MINI-REVIEW

Triadic Proteins of Skeletal Muscle

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Abstract

Biochemical approaches toward understanding the mechanism of muscle excitation have in recent years been directed to identification and isolation of proteins of the triad junction. The principal protein described—the junctional foot protein (JFP)[2]—was initially identified by morphological criteria and isolated using antibody-affinity chromatography. Subsequently this protein was described as the ryanodine receptor. It has been isolated and incorporated into lipid bilayers as a cation channel. This in its turn has directed attention toward the transverse (T)-tubular junctional constituents. Three approaches employing the JFP as a probe toward identifying these moieties on the T-tubule are described here. The binding of the JFP to the dihydropyridine receptor, which has been hypothesized to be the voltage sensor in excitation–contraction coupling, is also discussed. The detailed architecture and function of T-tubular proteins remain to be resolved.

Key Words: Triad junctions; transverse tubule; junctional foot protein; glyceraldehyde 3-phosphate dehydrogenase; dihydropyridine receptor.

Introduction

For all of the increase in our understanding of muscle activation, there is still not a single fully accepted hypothesis to describe the mechanism of excitation transmission into the interior of the fiber. An approach that has begun to elicit valuable information in the field is that of identifying, extracting, and

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[2]Abbreviations: DHP, dihydropyridine; GAPD, glyceraldehyde 3-phosphate dehydrogenase; IP$_3$, inositol 1,4,5-trisphosphate; JFP, junctional foot protein; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; TC, terminal cisterna; T-tubule, transverse tubule.

reforming the proteins of the triadic junction. Biochemical approaches in the past have been directed extensively towards describing and elucidating the mechanisms of calcium accumulation inside sacroplasmic reticulum (SR). More recent work beginning with the observations reported by Kasai and Miyamoto (1973) has been aimed at detecting Ca^{2+} release in isolated organelles and subsequently identifying the constituents responsible for this release. An alternative approach of identifying triadic constituents is complementary to the elucidation of the release mechanism, and is therefore interesting that these two approaches have now substantially melded as a consequence of research in the last 2–3 years. An additional goal of determination of triadic constituents is to elucidate the nature and role of the proteins in the transverse tubule (T-tubule) that participate in the junction and may be functionally liked to the SR in excitation. One basic hypothesis of muscle contraction is that a chemical transmitter–receptor system enables communication between the exterior membrane and the SR. Two putative transmitters are inositol 1, 4, 5-trisphosphate (IP_3) (Volpe *et al.*, 1985; Vergara *et al.*, 1985) and Ca^{2+} (Ford and Podalsky, 1970; Miyamoto and Racker, 1982; Fabiato, 1984). In the case of IP_3, it may be anticipated that the enzymes involved in synthesis of the transmitter and in its degradation or binding will be in the vicinity of the junction in order to provide the rapidity of release and inactivation required for muscle activation and relaxation. If Ca^{2+} is the transmitter, then proteins involved in binding and transport are likely to be triadic constituents. An alternative mode of muscle excitation is through direct mechanical coupling between the T-tubule and SR (Schneider and Chandler, 1973). In this latter case, clearly the voltage sensor for Ca^{2+} release must be in contact with the junctional foot processes. This review concentrates on the technical problems and solutions in evaluating the constituents of the triad and on the future directions of research in this field.

Identification and Isolation of Junctional Foot Protein

Successful subcellular fractionation of microsomal preparations from skeletal muscle into morphologically distinct and definable subfractions was first described by Meissner in 1975. By simple density-gradient centrifugation, he was able to distinguish two SR fractions, the heavier of which contained internal electron-dense matter and therefore appeared to be predominantly of terminal cisternae (TC) origin while the lighter fraction, which had a higher content of Ca^{2+}-stimulated ATPase, appeared to be of longitudinal reticulum origin. Further fractionation became possible when Caswell *et al.* (1976) determined that the external membrane marker,

