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Molecular Interactions of the Junctional Foot Protein and Dihydropyridine Receptor in Skeletal Muscle Triads

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Summary. Isolated triadic proteins were employed to investigate the molecular architecture of the triad junction in skeletal muscle. Immunoaffinity-purified junctional foot protein (JFP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), aldolase and partially purified dihydropyridine (DHP) receptor were employed to probe protein-protein interactions using affinity chromatography, protein overlay and crosslinking techniques. The JFP, an integral protein of the sarcoplasmic reticulum (SR) preferentially binds to GAPDH and aldolase, peripheral proteins of the transverse (T)-tubule. No direct binding of JFP to the DHP receptor was detected. The interactions of JFP with GAPDH and aldolase appear to be specific since other glycolytic enzymes associated with membranes do not bind to the JFP. The DHP receptor, an integral protein of the T-tubule, also binds GAPDH and aldolase. A ternary complex between the JFP and the DHP receptor can be formed in the presence of GAPDH. In addition, the DHP receptor binds to a previously undetected M_r 95 K protein which is distinct from the SR Ca^{2+} pump and phosphorylase *b*. The M_r 95 K protein is an integral protein of the junctional domain of the SR terminal cisternae. It is also present in the newly identified "strong triads" (accompanying paper). From these findings, we propose a new model for the triad junction.

Key Words excitation-contraction coupling · muscle-triad junction · junctional foot protein · dihydropyridine receptor

Introduction

The site of excitation-contraction coupling in skeletal muscle is the triad junction, where the terminal cisterna (TC) of the sarcoplasmic reticulum (SR) attaches to the invaginated transverse (T-) tubule system of the plasma membrane via the junctional feet. Cadwell and Caswell (1982) first identified a high M_r doublet as subunits of the junctional foot. Subsequently the upper band was demonstrated to be the native protein and the lower band a proteolytic product. The identity of the protein has been confirmed as the junctional foot protein (JFP) by immuno-electron microscopy (Kawamoto et al., 1986). Several laboratories (Hymel et al., 1987; Imagawa et al., 1987; Lai et al., 1988) have now demonstrated that the JFP is the ryanodine-sensitive Ca^{2+} release channel of the SR.

Two mechanisms for signal transduction at the junction have been proposed: (*i*) release of a second messenger such as Ca^{2+} (Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1970) or inositol trisphosphate (Vergara, Tsien & Delay, 1985; Volpe et al., 1985) from the T-tubule which activates the SR Ca^{2+} release channel, or (*ii*) electromechanical coupling between the voltage sensor on the T-tubule and the Ca^{2+} release channel of the SR (Schneider & Chandler, 1973). Rios and Brum (1987) have proposed that the dihydropyridine (DHP)-sensitive Ca^{2+} channel is the voltage sensor on the T-tubule. The simplest physical model for their hypothesis would propose a direct association between the DHP receptor and the JFP.

More complex models for the Rios and Brum (1987) hypothesis would propose that other junctional proteins could act as a linkage between the two integral membrane components, or alternatively act as modulators of the signal transduction. The glycolytic enzymes aldolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) may play such a role in the junction. These two proteins were first found to be major constituents of the electron-dense material on the cytoplasmic surface of the junctional complex of the SR (Brunschwig et al., 1982). Kawamoto et al., (1986) reported extraction of proteins in the M_r 35–40 K range from detergent-dissolved triads with immobilized JFP. Subsequently aldolase (M_r 40 K) was shown to bind to the isolated JFP and to be released by inositol polyphosphates (Thieleczek, Mayr & Brandt, 1989). GAPDH can promote junction formation between isolated T-tubules and TC (Corbett et al., 1985).

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In this paper we extend the understanding of the composition of the junction by examining the association of the putative junctional constituents. We have tested for the interactions of the JFP, DHP receptor, GAPDH and aldolase among themselves and with other constituents of the T-tubule and TC using affinity chromatography, protein overlay and crosslinking with heterobifunctional reagents as the experimental approaches. The results of these studies in conjunction with the data of the accompanying paper on the types of junctional association suggest a new model for the triad junction.

Materials and Methods

Materials

GAPDH was purified from rabbit skeletal muscle as described by Caswell and Corbett (1985). Aldolase, phosphofructokinase, triose phosphate isomerase, phosphoglycerate kinase and bovine serum albumin were purchased from Sigma. The glycolytic enzymes were of rabbit skeletal muscle origin. Calpain II isolated from bovine ventricular muscle was a gift from Dr. R. Mellgren, Medical College of Ohio. The production of monoclonal and polyclonal antibodies against the JFP followed protocols described previously (Kawamoto, Brunschwig & Caswell, 1988). Inositol trisphosphate was purchased from Boehringer-Mannheim. All other chemicals were of at least reagent grade. Na[^{125}I] was purchased from Amersham. All other radioactive ligands were purchased from New England Nuclear.

Membrane Isolation

Triads were isolated from rabbit skeletal muscle as described by Caswell, Lau & Brunschwig, (1976) and subsequently fractionated into T-tubule, light TC (LTC) and heavy TC (HTC) components as described by Brandt, Caswell and Brunschwig (1980). Nonjunctional SR membranes were separated from TC/triads by passing TC/triads through a French press and then adding cacodylate to cause reformation of the triad junction. The nonrejoining TC were separated from the reformed junctions by isopycnic centrifugation and routinely used as the longitudinal reticulum (LR) fraction of the SR. This fraction has a lower content of junctional membrane markers (e.g., calsequestrin, JFP) than the LR fraction from the microsome preparation. "Strong triads" were purified as described in the accompanying publication (Kim et al., 1990). All organelles were suspended in 250 mM sucrose, 2 mM histidine, pH 7.3 (sucrose/histidine buffer).

Isolation of the JFP

The JFP was isolated by a modification of previously reported protocols (Kawamoto et al., 1986; Kawamoto et al., 1988). Triads were dissolved in 2 mg Triton X-100/mg protein and 5 mM EGTA. The Triton-resistant fraction obtained by ultracentrifugation (100,000 × *g* for 1 hr) was dissolved in 1 M NaCl, 3 mM histidine, pH 7.3, 1 mM EGTA and 2 mg Zwitergent 3–14/mg protein. The dissolved proteins were incubated for 1 hr at 22°C with the monoclonal anti-JFP antibody affinity resin. The gel was rapidly washed with several volumes of 4 M NaCl, 5 mM Na_2HPO_4 (pH 7.0), 1 mM EGTA, 0.1% Zwittergent 3–14 with partial vacuum and the JFP then eluted with 4 M NaSCN buffered with 5 mM Na_2HPO_4, pH 7.0, 1 mM EGTA and 0.1% Zwittergent 3–14. This eluate was either immediately coupled to CNBr-activated Sepharose 4B (Pharmacia) for JFP affinity chromatography, or excess Zwittergent and NaSCN were removed by molecular sieve chromatography on a TSK 65W medium pressure column eluted with 30 mM NaCl, 10 mM Na_2HPO_4, pH 7.0. The TSK 65W eluate was concentrated in an Amicon ultrafiltration cell and the concentrated protein was used for production of the iodinated JFP probe. The yield from 40 mg Triton-resistant protein was routinely about 400 μg with a slight (~10%) decrease upon each use of the monoclonal anti-JFP antibody resin. Iodination was carried out by the Iodogen method (*vida infra*).

For some experiments, the junctional foot protein was isolated by a modification of the method of Inui, Saito and Fleischer (1987). TC/triads prepared in the presence of protease inhibitors (leupeptin, pepstatin, phenylmethyl sulfonyl fluoride and benzamidine) were suspended at 7 mg/ml in 250 mM sucrose, 2 mM histidine, pH 7.0 and 1.4% 3-[(3-cholamidoprophyl) dimethylammonio] 1-propanesulfanate (CHAPS), 0.28% egg phosphatidylcholine (PC). NaCl (0.8 M), KH_2PO_4 (0.075 M), pH 7.3 was added and the preparation was centrifuged at 80,000 × *g* for 1 hr. The supernatant was passed directly onto a hydroxyapatite column (1.5 cm diameter, 15 cm length). The column was washed with 20 ml 0.5% CHAPS, 0.1% PC (CHAPS-PC), 0.1 M KH_2PO_4, pH 7.3 in the presence of leupeptin, pepstatin and PMSF. The JFP was specifically eluted in the same medium in which the phosphate concentration was increased to 0.17 M. The eluate (approximately 10 ml) was diluted by the addition of a half volume of the same medium but lacking phosphate and applied to a heparin-agarose column (Sigma) (1.5 cm diameter, 2 cm length). This was washed with 10 ml of (0.15 M NaCl), CHAPS-PC. The JFP was eluted with a salt gradient from 0.15 to 1 M in the same detergent medium.

