J. Membrane Biol. 86, 267–276 (1985)

The Journal of
Membrane Biology


# Determinants of Triad Junction Reformation: Identification and Isolation of an Endogenous Promotor for Junction Reformation in Skeletal Muscle

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**Summary.** The junction of isolated triads can be mechanically broken by passage through a French press and subsequently reformed by incubation of the isolated organelles with certain salts of weak acids (e.g., K cacodylate, K propionate, and K butyrate). In contrast, other salts (e.g., KCl, K phosphate, and K benzoate) are ineffective in promoting triad formation. An endogenous factor obtained from a muscle homogenate acts in the same manner as these artificial compounds. When rabbit skeletal muscle is homogenized in a KCl solution and centrifuged to remove large cellular components and membrane fractions, an endogenous factor is extracted into the high speed supernatant which promotes the reformation of mechanically broken triads. A three-stage purification of this factor has been achieved using: (1) ammonium sulfate fractionation, (2) adsorption chromatography, and (3) molecular sieve chromatography. SDS-PAGE showed that the protein was purified to homogeneity and had a subunit $M_r$ of 34,000 daltons. This protein has the following characteristics: (1) it exists in 0.1 M KCl as a polymeric substance with an estimated $M_r$ = 123,000 on molecular sieve chromatography and a $M_r$ = 155,000 on sedimentation equilibrium; (2) it promotes the formation of triadic vesicles from isolated organelles in a low ionic strength medium; (3) Both this protein and cacodylate share the property of specifically catalyzing the association and aggregation of junctional proteins which had previously been dissolved by neutral detergent and salt; (4) it appears to be identical to an extrinsic constituent of terminal cisternae, which has been described as a protein of $M_r$ = 34K. It is not clear, however, whether this protein is a necessary and integral component of the junctional feet or whether it exerts predominantly a catalytic role in the formation of the triad junction.

**Key Words** triad junction · isolation · promotor · skeletal muscle · *T*-tubule · terminal cisternae

## Introduction

In skeletal muscle, the triad consists of the transverse tubule, which is physically joined to two terminal cisternae through an organized array of spanning proteins, or "feet" (Franzini-Armstrong, 1970). There is reason to believe that the triad junction, like the gap junction, plays a physiological role in muscle. Specifically, the mechanism of signal transfer from the transverse tubule to the sarcoplasmic reticulum during excitation-contraction coupling may involve the junctional proteins. Eisenberg and Gilai (1979) suggested that the formation and breakage of the triad junction was a dynamic process in intact muscle. They correlated an increase in the number of junctional units which completely spanned the gap between transverse tubules and terminal cisternae with increasing muscle stimulation. Franzini-Armstrong and Nunzi (1983) have recently argued that the triad junction is static and that incomplete closure of the gap by junctional feet is an artifact based on the angle of the section cut in electron microscopy.

Through the use of isolated triads, we have found that the triad can be dissociated into individual vesicles after mechanical breakage of the junction by passage through a French press and subsequently rejoined after addition of 0.6 M K cacodylate (Caswell et al., 1979). In this paper, we investigate the mechanism by which isolated triadic vesicles reassociate to form a triad. In our original experiments (Caswell et al., 1979) we sought to determine whether other salts were effective in promoting junction formation, but found that KCl, NaCl, K methyl sulfate and choline Cl were all ineffective. Nevertheless, it seemed unlikely that cacodylate exhibited such specific properties that it would not be mimicked by other compounds. In this paper we have reinvestigated the determinants of triad junction formation. The specificity of the process has suggested to us the possibility that junction formation may be promoted by an endogenous constituent of the muscle. An earlier communication (Caswell & Brandt, 1981) presented evidence that the supernatant of a muscle homogenate facilitated triad junction reformation. This paper is an extension of those earlier findings.

## Materials and Methods

### Isolation of Microsomal Organelles

The labeling of transverse (*T*) tubules of intact muscle with [$^3$H]-ouabain and the subsequent isolation of organelles has been described previously (Caswell, Lau & Brunschwig, 1976). A brief summary of the method follows.

*T*-tubules were labeled by injecting intact rabbit back muscle with a Krebs-Ringer solution containing 25 $\mu$Ci[$^3$H]ouabain (New England Nuclear) and incubating for 30 min. Crude microsomes were prepared by differential centrifugation and then separated on a continuous sucrose gradient. A light band at 28–33% sucrose has been shown to consist of longitudinal reticulum, while a heavy band at 39–41% sucrose was composed of a mixture of terminal cisternae (*TC*) and triads. *T*-tubules and light and heavy *TC* were prepared according to the procedure of Brandt, Caswell and Brunschwig (1980). The heavy microsomal fraction was passed through a French press at 8,000 psi. K cacodylate, pH 7.0 (0.5 M), was added and the suspension was centrifuged on a continuous density gradient. Two bands were recovered; the lighter is light *TC* and the heavier is a band of reformed triads. The heavy band was passed through a French press at 4,000 psi and again centrifuged on a continuous density gradient. The light band at 22–27% sucrose was collected. The [$^3$H]-ouabain label was used to track the *T*-tubules through the gradient.

### Preparation of High Speed Supernatant

A rabbit was stunned by a blow to the neck, bled, and the back muscle (sacrospinalis) excised. Each muscle was homogenized in 150–170 ml of a solution containing 100 mM KCl, 10 mM Tris Cl and 0.5 mM Tris EDTA, pH 7.0 for 1–1/2 min (3 × 30 sec periods with 30 sec rests) in a Waring blender at 4°C. The homogenate was centrifuged at 16,000 × *g* for 20 min to sediment large subcellular components. The supernatant was poured through 8 layers of cheesecloth to remove floating material and centrifuged at 125,000 × *g* for 60 min. The final supernatant (HSS) was retained.