ouabain, was associated with the dense fraction, suggesting that, after muscle homogenization, the T-tubules remained attached to the TC in the form of a triad. This was confirmed by electron microscopy that clearly showed an elongated vesicle apposed on each side by a circular vesicle that contained electron-dense material; this was very much in conformity with the appearance of the triad in intact muscle. Subsequently, Lau *et al.* (1977) showed that the T-tubule could be separated as a distinct organelle by breaking the triad in a French press followed by density-gradient centrifugation. The T-tubules again retained the morphology of the intact muscle, being elongate vesicles with a relatively small number of intercalated particles, while the TC, which were separated from the T-tubules, were largely spherical vesicles with considerable internal electron-dense matter and a high concentration of intercalated particles observed on the concave fracture (P) face (Lau *et al.*, 1979). This protocol has been effectively reproduced unchanged in several laboratories (Michalak *et al.*, 1980; Ikemoto *et al.*, 1984; Varsanyi *et al.*, 1986) or with inclusion of minor modifications to produce higher yields of triads versus free TC (Mitchell *et al.*, 1983). An alternative approach for the isolation of free T-tubules (i.e., those not associated initially with SR as triads) has been developed by Rosemblatt *et al.* (1981). A detailed comparison of the T-tubules obtained by the two methods has been recently published elsewhere (Caswell *et al.*, 1988).

Approaches to identifying the triadic structure as a molecular entity were fraught with a number of technical difficulties. Molecular constituents are most easily isolated if they can be identified through enzymic function or by ligand-binding assays; this has proved to be the major mechanism for isolation of the great majority of proteins found in biological systems. There were, however, no biochemical markers of triadic proteins, and even mild detergent treatments cause considerable disruption of the morphological appearance of the junction, so that electron-microscopic identification is problematic.

The approach of dissolving the apparent foot structure by salt treatment initially produced misleading results. Campbell *et al.* (1980) reported that the electron-dense matter associated with the cytoplasmic surface of the TC could be removed by treatment with hypertonic KCl. They proposed that these proteins of 34,000 and 38,000 DA were constituents of the foot processes. Brunschwig *et al.* (1982) subsequently demonstrated that, although KCl treatment dissolved these proteins and dispersed the dense aggregate in the lumen of the TC, the treatment did not disrupt the integrity of the triad junction. The low-molecular-weight proteins, since identified as glyceraldehyde 3-phosphate dehydrogenase (GAPD) and aldolase, respectively, probably play a role in the junction; they do not, however, constitute the main protein of the junctional foot.

Cadwell and Caswell (1982) provided a solution to this problem by indirectly identifying the electron-dense process that joins the two organelles. Caswell *et al.* (1979) had previously demonstrated that the broken triad could be reformed spotaneously from separated T-tubules and TC by the addition of high concentrations of the salt K cacodylate. In the identification of the electron-dense process, Cadwell and Caswell (1982) covalently labeled T-tubules with ^{125}I, using the catalyst iodogen. This procedure iodinates most proteins with exposed tyrosine residues. The labeled T-tubules were made to reform a triad junction in the presence of cacodylate with unlabeled TC, and the reformed triads were separated from free T-tubules by density-gradient centrifugation. The reformed triads were then broken a second time using a French press; components were separated on sucrose gradients and the distribution of the ^{125}I label was monitored. Most labeled proteins would be expected to migrate with the T-tubules under these conditions since it was T-tubule that was originally iodinated. However, the junctional foot process might migrate either with the T-tubule or the TC, depending upon the relative strength of the linkages of the foot protein process to the two vesicles when the triad was disrupted: *i.e.*, some degree of randomness can be expected in the way in which the foot process might separate. What was observed was that a small fraction of the iodine label originally from the T-tubule migrated with the TC after the junction was formed and then rebroken. Autoradiographic analysis of the SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) protein patterns showed that, although the original iodination of the T-tubules was quite nonspecific, only a single band was found to have migrated to the TC after junction formation and breakage. This band has a very high molecular weight, subsequently determined to be $\sim 300,000$ Da. The very high molecular weight was a very distinctive property of this protein since no other proteins of such large size were detectable in electrophoretic gels.

