125}I label to be present in the region of the gradient associated with TC/triads, demonstrating that rejoining of the labeled T-tubules with the unlabeled TC had occurred. In earlier experiments we observed full rejoining of T-tubules to TC (4). However, in the present experiments the T-tubules and TC have first been separated on a density gradient. Rejoining has been carried out about 2 h after initial breakage of the junction. The ability to reform the junction is labile after

French press treatment, and it is essential to carry out the rejoining as soon as possible after breakage. In the absence of K cacodylate (dashed trace), most of the ^{125}I label was present in the region of the gradient corresponding to free T-tubules, indicating that only a small amount of rejoining had occurred. The 38–42% sucrose region of each gradient was extracted and passed through the French press once again. The lower portion of Fig. 10 represents the subsequent density profile. When the separated vesicles had been mixed in the presence of 0.3 M K cacodylate, the transfer of ^{125}I label from labeled T-tubules to unlabeled TC was seen. Omission of K cacodylate from the medium (dashed trace) produced only a small amount of label transfer.

DISCUSSION

Determination of the physicochemical nature of the junctional feet and their role in excitation-contraction coupling is essential for understanding this physiological process in biochemical terms. As the junction is fractionated during isolation, the morphological identification of junctional in electron micrographs may become increasingly difficult. Classically, proteins are isolated on the basis of protein fractionation and enrichment of a particular fraction in the specific activity of the protein sought. The triad junction has not yet been shown to possess an enzymatic activity in this classic sense. Our inability to track the junctional feet either enzymatically or through quantitative electron microscopy necessitated the adoption of an unorthodox experimental approach. The specificity of T-tubules for TC in the rejoining process provides a novel means for manipulating junctional components.

The first proteolytic digestion experiments were designed with two goals in mind: (a) to test for susceptibility of the junction to proteolysis and (b) to determine whether the specificities of the particular proteolytic enzyme would provide information concerning the composition of the junctional feet. Trypsin, chymotrypsin, and pronase all caused separation of T-tubules from TC. This gives evidence that the junction is composed, at least in part, of protein, and that protein integrity is essential to integrity of the junction. The low concentration of enzyme required to break the junction is an indication that the junction is readily accessible to water-soluble enzymes.

Gel electrophoresis of triads digested for increasing lengths of time showed only a small number of protein bands affected by the protease treatment; the Ca^{2+} ATPase, a band of 80,000 M_r, and a doublet of high molecular weight were hydrolyzed. The high molecular weight doublet is particularly susceptible to proteolysis by both trypsin and chymotrypsin.

Labeling of the triads with ^{125}I was intended to take advantage of the accessibility of the junctional feet and to label selectively the individual organelles involved in the triad junction. Fig. 11 illustrates diagrammatically our view of the events that occur during label transfer. We consider that the French press breaks the junction in such a way that a majority, but not all, of the feet are associated with the TC. The data of Fig. 7 show that 3,200 dpm of label from the high M_r protein is present in the TC band after initial labeling of the triad whereas 470 dpm is present in T-tubules. If the labeling is random, then 13% of the high M_r protein is associated with T-tubules and 87% with TC. The rejoining reaction causes the formation of the junction such that the feet which had remained attached to the T-tubules are incorporated into the junction. We now propose (a) that the disposition of the feet in the reformed junction is the same whether they had remained associated
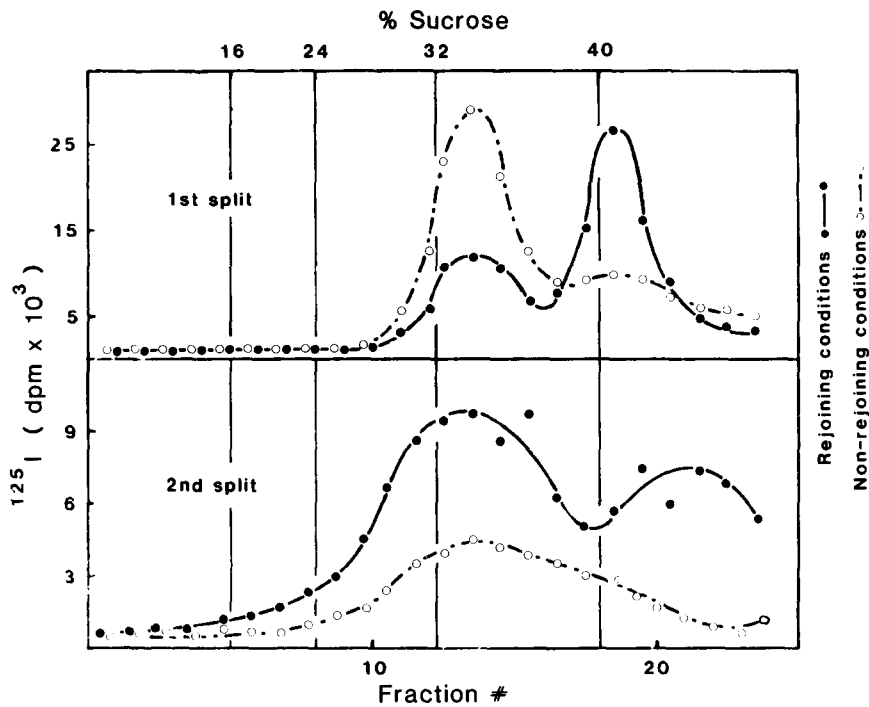
% Sucrose