The samples from the column fractions were analyzed for [^{3}H] ryanodine binding employing the procedure of Pessah et al. (1986). Specific binding to the purified protein was approximately 150 pmoles/mg. The yield from back muscles of one rabbit was approximately 2 mg. The purified CHAPS-PC JFP was iodinated using Iodobeads (Pierce). The protein was then passed onto a 1-ml heparin-agarose column, washed with 15 ml 0.15 M NaCl in CHAPS-PC and eluted with 0.6 M NaCl in the same medium.

Isolation of the DHP Receptor

The DHP receptor was purified according to the method of Curtis and Catterall (1984), incorporating the modifications described by Talvenheimo, Worley and Nelson (1987). T-tubule membrane proteins were solubilized in 1% (wt/vol) digitonin (4 mg per mg protein), 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$. The soluble membrane extract, containing 20–50 pmol DHP receptor per mg protein, was labeled with 2 nM [^{3}H]PN 200-110 and incubated with wheat germ agglutinin (WGA) Sepharose. The proteins specifically eluted with 100 mM N-acetylglucosamine were then chromatographed on a DEAE-Sephadex column eluted with a 20 to 200 mM NaCl gradient. The peak fractions containing bound [^{3}H]PN 200-110 were pooled and concentrated. The DHP receptor subunits (α_1, α_2, β and γ)

comprised over 70% of the protein as judged from Coomassie blue-stained SDS-PAGE gels.

The DHP receptor (100–200 μg) was iodinated with Na[^{125}I] (1 mCi) using Iodogen (Pierce) catalyst, or phosphorylated with 1 mCi [γ-^{32}P]ATP for 10 min at room temperature using the catalytic subunit of cyclic AMP-dependent protein kinase (Sigma), following the protocol of Curtis and Catterall (1985). The radiolabeled DHP receptor was then incubated with WGA-Sepharose and washed with 0.3% (wt/vol) CHAPS (Calbiochem), 0.15% (wt/vol) egg lecithin (Sigma) before elution with 100 mM N-acetylglucosamine in CHAPS medium.

Analytical Protocols

Protein was determined according to Bradford (1976) or with BCA Reagent (Pierce) when appropriate. Bovine serum albumin was used as standard. GAPDH activity was measured using the method of Tsai, Murphy and Steck (1982) as modified by Caswell and Corbett (1985).

Formation of Affinity Chromatography Gels

The JFP sample (300–400 μg) eluted from the monoclonal anti-JFP antibody column was adjusted to 91 mM $NaHCO_3$, pH 8.3, by adding 1/10 volume of 1 M $NaHCO_3$, pH 8.3. GAPDH (3.5 μg) was dissolved in 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3. The protein sample was then mixed with 1 ml CNBr-activated Sepharose 4B for 2 hr at 22°C. Tris-HCl, pH 8.3 (0.1 M) was subsequently added and the gel incubated at 4°C overnight to block unreacted sites. The gel was washed five times alternately with 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3 and 0.5 M Na acetate, pH 4.0, and finally with 10 volumes of 20 mM K gluconate, 20 mM Tris MOPS, pH 7.0 (G/M buffer). Control columns were prepared by an identical procedure except that the protein was omitted from the coupling reaction.

Protein Iodination

Proteins (except stated previously) were iodinated by incubation of 200 μl protein suspension (1 mg/ml) with 1 mCi Na[^{125}I] in glass tubes coated with 20 μg Iodogen. After incubation on ice for 20 min, the suspension was withdrawn; proteins were separated from NaI and I_2 by centrifugation through 3-ml Sephadex G-25 columns.

Electrophoresis and Western Blotting/Protein Overlay Experiments

SDS-PAGE was performed on 1-mm thick slab gels according to Laemmli (1970) using a 4% stacking gel and a 7% resolving gel unless otherwise stated. Gels were either stained with Coomassie blue or electroblotted onto nitrocellulose (Biorad) for 2 hr at 12 V in Laemmli buffer containing 20% CH_3OH for protein overlay experiments. Iodinated M_r standards were prepared by incubating a 1 mg high M_r standard mixture (carbonic anhydrase, ovalbumin, BSA, phosphorylase *b*, β-galactosidase and myosin) (Sigma) with 1 mCi Na[^{125}I] in the presence of Iodogen. Biotinylated M_r standards were prepared by incubation of 1 mg of the high M_r standards with NHS-Biotin (Pierce) according to manufacturer's directions.

For protein overlay experiments nitrocellulose membranes containing the blotted proteins were blocked overnight in G/M buffer with 3% BSA, then washed five times with G/M buffer containing 0.1% (wt/vol) Tween 20. The blots were incubated for 1 hr at 22°C or overnight at 4°C with radiolabeled proteins in G/M buffer as indicated in the figure legends. At the end of the incubation period, blots were washed five times with G/M buffer, 0.1% Tween 20, and air-dried. For antibody blotting experiments, all reactions were carried out in Tris-buffered saline (200 mM NaCl, 20 mM Tris-HCl, pH 7.4). Reactions with rabbit polyclonal anti-JFP antibody were carried out as described by Kawamoto et al. (1988).

Radioactivity on gels or nitrocellulose membranes was detected by autoradiography using Kodak X-OMAT film. Membrane or protein-bound radioactivity was quantitated by liquid scintillation counting. Individual labeled proteins used as probes in the overlay experiments were electrophoresed on SDS-polyacrylamide gels to confirm that the co-purifying radioactivity was covalently incorporated.

Crosslinking with the Heterobifunctional Reagent NHS-ASA

Four mg N-hydroxysuccinimidyl-4-azido salicyclic acid (NHS-ASA) (Pierce) was dissolved in 1 ml acetonitrile to which was added 33 μl 0.5 M Na_2HPO_4, pH 7.0, 67 μl chloramine T solution (7 mg/ml of 9 parts acetonitrile, 1 part dimethylformamide) and 2 mCi Na[^{125}I]. After 2 min at 22°C, the reaction mixture was dried under N_2 and 300 μg GAPDH in 250 μl of 5 mM imidazole/Cl, pH 7.0 was added. Coupling was carried out for 20 min at 4°C. Unreacted ester groups were blocked by addition of 1/10 volume of 1 M arginine for 10 min at 4°C. The coupled protein was then centrifuged twice through 3-ml Sephadex G-25 columns equilibrated in 250 mM sucrose, 10 mM MOPS, pH 7.3 to remove Na_2HPO_4 and unreacted ^{125}I. The final product contained 60 μg/ml protein and 10^6 cpm/ml.

GAPDH linked to the bifunctional reagent was incubated with 50-μg membranes in 250 mM sucrose, 10 mM MOPS in 50 μl final volume for 40 min at 22°C and then irradiated for 20 min with short wavelength UV light in 250-μl Beckman Airfuge tubes. The reaction mixtures were underlayed with 18% sucrose (wt/wt) and centrifuged 15 min at 30 psi. The pellets were resuspended in 30-μl Laemmli sample buffer and electrophoresed on 5–15% gradient gels. A portion of the coupled protein was irradiated prior to addition to the membrane suspension as a control. All steps prior to photoactivation were carried out under Na light to protect the arylazide group of the bifunctional reagent.

Results

Interaction of the JFP with T-Tubule Proteins

The isolated JFP has been employed as a probe to determine the sites of its attachment to T-tubules. Kawamoto et al. (1986) had previously demonstrated binding of junctional proteins to JFP by

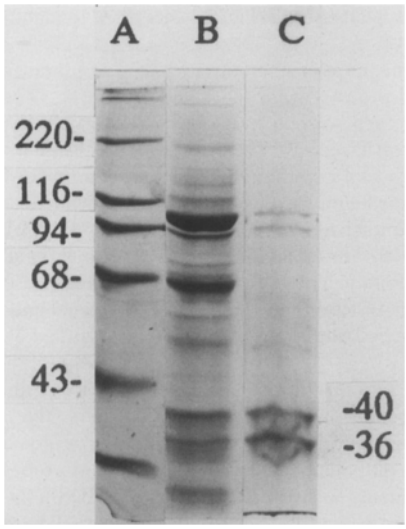

Fig. 1. Affinity chromatography of T-tubule proteins on JFP-Sepharose. T-tubules (8 mg/ml) were suspended in 300 μl of 250 mM sucrose, 5 mM Tris-HCl, pH 7.0, 5 mM Tris-EGTA, 2 mM $MgCl_2$, and Zwittergent 3–14 (2 mg/mg protein). This was followed by addition K gluconate to a final concentration of 0.3 M, and immediate dilution with 1.5 ml H_2O. The suspension was centrifuged for 30 min at 30 psi in a Beckman Airfuge and applied to a 0.5-ml column of JFP-Sepharose. The column was washed with 30 mM K gluconate, 2 mM Tris-HCl, pH 7.0, 0.1% Zwittergent, then eluted with the same buffer containing 1 M NaCl. Lane *A* contains M_r standards, lane *B* contains protein eluted by 30 mM K gluconate buffer and lane *C* contains proteins eluted by the 1 M NaCl buffer