### Assays for Reformation of Triad Junction

The assay for junctional reformation employed labeling and tracking the *T*-tubules with [$^3$H]ouabain. Labeled *TC*/Triads (approx. 3 mg protein/ml), suspended in 1 ml of 250 mM sucrose, 2 mM histidine, pH 7.0, were mechanically broken by a single passage through a French press at 8,000 psi. The vesicles were incubated with 0.5–1.0 ml of the appropriate rejoining factor at 4°C for 5 min. This mixture was layered onto a continuous sucrose gradient (12.5 to 50% wt/wt sucrose) and centrifuged in a Beckham SW 27 swinging bucket rotor at 120,000 × *g* for 5 hr. Each gradient was separated into 2-ml fractions and assayed for [$^3$H]ouabain and protein content. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as the standard. [$^3$H]ouabain was quantitated by liquid scintillation counting in a phophor which contained 45% Triton × 100.

### Isolation of Rejoining Factor

#### *Ammonium Sulfate Fractionation*

Ammonium sulfate was added slowly to 140 ml of HSS at 0°C until 75% saturation was reached. The suspension was stirred for an additional 20 min and then centrifuged at 12,000 × *g* for 20 min in a Sorvall SS-34 rotor. The supernatant was brought to 100% ammonium sulfate saturation, stirred for an additional 30 min and centrifuged as before. The pellets were resuspended in 10 ml of 5 mM histidine, pH 7.0, and centrifuged on a clinical centrifuge for 10 min to remove any sediment.

#### *Molecular Sieve Chromatography*

The HSS sample was loaded on a Pharmacia Sephacryl S-200 (2.5 cm × 60 cm) column which had been equilibrated in 5 mM histidine, pH 7.0, at 4°C and eluted with 5 mM histidine, pH 7.0, at a flow rate of 1.2 ml/min. Fractions (8 ml) were collected and adjusted to pH 7.0 with HCl or NaOH, if necessary. Protein content was determined either by monitoring absorbance at 280 nm or by the method of Lowry et al. (1951). The column was calibrated in the presence of 100 mM KCl using trypsin, ovalbumin, BSA, creatine phosphokinase and alcohol dehydrogenase (Sigma) as standards.

#### *Adsorption Chromatography*

The HSS sample was loaded on a Biorad Biogel HTPT (2.5 × 40 cm) column which has been equilibrated with 5 mM histidine, pH 7.0. The column was eluted with a phosphate gradient made by infusing 0.7 M $Na_2HPO_4$ (pH 7.0) into 550 ml of 5 mM histidine (pH 7.0) in a constant volume mixing chamber. The flow rate for the column was 2.5 ml/min and fractions were collected every 4 min. The column was washed and regenerated by sequential elution with one column volume of 1 M $Na_2HPO_4$ (pH 7.0), 1 volume of 1 M NaCl and four column volumes of 5 mM histidine.

### Gel Electrophoresis

SDS polyacrylamide gel electrophoresis followed the method of Laemmli (1970). Elution profiles from columns were run on gels which used 8% acrylamide, 0.21% N,N'-Methylene-bis-acrylamide (BIS). Samples of the gel were prepared by mixing the protein fraction with solubilizing medium (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.625 M Tris-HCl, pH 6.8, with bromophenol blue as tracking dye). After heating each sample in boiling water for 1 min, 20 $\mu$g of protein/lane were loaded and run. Slabs were stained with Coomassie blue (1 : 1 mix of 50% trichloracetic acid and 0.2% Coomasssie R-250 solution) for 1 hr and destained with 7.5% glacial acetic acid, 5% methanol.

Gels were scanned with a Zeineh Soft Laser Scanning Densitometer with a tungsten light source (LKB). Densitometric records of protein peaks were quantitated by cutting and weighing.

### Equilibrium Sedimentaiton Analysis

The purified promotor protein (10 ml) at a concentration of 1.2 mg/ml was dialyzed against 3 liters of 100 mM KCl, 5 mM histi-

dine (pH 7.0) at 4°C. Concentrations of 1.0, 0.5 and 0.25 mg/ml were placed in the inner, middle and outer compartments of a double sector cell of the Yphantis type, having three pairs of compartments of 3 mm length. Centrifugation was at 16,000 rpm in the Beckman Model ANH rotor at 5°C. Molecular weight was estimated using the meniscus-depletion method of Yphantis (1965). Photographs were taken using the Rayleigh interference optics and then measured with the Nikon comparator. Molecular weights were calculated by use of the computer program of Small and Resnik (1965), assuming a partial specific volume of 0.74 and solution density = 1.0044. In all cases the whole cell weight-average molecular weight was taken as the best estimate. The values for the three concentrations were then averaged.

### Electron Microscopy

Vesicles were prepared for thin section electron microscopy by tannic acid mordanting as described by Brunschwig et al. (1982). with one modification. The vesicles were diluted in 240 mM sucrose, 2.0 mM histidine, pH 7.0, and centrifuged at 10 psi for 5 min on a Beckman Airfuge. The pellets were then overlaid with 2.5% glutaraldehyde/4% tannic acid in 0.1 M Na-cacodylate, pH 7.2, 3% sucrose as described previously (Brunschwig et al., 1982).

