Journal of Bioenergetics and Biomembranes, Vol. 21, No. 2, 1989

MINI-REVIEW

Biochemical Properties of Isolated Transverse Tubular Membranes

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Received September 13, 1988; revised October 3, 1988

Abstract

This review addresses the major biochemical and structural characteristics of isolated transverse tubule (T-tubule)[4] membranes, including methods of isolation and morphology of purified membranes, evaluation of attendant membrane activities, including ion pumps and channels, and structural and compositional analyses of functionally relevant components. Particular emphasis is placed on the Mg^{2+}-ATPase, its localization in the T-system, its unusual kinetic properties, its possible functions, and its potential regulation by diacylglycerol and other biologically-relevant lipids. Conclusions are drawn with respect to the biochemical markers characteristic of T-tubule membranes and the criteria to be applied in the assessment of isolated T-tubule membrane purity.

Key Words: Transverse tubules; T-tubules; T-system; review; Mg^{2+}-ATPase; biochemical markers; composition; ion channels; muscle.

Introduction

In striated muscle cells, the contraction—relaxation cycle is controlled by a sarcotubular membrane system consisting of the transverse tubules

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[4]Abbreviations: CMC, critical micelle concentration; Con-A, concanavalin A; DHP, dihydropyridine; E–C, excitation–contraction; E–P, phosphoenzyme; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; immuno-EM, immunoelectron microscopic; IP_3, inositol 1,4,5-triphosphate; LPC, lysophosphatidylcholine; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diozole; NBD-F, 7-fluoro-NBD; NEM, *N*-ethylmaleimide; PL, phospholipid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SL, sarcolemma; SR, sarcoplasmic reticulum; STX, saxitoxin; TTX, tetrodotoxin; T-tubule, transverse tubule; WGA, wheatgerm agglutinin.

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(or T-tubules) and the sarcoplasmic reticulum (or SR). The T-tubules are continuous invaginations of the plasma membrane (sarcolemma or SL) (Smith, 1961; Franzini-Armstrong and Porter, 1964). As the T-tubules invaginate toward the interior of the cell, they periodically contact the terminal cisternae of the SR, forming junctional complexes known as dyads or triads. The junctional complexes are connected by electron-dense "feet" or "pillars" that span the 12-nm gap between the junctional faces of the SR and T-tubule membranes (Franzini-Armstrong, 1975). Because of the relatively low capacitance of muscle cells, it is generally presumed that no direct electrical continuity exists between the SR and T-tubule, although Golgi stain (but not lanthanum ion or ferritin) is able to penetrate from T-tubule lumen to the SR (Franzini-Armstrong and Peachey, 1981; Scales and Yasumura, 1982). The SR is an internal membrane system forming fenestrated sacs capable of sequestering Ca^{2+} via an ATP-energized Ca^{2+} pump (Ikemoto, 1982; Martonosi, 1984; Inesi, 1985). The release of Ca^{2+} from the SR is responsible for activating contraction, and the subsequent resequestration of Ca^{2+} into the SR is responsible for relaxation of the muscle cell (Martonosi, 1984).

The rapid time course of the radial spread of mechanical activation (Constantin, 1970; Gonzalez-Serratos, 1971), the occurrence of T-tubule Na^+ currents (Hille and Campbell, 1976; Mandrino, 1977), and the immunocytochemical localization of Na^+ channels in the T-system (Jaimovich *et al.*, 1987) suggest that the T-tubules are able to conduct regenerative, Na^+-dependent action potentials from the SL to the interior aspects of the fiber where SR–T-tubule junctions are located. In frog muscle fibers, T-tubule depolarization has been shown to elicit mechanical activation of both sides of the Z-line (Huxley and Taylor, 1956, 1958), presumably by causing the release of Ca^{2+} from the connecting elements of the SR that flank the Z-lines of frog muscle. Transient increases in intracellular Ca^{2+} subsequent to SR release have been observed using Ca^{2+}-sensitive photoprobes (Blinks *et al.*, 1978; Baylor, 1983; Eusebi *et al.*, 1983). More recent evidence from Ikemoto's laboratory has indicated that selective depolarization (through anion replacement) of the T-tubule component of isolated triads is capable of eliciting Ca^{2+} release from preloaded SR (Ikemoto *et al.*, 1984). Similarly, ionophore-induced depolarization of skinned fibers elicits SR Ca^{2+} release (Volpe and Stephenson, 1986). It is clear that, while several theories have been presented to explain the coupling mechanism by which T-tubule depolarization induces SR Ca^{2+} release (see reviews by Fabiato, 1983; Caille *et al.*, 1985; Martonosi, 1984; and Volpe *et al.*, 1986), the exact nature of the coupling mechanism remains obscure.

Transverse-Tubule Membrane Isolation

Much of our knowledge concerning the role of the T-tubules in excitation–contraction (E–C) coupling is inferential. This is partly due to the torturous nature and small size of the T-tubules (Franzini-Armstrong and Peachey, 1982), which often impedes direct *in situ* experimentation. Consequently, the focus of research in many laboratories has been upon isolating vesiculated T-tubule membranes and characterizing them structurally and functionally *in vitro*, with the goal that information derived from incisive structural and functional studies of isolated membranes will enhance the knowledge of the role of the T-tubules in E–C coupling. Surface membrane fractions enriched in T-tubule membranes have been isolated from rat (Smith and Appel, 1977; Barchi *et al.*, 1977, 1979; Beeler *et al.*, 1983), frog (Narahara *et al.*, 1979; Hidalgo *et al.*, 1986*b*; Pediconi *et al.*, 1987), rabbit (Caswell *et al.*, 1976; Lau *et al.*, 1977; Rosemblatt *et al.*, 1981; Moczydlowski and Latorre, 1983; Gilbert and Meissner, 1983; Michalak *et al.*, 1984; Saito *et al.*, 1984; Horgan and Kuypers, 1987), and chicken (Malouf and Meissner, 1979; Scales and Sabbadini, 1979; Sabbadini and Okamoto, 1983; Okamoto *et al.*, 1985) skeletal muscle. Isolated cardiac T-tubule preparations have also recently been described (Brandt, 1985; Brandt and Bassett, 1986; Varsanyi *et al.*, 1986; Doyle *et al.*, 1986). T-tubule membrane isolation techniques commonly rely on isopycnic density-gradient centrifugation as the primary means of separating T-tubule vesicles from SR and SL membrane fragments. T-tubule membranes are either isolated from triad-rich, high-density (35–45%) or lower-density (15–30%) sucrose gradient fractions. T-tubule membranes are commonly localized on gradients by means of their complement of enzymatic activities, receptor sites, or other biochemical markers that are distinguishable from those present in the SR, SL, or other organelle membranes (see below). Cytochemical or morphological criteria are often of value as well in the identification of T-tubule membranes.

Caswell and colleagues published one of the first methods for T-tubule isolation (Caswell *et al.*, 1976, 1979; Lau *et al.*, 1977). Using ^{3}H-ouabain as a proposed marker for the T-tubules, Caswell was able to identify triad-containing T-tubule membrane fragments. Subsequent disruption of the triads with a French press succeeded in releasing the ^{3}H-ouabain-bound T-tubules from the triad complex. The free T-tubules were then separated from the terminal cisternae by a second gradient in which the free T-tubules appeared at a lower isopycnic point. Junctional complexes could be reformed by combining the purified T-tubules with non-French-pressed terminal cisternae, but not with longitudinal SR (Caswell *et al.*, 1979). Other workers

have shown that T-tubules can also be released from triads by osmotic shock (Mitchell *et al.*, 1983*b*), by KCl treatment (Gilbert and Meissner, 1983; Malouf *et al.*, 1986), or by fractionation on ion-free sucrose gradients (Horgan and Kuypers, 1987). Because the isolation methods described above use triads as starting material, they likely produce junctional, rather than nonjunctional, T-tubule membranes.

A second popular approach to T-tubule membrane isolation involves separating T-tubule vesicles from heterogeneous microsomal preparations containing free T-tubule vesicles among mostly an excess of vesiculated SR fragments. In some of our earlier work (Sabbadini *et al.*, 1975; Scales *et al.*, 1977), we noted that, when conventional microsomal preparation techniques for rabbit SR were applied to chicken breast muscle, heterogeneous microsomal suspensions rich in T-tubule vesicles were obtained (Scales and Sabbadini, 1979). At that time, faced with the lack of definitive biochemical markers for the T-tubules, we developed a procedure for monitoring the distribution of T-tubules by their unique freeze-fracture morphology. Using freeze-fracture stereological methods (Weibel *et al.*, 1976; Losa *et al.*, 1978), it was possible to identify vesiculated T-tubule membranes in heterogeneous microsomal fractions of chicken breast muscle (Scales and Sabbadini, 1979) by comparing freeze-fracture particle-density distributions of the microsomes with freeze-fracture distributions of T-tubules, SL membranes, and SR membranes present in intact tissue. Estimates of the relative surface area for SR and T-tubule membranes in mixed microsomal preparations were obtained using statistical sampling techniques applied to calibrated micrographs of freeze-fracture replicas (Scales, 1981); the results showed that preparations were composed of SR and T-tubule membranes representing 73% and 27%, respectively, of the total surface area, with negligible contamination from SL vesicles.

The T-tubule membranes were separated from the SR vesicles by an iterative oxalate-loading, density-shift technique (Scales and Sabbadini, 1979) modified in Okamoto *et al.* (1985). This method was developed with the expectation that SR and not T-tubule vesicles were capable of forming intravesicular Ca^{2+}-oxalate deposits subsequent to Ca^{2+} uptake. The increased density of Ca^{2+}-oxalate-loaded SRs facilitates their separation from T-tubule vesicles during sucrose density-gradient centrifugation. Ikemoto and colleagues (Rosemblatt *et al.*, 1981) have applied a similar procedure to rabbit muscle microsomes in which contaminating SR is separated from T-tubule vesicles on sucrose gradients after active Ca^{2+}-phosphate loading. Yields as high as 5–10 mg/100 g of muscle were obtained with $< 10\%$ residual SR contamination. Polyclonal antibodies were developed against the purified rabbit T-tubule vesicles. After absorbing cross-reactive antibodies with serum proteins and with SR vesicles, the specific anti-T-tubule immunoglobulin G

(IgG) was localized immunocytochemically at the T-tubule-rich A–I band regions of sectioned rabbit muscle fibers (Rosemblatt *et al.*, 1981). The exact nature of the T-tubule-specific antigenic determinants recognized by their anti-T-tubule IgG has not been elucidated. Because these T-tubule membranes were obtained from low-density fractions instead of from the heavier, triad-rich fractions, it is often assumed that they are derived from nonjunctional T-tubules (Hidalgo, 1986; Horgan and Kiypers, 1987). However, this is an *a priori* assumption that lacks supportive evidence.