FIGURE 10 Effect of 0.3 M K cacodylate treatment (rejoining conditions) on the transfer of ^{125}I label from T-tubules to TC. In the upper portion of the figure (*1st split*) ^{125}I-labeled T-tubules were mixed with unlabeled TC in the presence (solid trace) and absence (dashed trace) of 0.3 M K cacodylate and layered onto a sucrose density gradient as described in Materials and Methods. In the lower portion of the figure (*2nd split*) the fractions corresponding to 38–42% wt/wt sucrose in the upper traces were passed through the French press and centrifuged on a sucrose density gradient as described in Materials and Methods.
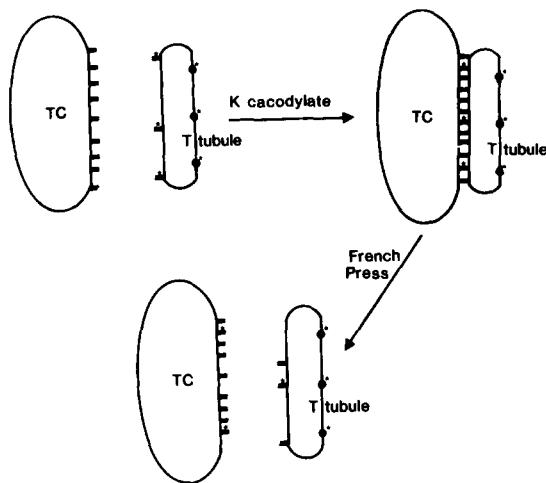

FIGURE 11 Proposed scheme to account for transfer of label from T-tubules to TC. When triads are broken in the French press, most junctional feet (represented by solid bars) remain associated with TC but some are attached to T-tubules. ^{125}I (represented by stars) labels both junctional protein and nonjunctional membrane protein (represented by circles) of T-tubules. K cacodylate causes reformation of the triad such that some label is incorporated into the junction. Breakage of this reformed junction in the French press occurs with random distribution of junctional feet so that many labeled feet from T-tubules remain with TC while some unlabeled feet are associated with T-tubules. Labeled nonjunctional proteins separate entirely with the T-tubules.

with the T-tubules or TC on initial breakage and (*b*) that the side of breakage for each foot caused by passage through the French press is random. We may then consider that when the junction is severed a second time the distribution of feet is the same as it was after the first breakage. Therefore, ~87% of the feet can be expected to remain with the TC and 13% with T-tubules. This explains why we readily observe label transfer from labeled T-tubules to TC although we have as yet been unable to observe transfer from labeled TC to T-tubules.

In making the proposal that the transfer of label occurs at the junctional feet, we have considered alternative interpretations of our data:

(*a*) The presence of label may be due to incomplete separation of the T-tubule from the TC after the second French press treatment. The data shown in Fig. 9 demonstrate that after French press treatment rejoined triads do not show significant [^{3}H]ouabain activity associated with the TC. This is also consistent with a similar observation by Brandt et al. (1). Furthermore, comparison of the gels in Fig. 7 B with those in Fig. 8 A shows that the former contains label in a large number of proteins whereas the latter shows only one high molecular weight band to be significantly labeled. This band is labeled to the same degree as in Fig. 7 B, yet the other labeled bands are not present, indicating that other material of T-tubule origin has been effectively removed.