## Results

### Salts That Promote Formation of the Triad Junction

*T*-tubules were labeled by injecting the intact muscle with [$^3$H]ouabain as described in Materials and Methods. *TC*/Triads were then prepared and the junction broken mechanically by passing the suspension through a French press. The preparation was incubated with 0.33 M salt as defined in Fig. 1 and centrifuged on a continuous density gradient. Although incubation with the salt was for a period of 5 min prior to centrifugation, the actual incubation time may be considerably longer since the vesicles are only slowly separated from the salt by the centrifugal force. The top trace in Fig. 1 shows the [$^3$H]ouabain distribution of broken *TC*/triads incubated with KCl. We have previously demonstrated (Caswell et al., 1979) that KCl does not promote reformation of the triadic junction. This treatment therefore can be employed as a control for the degree of breakage by the French press and for the buoyant density of free *T*-tubules. The major [$^3$H]-ouabain band occurs at 25% sucrose. We have previously identified this band as that of free *T*-tubules, which show an isopycnic point at 20–28% sucrose depending on the preparation. The shoulder at 35% may be triads that have been inadequately broken by the French press even though the position of the shoulder is slightly lower in density than native in-

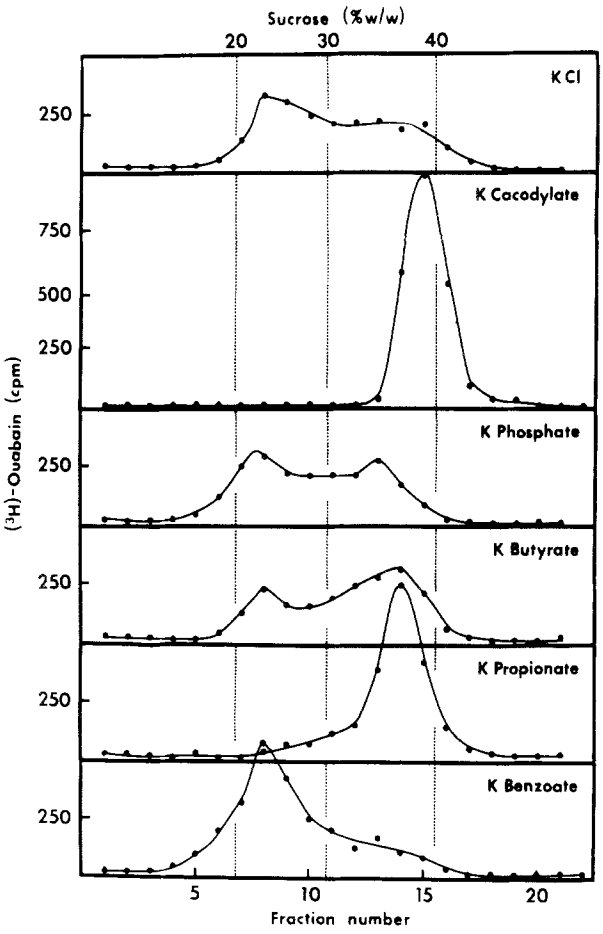



**Fig. 1.** [$^3$H] ouabain profile of density gradient of broken triads in presence of different salts. Labeled *TC*/triads were prepared, as described in Materials and Methods, and passed through a French press at 6,000 psi. This preparation was incubated with 0.33 M salt for 5 min at 0°C, as defined in each panel, and centrifuged on a continuous sucrose gradient

tact triads. In contrast, the ouabain profile after incubation of broken triads with 0.33 M K cacodylate shows a single sharp band at 40% sucrose, the isopycnic point of isolated triads. Full reformation of the triad junction has occurred. K phosphate-treated vesicles show a pattern similar to those treated with KCl, indicating that the K phosphate salt is also ineffective in promoting triad formation. K benzoate treatment causes a sharpening of the free T-tubule band at 24% sucrose. The content of unbroken triads, if anything, was decreased. K butyrate and K propionate, on the other hand, promote formation of the triad junction. In each case the ouabain content of the free *T*-tubule band has declined and the triad band has concomitantly increased. Therefore the action of cacodylate may be mimicked by simple organic acids.

We have carried out similar experiments to test

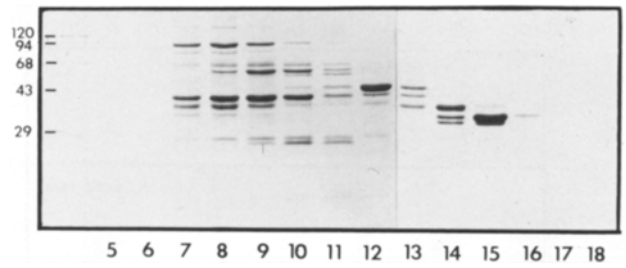



**Fig. 2.** Elution profile of HSS from Sephacryl S-200 molecular sieve column. A sample of HSS (15 ml) was loaded onto the molecular sieve column and eluted with 5 mM Histidine, as described in Materials and Methods. Aliquots (25 $\mu$l) from column fractions were prepared for analysis by SDS-PAGE as described in Materials and Methods. 50 $\mu$l containing up to 30 $\mu$g protein samples were added to each lane. Migration of standards (high molecular weight standards from Sigma Co.) are indicated by the $M_r$ numbers ($\times 10^{-3}$) on the left side