Morphology of Isolated T-Tubule Membranes

The most distinguishing morphological feature of intact T-tubules is their low density of 9-nm freeze-fracture protein particles as compared with SR and the SL (Scales and Sabbadini, 1979; Scales, 1981; also see Table I of this review). Approximately 97% of the vesicles in the purified

Table I. Major Characteristics of T-tubules (TT)

	Membrane source		
	TT	SR	SL
Structural characteristics			
Density of 9-nm freeze-fracture particles particles/μm^2 (concave face only)	680	6020	1630
Presence of 4-nm surface particles in negative satin	No	Yes	?
Enzymatic properties[a]			
Mg^{2+}-ATPase (U/mg; 300 μM vanadate)	2.67	0.073	0.20
Ca^{2+}-ATPase (U/mg)	< 0.005	2.42	< 0.005
(Na^+, K^+)-ATPase (U/mg; ouabain sensitive)	0.437	0.041	1.22
p-Nitrophenylphosphatase (U/mg)	0.000	0.25	0.83
5′-Nucleotidase (U/mg)	0.0683	0.0038	0.070
$P_i \leftrightarrow$ HOH exchange (μatoms/μmol ATP hydrolyzed)	< 0.05	1.21	2.15
E-P level (nmol/mg)	< 0.05[d]	3.5	0.09[e]
Other characteristics			
Dihydropyridine binding (pmol DHP/mg)[b]	50	0.01	1.50
Phospholipid content (μmol PL/mg)	2.51	0.79	1.55
Cholesterol content (mol Ch/mol PL)	0.86	0.17	0.38
Ryanodine binding (pmol/mg)[c]	0.57	3.3	–
DHP/ryanodine-binding ratio	87.7	< 0.03	–
(Na^+, K^+)-ATPase/cholesterol ratio	27.9	–	124

[a] 1U = 1 μmol/min.
[b] Data from Fosset *et al.* (1983).
[c] Data from Knudson *et al.* (1988).
[d] E-P under SR Ca^{2+}-ATPase conditions.
[e] E-P under sodium pump conditions.

T-tubule preparations display a paucity of 9-nm intramembranous protein particles and particle-density distributions similar to those of intact T-tubule (Scales and Sabbadini, 1979; Sabbadini and Okamoto, 1983). For example, the mean particle density of isolated T-tubule membranes (680 particles/μm^2, concave fracture face only), while similar to the P faces of intact T-tubules (690 particles/μm^2), are about one-tenth the mean density of SR (6020 particles/μ^2); additionally, T-tubule membranes have much lower particle densities than those of the SL (1630 particles/μm^2). Similar to the chicken T-tubules, isolated rabbit T-tubule vesicles have a low density of 9-nm protein particles in freeze-fractured preparations (Scales, 1981; Salviati *et al.*, 1982), and fish and frog T-tubules (intact) have the same distinct morphology (Franzini-Armstrong, 1975). Junctional T-tubules isolated from triads also possess low 9-nm particle densities when compared with SR; however, these vesicles often differ from other T-tubule preparations in that they exhibit disc- or discuslike shapes with aggregates of particles often forming ridgelike arrays (Lau *et al.*, 1979*a*). Freeze-fractured intact muscle reveals that the T-tubule junctional face region displays particles arranged in tetrads in opposition to the SR junctional feet (Block *et al.*, 1988). Curiously, intact cardiac T-tubule membranes of the rabbit have a much higher particle densities than skeletal muscle T-tubules, and they cannot be distinguished from cardiac SR (D. Scales, personal communication). The identity of the integral membrane protein responsible for the appearance of the 9-nm particle seen in freeze-fracture faces of skeletal muscle T-tubules has not as yet been elucidated; however, a chicken T-tubule integral membrane protein, the Mg-ATPase, which has at least one concanavalin A (Con-A)-reactive subunit with M_r in the area of 100,000 (Okamoto *et al.*, 1985; Hidalgo *et al.*, 1983), is a likely candidate for a common identity with the 9-nm particle of the nonjunctional T-tubule membrane face.

Because highly purified T-tubule vesicles can be prepared with little or no SR contamination and concomitant Ca^{2+}-ATPase activity (see below), negatively stained T-tubule preparations do not possess the 4-nm surface projections characteristic of SR vesicles. Thin sectioning and staining of T-tubule fragments freed from the junctional complexes show that many of the former junctional T-tubules retain their tubular appearance in isolated form and that numerous T-tubule membrane fragments exist that exhibit a more spherical morphology (Lau *et al.*, 1977; Brunschwig *et al.*, 1982; Mitchell *et al.*, 1983*b*). Other structural features of isolated junctional T-tubules include surface projections visualized after tannic acid mordanting (Brunschwig *et al.*, 1982). The projections are presumed to be remnants of the spanning protein. In addition, the interiors of thin-sectioned T-tubules often contain osmiophilic material of unknown origin (Mitchell *et al.*, 1983*b*).

T-tubule vesicles are sealed and commonly spherical, having diameters of $\sim$ 150–200 nm and isotope spaces of $\sim$ 1.5–2.0 μl/mg (Gilbert and Meissner,

1983; Okamoto *et al.*, 1985). The *in vitro* orientation of T-tubule vesicle components is of great importance, particulary to those interested in functional studies. Investigators have recently estimated that 80–95% of rabbit and frog T-tubule vesicles are sealed as measured by detergent-induced increases in ^{3}H-ouabain binding and/or (Na^{+}, K^{+})-ATPase activity (Brandt *et al.*, 1985*b*; Hidalgo *et al.*, 1986*b*). These data contrast with the 55–65% of sealed cardiac T-tubule vesicles obtained by permeabilizing the vesicles with sodium dodecyl sulfate (SDS) or alamethicin and measuring substrate accessibility to the (Na^{+}, K^{+})-ATPase (Doyle *et al.*, 1986). It has been estimated that all T-tubule vesicles that are sealed have inside-out orientations, since detergents were unable to increase ATP-dependent ^{3}H-digoxin binding to skeletal T-tubule vesicles (Brandt *et al.*, 1985*b*; Hidalgo *et al.*, 1986*b*); however, this contrasts with the 50–57% sealed inside-out orientation that can be calculated from the saponin-increased ^{3}H-tetrodotoxin (TTX) binding obtained in a companion publication (see Fig. 3 in Jaimovich *et al.*, 1986) and the 40–50% value obtained by others (Moczydlowski and Latorre, 1983) using the Rosemblatt–Hidalgo isolation procedure and measuring Lubrol-PX-induced increases in ^{3}H-saxitoxin (STX) binding to rat and rabbit vesicles. These data also contrast with the 30–45% sealed, inside-out population characteristic of cardiac vesicles (Doyle *et al.*, 1986). A mixture of both inside-out and right-side-out vesicles is perhaps not surprising when one considers that T-tubule vesicles are able to trap both serum proteins (Rosemblatt *et al.*, 1981) and sarcoplasmic proteins (Okamoto *et al.*, 1985; Beeler *et al.*, 1985).

Protein Composition

The protein composition of isolated T-tubules and/or fractions enriched in T-tubules have been reported from a number of sources (Lau *et al.*, 1977; Malouf and Meissner, 1979; Brandt *et al.*, 1980; Rosemblatt *et al.*, 1981; Beeler *et al.*, 1983; Hidalgo *et al.*, 1983, 1986*b*; Mitchell *et al.*, 1983*b*; Dombradi *et al.*, 1984; Okamoto *et al.*, 1985; Hidalgo, 1986; Doyle *et al.*, 1986; Horgan and Kuypers 1987, 1988; Chadwick *et al.*, 1988; Kirley, 1988). Generally, the isolated preparations exhibit 15–27 proteins upon 1-D SDS–polyacrylamide gel electrophoresis (SDS-PAGE).

As discussed above, T-tubule preparations may be mixed in terms of vesicle leakiness and sidedness with a significant number of right-side-out vesicles possibly containing trapped proteins derived from the cytoplasm as well as inside out vesicles containing extracellular proteins orginating from the T-tubule lumen. If one also considers possible contamination in T-tubule preparations arising from the SR, SL, mitochondria, and other sources, the overall protein composition of isolated T-tubular vesicles can be quite

complex, variable, and difficult to interpret. As an example, high-resolution SDS-PAGE of vesiculated fragments of the chicken T-tubule indicates at least 26 Coomassie blue-staining components, 12 of which have been identified as sarcoplasmic proteins (Okamoto *et al.*, 1985). Serum albumin ($M_r \sim 68{,}000$) appears to be a major contaminant of rabbit and rat preparations (Rosemblatt *et al.*, 1981; Beeler *et al.*, 1983). It is assumed that most of the serum and sarcoplasmic proteins are trapped during homogenization and vesiculation since many of them can be selectively released through the use of permeabilizing agents and detergents (Okamoto *et al.*, 1985; Beeler *et al.*, 1985). Curiously, however, there is some specificity of entrapment with regard to the general pool of soluble, glycolytic enzymes existing in the sarcoplasm (Okamoto*et al.*, 1985), suggesting that some of the sarcoplasmic proteins may have been membrane associated *in situ*. Several laboratories have in fact postulated structural roles for some of the glycolytic proteins that are associated with the T-tubules, particularly those that appear to be peripherally associated with the surface of triad-disrupted T-tubules. Evidence suggests that the triad junction may be formed or stabilized, in part, by some of the glycolytic enzymes; for example, such a structural role has been suggested for M_r 34–40,000 aldolase and glyceraldehyde 3-phosphate dehydro genase (Caswell*et al.*, 1988; Chadwick *et al.*, 1988), M_r 93,000 rabbit phosphorylase (Chadwick *et al.*, 1988), and M_r 128,000 phosphorylase kinase (Dombradi *et al.*, 1984).