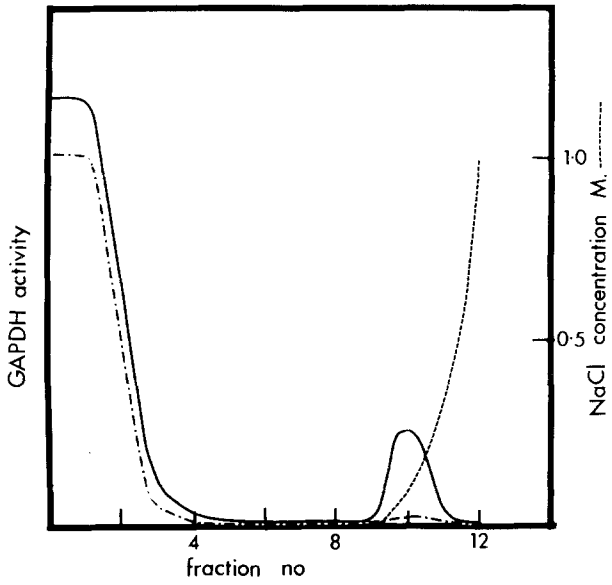

Fig. 2. NaCl gradient elution of GAPDH from JFP-Sepharose. GAPDH (20 μg) dissolved in 1 ml of 30 mM K gluconate, 2.5 mM Tris-HCl, pH 7.0, 1 mM Tris-EGTA, 0.1% Zwittergent 3–14 was applied to a 0.5-ml JFP-Sepharose column or a control column. The column was washed with the same buffer, then eluted with a NaCl gradient. NaCl concentration (- - - - -) was determined by refractive indices. GAPDH activity (——) was assayed as described in Materials and Methods and expressed in arbitrary units. GAPDH activity retained by a control column is shown by the broken line (· — ·)

passing detergent-solubilized triads over a JFP affinity column and eluting bound proteins with high salt concentrations. However, T-tubule proteins constitute only about 10% of the total protein in triad preparations. To test for interaction of the JFP with T-tubule-specific proteins, T-tubules were dissolved and chromatographed on JFP-Sepharose (Fig. 1). Lane *A* contains M_r standards, lane *B* contains T-tubule proteins which were not retained by the column and lane *C* contains the proteins adsorbed to the column and eluted by 1 M NaCl. The majority of T-tubule proteins, including prominent bands at M_r 26, 36, 40, 72, 94 and 102 K, pass directly through the JFP column (lane *B*). Bands at M_r 36 K and 40 K comprised about two thirds of the retained protein (lane *C*); the remainder was distributed over at least eight bands of M_r <110 K. Two T-tubule-specific proteins at M_r 26 and 72 K (Lau, Caswell & Brunschwig, 1977; Brandt et al., 1980; Cadwell & Caswell, 1982) were not bound to the JFP.

Previous experiments have shown that protein bands in T-tubules at 40 K and 36 K are aldolase and GAPDH. Aldolase has been shown to bind to JFP (Thieleczek et al., 1989). Figure 2 shows that GAPDH also binds JFP. The protein is eluted by a NaCl gradient in 30 mM K gluconate. A control column bound and released a negligible amount of GAPDH.

The protein overlay technique was employed to test for JFP binding to T-tubular proteins. For these experiments the blotted proteins were washed free of detergent to promote renaturation. In the experiment shown in Fig. 3 T-tubule membrane proteins and purified GAPDH were electroblotted. In Fig. 3*A* the overlay technique was employed with ^{125}I labeled JFP which had been prepared by the monoclonal affinity column which we originally employed. Recently we have used a modification of the technique of Inui et al. (1987) for isolation of the JFP which is shown in Fig. 3*B*. In the latter technique CHAPS is employed as the detergent for protein isolation (CHAPS-PC protein) and ryanodine binding is retained. Figure 3*A*, lanes *1* and *2* show the Amido Schwartz-stained protein for GADPH and T-tubule proteins, respectively. The T-tubule proteins include prominent bands at M_r 100 K, 72 K and 36 K. The 36-K T-tubule protein (lane *2*) co-

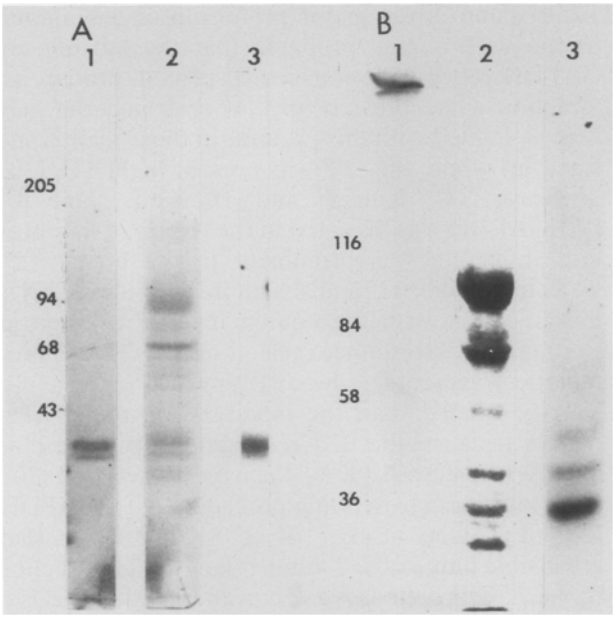

Fig. 3. Overlay of T-tubule proteins with [^{125}I]JFP. (*A*) GAPDH (2.5 μg, lane *1*) and T-tubules (20 μg, lanes *2* and *3*) were electrophoresed on 5–15% gradient gels and electroblotted on nitrocellulose. Lanes *1* and *2* were stained with Amido Schwartz. Lane *3* was incubated with 44 μg [^{125}I]JFP prepared by monoclonal antibody column and autoradiographed. (*B*) Lane *1* shows the autoradiogram of the [^{125}I]JFP. The JFP was purified in CHAPS-PC media. The protein was iodinated with [^{125}I]NaI after hydroxylapatite chromatography and then purified by heparin-agarose chromatography as described in Materials and Methods. This protein was then used to overlay T-tubules (Coomassie blue-stained gel, lane *2*; autoradiogram, lane *3*) overnight at 4°C in G/M media containing 0.5% CHAPS, 0.125% PC

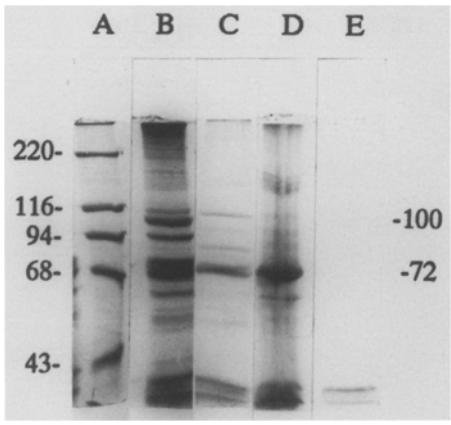

Fig. 4. Affinity chromatography of T-tubule proteins on GAPDH-Sepharose. T-tubules (55 mg in 300 μl of 250 mM sucrose, 2.5 mM Tris Cl pH 7.0) were dissolved with Zwittergent 3–14 (2 mg/mg protein) in 300 mM K gluconate, then diluted with 1.3 ml water and centrifuged as in Fig. 1. The solubilized T-tubule protein was applied to 0.5-ml GAPDH-Sepharose or control column. The column was washed and eluted as described for Fig. 1. Lane *A* contains M_r standards, lane *B* contains total T-tubule protein (before solubilization), lane *C* contains protein eluted from GAPDH-Sepharose by the low salt wash, lane *D* contains protein eluted from GAPDH-Sepharose by 1 M NaCl and lane *E* contains protein eluted from the control column by 1 M NaCl

electrophoreses with purified GAPDH (lane *1*). The autoradiogram of T-tubule proteins overlayed with [^{125}I]JFP is shown in lane *3*. Radioactivity was detected only at the position of GAPDH. [^{125}I]JFP binding to aldolase was not clearly discerned in this experiment, possibly because of the low content of aldolase in this T-tubule preparation, but has been observed in other T-tubule preparations. No interaction between the JFP and the M_r 72 K protein of T-tubules was detected.

Figure 3*B* shows that the method of isolating the JFP has little discernible influence on the results. Lane *1* shows the autoradiography of iodinated JFP. A single band is observed near the top of the gel. Lane *2* shows the Coomassie-stained T-tubules, while the overlay is presented in lane *3*. The major band overlayed by the CHAPS-PC-isolated JFP corresponds in position to that of GAPDH. A lesser band above it is in the position of aldolase, while a third minor band at M_r 50,000 is also visible. No bands could be detected corresponding to higher M_r proteins. Thus the CHAPS-PC-isolated protein, which has been shown to form a ryanodine-sensitive Ca^{2+} channel by Hymel et al. (1987), and that isolated in Zwittergent show essentially identical behavior in these overlay experiments.