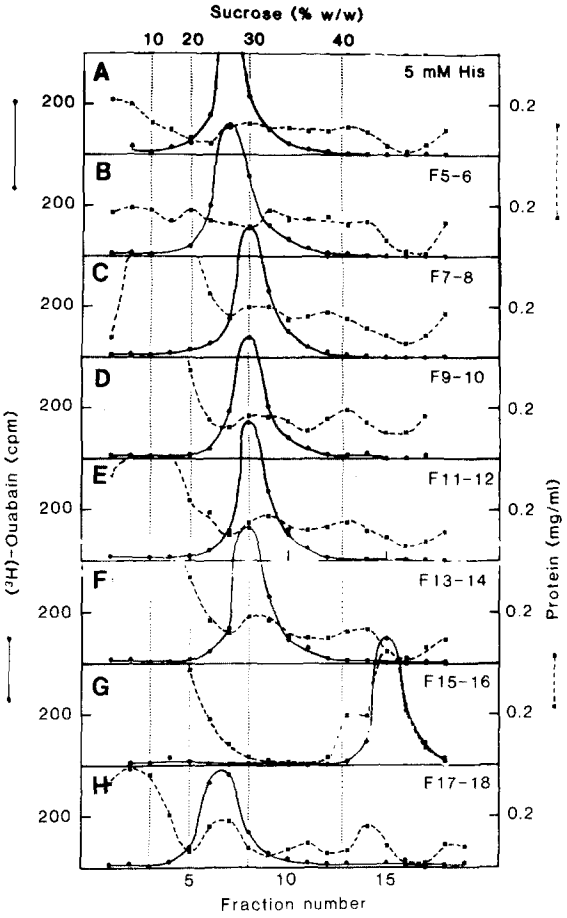



**Fig. 3.** Assay for triad junction formation using fractions from Sephacryl S-200 column. Aliquots (1 ml) from column fractions shown in Fig. 2 were incubated with mechanically broken labeled triads (6 mg), prepared as described in Materials and Methods. for 5 min. This mixture was layered onto a continuous sucrose gradient, centrifuged, separated into 2-ml fractions and assayed for [$^3$H]ouabain and protein content (by the method of Lowry). [$^3$H]ouabain and protein profiles are shown for column fractions and the control, 5 ml histidine, (panel *A*) as described in this figure

for the ability of different salts to promote junction formation and found that 0.5 M K acetate, K propionate, K 3-OH-propionate and K $HCO_3$ all cause significant reformation of the junction. On the other hand, K 2C1-propionate, K 3C1-propionate, K 2-OH-propionate, K Cl-acetate, K gluconate, K methanesulfonate and K OH-acetate are all without significant effect in promoting junction formation. There is a close correlation between the pK of the acid from which the salt was formed and its ability to catalyze junction formation. All the effective salts have pK's of 4.5 or greater, while the pK's of ineffective salts are 4.0 or below.

## Isolation of an Endogenous Factor that Promotes Junction Formation

Preliminary experiments have been described showing that a soluble extract (125,000 $\times$ *g* for 60 min) of muscle was capable of promoting the reformation of triads from disrupted junctions (Caswell & Brandt, 1981).

Figure 2 shows a SDS gel electrophoresis evaluation of the protein constituents of this high speed supernatant (HSS) which has been eluted through a Sephacryl S200 column. This column was eluted in a medium containing 5 mM histidine, pH 7. This low ionic strength environment was employed for two reasons: (1) We have found that the rejoining activity of HSS is inhibited by high salt environments and therefore junction formation is easier to assay in a low ionic strength medium. (2) The molecular sieve properties of the column are supplemented with a degree of ion exchange activity that assists the separation of proteins at low ionic strength. Many proteins elute close to the void volume of the column while a few, including a protein of subunit $M_r$ = 34 K, are retained longer on the column.

Figure 3 is an evaluation of the ability of these column fractions above to promote the reformation of the triad junction. The sucrose gradient assay of junction formation was carried out by incubating triads broken in a French press triads with aliquots of column fractions and monitoring the isopycnic density of transverse tubules labeled with [$^3$H]-ouabain through a sucrose gradient. A change in the isopycnic point of the *T*-tubules to that of the terminal cisternae indicated a reformation of the triad junction. A complete shift of the ouabain band to 43% sucrose is seen in Fig. 3*G*, corresponding to fractions 15–16 of the molecular sieve column. No

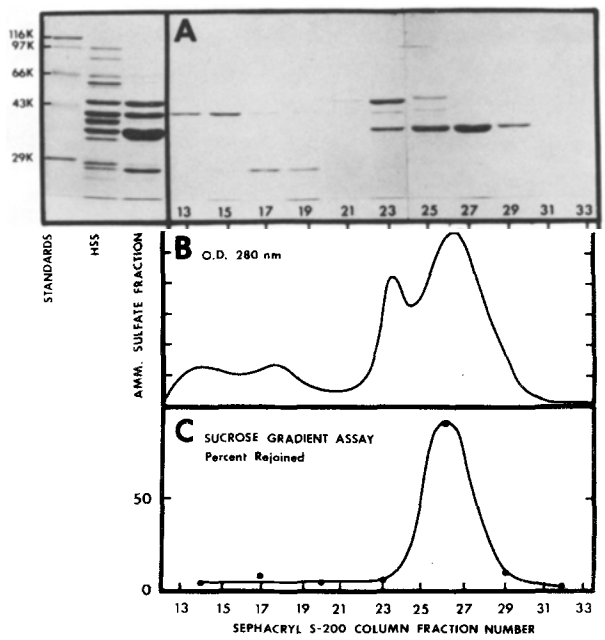



**Fig. 4.** Identification and partial purification of the endogenous promotor for triad junction reformation. HSS was fractionated by ammonium sulfate precipitation, loaded on a Sephacyl S-200 molecular sieve column and eluted with 5 mM histidine, pH 7.0, at 0°C. Fractions were collected, run on SDS-PAGE (panel *A*), assayed for protein content by monitoring absorbance at 280 nm (panel *B*) and the ability to reform the triad junction by sucrose gradient assay (panel *C*) as described in Materials and Methods. Percent rejoining was determined by dividing the number of [$^3$H]-ouabain counts in the region of 35–45% sucrose by the total counts in the gradient. Aliquots (20 $\mu$g/lane) of HSS before and after ammonium sulfate fractionation, along with 20 $\mu$l of the molecular sieve column fractions denoted were prepared for SDS-PAGE as described in Materials and Methods