Several reports have appeared bearing upon the integral membrane proteins of isolated, detergent-solubilized preparations of the T-tubules. Hidalgo *et al.* (1983) have reported 3–4 rabbit integral membrane proteins with the M_r 107,000 and 30,000 components comprising at least 80% of the protein pool. Horgan and Kuypers (1987, 1988) reported on a lysophosphatidylcholine (LPC) solubilized rabbit preparation that contained ~ 15 proteins with the M_r ($\times 10^3$) 104, 70, and 30 species (comprising 16, 16, and 31%, respectively, of the protein pool) that included that M_r 104,000 alpha subunit of the (Na^+, K^+)-ATPase. Kirley (1988) reported integral membrane proteins of M_r ($\times 10^3$) 176, 105, 70, and 44 for rabbit T-tubules. Isolated chicken T-tubule membranes typically possess at least 26 polypeptides. Subsequent to detergent extraction, solubilization, and chromatographic separation, the detergent-extracted chicken preparation possesses polypeptides at M_r ($\times 10^{-3}$) 105, 100, 89, 78, 76, and 38. The M_r 78,000 and 76,000 components may be related to the $M_r \sim 72{,}000$ T-tubule polypeptide proposed by Caswell *et al.* (1988) and Chadwick *et al.* (1988) to interact with the ryanodine receptor. Several laboratories report at least one integral membrane protein in the $M_r \sim 90{,}000$–$115{,}000$ range (Hidalgo *et al.*, 1983; Okamoto *et al.*, 1985; Damiani *et al.*, 1987*a*; Kirley, 1988) that may be related to the T-tubule Mg^{2+}-ATPase (see below). Even though the most recent,

highly purified T-tubule preparations are claimed to possess only negligible (1–3%) contamination by SR vesicles (Hidalgo, 1986*a*, *b*; Sabbadini and Okamoto, 1983; Damiani *et al.*, 1987*a*), caution must be employed in maintaining that a polypeptide at or near M_r 100,000 is not the SR Ca^{2+}-ATPase (Damiani *et al.*, 1987*a*; Kirley, 1988). A variety of criteria must be employed to exclude this possibility, including the determination of Ca^{2+}-ATPase, the absence of calsequestrin and ryanodine binding, and the evaluation of other SR-associated properties. Since the catalytic subunit of the (Na^+, K^+)-ATPase migrates at the same general location, careful analyses are required either to identify or to exclude this integral membrane protein component.

T-tubule vesicles derived from disruption of rabbit triads have been reported to contain M_r 77,000 and 88,000 membrane proteins that apparently are not abundant components in rabbit T-tubule vesicles obtained by other means (Lau *et al.*, 1977). Dombradi *et al.*, (1984) later showed that a M_r 72,000 polypeptide was specifically associated with triad-derived T-tubules that was probably related to the previously reported M_r 77,000 protein; a M_r 68,000 protein was also seen that most likely was albumin (Rosemblatt *et al.*, 1981). Caswell *et al.* (1988) and Chadwick *et al.* (1988) have reported a M_r 72,000 and 71,000 (4% wt/wt T-tubule protein) triad-derived T-tubule protein, respectively, that is associated with the ryanodine receptor of the opposing terminal cisternae membrane. The M_r 71,000 protein labeled by Chadwick *et al.* (1988) and the M_r 72,000 protein found by Caswell *et al.* (1988) are probably identical to the T-tubule-specific M_r 72,000 protein reported by Dombradi *et al.* (1984). Horgan and Kuypers (1987) have also identified a M_r 70,000 component, but this was released with mild LPC treatment as an extrinsic membrane protein.

In addition to the abundant proteins whose relative molecular weights are described above, an increasing number of low-abundance membrane-associated proteins being described in putative T-tubule membranes are detectable by means of their interaction with specific radioligands or immunoreagents. These include the insulin receptor [M_r 92,000 and 125,000 symmetric heterotetramer (Burdett *et al.*, 1987)], glucose transporter [M_r 40,0000 (Burdett *et al.*, 1987)], T-tubule triad-coupling protein [M_r 71,000 (Chadwick *et al.*, 1988)], dihydropyridine receptor [M_r ($\times 10^3$) 170 and 140 (Chadwick *et al.*, 1988); 175, 170, 52, and 32 (Leung *et al.*, 1988)], dystrophin [M_r ~ 400,000 (Leung *et al.*, 1988)], and anti-dystrophin-reactive protein [M_r 95,000 (Leung *et al.*, 1988)]. In addition to the above, apparent labeling of rabbit T-tubule membrane-associated glyceraldehyde 3-phosphate dehydrogenase (M_r ~ 38,000) and several other proteins of M_r 100,000, 29,500, and 23,500 have been noted by Chadwick *et al.* (1988) and of a M_r 34,000 protein noted by Caswell *et al.* (1988), both by means of a ryanodine receptor-mediated label transfer approach; based upon M_r comparisons of authentic skeletal

muscle proteins, the latter two components could be phosphoglucose isomerase and triose phosphate isomerase.

The recently discovered M_r 400,000 protein dystrophin, which has been identified as the missing gene in mouse and human muscular dystrophy (Monaco *et al.*, 1986; Hoffman *et al.*, 1987*a*, *b*; Koenig *et al.*, 1987), was recently identified as a T-tubule-specific protein by immunoblotting triad-disrupted T-tubules with an antidystrophin IgG (Knudson *et al.*, 1988; Watkins *et al.*, 1988); however, immunocytochemical and electron-microscopic evidence indicates that dystrophin is principally associated with the SL (Zubrzycka-Gaarn *et al.*, 1988; Bonilla *et al.*, 1988). Consequently, the presence of antidystrophin antibody-reactive bands in Western immunoblots of T-tubule preparations (Knudson *et al.*, 1988) may be due to contamination of the preparation by SL. However, a recent report suggests that dystrophin may be specifically associated with junctional T-tubules (Salviati *et al.*, 1989).

Phosphorylation studies employing nitrendipine-receptor enriched, T-tubule-containing fractions from cardiac ventricular tissue show cAMP-stimulated phosphoproteins at M_r ($\times 10^3$) 155, 130, 102, 93, 48, 44, 32, and 20 (which are in addition to those found at 275 and 250 in the presence of calmodulin/Ca^{2+}) for the bovine system and cAMP-stimulated phosphoproteins at M_r ($\times 10^3$) 200, 182, 155, 145, 120, 100, 48, and 37 for the sheep system (Doyle *et al.*, 1986). Dombradi *et al.* (1984) have shown the labeling of a M_r 143,000 protein by endogenous and exogenous protein kinases: labeling of groups of proteins also occurred between M_r 80,000 and 90,000 and between 60,000 and 64,000. Since the functions of these components are largely unknown, the significance of their abilities to serve as substrates for phosphorylation is unclear.

Chicken T-tubule vesicles contain M_r ($\times 10^3$) 175, 140, and 89 putative glycoproteins that are reactive with periodate–dansylhydrazine, Con-A, and wheatgerm agglutinin (WGA) (Okamoto *et al.*, 1985). Rabbit T-tubule vesicles contain a M_r 140,000 protein that is reactive with periodate–Schiff reagent (Hidalgo *et al.*, 1983) and other Con-A-recognized proteins, including a M_r 105,000 component thought to be purified Mg^{2+}-ATPase (Kirley, 1988). Cifuentes *et al.* (1987) have reported fluoresceinated Con-A recognition of M_r 130–150,000 and 102,000 rabbit T-tubule polypeptides.

Lipid Composition

Complete phospholipid (PL) composition analyses have been performed on T-tubule membranes obtained from rat (Smith and Appel, 1977), chicken (Sumnicht and Sabbadini, 1982), rabbit (Lau *et al.*, 1979*a*; Rosemblatt *et al.*, 1981), and frog (Pediconi *et al.*, 1987) muscle. While T-tubule membranes are relatively rich in phosphatidylcholine (50% of total PL), they routinely

display 15–20% lower contents of phosphatidylcholine when compared with SR. Significantly higher sphingomyelin levels are also consistently found for T-tubule membranes. For example, the sphingomyelin content of T-tubules is typically five- to eightfold higher than SR. Rabbit and frog, but not chicken, T-tubules have higher phosphatidylethanolamine and phosphatidylserine contents when compared with SR (Rosemblatt *et al.*, 1981; Pediconi *et al.*, 1987). In contrast, SL and T-tubule membranes from chicken fast skeletal muscle do not differ in their distributions of various PL classes (Sumnicht and Sabbadini, 1982).

One important distinguishing feature of T-tubule membranes is their unusually high total lipid contents (Table I). This is due to both the high PL and cholesterol contents. PL phosphorous/protein ratios of 1.1–1.6 and molar ratios of cholesterol/PL of 0.4–0.9 are generally reported (Smith and Appel, 1977; Lau *et al.*, 1979*a*; Rosemblatt *et al.*, 1981; Sumnicht and Sabbadini, 1982; Saito *et al.*, 1984). Because ~50% of the protein present in T-tubule membranes can be accounted for by the presence of trapped or loosely bound proteins of nonmembrane origin (Beeler *et al.*, 1983; Okamoto *et al.*, 1985), a PL phosphorous/integral membrane protein ratio of 2.5 probably represents a more accurate figure for the T-tubules (Okamoto *et al.*, 1985); the higher lipid content is more consistent with the low density of 9-nm-protein particles characteristic of freeze-fractured T-tubule membranes (Scales and Sabbadini, 1979; Scales, 1981; Sabbadini and Okamoto, 1983).

T-tubule membranes possess molar ratios of cholesterol/PL that are, respectively, eightfold and 2.2-fold higher than those found in the SR and SL membranes isolated from the same species and analyzed in the same laboratory (Sumnicht and Sabbadini, 1982). This agrees with the observation that the T-tubule sphingomyelin content is nearly eightfold higher than that in SR membranes (Sumnicht and Sabbadini, 1982; also see Table I). Extracellular membranes often contain high amounts of both sphingomyelin and cholesterol (Patton, 1970). Sphingomyelin is thought to parallel cholesterol content in order to prevent the formation of reverse hexagonal (H_{II})-phase structures that can occur in cholesterol-rich PC membranes (Cullis and DeKruijff, 1979). The high cholesterol content of T-tubule membranes has also been implicated by the presence of filipin-induced cholesterol complexes in freeze-fractured whole muscle (Severs, 1981). Further, cholesterol content varies with the proportion of T-tubular vesicles in mixed microsomal preparations from fast and slow muscle (Salviati *et al.*, 1982). It was originally proposed quite early that cholesterol content may be a useful biochemical marker to follow T-tubules during membrane purification (Headon *et al.*, 1977), and it has recently been suggested that the ratio of (Na^+, K^+)-ATPase activity to cholesterol content might be a useful measure to distinguish T-tubules from SL (Hidalgo, 1986; Hidalgo *et al.*, 1986*b*).