The approach described above was quite indirect, but, in the course of time, other indicators suggested that this protein was indeed the JFP. Triad junctions could be fragmented further by treatment of triadic vesicles with the detergent Triton X-100 (Brunschwig *et al.*, 1982). This detergent dissolved the nonjunctional regions of the membrane, but left the triadic portion of the membrane almost intact. Thin-section electron micrographs showed the following features: the presence of fragments of T-tubules that retained to a high degree the trilaminar structure observed in the intact membrane, processes separating the T-tubule from the TC membrane, a weakly preserved TC trilaminar structure, and the electron-dense material of the SR lumen. A junctional complex was described consisting of the foot protein and associated structures in the TC and T-tubules. A more complete study of this selective dissolution was reported subsequently by Costello *et al.* (1986), who

applied this protocol of Triton treatment to free TC and demonstrated morphological preservation of the junctional complex. Both studies suggested, on morphological grounds, an association of the junctional foot protein with calsequestrin. Whether this is a direct linkage or through auxiliary proteins (Mitchell *et al.*, 1988) remains to be resolved. In either case, a functional linkage between the calcium-binding protein (calsequestrin) and the release channel (the JFP) may be anticipated.

Further fractionation could be obtained by treating the detergent-resistant fragments with either KCl or EGTA (Brunschwig *et al.*, 1982; Caswell and Brunschwig, 1984; Costello *et al.*, 1986). In both cases, a substantial dissolution of the luminal electron-dense matter of the TC occurred. Through this whole protocol of enriching in triadic constituents, the high-molecular-weight protein was itself enriched, and electron microscopy has revealed the presence of square particles that had the approximate dimensions of the junctional feet as described *in vivo* or in isolated organelles (Costello *et al.*, 1986).

A more definitive identification would require the isolation of the foot protein itself. It was possible to extract this protein using a combination of neutral detergent and hypertonic salt concentrations. The detergent that causes the best dissolution of the junction under these conditions was Zwittergent (Caswell and Brunschwig, 1984). The foot protein could be enriched to $\sim 50\%$ purity by molecular-sieve chromatography and its native molecular weight was estimated to be in the range of 1.2×10^6 Da. This enriched fraction was employed to immunize mice in order to prepare monoclonal antibodies against the protein (Kawamoto *et al.*, 1986). A hybridoma clone was prepared containing an immunoglobulin-G (IgG) antibody that, although unsuitable for Western blot analysis, was identified by its immunogenic response to fractions from a molecular-sieve column to be a likely antibody to the JFP. Confirmation of this supposition was provided by the ability of the antibody to retain specifically the JFP when the antibody was attached to a CNBr–Sepharose column. The foot protein was eluted by employing 4 M NaSCN, giving rise to an effective one-stage purification of the protein from isolated TC triads.

The confirmation that the isolated 300-kDa protein was a part of the junctional foot structure was provided by immunoelectron microscopy. Indirect immunoelectron microscopy employing polyclonal antibody and gold-tagged IgG showed the presence of particles in the junctional region of sections of intact rabbit sacrospinalis muscle. The technique has an intrinsic ambiguity of the position of label with respect to the visible structure being tagged, but the average disposition of particles was very close to the junctional gap between the T-tubule and TC. Subsequent observations on isolated organeles revealed the presence of gold particles in the vicinity of visible

junctional foot structures. Treatment of the vesicles with trypsin removed the visible projections from the TC vesicles and caused the complete loss of antigenic sites from the exterior surface as observed by gold labeling (Kawamoto *et al.*, 1988). On the other hand, luminal antigenicity in the TC was observed in thin sections labeled with antibody that were little affected by trypsin treatment of internal vesicles, indicating that the 300-kDa protein is the JFP, which also has transmembrane segments.

Subsequent to the original Cadwell and Caswell (1982) experiments, Seiler *et al.* (1984) identified a similar protein in dog cardiac junctional SR. They demonstrated that both the skeletal muscle and cardiac proteins bound calmodulin and ATP. In addition, both were phosphorylated by cAMP kinase and by an endogenous Ca^{2+}–calmodulin kinase. Interestingly, the faster-running species of skeletal muscle triads, which has since been shown to be a proteolytic product of the JFP, appeared to be the better substrate. The JFP-like protein has now been identified in junctional SR–dyad preparations from cat (Brandt, 1985), rabbit (Brandt, 1985), cow and sheep (Doyle *et al.*, 1986) ventricular muscle.