Interaction of GAPDH and Aldolase with Junctional Proteins

Because the JFP appears to bind selectively to GAPDH associated with T-tubule membranes, we determined whether GAPDH interacts with specific proteins in the T-tubule membrane. Figure 4 shows the electrophoretic pattern of T-tubule proteins solubilized in K gluconate and Zwittergent and extracted by GAPDH immobilized on Sepharose. Lane *A* contains M_r standards. Lane *B* shows the protein pattern for native T-tubules prior to solubilization; prominent bands are the M_r 100 K ATPase and the 72 K T-tubular protein, as well as aldolase and GAPDH. Lane *C* shows the Zwittergent-extracted proteins which pass through the GAPDH-Sepharose column in low salt medium. Note that the relatively mild conditions of detergent solubilization necessary for this experiment dissolved only a portion of the T-tubular proteins. The M_r 72 K

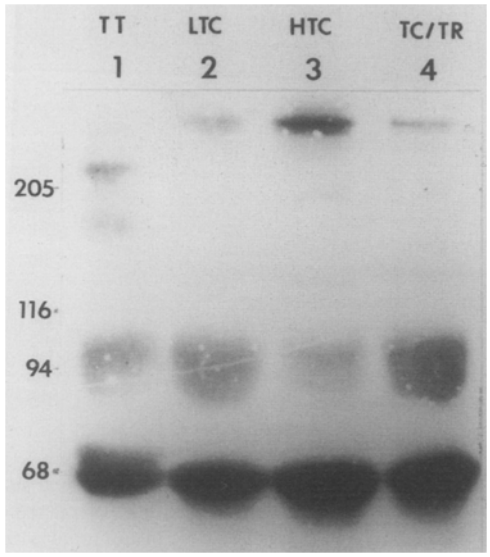

Fig. 5. Overlay of T-tubule and SR membranes with [^{125}I]-GAPDH. T-tubules, (lane *1*), light TC (lane *2*), heavy TC (lane *3*) and TC/triads (lane *4*) were electrophoresed (50 μg per lane) electroblotted onto nitrocellulose. The blot was overlayed with 480 μg [^{125}I]GAPDH (3.5×10^{-4} cpm/μg) in 10 ml G/M buffer, and then autoradiographed

protein, aldolase, and GAPDH were detected in the eluate, while the M_r 100 K band remained largely in the pellet after detergent treatment. Lane *D* shows the proteins retained by the gel and subsequently eluted with high salt. The GAPDH-Sepharose column bound GAPDH, aldolase and the M_r 73 K proteins as well as about 10 minor bands. In addition a set of proteins between M_r 140 and 170 K were selectively retained by GAPDH-Sepharose. These proteins are barely detectable in the native T-tubules (lane *B*). The α_1 and α_2 subunits of the DHP receptor electrophorese with a similar mobility; however, positive identification was not made in this experiment. Control columns without immobilized GAPDH retained very little protein (lane *E*).

The protein overlay technique was employed with ^{125}I-labeled GAPDH as the probe protein (Fig. 5). T-tubules, (lane *1*), light TC (lane *2*), heavy TC (lane *3*) and TC/triads (lane *4*) were electrophoresed and blotted onto nitrocellulose, which was then overlayed with [^{125}I]GAPDH in low salt medium. The autoradiogram shows that GAPDH binds most strongly to calsequestrin (M_r 63 K) in HTC and TC/triads. The band at 63 K in T-tubules and light TC (consisting primarily of nonjunctional SR) which is labeled by GAPDH is most likely contaminating calsequestrin which has been released from the TC during triad disruption. A broad band is labeled in the region of the SR Ca^{2+} pump (M_r 102 K). The M_r 102 K band is the major protein in all membrane fractions. It seems probable that the labeling by GAPDH represents nonspecific protein-protein interaction since the labeling is weak and the gels heavily loaded with this protein. In those membrane fractions where the JFP was present (light TC, lane *2*; heavy TC, lane *3*; and TC/triads, lane *4*), [^{125}I]GAPDH was detected in the region of that protein. Labeling was prominent in the heavy TC, weak in the light TC and absent in T-tubules. This is in agreement with the content of JFP as seen in Coomassie blue-stained gels. The M_r 270 K proteolytic fragment of the JFP appears to be more heavily labeled than the intact JFP protein. This occurs because the 270 K fragment is more efficiently transferred (90%) than the intact JFP (30–70%) in the electroblotting procedure. [^{125}I]GAPDH labeled proteins of M_r 72 K, 170 K and 230 K that are visible only in the T-tubule (lane *1*). The labeling of the 72 K protein is weak considering that this is a major T-tubular protein. The band at 170 K runs in a similar position to the α_1 subunit of the DHP receptor. The binding of ^{125}I-GAPDH to all proteins was considerably reduced but not eliminated when KCl (100 mM final concentration) was added to the binding medium.

Figure 6 shows an overlay experiment employing ^{125}I-labeled aldolase. Panel *A*, lane *1* shows the binding to TC/triads, while lane *2* shows binding to T-tubules. Aldolase binds predominantly to calsequestrin (M_4 63 K), a luminal component of the TC/triads and a contaminant of the T-tubules. Prominent binding to the JFP and its proteolytic product at M_r 270 K is seen in the TC/triads (lane *1*). As in Fig. 5, the relative intensities of the two JFP bands are misleading since the intact JFP is poorly transferred in the electroblot. [^{125}I]aldolase also labels bands of M_r 36 K, 40, 80 and 200 K. In the T-tubules (lane *2*), in addition to those proteins in common with TC/triads, a band at M_r 170 K is labeled. This is probably the same T-tubule protein which binds GAPDH and may be the α_1 subunit of the DHP receptor.

Figure 6*B* shows the effect of selective proteolytic cleavage of the JFP on binding of aldolase to this protein. Ca^{2+}-dependent proteases are known to hydrolyse the JFP without apparently degrading other proteins of the SR (Seiler et al., 1983). We have demonstrated that the Ca^{2+}-dependent protease, calpain II, breaks the (weak) triad junction, which permits subsequent separation of the component organelles (Kim et al., 1990). Lanes *1* and *3* in Fig. 6*B* are TC/triad Western blots which have been overlayed by polyclonal antibody against the JFP and developed by ELISA techniques. Lanes *2* and *4* are aldolase overlays of blots from

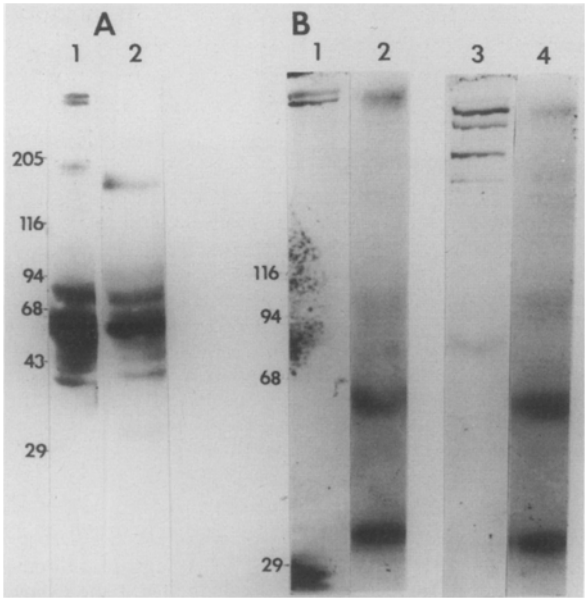

Fig. 6. Overlay of TC/triads, T-tubules and calpain fragments of TC/Triads with [^{125}I]aldolase. (*A*) TC/Triads (30 μg) or T-tubules (30 μg) were electrophoresed and electroblotted onto nitrocellulose. The blots were overlayed with 20 μg [^{125}I]aldolase (3.7×10^5 cpm/μg) in 5 ml G/M buffer and autoradiographed. (*B*) TC/triads were resuspended at 1 mg/ml in 250 mM sucrose, 20 mM MOPS/Tris, pH 7.3, 2 mM $CaCl_2$, 3 mM dithiothreitol and incubated for 30 min in the absence (lanes *1* and *2*) or presence (lanes *3* and *4*) of 0.01 mg calpain II/mg triads at 20°C. Incubation was stopped by addition of an equal volume of Laemmli sample buffer; 50 μl (50 μg) was electrophoresed on each gel lane. Nitrocellulose blots containing protein from control and calpain-treated triads were incubated with polyclonal anti-JFP antibody (lanes *1* and *3*) or overlayed with 15 μg [^{125}I]aldolase (lanes *2* and *4*) in 10 ml G/M buffer. Antibody binding was detected using a second antibody, as described previously (Kawamoto et al., 1986). Radioactivity was detected by autoradiography