The lipid distribution can be of value in predicting biophysical and biochemical properties of the native and isolated membranes. Predictably, the high T-tubule cholesterol content is associated with the high degree of order and low-fluidity characteristic of T-tubular membranes as revealed by election spin resonance (Hidalgo, 1985). The T-tubule cholesterol content may explain why saponin and digitonin, which bind specifically to 3-beta-hydroxy steroids like cholesterol, are preferred permeabilizing agents for T-tubule membranes (Hidalgo *et al.*, 1986*b*). The unique lipid composition of T-tubule membranes may also explain the unusual temperature dependency of the chicken T-tubule Mg^{2+}-ATPase (Malouf and Meissner, 1979; Sabbadini and Okamoto, 1983); the chicken T-tubule Mg^{2+}-ATPase displays a concave-down Arrhenius plot, yielding E_{act}'s of 4.5 and -11.5 kcal/mol, below and above, respectively, the transition temperature of 27°C (Moulton *et al.*, 1986). Further, the characteristic effects of Con-A on the Mg^{2+}-ATPase kinetics and temperature response (see below for details) may also be explained by an effect of the lectin on lipid mobility and resulting lipid–protein interactions (Beeler *et al.*, 1983; Moulton *et al.*, 1986).

Phosphoinositide Metabolism

It has recently been postulated that the phosphoinositide intermediate, inositol 1,4,5-triphosphate (IP_3), may be a chemical messenger involved in triggering or otherwise modifying the release of Ca^{2+} from the SR during E–C coupling (Vergara *et al.*, 1985; Volpe *et al.*, 1986). Significantly, IP_3 elicits force production in skinned skeletal fibers and is capable of releasing Ca^{2+} from isolated SR terminal cisternae (Volpe *et al.*, 1986), suggesting a direct relationship between the two phenomena. In addition, it has recently been argued that the observed GTP γ-S-induced tension development is mediated by the T-tubules and not the myofibrils or the SR (Di Virgilio *et al.*, 1986), further implicating T-tubule-generated IP_3 in the Ca^{2+} trigger hypothesis.

Both frog (Hidalgo *et al.*, 1986*b*) and rabbit (Varsanyi *et al.*, 1986) T-tubules possess the kinases responsible for phosphorylating phosphatidylinositol to phosphatidylinositol 4-monophosphate and to phosphatidylinositol 4,5-biphosphate, the first to steps preceding IP_3 production. Further evidence for an active role of the T-tubule membranes in phosphoinositide metabolism is the discovery of G-proteins in isolated rabbit T-tubules, SR membranes, and SL membranes, coupled with the discovery that T-tubules display the highest degree of G-protein labeling by the ADP-ribosylation method (Scherer *et al.*, 1987). It is well established that beta receptors and the adenylate cyclase system are coupled to each other via G-proteins (Gilman, 1984), so it is not surprising to find that T-tubule membranes display significant

beta-adrenergic binding and adenylate cyclase activities (Caswell *et al.*, 1978; Mitchell *et al.*, 1983*a*). It is not clear at this time whether a relationship exists between G-proteins as couplers for hormone action and as putative transducers for the chemical mediation hypothesis of E-C coupling. A possible role for diacylglycerol, the companion product of phosphoinositide metabolism and IP_3 production has been ignored. However, we have recently obtained evidence for specific, high affinity inhibition of the T-tubule Mg^{2+}-ATPase by diacylglycerols and phorbol esters, the potential significance of which is explored in the last section of this review.

Ion Channels and Pumps in T-Tubule Membranes

Sodium Channels and Pumps

As discussed above, there is much indirect evidence that the T-tubules are capable of conducting sodium-dependent action potentials radially into the interior of the cell. The availability of highly purified T-tubule membrane preparations has enabled a molecular analysis of T-tubule sodium channels (Jaimovich *et al.*, 1983, 1986; Moczydlowski and Latorre, 1983; Doyle *et al.*, 1986) that have recently been purified from T-tubules and reconstituted into liposomes (Kranen *et al.*, 1985). The maximum binding capacity of tetrodoxin (TTX) to T-tubule vesicles (B_{max} = 4.8 pmol/mg) is about half that of crude SL membranes (9.0 pmol/mg) (Jaimovich *et al.*, 1983, 1986). Earlier studies comparing TTX binding with intact vs. detubulated frog muscle indicated that the T-tubules possess one-fourth the density of TTX sites relative to the SL (Jaimovich *et al.*, 1976). Isolated skeletal T-tubule vesicles apparently have only one class of binding site while the crude SL fractions possess two receptor subtypes characterized by diverse affinities for TTX derivatives (Jaimovich *et al.*, 1983, 1986). Distinct subtypes of SL and T-tubule sodium channels have also been distinguished immunocytochemically (Jaimovich *et al.*, 1987) and electrophysiologically (Jaimovich *et al.*, 1982; Barhanin *et al.*, 1984). In contrast, however, both low- and high-affinity saxitoxin (STX)-binding sites are found on T-tubules as well as SL membranes of the heart (Doyle *et al.*, 1986).

Chemical gates for sodium have also been observed in T-tubule preparations as measured by the binding of a cholinergic blocker, ^{125}I-bungarotoxin, to vesicles (Jaimovich *et al.*, 1986). This finding also agrees with the demonstration of acetylcholinesterase activity in isolated T-tubule membranes (Horgan and Kuypers, 1987, 1988); however, there is no supporting evidence from electrophysiological, immunocytochemical, or other techniques to suggest that cholinergic receptors in T-tubule preparations represent other than contamination from motor endplate fragments.

Active Na^+ pump activity and ouabain-sensitive (Na^+, K^+)-ATPase have been described for purified T-tubule vesicles, along with ATP-energized $^{86}Rb^+$ (a K^+ congener) and $^{36}Cl^-$ uptake (Lau *et al.*, 1979*b*). ^{3}H-oubain-binding stoichiometries as high as 49–215 pmol/mg have been reported for rabbit T-tubules purified by the Ca^{2+}-phosphate-loading method (Moczydlowski and Latoree, 1983; Jaimovich *et al.*, 1986). It has been estimated from ouabain binding to intact vs. detubulated frog muscle that the T-tubules possess only 4–5% of the density of ouabain sites of SL membranes (Venosa and Horowicz, 1981). Because the density of ouabain sites is > 20-fold higher in the SL and because the *in situ* density of TTX sites is fourfold higher in the SL than in the T-tubules (Jaimovich *et al.*, 1976), T-tubule preparations are presumed to have lower ouabain/saxitoxin-binding ratios compared with SL (Moczydlowski and Latorre, 1983). this presumption has been used to distinguish T-tubules from SL in sucrose gradient fractions (Moczydlowski and Latorre, 1983).

While crude skeletal muscle SL fractions possess (Na^+, K^+)-ATPase activity rates as high as 1.2 U/mg (Seiler and Fleischer, 1982; Sabbadini and Okamoto, 1983), T-tubular fractions usually exhibit much lower (Na^+, K^+)-ATPase activity rates (Table I); however, the reported T-tubule values vary considerably from $\sim$0.05 U/mg to nearly 1 U/mg (see Table I in Hidalgo, 1986). For the purposes of this review, a unit will be defined as 1 μmol/min.

Calcium Channels and the 1,4-Dihydropyridine Receptor

Voltage-clamp studies of skeletal muscle cells have demonstrated the presence of slow, voltage-dependent, inward Ca^{2+} currents (Sanchez and Stefani, 1978) that are localized predominantly in the T-tubules (Nicola Siri *et al.*, 1980; Almers *et al.*, 1981). Conductance through these Ca^{2+} channels can be blocked with the Ca^{2+} antagonists such as the dihydropyridines (DHPs), nitrendipine and nifedipine (Almers *et al.*, 1981; Lee and Tsien, 1983; Chiardini and Stefani, 1983), and the papaverine derivative, D600 (methoxyverapamil) (Almers *et al.*, 1981).

Receptors with high affinities (nM) for the DHPs are preferentially localized in isolated T-tubule membranes of skeletal muscle (Fosset *et al.*, 1983; Glossman *et al.*, 1983). Isolated T-tubules are one of the richest sources of Ca^{2+} channels with binding-site stoichiometries that are 50 to 100-fold greater than other tissues. Binding stoichiometries as high as 131 pmol/mg membrane protein have been reported for skeletal muscle T-tubules (see the review by Hidalgo, 1986). Skeletal T-tubule membranes possess DHP sites that are 30-fold higher in density than SL membranes (Fosset *et al.*, 1983) while highly purified SR membranes display negligible DHP-binding capacities (Sariemento *et al.*, 1982; Fosset *et al.*, 1983; also see Table I). A monoclonal antibody specific for the rabbit skeletal T-tubule DHP receptor

has been immunocytochemically localized specifically at the A–I junctions where the T-tubules are found, and the antibody was not seen on the SL (Malouf *et al.*, 1987). Junctional T-tubules obtained from disrupted triads are rich in DHP sites (Brandt, 1985; Brandt *et al.*, 1985*a*; Horgan and Kuypers, 1987); however, it is controversial whether their maximum binding capacity differs from the density of sites reported for "free" skeletal T-tubules separated from SR on sucrose density gradients (Damiani *et al.*, 1989). The DHP receptor appears to have multiple subunits (see the review by Catterall *et al.*, 1988). The major functional subunit of the DHP receptor is a M_r 170,000 protein that has recently been cloned and sequenced for rabbit muscle (Tanabe *et al.*, 1987).

Cardiac T-tubules also possess DHP receptors (Campbell *et al.*, 1984; Brandt, 1985; Brandt and Bassett, 1986; Doyle *et al.*, 1986); however, the maximum binding capacity of the cardiac membranes is at least 2 orders of magnitude lower than skeletal membranes. In cardiac muscle, the membrane distribution of DHP receptors differs from that of the skeletal muscle. The density of DHP receptors is, in fact, higher in the SL membranes relative to the cardiac T-tubules (Brandt, 1985; Doyle *et al.*, 1986), and it has been suggested (Doyle *et al.*, 1986) that the higher ratio of DHP to STX binding characteristic of the SL may be a useful marker to distinguish cardiac T-tubules from SL. Another interesting, but enigmatic, feature of heart muscle DHP receptors is that heart mitochondria, while displaying negligible DHP binding in the nanomolar range, nevertheless possess low-affinity (K_d = 76 nM) DHP sites that have high maximum binding capacities (Brush *et al.*, 1987). The physiological significance of the lower-affinity mitochondrial sites is unknown.