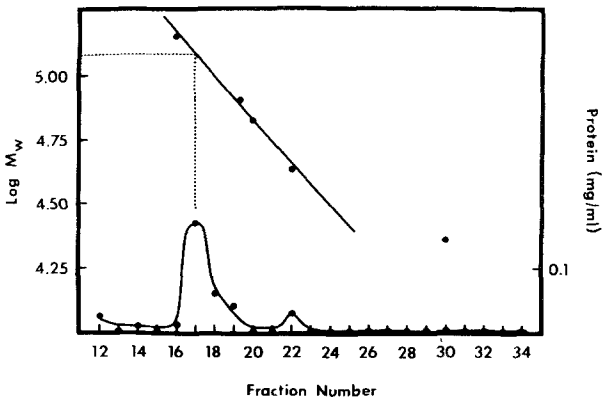


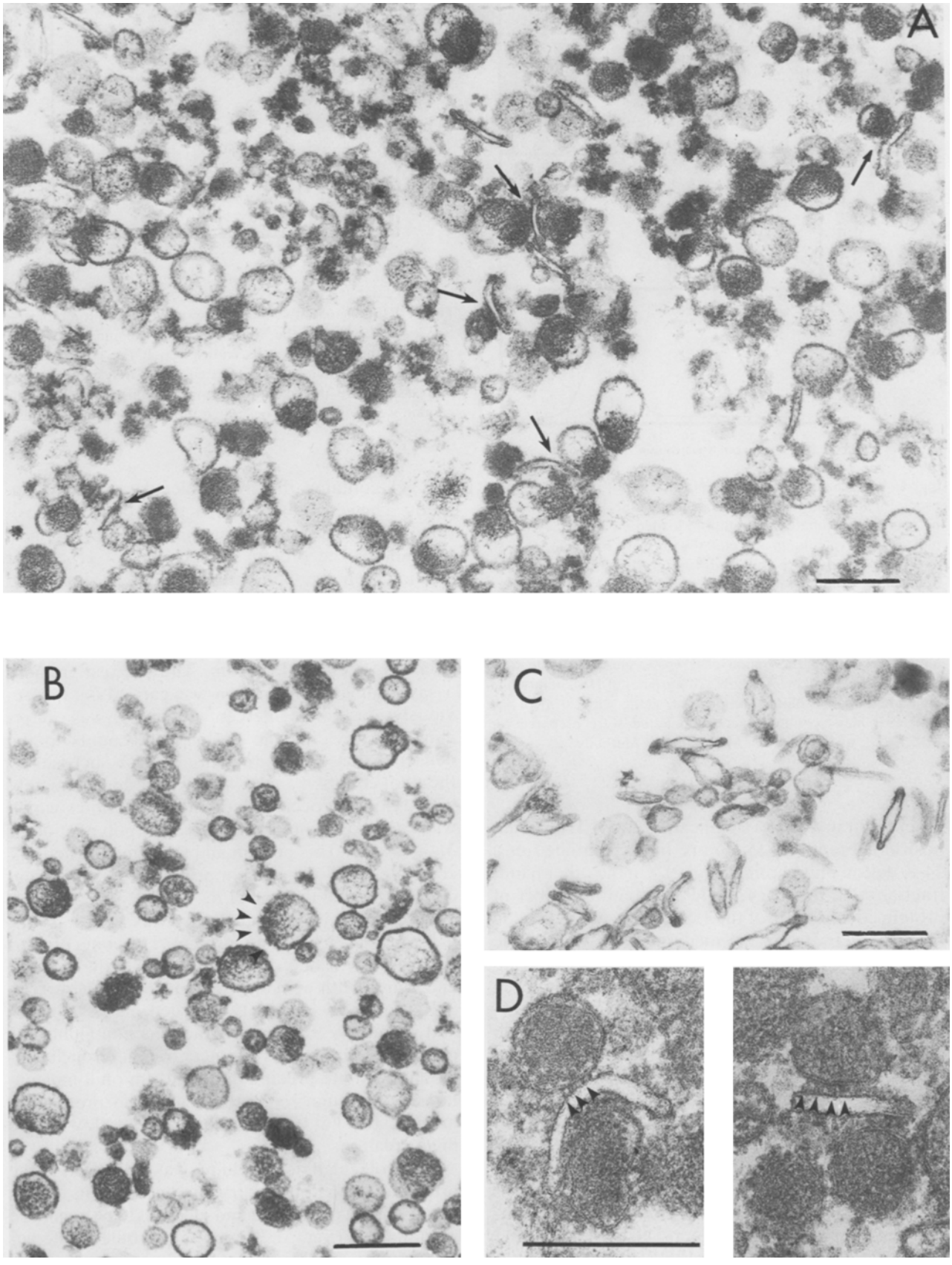

**Fig. 5.** Estimate of native molecular weight of the promotor protein by calibration of the Sephacryl S-200 column. The elution profile of the 34K protein is shown across the bottom of the graph, while the standards are plotted across the center. Protein was assayed by the method of Lowry et al. (1951), and the column was calibrated as described in Materials and Methods

other fractions show a significant shift in [$^3$H]ouabain position. Fractions 15–16 are highly enriched in a 34-K dalton polypeptide (Fig. 2). On the other hand, fractions 7 through 14, which contain a number of protein constituents of the HSS, show no detectable rejoining activity. In the medium employed for the rejoining assay in this experiment the [$^3$H]ouabain of rejoined triads occurs at 42% sucrose while the native triads normally have isopycnic point at 38–40% sucrose. We subsequently discovered (Figs. 6 and 7) that inclusion of 20–30 mM K gluconate in the medium largely prevents this enhanced density while still permitting full rejoining.