Morphology of the Triad Junction

Since the original work of Revel (1962) in describing the junctional foot processes that separate and join the T-tubules to the TC, a fair picture has emerged of the triadic composition. The feetlike structures are present in skeletal muscle in the form of tetragonal arrays in mammalian muscle (Franzini-Armstrong, 1970) as well as in some invertebrates (Smith, 1966). These form two lines along the T-tubule–SR junction with a thickness of ~200 Å and a transverse depth between the two membranes of ~100 Å. Foot structures were identified in isolated vesicles by Caswell *et al.* (1976), who described individual projections from TC to T-tubules. Campbell *et al.* (1980) described them as protruding structures from preparations of free TC. Both Campbell *et al.* (1980) and Brunschwig *et al.* (1982) described the apparent diminution in their number after KCl treatment. In retrospect, the reason for this is unclear since KCl does not dissolve the feet unless detergent is also present. The visible particles might include significant portions of extrinsic proteins. Saito *et al.* (1984) reported organized arrays of particles of width 200 Å in isolated TC. Ferguson *et al.* (1984), using shadow casting, have observed a subunit structure such that the full foot protein process consists of four units arranged in a cloverleaf pattern with a central core. Evidence for a specific structure within the membrane of the TC associated with the junction was first provided by Kelly and Kuda (1979), who described an array of bumps in freeze-fracture process. These bumps were of dimensions

and separation in register with the foot processes observed in thin section. Evidence of a specific organization within the membrane of the T-tubule has been considerably more difficult to obtain. Franzini-Armstrong *et al.* (1987) reported the occasional presence of intercalated particles in freeze-fracture views of the T-tubule in which two particles were visible for each junctional foot. These intercalated particles had a high profile. No such correlates have yet been observed in isolated T-tubules. However, the isolated vesicles display high-profile intercalated particles on convex and concave faces and, in a number of instances, the number of particles are condensed to form a long row with a thickness corresponding to two or three particles (Lau *et al.*, 1979). Whether these match the intercalated particles described by Franzini-Armstrong *et al.* (1987) is not currently clear. This ambiguity can, however, be expected as the organization may have been altered in the isolated T-tubule preparation where the JFP has been removed.

Identification of Junctional Foot Protein as Ryanodine Receptor

Ryanodine is a plant alkaloid that inhibits contraction of skeletal muscle bimodally. At low concentrations, it enhances Ca^{2+} permeability in SR while, at higher concentrations, the drug blocks Sr Ca^{2+} release (Lattanzio *et al.*, 1987). Three groups (Waterhouse *et al.*, 1984; Fleischer *et al.*, 1985; Sutko *et al.*, 1986) played a critical role in developing radioactive forms of this drug to enable its usage as a ligand to identify and extract the putative Ca^{2+} release channel from SR. pessah *et al.* (1985) demonstrated saturable binding of the drug to SR vesicles and established some of the conditions that favor optimal binding of the drug. They also showed that the binding of the drug to its receptor survives certain detergent treatment, and suggested that the molecular size of the receptor was very large (Pessah *et al.*, 1986). Thus, they were able to establish the feasibility of isolating the receptor. Subsequently, three groups, almost simultaneously, have isolated the ryanodine receptor and identified it as a high-molecular-weight entity. The approaches in each case have been somewhat different: Inui *et al.* (1987) employed conventional chromatographic approaches; Campbell *et al.* (1987) employed a monoclonal antibody in affinity chromatography; and Lai *et al.* (1987) extracted the protein in a one-step procedure using rate zonal density-gradient centrifugation.