In intact skeletal muscle fibers, $>95\%$ of the high-affinity DHP sites appear to be nonfunctional as Ca^{2+} channels, since PN-200/110 has only a minimal ability to block Ca^{2+} currents at concentration levels that saturate nearly all of the DHP sites (Schwartz *et al.*, 1985). These data plus the suggestion that an insignificant amount of Ca^{2+} probably enters the cell through the Ca^{2+} channels (Stefani and Chiarandini, 1982) and that blockage of the slow Ca^{2+} current does not abolish E–C coupling and force generation (Gonzales-Serratos *et al.*, 1982; Eisenberg, 1987) question the functional significance of the transmembrane Ca^{2+} fluxes in T-tubules and represent a problem for the Ca^{2+}-induced Ca^{2+} release mechanism of E–C coupling (Stephenson, 1981; Fabiato, 1983) as it applies to skeletal muscle. In light of these reservations, the ability of Ca^{2+} channel blockers, D600 and diltiazem, to block force development effectively has been recently explained (Eisenberg, 1987; Luttgau *et al.*, 1987) in terms of the ability of Ca^{2+} antagonists to block nonlinear charge movement (Hui *et al.*, 1984; Rios and Brum, 1987) presumably by binding to a distinct T-tubule membrane protein that is acting as a "voltage sensor" rather than a Ca^{2+} channel.

While the role of the cardiac DHP receptor/Ca^{2+} channel in mediating the inotropic effect of beta-adrenergic ligands is fairly well established (Fleckenstein, 1977), the physiological significance of the T-tubule DHP receptor in skeletal muscle appears uncertain at this time. Nevertheless, the preferential localization of the high-affinity DHP receptor in skeletal T-tubule membranes is clearly a useful tool in following T-tubule membranes during isolation and in judging the extent of T-tubule contamination in SL and SR preparations.

Ca^{2+}-ATPase, Ca^{2+} Pump, and Na^+/Ca^{2+} Exchange Activities

An important aspect of T-tubule research has been to characterize the isolated membranes with regard to their contents of ATPases and associated transport activities. Some of the earliest papers reported that T-tubule vesicles contained moderately high levels of an SR-like Ca^{2+}-ATPase that was stimulated by Ca^{2+} in the micromolar range in the presence of MgATP (Lau *et al.*, 1977; Sabbadini and Scales, 1979; Brandt *et al.*, 1980; Rosemblatt *et al.*, 1981; Kirley and Schwartz, 1984). Other reports, many of which have appeared in the more recent literature, have shown that highly purified, well-characterized T-tubule membrane preparations can be made that possess either little or no Ca^{2+}-ATPase activity (Narahara *et al.*, 1979; Malouf *et al.*, 1981; Beeler *et al.*, 1983; Hidalgo *et al.*, 1983, 1986*a*, *b*; Sabbadini and Okamoto, 1983; Okamoto *et al.*, 1985). Low-density microsomal fractions cautiously characterized only as "surface membranes" displayed very low Ca^{2+}-ATPase levels (Malouf and Meissner, 1979) and, although not specified at the time, their preparation was probably rich in T-tubules and devoid of SR contamination.

We and others (Sabbadini and Okamoto, 1983; Hidalgo *et al.*, 1983, 1986*a*; Damiani *et al.*, 1987*a*) have suggested that many, if not all, of the earlier T-tubule preparations, including our own, may have been more heavily contaminated with SR than was assumed at the time. In fact, it was only after we improved our membrane separation and isolation techniques that we produced T-tubules with little or no Ca^{2+}-ATPase activity (Sabbadini and Okamoto, 1983; Okamoto *et al.*, 1985). We are currently of the opinion that most, if not all, of the Ca^{2+}-ATPase seen in T-tubule preparations is SR derived. Although one laboratory maintains that the T-tubules possess a low-turnover Ca^{2+}-ATPase activity that is insensitive to inhibition by μM vanadate and is, therefore, not of SR or SL origin (Michalak *et al.*, 1984), others have argued that the T-tubules may possess a latent Ca^{2+}-ATPase that is not abundant and is easily masked by the exceptionally high catalytic rate of the T-tubule Mg^{2+}-ATPase (Hidalgo *et al.*, 1986*a*). The occasional detection of a non-SR-like Ca^{2+}-ATPase in T-tubule preparations might be

due to SL contamination, estimated in some frog T-tubule preparations to be as high as 30% (Moczydlowski and Latorre, 1983). However, it is not clear whether the SL possesses its own Ca^{2+}-ATPase. For example, Fleischer considered that the negligible Ca^{2+}-ATPase seen in their rabbit SL preparation was SR derived (Seiler and Fleischer, 1982), while others have characterized a calmodulin-sensitive Ca^{2+}-ATPase and associated Ca^{2+} transport activity (Michalak *et al.*, 1984). The development of a well-defined SL preparation would aid in resolving this and other controversies.

Currently, several laboratories have proposed that highly purified T-tubules display ATP-energized Ca^{2+} transport activity that cannot be accounted for by SR contamination (Brandt *et al.*, 1980; Hidalgo *et al.*, 1983, 1986*a*; Michalak *et al.*, 1984). Two investigators report that the transport activity is energized by a detectable Ca^{2+}-ATPase (Brandt *et al.*, 1980; Michalak *et al.*, 1984). An earlier paper by Hidalgo suggested that T-tubule Ca^{2+} transport may be energized by the prominent T-tubule Mg^{2+}-ATPase (Hidalgo *et al.*, 1983), but Hidalgo now argues that the transport activity is energized by a nondetectable, latent Ca^{2+}-ATPase (Hidalgo *et al.*, 1986*a*).

The agreed-upon characteristics of the putative T-tubule Ca^{2+} pump include (1) a high affinity for Ca^{2+}; (2) a strict requirement for MgATP without support by alternate substrates such as *p*-nitrophenylphosphate; (3) a slow rate of transport (7–30 nmol/min/mg) by comparison to SR (300 to 500-fold lower), but comparable steady-state transport levels of ~100–200 nmol/mg taking 10–30 min to reach saturation level; (4) transport that is stimulated by calmodulin [although Michalak *et al.* (1984) report that the T-tubule Ca^{2+}-ATPase is not affected by calmodulin or its inhibitors]; and (5) oxalate or P_i are unable to increase steady-state transport levels [although Brandt *et al.* (1980) admit that their transport was increased twofold by oxalate, they argued that it was insignificant compared with the ability of oxalate to stimulate SR Ca^{2+} transport]. There is also disagreement as to whether vanadate inhibits (Hidalgo *et al.*, 1986*a*) or does not inhibit (Michalak *et al.*, 1984) transport.

Based on the relative lack of oxalate stimulation, the vanadate insensitivity of the transport system, the calmodulin effect, and the inability of alternate substrates to support transport activity, it is argued that the T-tubule Ca^{2+} transport is not SR derived (Hidalgo *et al.*, 1986*a*). However, complete agreement on these characteristics is lacking and, if, in the final analysis, the putative T-tubule Ca^{2+} pump resembles the SR pump, it should be kept in mind that as little as 0.2% SR contamination of the T-tubule preparation can account for the reported 300 to 500-fold lower rates of transport. According to Carafoli (Michalak *et al.*, 1984), the T-tubule Ca^{2+} transport is not SL derived and is distinguishable from that of the SL because T-tubule Ca^{2+} transport is vanadate insensitive while SL transport

is sensitive to vanadate inhibition. Because Hidalgo's group indicates that the T-tubule pump is vanadate sensitive, however, it is not possible at this time to claim firmly that the T-tubule pump cannot be accounted for by SL contamination.

An additional troublesome feature of the T-tubule Ca^{2+} pump story is that it has a strict requirement for MgATP (Hidalgo *et al.*, 1983). Although the authors now disclaim their original proposal that the prominent T-tubule Mg^{2+}-ATPase is responsible for the Ca^{2+} transport, we believe that this original proposal may still have merit. Recalling that the Mg^{2+}-ATPase is a very active enzyme, displaying hydrolysis rates as high as 17 U/mg under optimum conditions (Damiani *et al.*, 1987*a*), one can calculate that, within the first few seconds after ATP addition, the T-tubule vesicles would have generated micromolar concentrations of P_i that could be precipitated by Ca^{2+} after the latter's diffusion into the intravesicular space. This process would be ATP dependent, not supported by alternate substrates, unaffected by exogenous additions of oxalate or P_i, and could saturate within the observed time frame (10–30 min). In support of this argument is the fact that 3 min were required for the Ca^{2+} ionophore, A23187, to release 85% of the transported Ca^{2+} (Hidalgo *et al.*, 1983). Alternately, if the T-tubule vesicles support ATP-energized proton gradients across the T-tubule membrane, as was suggested by some initial experiments performed in our laboratory (Damiani *et al.*, 1987*a*), then the T-tubule Ca^{2+} transport may be indirectly supported by a MgATP-dependent H^+/Ca^{2+} exchange. It might be useful to repeat some of the Ca^{2+} transport studies in the presence of protonophores. The discovery of a specific and effective inhibitor of the T-tubule Mg^{2+}-ATPase would also aid in answering this question.

The question also arises as to whether the observed T-tubule Ca^{2+} transport could be account for by a Na^+/Ca^{2+} exchange energized by a T-tubule Na^+ pump (Lau *et al.*, 1979*b*). Arguing against this possibility is the observation that digitoxin and monensin (which prevent Na^+ gradient formation) could not prevent T-tubule Ca^{2+} transport (Brandt *et al.*, 1980) and that Na^+ replacement of the extravesicular medium after active Ca^{2+} loading did not cause Ca^{2+} release (Hidalgo *et al.*, 1986*a*). Importantly, these data would suggest that a Na^+/Ca^{2+} exchange system does not operate in rabbit skeletal T-tubules as it does in rabbit skeletal SL (Gilbert and Meissner, 1982; Michalak *et al.*, 1984), and that the presence or absence of such an exchanger might aid in distinguishing rabbit T-tubules from SL. A recent preliminary report by Hidalgo's group (Donoso and Hidalgo, 1988) confirms that rabbit T-tubules do not possess a Na^+/Ca^{2+} exchange system. Interestingly, however, they also report that frog T-tubules appear to display significant exchange activity. Since frog T-tubule preparations can be more heavily contaminated with SL vesicles when compared with rat or rabbit T-tubules (Moczydlowski and Latorre, 1983), the Na^+/Ca^{2+} activity of the

frog might not be T-tubule derived. Morcos (1982) reports that Na^+/Ca^{2+} exchange activity of cardiac microsomes appears in a different sucrose gradient fraction from Mg^{2+}-ATPase.