The active factor of HSS was partially purified in a two-stage protocol as illustrated in Fig. 4. The first stage of purification and concentration was effected by ammonium sulfate precipitation described in Materials and Methods. The concentrated solution was applied to a Sephacryl S200 column and eluted with 5 mM histidine (pH 7.0). The first lane of the gel contains the five molecular weight standards. The second lane shows the protein composition of the high speed supernatant (HSS). The third lane shows the protein composition of HSS after fractionation by ammonium sulfate precipitation. Four major proteins remained in this fraction (lane 3) with subunit $M_r$'s = 43K, 38K, 34K and 26K. The remaining gels (panel *A*) show the elution profile from the Sephacryl column. The sucrose gradient assay of junction formation was carried out as previously described. Percent rejoining was determined by dividing the [$^3$H]ouabain counts in the denser region of the gradient (35–45% sucrose) by the total counts. Panel *C* shows that approximately 97% rejoining occurred in those fractions which were composed predominantly of a polypeptide of $M_r$ = 34,000. This protein and the rejoining activity correlates well with a major protein absorption band detected at 280 nm (panel *B*).

Adsorption chromatography was added as a final step to purify the 34K protein. The concentrated sample was loaded onto a hydroxyapatite column and eluted with a shallow phosphate gradient. The 34K protein is selectivity retarded on this column and is roughly 97% pure. Fractions containing the promotor protein were concentrated through ammonium sulfate precipitation and run on a molecular sieve column to remove salt. The protein remained stable at 0°C for several days following the final isolation.

A molecular sieve column was calibrated in the presence of salt using trypsin, ovalbumin, BSA, creatine phosphokinase and alcohol dehydrogenase as standards (Fig. 5). The elution profile of the native promotor as determined by Lowry assay (1951)


A
B
C
D

is shown across the bottom of the graph, corresponding to an apparent $M_r$= 123,000. Sedimentation equilibrium analysis was performed on the purified protein in the presence of 0.1 M KCl, 5 mM histidine, pH 7.0. An average molecular weight estimate of 155,000 was obtained, suggesting that the native protein is composed of four subunits of $M_r$ = 34K.

## Electron Microscopy

Figure 6*A* shows a low magnification field view of triads reformed by incubation of broken triads with the 34K protein, and isolated by density gradient centrifugation to remove free *T*-tubules. Assay of the presence of *T*-tubules in the *TC*/triad fraction by [$^3$H]ouabain assay in this experiment indicates that 50% of *T*-tubules have associated with the *TC* fraction at 38–42% sucrose. The panel shows visually the presence of many elongate vesicles in close proximity to spherical vesicles (arrows). The presence of electron-dense matter in the spherical vesicles is diagnostic of terminal cisternae while the elongate shape of vesicles is diagnostic of *T*-tubules. The micrograph therefore indicates morphologically that triads have been reformed, consisting of elongate *T*-tubules in association with *TC*. The appearance of the reformed triads is hardly distinguishable from that of native triads shown previously (Lau, Caswell & Brunschwig, 1977). A few *T*-tubules are not associated with well defined *TC*, but this may reflect the limitations of the plane of sectioning.

Panel *B* shows protein triads that have been treated identically to panel *A* except for the omission of the rejoining factor. [$^3$H]ouabain analysis shows that this fraction at 38–42% sucrose contains 8% of the total *T*-tubule population. Discernible *T*-tubules are almost completely absent from the field. On the other hand, most vesicles show an approximately circular shape with enclosed electron-dense material. Thickening of the membrane described previously (Campbell, Franzini-Armstrong & Shamoo, 1980; Brunschwig et al., 1982) can be discerned in several vesicles. Sometimes the thickened membrane shows discrete regular structures similar in appearance to triadic feet (arrow heads)

Panel *C* is a view of the fraction sedimenting at 22–26% sucrose in isopycnic centrifugation employing the same protocol as panel *B*. This is the isopycnic point of free *T*-tubules. The field shows a large number of elongate vesicles. This *T*-tubule preparation has been described before and analyzed as consisting of discus-shaped vesicles (Lau et al., 1979).

Panel *D* represents higher magnification examples of reformed triads in which the individual junctional feet are well defined (arrow heads). In some cases the spanning structures appear in the form of solid rectangles, while in others the structures are thin with an apparent translucent core. The physical appearance of feet may be influenced by the sectioning conditions.

## Association of 34K Protein with Triadic Structures

We have evaluated the specificity of interaction of *T*-tubules with microsomal subfractions in the presence of the 34K protein. *T*-tubules labeled with [$^3$H]-ouabain were incubated with a separate preparation of microsomes to which no label had been applied. Incubation was carried out in the presence of increasing concentrations of the purified 34K protein, which had previously been dialyzed against a solution of 5 mM histidine. The results of this experiment are shown in Fig. 7; the numbers in the boxes represent the concentration of the 34K protein in $\mu g$/ml present in the incubation mixture. After incubation, the vesicles were centrifuged on a sucrose density gradient as shown. The dashed line representing protein indicated three bands in the gradient. The large protein band, at isopycnic point at 18% wt/wt sucrose, represents soluble protein which is being extracted from the vesicles as a consequence of the incubation in the presence of 20 mM K gluconate. The second main peak at 30% sucrose represents longitudinal reticulum and the third band which, in the absence of the 34K protein, is a shoulder at 38% sucrose represents terminal cisternae.