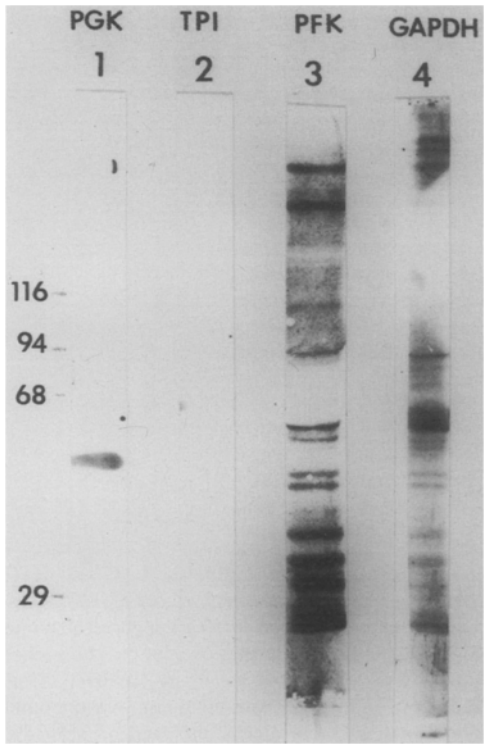

Fig. 7. Overlay of TC/triads with [^{125}I] glycolytic enzymes. TC/triads (50 μg) were electrophoresed and electroblotted onto nitrocellulose. The blots were overlayed with phosphoglycerate kinase (lane *1*, 3.2 μg, 3.8×10^5 cpm/μg); triose phosphate isomerase (lane *2*, 2 μg, 1.2×10^6 cpm/μg); phosphofructokinase (lane *3*, 1.0 μg, 3.5×10^5 cpm/μg); and GAPDH (lane *4*, 13 μg, 2×10^5 cpm/μg). All blots were autoradiographed for 20 hr

the same preparation. The immunoblot shows two bands in the native triad preparation (lane *1*), corresponding to the intact protein and the 270 K proteolytic fragment. After calpain treatment (lane *3*), the TC/triad immunoblot shows less of the intact protein, a slight enhancement in the M_r 270 K fragment, and the appearance of new bands of M_r 250, 220 and 190 K. This treatment did not cause discernible loss of ryanodine binding (Kim et al., 1990). The aldolase overlay shows binding to the intact protein and to the proteolytic product at M_r 270 K, but not to the smaller fragments. More extensive calpain digestion causes a more complete disappearance of the intact and M_r 270 K species.

The selectivity of interaction of TC/triads with several glycolytic proteins is shown in the protein overlay experiment of Fig. 7. In lane *4* [^{125}I]GAPDH clearly labels the JFP and the M_r 270 K product. In contrast, neither phosphoglycerate kinase (lane *1*) nor triose phosphate isomerase (lane *2*) bound significantly to triad proteins, although they were labeled to similar specific activities (0.4 to 1.0×10^6 cpm/μg). After a 10-fold longer autoradiographic exposure, a faint labeling of GAPDH and aldolase was detected. Lane *3* shows that [^{125}I]PFK binds to several triad proteins which are also labeled by GAPDH. In addition, it binds strongly to two proteins at M_r 250 and 200 K. However, PFK does not bind to the JFP or to its 270 K fragment.

To test whether GAPDH binds to the JFP in native organelles, GAPDH was coupled to the heterobifunctional reagent [^{125}I]N-hydroxysuccinimidyl-4-azidosalicylic acid ([^{125}I]-NHS-ASA) (Fig. 8). This reagent does not have a cleavable bridge between the amide-linked GAPDH and the arylazide group; thus all cross-reacted proteins will appear in the autoradiogram at an M_r increased by 36 K (or multiples thereof). The advantage of this reagent over cleavable bifunctional reagents such as sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-

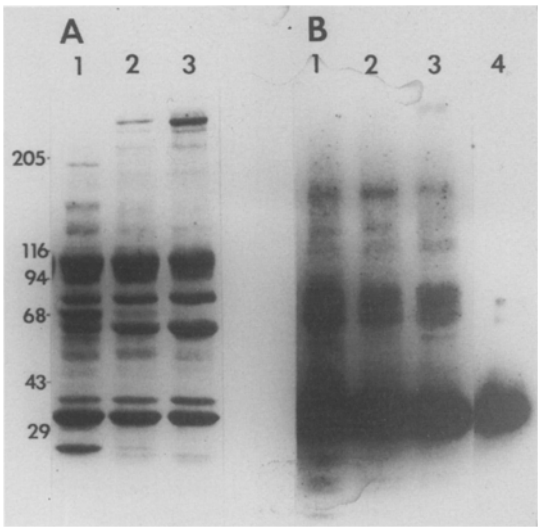

Fig. 8. Crosslinking of GAPDH to T-tubules, LTC and HTC using NHS-ASA. Fifty μg each of T-tubules (lane *1*), LTC (lane *2*) and HTC (lane *3*) were incubated in 50 μl sucrose/histidine buffer containing 1.5 μg GAPDH-NHS-ASA adduct. The adduct had a specific activity of 1.3×10^4 cpm ^{125}I/μg GAPDH. After incubation, irradiation and centrifugation to remove unbound GAPDH, membrane pellets were electrophoresed on a 5–15% gradient gel. (*A*) shows the Coomassie blue-stained gel. (*B*) shows the corresponding autoradiogram. Lane *4* shows the autoradiogram of 1.25 μg of the GAPDH-NHS-ASA adduct

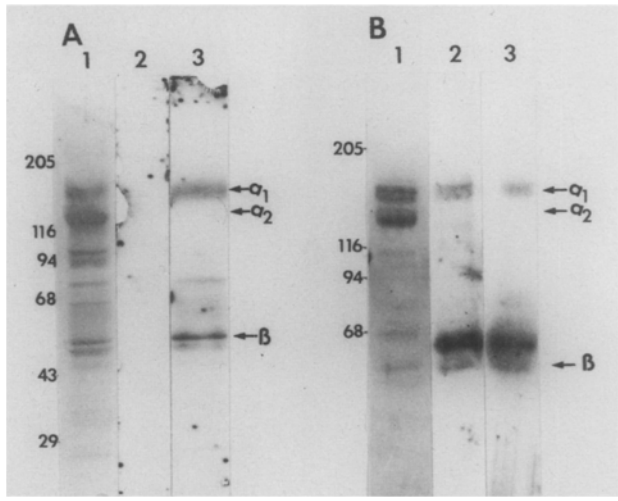

Fig. 9. Overlay of the DHP receptor with JFP, GAPDH and aldolase. (*A*) DHP receptor purified by WGA-Sepharose chromatography (40 μg) was electrophoresed and blotted. Lane *1* shows the blotted DHP receptor stained with Amido Schwartz; lane *2* contains DHP receptor overlayed with 200 μg [^{125}I]JFP in 3 ml G/M buffer; and lane *3* shows DHP receptor first incubated with 200 μg GAPDH, then washed and overlayed with 200 μg [^{125}I]JFP. (*B*) DHP receptor (5 μg) purified through two steps, as described in Materials and Methods, electrophoresed and blotted. Lane *1* shows the Coomassie blue-stained DHP receptor from a gel lane adjacent to the electroblotted lanes; lane *2* shows blotted DHP receptor overlayed with 140 μg GAPDH (3.6×10^5 cpm/μg) in 10 ml G/M buffer; and lane *3* shows DHP receptor overlayed with 25 μg aldolase (3×10^6 cpm/μg) in 10 ml G/M buffer

1,3′-dithiopropionate (SASD) is that membrane protein labeling through disulfide exchange cannot occur (*cf.* Chadwick, Inui & Fleischer, 1988). Figure 8*A* shows the Coomassie blue pattern for T-tubules (lane *1*), light TC (lane *2*), and heavy TC (lane *3*), with the corresponding autoradiograms shown in Fig. 8*B*. The JFP is clearly enriched in the HTC fraction (lane *A3*). The corresponding autoradiogram (lane *B3*) shows radioactivity in a band of slightly higher M_r, as expected for an adduct of GAPDH with the JFP. This band was also detected in the light TC fraction in the original autoradiogram although at a much lower level; it was not detected in the T-tubules. The intensity of this labeled band in the three membrane fractions corresponded to the content of JFP as detected with Coomassie blue.

Lane *B4* is the autoradiogram of the photoactivated GAPDH adduct in the absence of vesicles and shows labeling of GAPDH as well as polymeric products of the crosslinking. The major labeled band in all membrane fractions corresponds to the GAPDH-[^{125}I]NHS-ASA adduct. Other bands common to all three membrane fractions may represent GAPDH cross-linked adducts, but cannot be assigned to specific proteins.