The *in vivo* ability of T-tubules to transport Ca^{2+} either through a Na^+/Ca^{2+} exchange mechanism or via an ATP-dependent Ca^{2+} pump process would have important physiological implications. Both of these mechanisms could aid the SL in the maintenance of low intracellular free Ca^{2+} that would otherwise progressively increase to a level where contracture would set in. The large surface area of the T-system, plus its proximity to the interior sarcoplasm would enable these membranes to provide an efficient means of controlling intracellular calcium as an adjunct to the SL and SR. In addition, the presence of Ca^{2+} in the T-tubule lumen of intact fibers is required as a source of trigger Ca^{2+} for E–C coupling, particularly if the Ca^{2+}-induced Ca^{2+} release mechanism is operating (Stephenson, 1981; Fabiato, 1983). However, direct measurements of T-tubule Ca^{2+} concentrations have not been made, principally because of the small size of the T-tubule lumen and the difficulty in making X-ray microprobe analyses of this compartment. Further, elevation of T-tubule luminal Ca^{2+} that is postulated to occur during repetitive activity (Bianchi and Narayan, 1982*a*, *b*; Howel and Oetliker, 1987) may be responsible for fatigue by causing either a failure of the T-tubule action potential (Gonzales-Serratos *et al.*, 1978; Howell and Oetliker, 1987) or by raising the mechanical threshold for E–C coupling (Bianchi and Narayan, 1982*a*).

Ion Permeabilities and Membrane Potentials of T-tubule Vesicles

T-tubule vesicles isolated by a KCl triad disruption procedure are relatively impermeable to Tris, Na^+, K^+, and H^+ and Cl^- when compared with SR vesicles (Gilbert and Meissner, 1983). Using the fluorescent membrane potential dye, DiS-C_3, we have observed similar results with T-tubule vesicles purified by the iterative oxalate-loading method (unpublished observations); in fact, a substantial percentage of the total vesicles in the T-tubule fraction have preestablished, acid-stable membrane potentials that can be dissipated with ionophores. In contrast to SR vesicles, T-tubule vesicles are relatively impermeable to ^{14}C-oxalate, which partially explains why T-tubule vesicles cannot be actively loaded with Ca^{2+} oxalate.

Conclusions Regarding the Biochemical Markers Characteristic of Isolated T-tubules

A major difficulty faced by workers in this field has been to assess the purity of the isolated T-tubule membranes using reliable markers that are

characteristic of the various membrane classes. Because SR, T-tubule, and SL vesicles possess similar buoyant densities, and because they can aggregate during isolation, isopycnic density-gradient centrifugation does not easily separate the three membrane classes. As a result, cross-contamination of T-tubules, SR membranes, and SL membranes may be substantial. For example, microsomal fractions thought to contain only fragments of SR are often contaminated with T-tubule vesicles (Sabbadini and Scales, 1979; Campbell *et al.*, 1980; Scales, 1981; Kosk-Kosicka *et al.*, 1982; Mrak and Fleischer, 1982; Salviati *et al.*, 1982) and sometimes SL membrane contamination is present in SR preparations (Jones *et al.*, 1979). As discussed earlier, isolated T-tubules, particularly some of the earlier preparations, can be contaminated with SR (Sabbadini and Okamoto, 1983; Hidalgo *et al.*, 1983, 1986*a*; Damiani *et al.*, 1987*a*) and some T-tubule preparations contain SL fragments (Moczydlowski and Latorre, 1983). Measuring the distribution of enzymatic activities, drug binding, and lipid and glycoprotein content often aids in identifying membranes on gradients; however, it is often difficult to find markers that can be exclusively assigned to a single membrane class. For example, T-tubule membranes have been found to contain significant levels of (Na^+, K^+)-ATPase and Na^+ transport activity, adenylate cyclase activity, G-proteins, beta receptors, cholinergic receptors and acetylcholinesterase activity, sodium channels, and 5′-nucleotidase activity. Significantly, all of these activities have also been associated with "highly purified" SL preparations.

The difficulty then arises as to what biochemical and/or morphological markers can be used with confidence to assess T-tubule membrane purity and to distinguish T-tubule membranes from the other two membrane systems that actually make contact with the T-tubules *in situ*. Regarding SR contamination, it has become clear that T-tubules do not possess an SR-like Ca^{2+}-ATPase, characterized by μM Ca^{2+} stimulation in the presence of mM Mg^{2+}, an acid-stable phosphoenzyme (E-P), and vanadate sensitivity. Consequently, highly purified T-tubules should not display this type of Ca^{2+}-ATPase, nor should highly purified T-tubule vesicles possess calsequestrin (associated with the longitudinal or nonjunctional SR) or other SR protein components. For example, ^{3}H-ryanodine binding is exclusively associated with the junctional SR membranes and not the T-tubules (Campbell *et al.*, 1987; Knudson *et al.*, 1988). Since evidence clearly exists that skeletal T-tubules and not the SR possess DHP receptors (Sariemento *et al.*, 1982; Fosset *et al.*, 1983), Campbell and colleagues (Knudson *et al.*, 1988) have used the ratio of ^{3}H-PN200-110 to ^{3}H-ryanodine to distinguish junctional SR from T-tubules. In accordance with Campbell's work, we have calculated that this ratio is several orders of magnitide higher for the T-tubules (see Table I). Consequently, highly purified T-tubules can be easily judged

free of SR contamination if they display high DHP/ryanodine-binding ratios and display no Ca^{2+}-ATPase activity.

Assessing the extent of SL contamination in T-tubule fractions is somewhat more complicated, mostly because a well-characterized SL preparation has not been developed and because the SL and the T-tubules may share common characteristics. Only a few laboratories have attempted to distinguish T-tubule membranes and SL membranes isolated from the same tissue (Smith and Appel, 1977; Barchi *et al.*, 1977, 1979; Sumnicht and Sabbadini, 1982; Sabbadini and Okamoto, 1983; Moczydlowski and Latorre, 1983; Fosset *et al.*, 1983; Brandt, 1985; Doyle *et al.*, 1986). With regard to skeletal muscle membranes, we now know that SL and T-tubules can be distinguished by the absence and presence of DHP receptors, respectively (Fosset *et al.*, 1983), but this was generally known only after most of the studies listed immediately above were performed. From two of the more recent studies (Brandt, 1985; Doyle *et al.*, 1986) on cardiac tissue, we know that cardiac SL displays higher stoichiometries of DHP binding than are found in the cardiac T-tubules. In fact, cardiac SL can be distinguished from cardiac T-tubules not based on the absence of DHP binding as is the case for skeletal muscle SL, but, conversely, based on a higher DHP/STX ratio for the SL when compared with the T-tubules (Doyle *et al.*, 1986).

Other attempts have been made to distinguish T-tubules from SL. For example, Hidalgo (Hidalgo, 1986; Hidalgo *et al.*, 1986*b*) suggests that T-tubules differ from SL by the lower (Na^+, K^+)-ATPase/cholesterol ratios of the T-tubules. Hidalgo calculated that the ratio is < 55 for the T-tubules and that the (Na^+, K^+)-ATPase/cholesterol ratio is commonly > 55 for the SL when expressed in μmol ATP hydrolyzed/μmol cholesterol/mg. Based on the *in situ* binding studies of Jaimovich *et al.* (1976) and Venosa and Horowicz (1981), Moczydlowski and Latorre (1983) have argued that skeletal T-tubules should display higher ratios of ouabain/STX binding. Using this assumption, these workers have estimated that their frog (but not rabbit) T-tubules were heavily (30%) contaminated with SL. In other studies, Brandt (1985) suggests that the ratios of DHP to muscarinic receptors is greater than sixfold higher in SL when compared with T-tubules; if this ratio is to be used as a criterion, however, it would only apply to cardiac membranes since the cardiac, but not skeletal muscle SL displays sixfold higher DHP-binding stoichiometries (Brandt, 1985).

In our laboratory, we have observed that T-tubules display freeze-fracture particle densities that are distinctly different from that of the SL (Scales and Sabbadini, 1979; also see Table I). We have also noted that crude preparations of SL could be distinguished from the T-tubules (and SR as well) by differences in the relative distributions of various cation-stimulated ATPases (Sabbadini and Okamoto, 1983) and the higher cholesterol content

of the T-tubule membranes (Sumnicht and Sabbadini, 1982). Using the criteria of Hidalgo (1986), our chicken skeletal muscle membranes display (Na^+, K^+)-ATPase/cholesterol ratios of 27.9 for the T-tubules compared with 124 for the SL (Table I). The T-tubules were also distinguished from the SL by the tenfold higher Mg^{2+}-ATPase rates found for T-tubules (Sabbadini and Okamoto, 1983). The low levels of Mg^{2+}-ATPase activity often seen in skeletal SL preparations (Seiler and Fleischer, 1982, for example) might be due to T-tubule contamination; however, rigorous studies addressing this point have not been undertaken and it would be prudent to include DHP-binding measurements in future studies working with skeletal SL. It is worthy to note, however, that, in our hands, the SL Mg^{2+}-ATPase displays all of the characteristics (vanadate insensitivity, lectin stimulation, temperature dependence, etc.) of the T-tubule enzyme (see the next section).

Consequently, it may be argued that the vanadate-insensitive Mg^{2+}-ATPase can be used as a T-tubule marker in conjunction with other criteria such as a high DHP receptor density, a high cholesterol content, and low (Na^+, K^+)-ATPase/cholesterol ratio as well as the low density of 9-nm freeze-fracture particles seen in electron micrographs. Further, highly purified T-tubules should not display SR-like Ca^{2+}-ATPase or *p*-nitrophenylphosphatase activity, and the prominent Mg^{2+}-ATPase should be resistent to vanadate, ouabain, azide, and oligomycin inhibition.