Some differences in the preparations exist. Inui *et al.* (1987) and Lai *et al.* (1987) suggested that the receptor had multiple subunits of different molecular sizes. It is now generally accepted that the protein consists of a single unit and that the different molecular sizes observed originally occurred through proteolytic breakdown. The estimates of molecular weight of the

subunit have varied from 350,000 to 450,000. All three groups (Inui *et al.*, 1987; Campbell *et al.*, 1987; Lai *et al.*, 1987), however, have employed digitonin or CHAPS as the solubilizing detergent. Treatment with Zwitter-gent, on the other hand, may itself disrupt ryanodine-binding sites and appears to break the polymeric form to give rise to the monomer (Kawamoto *et al.*, 1986). The CHAPS-solubilized protein has been shown by all groups to evidence Ca^{2+} conductance in lipid bilayers blockable by ruthenium red. However, the ability of ATP, Mg^{2+}, and ryanodine to modulate this con-ductance and the number of putative conductive substates is not consistent among these three preparations.

T-Tubular Junctional Proteins

The identification and isolation of the JFP have established the Ca^{2+} release phase of muscle activation, but leave open to question the mechanism of activation of the release protein. The biochemical data indicate that Ca^{2+} at micromolar concentrations and ATP synergistically activate channel opening while Mg^{2+} at millimolar concentrations is an inhibitor. It is nevertheless not known whether any of these compounds control opening *in vivo*. In addition, the JFP maintains physical communication with the T-tubule, and the T-tubule must provide the original signal for Ca^{2+} release. it is therefore critical to characterize the T-tubular side of the triad molecular structure.

Some information on the T-tubular constituents has been provided by investigation of the conditions that determine the junctional integrity. The junction may be broken by a variety of proteases including trypsin, chymo-trypsin, pronase (Cadwell and Caswell, 1982) and calpain (Seiler *et al.*, 1984). These proteases all hydrolyze the JFP preferentially. In addition, hypertonic solutions of salts may facilitate junction breakage (Caswell and Brandt, 1981). The order of potency of salts is $KSCN > KNO_3 > KCl = K$ gluconate. This is the same as the order of chaotropic potency of anions. These observations imply that ionic interactions play an important role in maintaining the association of the junction. If the junction is held together *in vivo* by only moderately strong forces, the cytoplasmic salt milieu may be such as to weaken the forces of association into a quite limited extent of bonding.

Some salts are able to promote the association of the junction from the mechanically broken triads (Caswell *et al.*, 1979; Caswell and Brandt, 1981; Corbett *et al.*, 1985). The order of potency is cacodylate > propionate = 3 OH propionate > HCO_3 = acetate, in media buffered to pH 7.2. These are salts of weak acids with pKs in the range of 6.3–4.8. The mode by which they promote association is still unclear. Spontaneous rejoining occurs in acidic

media with half-maximal effect at pH 6.2 (Caswell and Brandt, 1981). Disruption occurs in basic media and the process appears to display hysteresis.

The original finding (Caswell *et al.*, 1979) that the junction could be reformed suggested that endogenous promoters may be present in the muscle that cause the junction to form. Corbett *et al.* (1985) demonstrated that a supernatant fraction from high-speed centrifugation of a muscle homogenate was able to promote such an association of the triad after it had been broken in a French press. This factor was purified from the supernatant and identified as a protein of subunit $M_r = 36,000$. Subsequent N-terminal sequencing (Caswell and Corbett, 1985) demonstrated a complete identity to the glycolytic enzyme, GAPD.

The identification of proteins that bind to the JFP was first approached by affinity-chromatography techniques (Kawamoto *et al.*, 1986). Triads were dissolved in Zwittergent and low salt (30 mM K gluconate) and the incubated with JFP attached to Sepharose. Proteins of 34–40,000, 65,000, and 170,000 were retained and could be eluted only with high salt. The low-M_r protein(s) could have been either GAPD or aldolase. The 65,000 and 170,000 proteins stained blue with Stains All, implying that these were calsequestrin and the M_r 170,000 clasequestrinlike protein.