**Fig. 6** (*facing page*). Thin section electron micrographs of reformed triads and diads. *TC*/triads containing [$^3$H]ouabain were prepared as described in Materials and Methods and passed through a French press at 6,000 psi. Incubation was carried out at 0°C in a medium containing 15 mg protein of broken triads in 4.5 ml of 250 mM sucrose, 2 mM histidine, 30 mM K gluconate, pH 7.0. The rejoining medium contained 2.8 mg purified promoter protein which was omitted from the control. After 10 min incubation the samples were loaded on continuous sucrose gradients and centrifuged at 100,000 × *g* overnight. Samples (1.8 ml) were withdrawn from the gradient and assayed for [$^3$H]ouabain content. Fractions containing rejoined triads were combined, diluted with water and concentrated by centrifugation. Similarly control samples of *T*-tubules and terminal cisternae from gradient in which promotor protein had been omitted were combined and concentrated. Subsequent treatment for electron microscopy is described in Materials and Methods. Panels *A* and *D* were obtained by incubation with promotor protein while panels *B* and *C* were from preparations in the absence of this protein. *A* and *D* are reformed triads, *B* is terminal cisternae and *C* is *T*-tubules. Arrows indicate *T*-tubules. Arrowheads represent extensions from the terminal cisternae membrane, which may represent junctional feet. Bars represent 250 nm

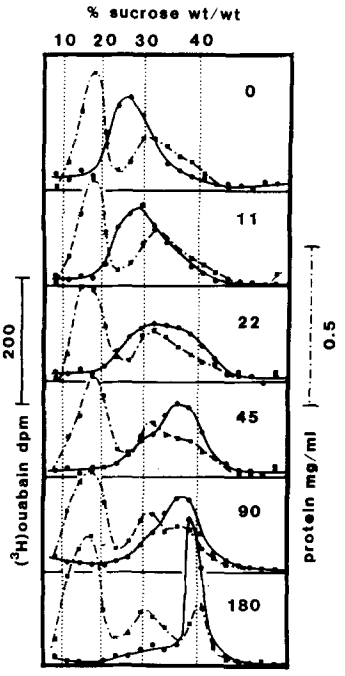



**Fig. 7.** Specificity of association of *T*-tubules to SR fragments. *T*-tubules were prepared with entrapped [$^3$H]ouabain as described earlier. Microsomes without [$^3$H]ouabain were prepared separately. The incubation mixture contained 4 ml of 250 mM sucrose, 5 mM histidine, 1 mM dithioerythritol, 20 mM K gluconate, pH 7.0, *T*-tubules 0.1 mg protein/ml, microsomes 2 mg protein/ml. 34K protein was added to a final concentration in $\mu$g/ml as indicated in each box and incubated for 10 min at 0°C. Each sample was layered on a continuous sucrose density gradient in a SW27 rotor and centrifuged for 5 hr

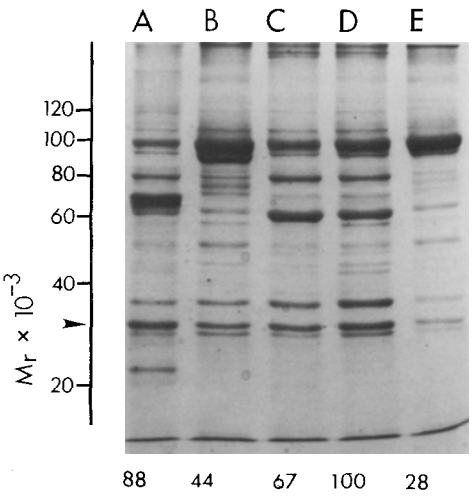



**Fig. 8.** SDS PAGE of microsomal subfractions showing intensity of protein $M_r$ = 34,000. Each lane contained 20 $\mu$g of protein. Lanes are: *A*, *T*-tubules; *B*, longitudinal reticulum; *C*, heavy terminal cisternae; *D*, *TC*/triads; *E*, nonrejoining light terminal cisternae. *B* and *D* were subfractions of microsomes subjected to density gradient centrifugation. *A*, *C* and *E* were prepared as follows: *TC*/triads were passed through a French press and treated with 0.3 M K-cacodylate, pH 7.0, to reform triads. The vesicles were centrifuged on a sucrose density gradient. The light band was nonrejoining light terminal cisternae (*E*). The heavy band of reformed triads were passed through a French press and centrifuged on a density gradient. The light band was *T*-tubules (*A*) and the heavy band heavy terminal cisternae (*C*). The method is described in detail by Brandt et al. (1980). The numbers in each lane refer to the densitometric intensity of the 34,000 $M_r$ band (arrowhead) referred to *TC*/triads (100)

Because the *T*-tubules were added in considerable lower concentration than the microsomes their presence is not apparent in the protein assay. The [$^3$H]-ouabain assay shows that, in the absence of the 34K protein, the *T*-tubules have an isopycnic point of about 26% sucrose. Increasing concentrations of the 34K protein are responsible for causing the *T*-tubular [$^3$H]-ouabain profile to separate into two bands with isopycnic points of about 26% sucrose and about 38% sucrose. Higher concentrations of the 34K protein caused diminution in the 26% band and enhancement in 38% band. At the same time the protein shoulder, observed in the absence of the 34K protein, is transformed by the 34K protein into a distinct peak associated with a sharpening of the terminal cisternae band. The longitudinal reticulum band is visually, and through the protein assay, unaffected by the presence of the 34K protein. At the highest concentration of 34K protein employed, 85% of the ouabain is associated with the *TC*. It is not clear whether the remaining 15% is associated predominantly with the longitudinal reticulum or represents free *T*-tubules. This figure gives evidence of a considerable specificity of interaction of *T*-tubules with terminal cisternae as distinct from longitudinal reticulum. The potency of interaction of the 34K protein with the organelles substantially is influenced by factors such as ionic strength, and it is probably not useful to attempt any evaluation of the affinity of $K_m$ of the 34K protein in initiating junction reformation.