The T-tubule Mg^{2+}-ATPase

One of the most prominent enzymatic activities associated with highly purified T-tubule vesicles is a calcium- or magnesium-stimulated ATPase (Ca^{2+} or Mg^{2+}-ATPase, EC 3.6.1.3), often simply referred to as the Mg^{2+}-ATPase. This enzyme is unique to the T-tubule membrane and possesses a number of physical, enzymatic, kinetic, and thermodynamic properties that easily distinguish it from other major divalent-cation-dependent ATPases present in the SR and the SL. This section of the review focuses upon its properties, its cytological localization, and its relationship to other membrane ATPases as well as important aspects bearing upon its structure and function. The major points to be stressed are (1) that the Mg^{2+}-ATPase is a specific component of the skeletal muscle T-system and is not a component of the SR, nor is it a prominent component of the SL, and that, as a result, under appropriate conditions, the Mg^{2+}-ATPase can be used as a membrane marker for the T-tubules; (2) that the Mg^{2+}-ATPase may be exclusively localized at junctional regions and may play an important role in contraction–relaxation; and (3) that the unusual properties of the Mg^{2+}-ATPase

reflect an elaborate mechanism designed to regulate microenvironmental, intrajunctional, sarcoplasmic ATP levels.

Headon *et al.* (1977) were the first to propose that the "basal" Mg^{2+}-ATPase may be associated with cholesterol-rich membranes that were enriched in T-tubules, and that the Mg^{2+}-ATPase might, in turn, provide a convenient biochemical marker in monitoring the distribution of T-tubules during membrane purification. In their studies employing continuous sucrose gradients, the distribution of SR-derived Ca^{2+}-dependent ATPase activity was shown to be different from the distribution of low-density microsomes (now known to be T-tubules) that were also substantially enriched in cholesterol and contained "basal" Mg^{2+}-ATPase activity and 5′-nucleotidase activity (Flaherty *et al.*, 1975; Headon *et al.*, 1977; Burnett *et al.*, 1980). Subsequently, studies by Malouf and Meissner (1979) showed that low-density microsomes were virtually devoid of Ca^{2+}-ATPase and exhibited high levels of "basic" Mg^{2+}-ATPase; the latter were cytochemically identified with the T-system and the SL. In determining the subcellular membrane distribution of the Mg^{2+}-ATPase activity, we noted that, while chicken T-tubule membranes contained high levels of Mg^{2+}-ATPase activity, only negligible activity was present in the SR or SL fractions (Sabbadini and Okamoto, 1983) (Table I). Moreover, rabbit SR membranes can be isolated that are devoid of Mg^{2+}-ATPase activity (Fernandez *et al.*, 1980). The relative absence of the Ca^{2+}-ATPase in T-tubule and SL fractions and its presence in highly purified SR fractions show that the active Ca^{2+}-ATPase is uniquely associated with the SR membrane system of skeletal muscle.

The predominence of the Mg^{2+}-ATPase activity in T-tubule preparations from a variety of tissue sources has led investigators to argue that the Mg^{2+}-ATPase is a T-tubule-specific enzyme (Hidalgo *et al.*, 1983; Beeler *et al.*, 1983; Sabbadini and Okamoto, 1983). However, the divergent results in Mg^{2+}-ATPase specific activity observed between different laboratories have caused some investigators to speculate that the Mg^{2+}-ATPase is inactivatible or extractable and therefore not a reliable index of the amount of T-tubule in a membrane fraction (Hidalgo, 1986; Mitchell *et al.*, 1983*c*).

Common Identity between the Mg^{2+}-ATPase and the "Basic" ATPase Observed in Crude SR Fractions

A Mg^{2+}-ATPase activity, variously referred to as "basic" ATPase, "basal" ATPase, Ca^{2+}-independent ATPase, or (Ca^{2+} or Mg^{2+})-ATPase (which in contrast to the SR Ca^{2+}-ATPase, is active in the presence of Mg^{2+}, even when the pCa^{2+} is lowered to < 8 by the use of EGTA) has been known to be present in skeletal muscle mixed microsomal preparations that were predominantly composed of vesicularly fragmented SR (Hasselbach and

Makinose, 1961, 1963; Hasselbach, 1964; Makinose and Hasselbach, 1965; Makinose and The, 1965; Engel and Tice, 1966; Weber *et al.*, 1966; Inesi *et al.*, 1967; Duggan, 1968). This enzyme activity exhibited properties profoundly different than those of SR Ca^{2+}-ATPase: a relatively high K_m for ATP, a low nucleotide specificity, lack of inhibition by ADP, relative insensitivity to *N*-ethylmaleimide (NEM), and sensitivity to several detergents, including Triton X-100 (Hasselbach, 1974; Fernandez *et al.*, 1980). For the purposes of this section, the term "basic" Mg^{2+}-ATPase will be used to designate the Mg^{2+}-ATPase of nonmitochondrial origin that is found in crude SR microsomal fractions.

For a number of years after its detection, the origin of the "basic" activity remained obscure; it was thought in some circles to be derived from the SR and possibly interconvertible with the SR Ca^{2+}-ATPase (Inesi *et al.*, 1976; Heilman *et al.*, 1977). In recent years, it has been become increasingly clear that most, if not all, of the "basal" ATPase of mixed microsomal SR preparations arises from coexisting fragments of the T-system, the T-tubule vesicles. Several studies showed that standard SR preparations are contaminated with T-tubule membrane vesicles (Scales and Sabbadini, 1979; Scales, 1981; Salviati *et al.*, 1982). Additionally, highly purified SR preparations could be prepared that were fully devoid of the "basic" Mg^{2+}-ATPase (Fernandez *et al.*, 1980; Okamoto and Sabbadini, 1983). Further, the cytochemical, immunochemical, and immunoelectron-microscopic (immuno-EM) data show the absence of a Mg^{2+}-ATPase associated with the SR, supporting the conclusion that the SR-derived "basic" Mg^{2+}-ATPase represents contamination of the SR microsomal fraction with T-system-derived membrane vesicles (Malouf and Meissner, 1979, 1980, 1984; Damiani *et al.*, 1987*b*).

On the basis of comparative nucleotide and divalent cation specificities (Makinose and The, 1965; Weber *et al.*, 1966; Inesi *et al.*, 1967, 1976; Duggan, 1968; Hidalgo *et al.*, (1983), thermodynamic and kinetic properties (Inesi *et al.*, 1976; Hidalgo *et al.*, 1983), inhibitor specificities (Hasselbach and Makinose, 1961; Hasselbach and Seraydarian, 1966; Inesi *et al.*, 1967; Duggan, 1968; Walter and Hasselbach, 1973; Malouf and Meissner, 1979; Fernandez *et al.*, 1980; Okamoto and Sabbadini, 1983), and detergent responses (Yamamoto and Tonomura, 1967; McFarland and Inesi, 1970; Hasselbach, 1974; MacLennan and Holland, 1975; Inesi *et al.*, 1976; Martonosi, 1984), there is a nearly absolute correspondence between the properties of, and a common identity between, the SR fraction-derived "basic" Mg^{2+}-ATPase and those of the T-tubule-derived Mg^{2+}-ATPase.

Enzyme Localization

Cytochemical and immuno-EM analyses of the distribution of the Mg^{2+}-ATPase show exclusive localization of the Mg^{2+}-ATPase in the T-system.

Using a rabbit antichicken Mg^{2+}-ATPase IgG (Damiani *et al.*, 1987*b*), we have also employed immunocytochemical and immuno-EM approaches to determine the subcellular distribution of the Mg^{2+}-ATPase. Importantly, the antibody both inhibits Mg^{2+}-ATPase and reduces the extent of Con-A stimulation. Localization of antibody in longitudinal crosssections of breast muscle was determined by indirect immunofluorescence, and antibody was found to be restricted to I-band regions (where the T-tubules are located) with no labeling of the SR-rich A-band regions (Damiani *et al.*, 1987*b*). Insignificant labeling of the SL occurred, in contrast to the cytochemical reaction product distribution results of Malouf and Meissner (1979, 1980, 1984), who showed some Mg^{2+}-ATPase activity also associated with the SL cytoplasmic surface. The discontinuous and punctate distribution of the label suggests that the Mg^{2+}-ATPase was not uniformly distributed along the tortuous T-tubular system.

In electron micrographs, indirect immunoferritin labeling with anti-Mg^{2+}-ATPase IgG was concentrated in the intermyofibrillar regions of I-bands and was prominently associated with T-tubule-containing triads (Damiani *et al.*, 1987*b*; see also Fig. 1). Neither junctional nor nonjunctional

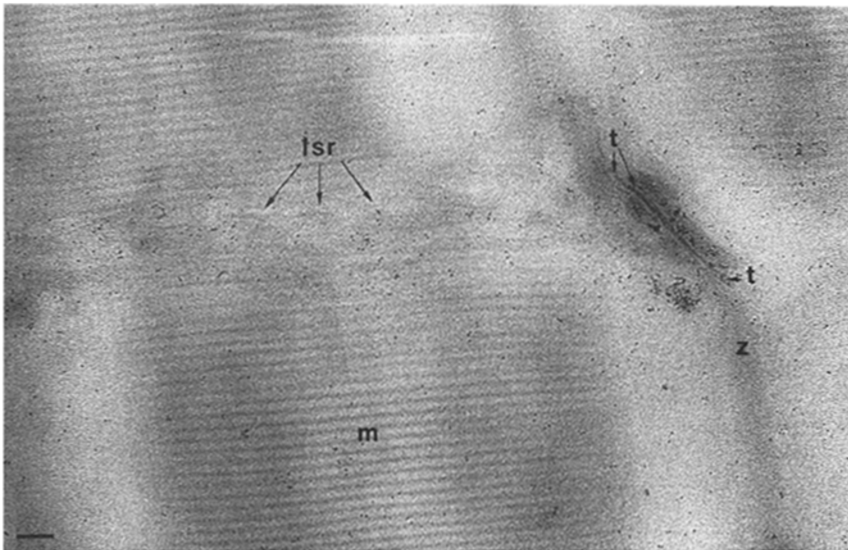

Fig. 1. Electron micrograph of a longitudinal muscle cell cryosection, absorption-stained with 0.2% uranyl acetate and indirectly ferritin-labeled with a rabbit antichicken IgG developed against the T-tubule Mg^{2+}-ATPase. This IgG does not recognize SR proteins in Western immunoblots or in ELISA assays (Damiani *et al.*, 1987*b*). The exposed region of fenestrated, longitudinal SR (lsr) between two myofibrils is devoid of significant label, while the ferritin-labeled anti-Mg^{2+}-ATPase IgG particles are more heavily concentrated near the tangentially sectioned T-tubules (t) of the triad junction. Bar, 0.1 μm.

elements of the SR were appreciably labeled with the anti-Mg^{2+}-ATPase IgG, in substantial contrast to results obtained with anti-Ca^{2+}-ATPase IgG (Jorgensen *et al.*, 1982). The data have provided additional morphological evidence for the T-tubule membrane localization of the Mg^{2+}-ATPase and the first demonstration that the Mg^{2+}-ATPase is highly concentrated in junctional or perijunctional T-tubules near where the T-tubule and the SR form triadic complexes. While the function of the Mg^{2+}-ATPase has not been firmly established (see below), its localization near junctional regions plus its high catalytic rate as well as other unusual features indicate that the Mg^{2+}-ATPase may play an important role in contraction–relaxation.