Attempts to define the T-tubular constituents of the junction by more direct approaches are still at the preliminary stage. We have employed three approaches dependent upon the use of the isolated JFP as probe for T-tubular-associated proteins. There are three approaches: (1) Affinity chromatography in which the isolated foot protein is attached to a solid support and detergent-dissolved T-tubular proteins are passed through the column (2) Protein overlays in which T-tubular proteins are electrophoresed on SDS gels and then electroblotted onto introcellulose. After blocking with bovine serum albumin, the blot is washed with renaturing medium and then incubated with purified proteins labeled by iodination. After extensive washing to remove unbound protein, the blot is autoradiographed (3) Intermolecular cross-linking with hetero-bifunctional reagents composed of amino-reactive and photo-activated groups linked by a disulfide bridge. The ring of the aryl-azide group is first iodinated with ^{125}I. The isolated JFP is attached through its free amino groups to the bifunctional reagent and then incubated with T-tubular vesicles. The JFP can then be cross-linked to neighboring groups by photo activation of iodinated aryl-azide. The neighboring proteins will thus retain the iodine label after cleavage of the linking reagent with dithiothreitol. Each of these three approaches provided evidence that the JFP attaches specifically to GAPD. The affinity-column procedure also suggested an association between the JFP and aldolase. Pure GAPD and pure aldolase bind to the immobilized JFP at low ionic strength; aldolase can be specifically eluted with micromolar concentrations of IP$_3$ (Thieleczek

et al., 1989), which dissociates aldolase from triads and TC, but not from isolated T-tubules, suggesting that the enzyme is not involved in anchoring the JFP to the T-tubule membrane.

The observations described above combined with the fact that GAPD can promote reformation of triads from isolated components (Corbett *et al.*, 1985) suggest that GAPD may serve as a mediator promoting association between the JFP and T-tubular constituents. This proposition, in its turn, raises the question as to what the GAPD reacts with in the T-tubular membrane. Applying similar protocols, evidence has been generated for association between GAPD and two proteins in the T-tubule of M_r 72,000 and 100,000. In addition, using protein overlay techniques, it has been possible to demonstrate binding of GAPD to the 170,000 (α_1) subunit of the partially purified dihydropyridine (DHP) receptor. Under identical conditions, the JFP does not bind detectably to any DHP receptor subunits. However, when the blotted receptor was first incubated with unlabeled GAPD and then with ^{125}I-labeled JFP, the α_1 subunit acquired radioactivity. The picture emerging from these studies is that the T-tubular portion of the triad is quite complex and involves both extrinsic and intrinsic proteins. The hope that there might be a simple bimolecular linkage between the foot protein and the T-tubular voltage sensor (Schneider and Chandler, 1973; Rios and Brum, 1987) is probably not realized. None of the data above, however, preclude the possibility that a protein acting as a voltage sensor participates as one of the components of the triad junction.

Future Directions

The molecular information available to us at present concerning the constituents of the triad junction, though still limited, points the way to the resolution of a number questions on the mechanism of excitation–contraction coupling. These can be broadly classified as follows: (1) What is the order and specificity of attachment of T-tubular proteins to the junction? (2) Which moieties are involved in voltage sensing and in transmissions to the JFP? (3) What is the mechanism of signal transmission?

(1) The answer to the first question will probably be obtained in the near future as we become more adept at paring the junction down to its minimal constituents. The easiest situation to resolve would be one in which a single entity in the T-tubule forms a direct association with its complement in the SR and that entity is the voltage sensor gating the SR Ca^{2+} release. In terms of mechanisms of action, the hypothesis of Rios and Brum (1987) may be

extrapolated to propose that the DHP receptor directly associates with the JFP and serves to transmit excitation. Preliminary data from our laboratory, however, indicate a more complex interaction at the T-tubular interface. While it is likely that the DHP receptor is present at the junction, and is associated with the JFP, that linkage is indirect. Furthermore, it is questionable whether this association through GAPD is sufficiently specific to constitute a single hypothesis of muscle activation.

The alternative hypotheses of chemical transmission do not require a linkage between the voltage-sensor–messenger-producing system and the SR Ca^{2+} release channel. The sensor and transmitter-releasing protein(s), however, must be in close proximity to the junctional feet to produce a high local concentration of messenger within milliseconds. Furthermore, a mechanism for rapid removal of the messenger from the junctional space is required so that repetitive muscle contractions can occur.