Interaction of DHP Receptor with Junctional Proteins

A protein of M_r 170 K present only in the T-tubule component of the triad binds both GAPDH and aldolase in overlay experiments (Figs. 5 and 6). At least one protein in that mobility range was extracted by GAPDH-Sepharose from solubilized T-tubules (Fig. 4). The possibility that one or more of these bands was the DHP receptor was tested by the protein overlay technique (Fig. 9). The DHP receptor was purified through a single WGA-Sepharose step, then electrophoresed and blotted onto nitrocellulose (Fig. 9*A*). The Amido Schwartz-staining pattern is shown in lane *1*. The α_1 (170 K), α_2 (140 K), and β (54 K) subunits are clearly visible. The γ subunit (33 K) is also present but does not stain well with Amido Schwartz. When the nitrocellulose strip was overlayed with [^{125}I]JFP (lane *A2*), little or no binding was observed with the possible exception of a faint band at M_r 36 K. The strip of lane *A3* was first incubated with unlabeled GAPDH and washed extensively before incubation with

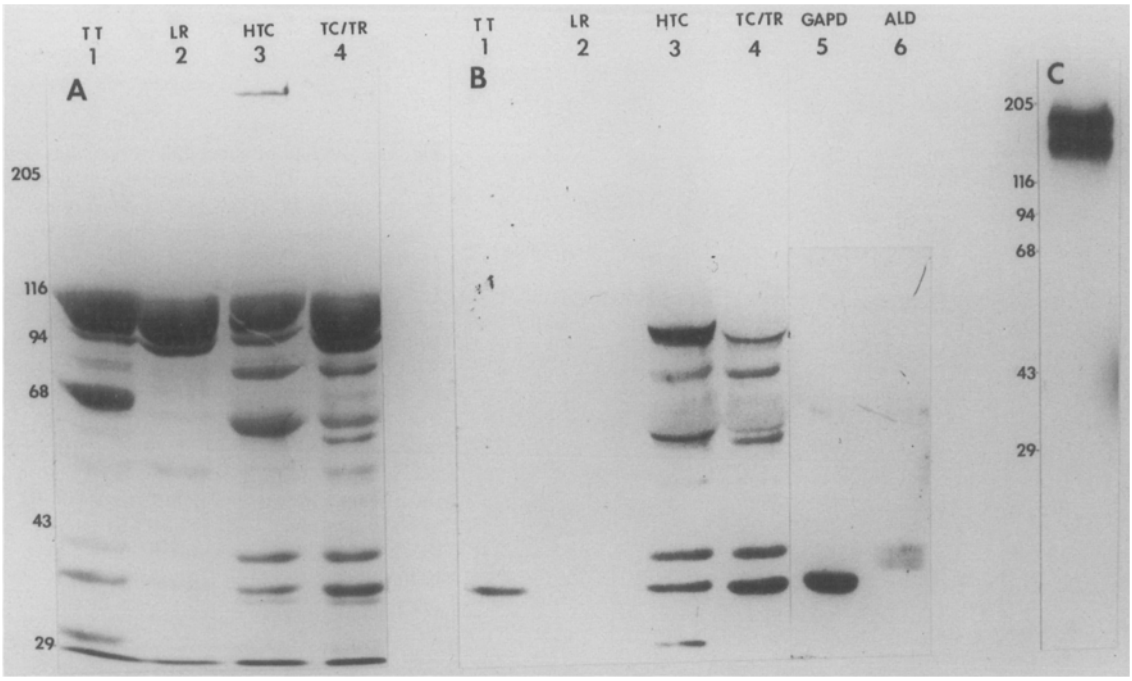

Fig. 10. Overlay of subcellular organelles and glycolytic enzymes with [^{125}I]DHP receptor. (*A*) Coomassie blue-stained gel of 50 μg T-tubules (lane *1*), LR (lane *2*), HTC (lane *3*) and TC/Triads (lane *4*). (*B*) Autoradiogram of electroblotted T-tubules (lane *1*, 50 μg), LR (lane *2*, 50 μg), HTC (lane *3*, 50 μg), TC/triads (lane *4*, 50 μg), GAPDH (lane *5*, 3 μg), and aldolase (lane *6*, 3 μg) overlayed with 77 μg [^{125}I]DHP receptor (1.2×10^6 cpm/μg) in 10 ml G/M buffer, 0.05% CHAPS, 0.013% PC. (*C*) Autoradiogram of 5 μg [^{125}I]DHP receptor electrophoresed on an 8% separating gel

[^{125}I]JFP. The major labeled bands at M_r 170 and 54 K co-migrate with the α_1 and β subunits (lane *A1*). No ^{125}I was detectable at the position of the α_2 subunit. In addition, a band at M_r 72 K, which is a contaminant in the DHP receptor preparation, was labeled. The [^{125}I]JFP employed in this experiment had been prepared using Zwittergent as detergent but we have also employed JFP prepared using CHAPS as detergent and have similarly observed binding to the α_1 and β subunits if the blots are preincubated with GAPDH.

Figure 9*B* shows an overlay of the purified DHP receptor by [^{125}I]aldolase (lane *B2*) and [^{125}I] GAPDH (lane *B3*). Lane *B1* is the Coomassie blue-stained receptor preparation showing α_1, α_2 and β subunits. Both GAPDH and aldolase bind to the α_1 subunit, but not the α_2 subunit. In addition GAPDH and aldolase bind to proteins of M_r 63 K and 54 K. The labeled M_r 54 K protein coincides with the position of the β subunit. The M_r 63 K protein co-migrates with calsequestrin, which may be a contaminant in the receptor preparation and which binds GAPDH and aldolase strongly. T-tubule and HTC proteins were electrophoresed and blotted in adjacent lanes (*data not shown*) and overlayed with [^{125}I]aldolase and [^{125}I]GAPDH. A protein which co-migrated with the α_1 subunit was labeled in the T-tubule preparation by both enzymes, but not in the HTC sample. This is consistent with the location of the DHP receptor in T-tubule membranes. Thus a ternary complex may be formed between the JFP, GAPDH and the DHP receptor. To investigate further the interactions of the DHP receptor with junctional proteins, the receptor was purified and radiolabeled with ^{125}I or ^{32}P, for use as the probe in protein overlay experiments. Figure 10 shows the results of an overlay experiment using [^{125}I]DHP receptor. The autoradiogram of the DHP receptor used as the probe is shown in panel *C*. The α_1 and α_2 subunits are the dominant polypeptides iodinated with little or no labeling of other subunits or of any impurities. The Coomassie blue-stained proteins from the isolated membrane fractions are shown in panel *A*. The content of the JFP is enriched in the HTC (lane *A3*) compared to the TC/triads. GAPDH and aldolase are present in junctional membranes and very low in LR; GAPDH is the predominant glycolytic protein of T-tubules. Panel *B* shows the overlay of the organelles by DHP receptor. Lane *B1* shows very little interaction of the DHP receptor with blotted T-tubule proteins with the exception of the band at M_r 36 K corresponding to GAPDH.

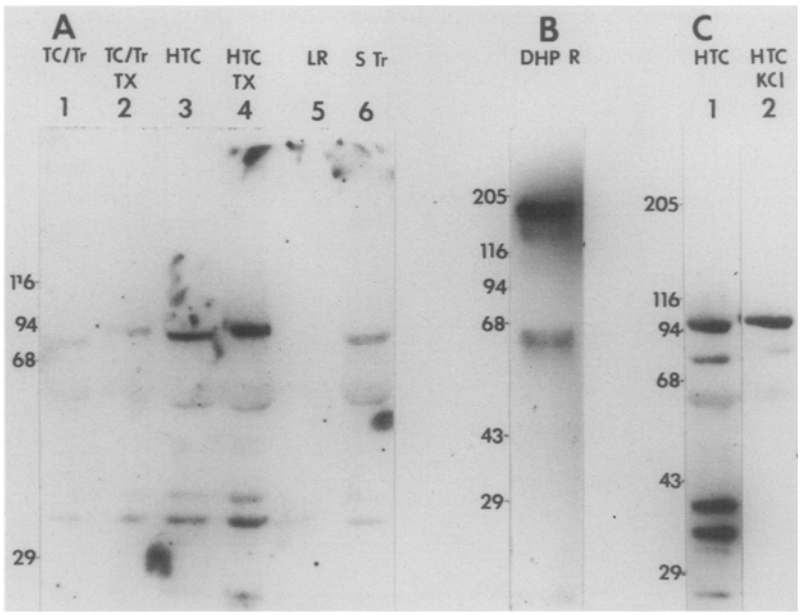

Fig. 11. Overlay of subcellular organelles with DHP receptor. (*A*) Autoradiogram of 30 μg electroblotted TC/triads before (lane *1*) and after extraction with Triton X-100 (2 mg/mg protein) (lane *2*); 30 μg HTC before (lane *3*) and after extraction with Triton X-100 (2 mg/mg protein) (lane *4*); 30 μg LR (lane *5*); and 30 μg strong triads (lane *6*) overlayed with 100 μg [^{32}P]DHP receptor (1250 cpm/μg) in 20 ml G/M buffer, 0.01% digitonin. (*B*) Autoradiogram from 5 μg of the [^{32}P]DHP receptor electrophoresed on an 8% separating gel. (*c*) Autoradiogram of 50 μg HTC before (lane *1*) and after (lane *2*) extraction with 0.5 M NaCl overlayed with 100 μg [^{125}I]DHP receptor (1.3×10^5 cpm/μg) in 10 ml G/M buffer, 0.05% CHAPS, 0.013% phosphatidylcholine