(*b*) The material being transferred may be a soluble protein moving in solution from one vesicle to the other. However, exposure to K cacodylate and rejoining are necessary to transfer large amounts of label as shown in Fig. 10. This figure shows that in the absence of K cacodylate in the rejoining medium only a small shoulder of ^{125}I is associated with TC (38–42% sucrose). When the TC band is subjected to a second French press treatment most of the label becomes associated with the T-tubule region of the density gradient. Thus label transfer is minimal. Moreover, if the protein were truly soluble, it should be separated from the vesicles by the centrifugation steps (pelleting and gradient) that follow the French press treatments. We do not find ^{125}I activity present in the upper region of the density gradient associated with free protein.

(*c*) Label transfer may be due to the presence of unreacted free iodine. We have carried out extensive procedures to remove unreacted label. Iodine obtained from New England Nuclear is carrier-free. Removal of catalyst halts the production of reactive iodine. It is unlikely that activated iodine would remain unreacted for long since I_2 reacts rapidly with sulfhydryl and imidazole groups as well as tyrosine residues on proteins. The vesicles are thoroughly washed, first by passage through

a Sephadex G-50 column and then by dialysis overnight against two changes of 1,000 vol of buffer. Centrifugation on a sucrose density gradient preceded any contact of unlabeled vesicles with labeled ones. We believe that this washing protocol is adequate to remove all unreacted iodine.

(d) Portions of the T-tubule membrane may be transferred to the unlabeled TC. However, we find no evidence of [^{3}H]ouabain release from vesicles after French press treatment nor do we see significant [^{3}H] activity transferred to the TC. Furthermore, electron micrographs of T-tubules and TC after passage through the French press show discrete enclosed vesicles of distinct morphology without fragments of membrane attached to the outside (see preceding article).

On the basis of these data, we conclude that the appearance of a heavily labeled, high molecular weight band in a previously unlabeled TC fraction arises from the transfer of protein from T-tubules to TC. Two other observations, although neither of them is individually diagnostic, give support for our identification. (1) The junction may be broken by proteolytic enzymes. These enzymes attack only a small number of proteins in the vesicles and among these is the protein identified by label transfer. Of other proteins attacked by proteases, the Ca pump is not a likely contender for the junctional feet, and the $M_r = 80,000$ protein remains the other major protein attacked. (2) The accompanying paper demonstrates that the association of TC and T-tubule membrane survives treatment with Triton X-100 and KCl. This treatment also causes enrichment of the high M_r doublet while a number of other proteins are lost or diminished. We therefore present one positive and two supportive arguments that the high M_r protein associated with label transfer is a portion of the junctional feet.

By using 4.5% Laemmli acrylamide gels, we have resolved the protein as a doublet of approximately 300,000 and 325,000 daltons. Both of these bands contained ^{125}I label when separated and counted (data not shown). The junctional feet of rabbit back muscle have a length of 100 Å and a diameter of 150 Å and occupy a volume of $1.8 \times 10^{-6} \mu m^3$ (see preceding article). A protein of density 1.34 and M_r 300,000 daltons occupies $3.7 \times 10^{-7} \mu m^3$. Therefore, although the protein is of sufficient size to span the junctional gap, taken as a single unit it does not account for the full volume of the feet as observed in electron micrographs. However, our knowledge of the geometry of the junction is limited and it is possible that the junction is composed of an aggregate of molecules. Rough calculations based on morphometric data of intact muscle and isolated SR (7, 9, 16) suggest that 0.4 junctional feet occur per 100 intercalated particles of SR or 1.4 feet per 100 particles of TC. Taking account of the size of the feet and of intercalated particles, the volumetric ratio of feet to particles in TC is 6.5%.

It may be presumed that the majority of intercalated particles are Ca pump. Although some variability of high M_r protein content in gels is seen, this ratio is in approximate accord with the content of high molecular weight protein compared with Ca pump.

In conclusion, we have presented evidence that the junctional feet of the skeletal muscle are composed, at least in part, of two proteins of 300 and 325 kdaltons. The junction has been identified positively on the basis of the specificity of T-tubules for TC in the rejoining process. We are currently actively seeking corroborative confirmation of our analysis through conventional approaches.

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