Campbell et al. (1980) have described a protein of *TC* which has a subunit of $M_r$ of 34,000 and may be identical to the promotor protein. Figure 8 shows a SDS PAGE pattern of proteins in different fractions from a preparation of microsomes. A protein of $M_r$ = 34,000 is present in these gels (arrow). This protein has an electrophoretic mobility identical to the promotor protein (*data not shown*). The content of this protein in different microsomal subfractions was estimated by a densitometric scan. The numbers on the figure (relative to the content in *TC*/triads) indicate the content. The protein is considerably higher in the *TC*/triad fraction than the *LR* fraction. After the *TC*/triad has been passed through the French press the protein is found both

in *T*-tubules and in heavy *TC*. It is present in very low amount in light *TC*. This latter fraction is that which does not attach to *T*-tubules after treatment of the disrupted triads with K cacodylate. Proteins of $M_r$ = 38,000 and 80,000 are similarly depleted in this fraction.

## Discussion

In previous work we have demonstrated that a hypertonic solution of K cacodylate is capable of causing isolated *T*-tubules and *TC* to reassociate in the form of a triad. We demonstrate here that this promotor property of cacodylate is also shared by other salts of weak acids. This suggests that the action of cacodylate, rather than being a specific action exhibited by this organic arsenic salt, is a property shared by salts of weak acids which are not chemically analogous. The mechanism by which this action is manifested is still not clear. We have demonstrated in an earlier paper (Caswell & Brunschwig, 1984) that an intact vesicular structure is not necessary to facilitate junction formation. Therefore these salts are not acting simply to alter the pH of the lumenal space. In this paper we extend these findings through the observation of an endogenous factor that also promotes the formation of the triadic junction from the isolated vesicles. A three-stage purification employing ammonium sulfate precipitation, hydroxyapatite and molecular sieve chromatography is able to give an electrophoretically pure preparation of a protein of subunit molecular weight 34,000 which promotes junction formation.

This paper illustrates the degree of specificity of the 34K protein in initiating junction formation between the *T*-tubule and the *TC*. We have found no other fraction from a muscle extract which exhibits comparable activity in promoting junction formation. Our assay is not sensitive enough to preclude an action of another protein, but it does demonstrate that no other protein is present in the extract at a similar concentration which is as effective as the 34K protein. The high specificity is also illustrated in another way through the observation (Fig. 7) that *T*-tubules bind specifically with *TC* and show apparently little or no tendency to associate with *LR*. The content of a protein of $M_r$ = 34,000 which may be identical to the promotor protein in isolated vesicles indicates a preponderant concentration in junctional fragments (heavy *TC*, *TC*/triads, *T*-tubules). The content in *LR* is lower and that in *LTC* which do not form a junction with *T*-tubules is still lower. Our data suggest that the isolated *T*-tubules and *TC* contain 34K protein and yet do not normally spontaneously associate. The most likely explanation is that the concentration is inadequate.

In our early experiments we carried out the rejoining reaction in a low ionic strength medium because we found that high ionic strength (0.1 M KCl) inhibited rejoining. In low ionic strength the reformed triads had a higher isopycnic density than the native preparation. Subsequently, we included 20–30 mM K gluconate in the medium and very little shift in density of the triads occurred. The explanation for this concerns the properties of the promotor protein. In a separate publication we demonstrate that this protein is glyceraldehyde phosphate dehydrogenase and that it exhibits attachment sites to certain specific proteins of the *TC* (Caswell & Corbett, 1985). This enzyme is an extrinsic enzyme, and therefore it may attach to the *TC* and render them heavier. In the presence of K gluconate the enzyme is released from the membrane.

A further feature of the reformation of triads either by salts or by the promotor protein is that the triad band becomes sharper. The native *TC*/triad preparation is sharpest in the gradient when the preparation is richest in *T* tubules. French press treatment or digestion of the junction by proteases (Cadwell & Caswell, 1982) causes diffusion of the *TC*. A possible explanation for this is that the *T* tubules cause association of two or more *TC* vesicles in the triad, and therefore the density is averaged for several vesicles. Although *T* tubules are lighter than *TC* we have found no discernible difference in density between *TC* and triads, probably because the small sized *T* tubule vesicles contribute little to the overall density of the triads.

The 34K protein may be exerting a structural role on the triad so that it serves as the glue to join the two triadic vesicles together. An alternative hypothesis is that the protein serves a catalytic role and that its presence is required to facilitate the formation of the junction but is not a necessary constituent of the junctional processes. Campbell et al. (1980) proposed that two extrinsic proteins of molecular weights 34K and 38K are the junctional processes of the triad. The evidence in favor of this hypothesis is that *TC* contain visible electron-dense processes on the cytoplasmic side and that KCl causes both extraction of these proteins and removal of the junctional processes.

An alternative mechanism of action for both K cacodylate and the 34K promotor protein is that each interacts with specific proteins of the triadic junction and organizes them into arrays, both in the *TC* and in the *T*-tubules. The organization of these proteins in arrays may then promote the association of the two vesicles by encouraging a lock and key fit between them. We have previously identified a pro-

tein of molecular weight 1.2 million (subunit molecular weight approximately 300K) which may represent the connecting junctional process (Cadwell & Caswell 1982; Caswell & Brunschwig, 1984). This protein is less susceptible to extraction from the membranes by salt treatment.

The 34K promotor protein is a major constituent of the muscle. In another communication we identify the promotor protein as the enzyme, glyceraldehyde-3-phosphate dehydrogenase. The specific site of action of this enzyme with sarcoplasmic reticulum proteins is addressed in this other paper.

We wish to thank Dr. J.F. Woessner for performing the sedimentation equilibrium analysis, Darshan V. Tolat and Robert D. Bonnel for their excellent assistance in the electron microscopy work, and Lori Link for typing the manuscript.

This work was supported by NIH Training Grant HL-07188 and NIH Grants AM-21601 and HL-28804.

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Received 20 February 1985; revised 29 April 1985