Binding of DHP receptor to LR proteins was not discernible (lane *B2*). In contrast, the HTC (lane *B3*) and the TC/triads (lane *B4*) show extensive binding of the iodinated receptor. The bands at M_r 36 and 40 K are GAPDH and aldolase, as indicated by the binding of the DHP receptor to the isolated glycolytic enzymes (lanes *B5* and *B6*, respectively). Both the HTC and TC/triads show an intense labeling at M_r 95 K. This band is more prominent in the isolated HTC than in the intact triad complex. The M_r 95 K band, which is labeled only in the HTC and TC/triad fractions, is not distinguishable as a separate band in the Coomassie blue-stained gel. It is most likely obscured by the large amount of Ca ATPase which runs at M_r 102 K. The fact that this M_r 95 K band is not the SR Ca pump is evidenced by its absence from the LR (lane *B2*). It is also distinct from the band co-migrating with phosphorylase *b* (M_r 94 K), which is present in both T-tubules and SR. Two other bands at M_r 80 and 60 K are also labeled in the HTC and TC/triads. The labeling of these bands is variable. The M_r 60 K band is obscured in the Coomassie blue-stained gels by calsequestrin, which did not bind the DHP receptor. No labeling of the JFP by the DHP receptor was discerned.

Figure 11 shows a more extensive investigation of the proteins interacting with the labeled DHP receptor. For part of this experiment the DHP receptor was phosphorylated with [γ^{32}P]ATP, which specifically labels the α_1 and β subunits. A low level of labeling of the α_2 subunit occurred under our reaction conditions (Fig. 11*B*). Figure 11*A* shows an autoradiogram of isolated membrane fractions overlayed with the [^{32}P]DHP receptor. Lanes *A1* and *A2* contain native and Triton X-100 extracted TC/triads, respectively; lanes *A3* and *A4* contain native and Triton X-100 extracted heavy TC, respectively, lane *A5* is intact LR, and lane *A6* contains strong triads which are characterized in the accompanying publication (Kim et al., 1990). Lane *A1* shows faint labeling of proteins at M_r 95, 60 and 36 K. The TC/triads were treated with Triton X-100 to extract more than 90% of the SR Ca ATPase (M_r 102 K) while leaving the junctional complex intact. The extracted membranes (lane *A2*) show more intense labeling of the M_r 95 K protein. This migrates with a slightly higher apparent M_r, probably because in the intact triad the high content of Ca ATPase "pushes" the band further into the gel. The Triton X-100-solubilized proteins did not show labeling in the M_r 100 K region (*data not shown*), supporting the conclusion that the M_r 95 K protein is distinct from the SR Ca ATPase.

The heavy TC are substantially enriched in SR specific components of the junctional structure compared to the TC/triads. The native heavy TC (lane *A3*) show considerably more intense labeling of the M_r 95 K protein. In addition, the DHP receptor bound to bands at M_r 36 K (GAPDH), and M_r 40 K (aldolase) and faintly labeled bands in the position of calsequestrin and at M_r 60 K. Following Triton X-100 extraction, labeling of the M_r 95, 40 and 36 K proteins by the [^{32}P]DHP receptor is even more pronounced (lane *A4*). In contrast, the DHP receptor does not bind to any proteins in the LR

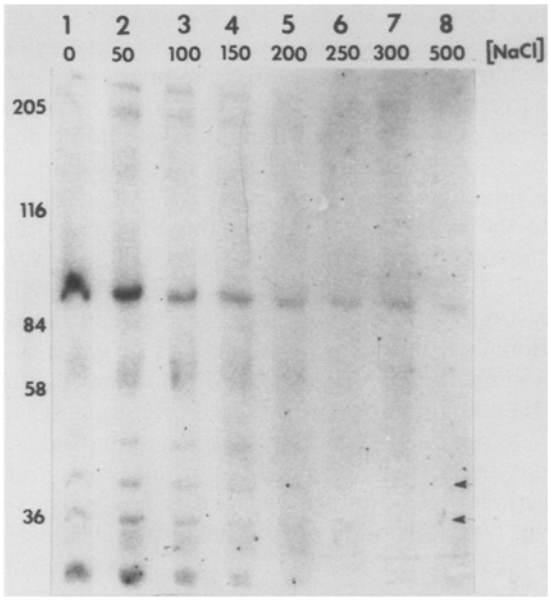

Fig. 12. Effect of NaCl on [^{125}I]DHP Receptor Overlays of HTC. HTC (24 μg/lane) was electrophoresed and blotted onto nitrocellulose. After blocking with 3% BSA in G/M buffer, the nitrocellulose was aligned in a "Mighty Small Deca Probe" apparatus (Hoefer Scientific Instruments) and each lane incubated with 11 μg [^{125}I]DHP receptor (12,000 cpm/μg) in 1 ml G/M buffer containing 0.15% CHAPS, 0.06% phosphatidylcholine and the concentration of NaCl indicated above each lane

fraction (lane *A5*), with the exception of very faint labeling of GAPDH. The strong triads (lane *A6*) show more intense labeling of the M_r 95 K protein than do the TC/triads.

From the data described so far it appears that the DHP receptor binds selectively to a TC protein of the triad junction and that protein is distinct from the JFP. To determine whether this M_r 95 K protein is an intrinsic membrane protein, heavy TC membranes were washed with high salt to remove extrinsic membrane-bound proteins. Untreated HTC (lane *C1*) again shows intense labeling of the M_r 95 K protein, as well as of GAPDH, aldolase, and an 80 K protein by the [^{125}I]DHP receptor. The band at M_r 60 K is not seen in this preparation and may therefore be a proteolytic product. When the heavy TC are extracted with hypertonic NaCl (lane *C2*), labeling of the extrinsic proteins GAPDH and aldolase is not observed and the M_r 80 K protein is barely detectable. These proteins are greatly diminished in the corresponding Coomassie blue-stained lanes (*not shown*). In contrast, the intensity of the M_r 95 K protein was not visibly diminished by the salt treatment, indicating that it is an intrinsic protein. Furthermore, treatment of the HTC with Triton X-100 and EGTA, which does extract calsequestrin, did not solubilize the M_r 95 K protein (*data not shown*).

In overlay experiments using JFP and GAPDH as probe proteins, the overlay medium contained low salt concentrations since we found that inclusion of 100 mM KCl in the incubation medium extensively diminished the binding of the probes. Figure 12 shows the influence of NaCl in the incubation and wash media on the overlay of [^{125}I] labeled DHP receptor onto HTC. The major overlay band at 95 K Da is little influenced by the presence of 50 mM NaCl in the media. Higher NaCl concentrations cause a progressive loss of binding, but a band is still discernible even in the presence of 500 mM NaCl. Similarly the minor bands, including that of GAPDH and aldolase (arrow heads), retain binding to the DHP receptor even at elevated NaCl concentrations. Thus the binding of DHP receptor to GAPDH has an ionic bond character which is stronger than that of JFP to GAPDH and aldolase.

Discussion

We have presented in this paper evidence on the nature of the association of junctional proteins using three technical approaches: affinity chromatography, protein overlay and crosslinking with a heterobifunctional reagent. Because there is no simple method for defining the protein-protein associations in the intact triad, our approach has been to look for specific interactions between the purified JFP, DHP receptor, and proteins in isolated membrane fractions. With one exception, we have demonstrated selected pairs of protein-protein interactions with each member of the pair being used as the probe. For example, we have used [^{125}I]JFP and [^{125}I]GAPDH to overlay electroblotted organelles. [^{125}I]JFP labeled GAPDH in T-tubules while [^{125}I]GAPDH overlayed the JFP in HTC and TC/triads. The one exception is the M_r 95 K protein which has not yet been purified. We have not detected a direct association between the JFP and the DHP receptor when either protein was employed as the probe in overlay experiments, or by JFP affinity chromatography. While we cannot exclude the possibility that these proteins lose their ability to interact directly upon isolation, our results suggest an alternative model in which GAPDH may serve the role of forming a ternary complex with the JFP and integral T-tubule proteins. In addition our techniques have not detected a direct interaction between the JFP and the 72 K Da protein of T-tubules described by Chadwick, Inui and Fleischer (1988).