The biochemical composition of the T-tubule still supports the possibility of a Ca^{2+}-induced Ca^{2+} release mechanism. Voltage-gated Ca^{2+} channels (Siri *et al.*, 1980) and a Ca^{2+} extrusion pump (Brandt *et al.*, 1980) are components of the T-tubule. However, it is hard to reconcile a Ca^{2+}-induced Ca^{2+} release mechanism with the fact that SR release can be triggered in the presence of high levels of Ca^{2+} chelators inside the fibre (Baylor and Hollingworth, 1988).

Current data do not permit a definitive evaluation of the IP_3 transmitter hypotheses. An active phosphatidylinositol phosphate kinase (Carrasco *et al.*, 1988; Varsanyi *et al.*, 1988) and phospholipase C (Varsanyi *et al.*, 1989) have been localized to the T-tubule membrane. The phospholipase C requires micromolar free Ca^{2+} in the absence of Mg^{2+} for activity. The half-maximal activity is decreased and shifts to a higher Ca^{2+} requirement at physiological $[Mg^{2+}]$. The problems associated with IP_3 serving as a chemical messenger in skeletal muscle are those of time resolution since the full time course of activation and relaxation can be completed in 50 msec. This requires highly active enzymes for both generation and inactivation of the messenger substance. The mechanism of IP_3 inactivation is currently unknown. It could involve degradation by a phosphatase, conversion to an inactive product through a kinase or binding to a cytoplasmic site. This last possibility is enhanced by the finding that aldolase binds IP_3 with a K_0 of $3\,\mu M$ (Koppitz *et al.*, 1986) and this enzyme is associated with the junction (Thieleczek *et al.*, 1989).

(2) The answer to the identity of the voltage sensor is still open. The proposed candidates include the DHP receptor and phospholipase C. In each case, the evidence to favor either protein is limited. In the case of the DHP receptor, it is clear that the protein responds to alterations of E_m by varying

the P_{open} of the Ca^{2+} channel. The difficulty is in linking the slow Ca^{2+} channel that is opened during muscle activation. In the model of Rios and Brum (1987), the DHP receptor serves a second function, which is direct transmission. This in part answers the proposal that the content of receptors in the T-tubule is in considerable excess over that required to carry the I_{Ca} of the membrane. It should be stated, however, that there is no need for the voltage sensor to exhibit any channel activity and we may therefore not have identified the protein. If the DHP receptor serves as the direct transmitter to the SR, then presumably its rate of transmission is greater than that observed for opening the Ca channel.

Whether the voltage sensor is a direct part of the triad is also unanswered. Regardless of the mechanism of transmission, it is certainly geometrically simple and biochemically consistent that the sensor should juxtaposed with the JFP.

(3) The mechanism of transmission is intimately linked with the issues of the triadic composition and identity at the voltage sensor. Present biochemical evidence indicates that the JFP responds to ATP and Ca^{2+} by opening the channel and to Mg^{2+} by closing it. This is not fully satisfactory for a physiological process. ATP is present in the cell at a high and uniform concentration that is buffered throughout the twitch. Although Ca^{2+} could be a trigger for excitation, the Ca^{2+} level required to activate the SR channel is in the micromolar range. This level is too high to be a suitable trigger unless the Ca^{2+} is sequestered or confined to the junctional region. The inhibition of Ca^{2+} release by Mg^{2+} is also troublesome since Mg^{2+} is present at a high (1 mM) and uniform concentration. Two possible resolutions are (1) the coupling to the T-tubule provides the physiological control of channel opening that has not been described before because the coupling is lost in the experimental systems studies such as skinned muscle or isolated organelles, and (2) transmitters are normally occluded from access to the channel gate when in the cytoplasm and all transmitter receptor function is confined in the intact muscle to the junctional foot structures and attached membrane constituents. Each of these possibilities is rendered more probable by the finding that the junctional foot is also the Ca^{2+} channel of SR.

Acknowledgements

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