In most of our experiments it has been necessary to employ detergents for protein purification,

electrophoresis and Western blotting. Where possible, the experimental conditions have been designed to preserve the function of the solubilized proteins used as probes in the overlay experiments, and to promote renaturation of protein structure after electroblotting. Certain protein-protein interactions which depend upon quaternary structure may not be detectable by the protein overlay approach. However, there is considerable evidence that proteins frequently retain or regain biochemical function even after SDS solubilization, as evidenced by reports that Western blotted proteins retain enzymatic activity (Sock & Rohringer, 1988) and bind specific ligands such as Ca^{2+} (Zorzato & Volpe, 1988) and insulin-like growth factor (Hossenlopp et al., 1986), as well as antibodies. Thus, this approach can provide information about protein interactions which may occur in the intact triad. For all experiments we employed media of sufficient ionic strength to inhibit binding of glycolytic enzymes to acid lipids (Pierce & Phillipson, 1985) and to inhibit extensive GAPDH self-association (Caswell & Corbett, 1985), while maximizing protein-protein interactions. We obtained corroborative evidence for these interactions by using the JFP and GAPDH as affinity ligands immobilized on Sepharose. Finally, we have shown by crosslinking that GAPDH can bind to the JFP in native membranes.

These observations are consistent with our prior knowledge of the junctional structure and our new finding (Kim et al., 1990) of "weak" and "strong" subpopulations of isolated triads. (*i*) Addition of GAPDH causes isolated T-tubules to reassociate with terminal cisternae (Corbett et al., 1985). Our data suggest that the JFP binds to the DHP receptor only when GAPDH is also present. (*ii*) The "weak" subpopulation of triads are disrupted by hypertonic salt solutions (Caswell & Brandt, 1981; Kim et al., 1990) in the same range as that required to dissociate GAPDH from the immobilized JFP. (*iii*) "Weak" triads are disrupted by calpain (Kim et al., 1990), which selectively degrades the JFP (Seiler et al., 1983). Our data show that calpain removes the aldolase-binding domain on the JFP. If aldolase and GAPDH bind to the same domain on the JFP, cleavage of this domain could break the ternary complex between the JFP, GAPDH or aldolase, and the T-tubule membrane, disrupting the triad.

Two criteria must be satisfied for GAPDH or aldolase binding to the JFP and the DHP receptor to be physiologically relevant: (*i*) the binding sites of the JFP and the DHP receptor must be accessible from the cytosol, and (*ii*) the enzymes must bind under physiological conditions. The fact that GAPDH can be crosslinked to the JFP in intact organelles shows that the GAPDH-binding domain on the JFP is cytosolic, since the cytosolic surfaces of the membranes of both triad components are exposed to the external medium. This localization is supported by the fact that the aldolase-binding domain of the JFP is accessible to digestion by calpain in the intact vesicle. Both GAPDH and aldolase bind to the α_1 and β subunits of the DHP receptor which are reported to have cytosolic domains (*see* Catterall, Seagar & Takahashi, 1988 for review). The α_2 subunit, which is not known to have intracellular domains, does not bind GAPDH or aldolase. The binding domain of proteins which interact with GAPDH and aldolase is considered to consist of an extensive region of negatively charged amino acids. This has been shown for the N-terminal region of the erythrocyte anion channel and is likely to explain the very strong *in vitro* binding of calsequestrin to these enzymes. The binding of calsequestrin will not occur, however, in intact vesicles owing to lack of accessibility between the calsequestrin in the lumen of the TC and the cytoplasmic enzymes. The sequence of the α_1 subunit of the DHP receptor does not show a predominance of acidic amino acids in the putative cytoplasmic domains (Tanabe et al., 1987). It is possible, however, that a localized high density of negative charges, e.g., amino acids 730–750 may provide a binding locus.

Both GAPDH and aldolase are tetrameric enzymes consisting of four identical subunits, each of which may provide a site for protein-protein interaction. Their association with the JFP appears to be selective, since the other glycolytic enzymes tested showed no binding to the JFP. However, direct competition between GAPDH and aldolase for the same binding sites on the JFP and the DHP receptor has not been tested. Although both GAPDH and aldolase can be dissociated from triads by P_i, ATP^{4-}, and fructose-1,6-*bis*phosphate, only aldolase is displaced from the JFP by physiological levels of inositol polyphosphates (Thieleczek et al., 1989; N.R. Brandt & R. Thieleczek, *unpublished observations*). However, virtually complete removal of aldolase with 50 μM inositol trisphosphate does not disrupt the junction (Thieleczek et al., 1989).

The results of these studies do not generate the simple model of direct communication between the putative voltage sensor (the DHP receptor) and the JFP-Ca^{2+} release channel. It seems unlikely that the DHP receptor communicates a conformational change to the JFP through glycolytic enzymes. On the other hand, aldolase binds inositol polyphosphates (Koppitz, Vogel & Mayr, 1986) and may play a role in modulating Ca^{2+} permeability of SR or Ca^{2+} influx through the DHP Ca^{2+} channel (Vilvèn

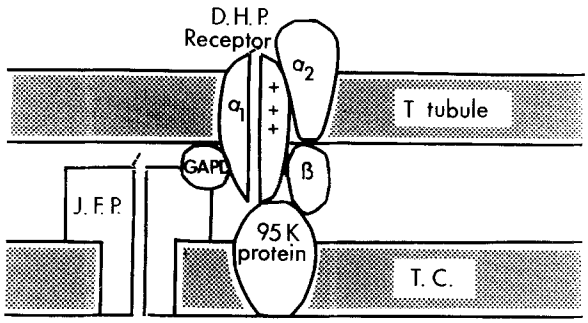

Fig. 13. A model for the triad junction. The JFP and M_r 95 K protein are integral proteins of the junctional TC membrane. The α_1 subunit of the DHP receptor is shown forming a ternary complex with the JFP protein through GAPDH and the DHP receptor is shown directly interacting with the M_r 95 K protein. Aldolase may substitute for GAPDH. It has been proposed that basic residues on the α_1 subunit (+ + +) of the DHP receptor are involved in excitation-contraction coupling (Tanabe et al., 1987)

& Coronado, 1988) by binding or buffering inositol trisphosphate. These modulatory mechanisms may be of consequence in sustained altered metabolic activity associated with tetanic contraction (Thieleczek et al., 1989).

Figure 13 illustrates a model for the triad structure incorporating an important finding of this report: the existence of two possible bridging structures in the junction. The first structure is the JFP-GAPDH-DHP receptor ternary complex. For simplicity the α_1 subunit of the DHP receptor is depicted as the T-tubule integral protein anchoring the ternary complex although our data also indicate that the β subunit can form a ternary complex. The second potential bridging structure consists of the DHP receptor and the M_r 95 K protein in the junctional domain of the SR. Work is currently in progress to determine the conditions in which this association occurs and to define which subunits of the DHP receptor are involved.

This biochemical model must be reconciled with our current knowledge about the triad junction. In the accompanying paper (Kim et al., 1990), we identify two classes of triad junction: the "weak" triad, which can be dissociated by high salt, protease treatment or French press treatment; and the "strong" triad, which is resistant to breakage by these procedures. The JFP association with the glycolytic enzymes can be disrupted by high salt and by proteolytic digestion with calpain, suggesting that this interaction may be the predominant bridging structure in weak triads. Strong triads are enriched in DHP receptors compared to the JFP. This observation suggests that a direct interaction of the DHP receptor with a junctional SR protein is involved in strong triad formation. The M_r 95 K protein is a candidate for this role. Consistent with this role is our observation here that even high ionic strength only partly prevents association between the DHP receptor and the 95 K Da protein.

Block et al. (1988) have clearly shown tetradic structures in freeze fracture views of the junctional face of the T-tubule membrane. They proposed that these junctional T-tubule tetrads are formed by the DHP receptor, which is directly linked to the tetrameric JFP. In their model one tetrad of DHP receptors interacts with every second JFP tetrad. This linkage, however, cannot be directly demonstrated by current electron microscopic techniques. We propose that the junctional T-tubule tetrads link to the M_r 95 K protein in the TC membrane, and that these structures interdigitate with the "junctional foot".

The model presented in Fig. 13 also suggests a mode of excitation-contraction coupling. If the DHP receptor acts as the voltage sensor for muscle excitation, it may deliver the signal through the 95 K protein. This protein may interact directly or indirectly through a second linkage with the JFP to open the SR Ca^{2+} channel. An alternative mode of transmission may occur if the physical interaction of the T-tubule with the TC is altered by a conformational change in the DHP receptor-95 K protein complex, e.g., the separation distance of T-tubule and SR may be altered. Such a physical separation during tetanus has been described by Eisenberg and Gilai (1979). This conformational change may alter the nature of the interaction of the JFP with the GAPDH or aldolase by physically modifying the JFP-glycolytic enzyme-T-tubule ternary complex. Aldolase is known to bind the putative transmitter inositol trisphosphate, while GAPDH binds ATP^{4-} which is known to activate the SR Ca^{2+} channel. Thus either enzyme may release bound transmitter to act on the JFP when the physical conformation of the junction is altered. In addition the DHP receptor activation may deliver Ca^{2+} directly to the JFP in the confined space of the junctional proteins to provide the second required activator of the Ca^{2+} release channel.

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