Ionic Requirements and Substrate Specificity of the Mg^{2+}-ATPase

The Mg^{2+}-ATPase requires either Mg^{2+} or Ca^{2+} as a cosubstrate at millemolar concentrations; the high Ca^{2+} concentrations required for expression of hydrolytic activity are nonphysiological, and it is clear that, under these conditions, Ca^{2+} is merely serving as a Mg^{2+} congener, particularly since Ca^{2+} cannot stimulate the presence of Mg^{2+} (Sabbadini and Okamoto, 1983). Free Mg^{2+} or Ca^{2+} (10 mM) in 33-fold excess over nucleotide is not inhibitory toward the rabbit or chicken enzyme. The divalent cation specificities of Mg^{2+}-ATPase from several sources have been determined (Malouf and Meissner, 1979, 1980; Beeler *et al.*, 1985). In general, the enzyme is activated by $Mg^{2+} > Ca^{2+} > Mn^{2+}$. Beyond an appreciable ionic strength effect, the enzyme from a variety of sources is not responsive to Na^+ or K^+, either as chlorides, sulfates, or nitrates, and is unaffected by P_i up to 300 mM.

One of the early-recognized characteristic features of the Mg^{2+}-ATPase is its broad substrate specificity with preferential activity toward MgATP (Malouf and Meissner, 1979). The apparent K_M's at 25°C of the rabbit enzyme for MgATP and CaATP have been determined to be 170 and 180 μm, respectively, which were changed to 220 and 340 μM after solubilization with LPC (Hidalgo *et al.*, 1983). The K_M's of rat enzyme for MgATP were 200 and 340 μM. in the absence and presence of lectins, respectively, at an unspecified temperature (Beeler *et al.*, 1983). The apparent K_M of the chicken enzyme for MgATP at 37°C was found to be 14 μM, which was elevated 12-fold to 166 μM in the presence of activating Con-A (Moulton *et al.*, 1986); these data are relative to the apparent K_M's for MgATP and MgITP of 25 and 300 μM, respectively, previously determined by Meissner for the chicken enzyme at 25°C (Malouf and Meissner, 1979). The reason for the 10 to 25-fold higher affinity of the chicken enzyme for MgATP compared with the rat and rabbit enzymes is not understood, but several proposals are made in a later section that address the issue.

The relative Mg^{2+}-ATPase V_{max}'s of native T-tubule preparations have been tabulated recently (Mitchell *et al.*, 1983*c*; Hidalgo, 1986; Hidalgo *et al.*, 1986*a*; Horgan and Kuypers, 1987). In the absence of external agents, the native rates range from 0.052 to 12.7 U/mg. Relevant to a later section, in the presence of lectins, the chicken enzyme can be activated six- to eightfold at 37°C and even greater at higher temperatures, resulting in activities of ~17 U/mg (Moulton *et al.*, 1986). All workers reporting high-specific-activity Mg^{2+}-ATPase in native vesicles have employed isolation procedures that are presumed to isolate nonjunctional T-tubule membrane vesicles, while those who have used a technique that selects T-tubule vesicles subsequent to disruption of isolated junctional complexes have reported unusually low specific activities. These observations suggest that (1) activity has been lost due to the physical treatment [e.g., triad-disrupted SR no longer transports Ca^{2+} (Brandt *et al.*, 1980)], or (2) there is a structural as well as a functional heterogeneity between *in situ* "free" and junctional T-tubules reflected in different levels, or turnover numbers, of the Mg^{2+}-ATPase in the two locales.

The inability of the T-tubule Mg^{2+}-ATPase to hydrolyze alternative substrates or pseudosubstrates offers an additional approach in distinguishing the Mg^{2+}-ATPase from other membrane ATPases. The SR Ca^{2+}-ATPase and (Na^+, K^+)-ATPase hydrolyze a variety of organophosphorous compounds (Hasselbach, 1974; Shaffer *et al.*, 1977; Inesi, 1984; Martonosi, 1984). The Mg^{2+}-ATPase from chicken, rat, and rabbit do not exhibit detectable *p*-nitrophenylphosphatase activity ($< 1\%$ of the ATP hydrolytic rate) (Malouf and Meeissner, 1979; Hidalgo *et al.*, 1983; Beeler *et al.*, 1985; Norton *et al.*, 1986). In our laboratories, with highly purified chicken T-tubule vesicles free of any Ca^{2+}-ATPase, the rate of *p*-nitrophenylphosphatase was found to be $< 10^{-5}$ of the ATPase rate (Table I); as seen below, the chicken enzyme apparently does not catalyze any other partial reaction. Hydrolysis of phosphorylated compounds by T-tubule membrane vesicles has been reported by others, but, in light of more recent studies, it is apparent that these fractions were substantially contaminated by SL- or SR-derived membranes (as judged by the presence of Ca^{2+}-ATPase) (Brandt *et al.*, 1980; Horgan and Kuypers, 1987).

Kinetic Variations of T-Tubule Mg^{2+}-ATPases

The T-tubule Mg^{2+}-ATPases exhibit several unusual kinetic phenomena that support the proposed existence of at least one separate nucleoside triphosphate-binding site in addition to the catalytic site. The function of this site is closely associated with the ability of the Mg^{2+}-ATPase to respond to lectins and putative lipid regulatory agents (q.v., below). Rat T-tubule membrane vesicle Mg^{2+}-ATPase exhibits prominent nonlinear reaction kinetics,

which has been interpreted to be due to an ATP-dependent inactivation, an effect mimicked by AMPPNP (Beeler *et al.*, 1983, 1985). This slow inactivation could be retarded up to 600-fold by pretreatment with lectins, glutaraldehyde, or rabbit anti-T-tubule IgG; reversal of inactivation by addition of the above reagents after ATP was not seen (Beeler *et al.*, 1983, 1985). Significantly, although lectins protected against ATP-mediated inactivation, when they were added prior to ATP at a ratio of 25 to 35 : 1 over T-tubule protein, a net inhibition of the rat ATPase was observed, ranging from 23 to 33% (Beeler *et al.*, 1983). The apparent K_d for AMPPNP-induced inactivation was close to that of ATP (260 μM) (Beeler *et al.*, 1985); the energy of activation for ATP-dependent inactivation was 14.4 kcal/mol (Beeler *et al.*, 1983). The frog enzyme has also been reported to exhibit nonlinear kinetics, but this enzyme is less well studied (Hidalgo *et al.*, 1986*a*).

The chicken and rabbit enzymes do not exhibit this type of slow, ATP-dependent inactivation, and, in the case of the chicken Mg^{2+}-ATPase, only linear reaction kinetics are seen; more importantly, the chicken Mg^{2+}-ATPase manifests a highly unusual, complex dependency upon MgATP (Moulton *et al.*, 1986). In contrast to the apparent simple Michaelis–Menton kinetics observed with several other T-tubule Mg^{2+}-ATPases (Beeler *et al.*, 1983; Hidalgo, 1983), the chicken enzyme manifests highly unusual complexity reversals in double reciprocal space with a combination of both negative cooperativity and substrate inhibition (Moulton *et al.*, 1986). The apparent affinity of the hydrolytic site for MgATP (14 μM) was far higher than that for the substrate inhibition site (10.2 mM) (Moulton *et al.*, 1986). It has been estimated that a minimum of 22% of all known enzymes deviate from simple Michaelis–Menton kinetics with 54% of these enzymes exhibiting negative, positive, or mixed cooperativity and with the remaining 46% exhibiting substrate inhibition (Hill *et al.*, 1977; Neet, 1980). Combined negative cooperativity and substrate inhibition is a most unusual phenomenon that can be rationalized from the most simple perspective by a mechanism in which one monomeric enzyme molecule with one active site and one major conformational change cycles catalytically (the homotropic substrate modifier mechanism), a representation of which is seen in Figure 2 (Bardsley *et al.*, 1980; Moulton *et al.*, 1986); in this case, in addition to the substrate catalytic site, another binding site exists for the substrate, and, when this site is occupied, catalytic features of the enzyme are altered with regard to substrate binding or product release, leading to a variety of phenomena, including substrate inhibition or activation and the formation of abortive, dead-end complexes (Bardsley, 1977; Bardsley *et al.*, 1980; Moulton *et al.*, 1986). Although the kinetic data manifested by the chicken enzyme are reflective of a homotropic substrate modifier mechanism, they do not exclude the existence of more elaborate mechanisms involving either a homooligomeric

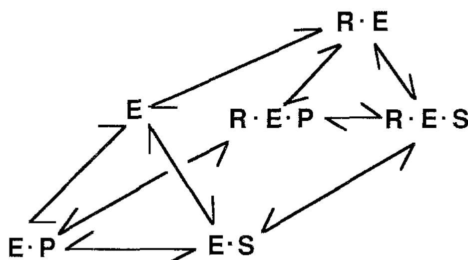

Fig. 2. The homotropic substrate modifier mechanism. Combined substrate inhibition and negative cooperativity can occur through the indicated mechanism. The catalytic site can cycle through either the front or back triangular faces, depending upon the presence of the regulatory ligand R at a binding site distinct from the catalytic site. When R is MgATP, both substrate inhibition and negative cooperativity can be produced with those MgATP-dependent enzymes exhibiting this mechanism. In the simplest case, a single polypeptide may contain both sites; the catalytic and regulatory sites could also exist on separate polypeptides within a heterooligomeric Mg^{2+}-ATPase complex. In the case of the T-tubule Mg^{2+}-ATPase, R can also represent another ligand, including Triton and regulatory lipids.

or a heterooligomeric enzyme. As described below, the ligand R in Figure 2 may also represent high-affinity regulatory ligands other than low-affinity nucleoside triphosphates. Importantly, the slow, nucleotide-dependent inactivation of the rat T-tubule Mg^{2+}-ATPase may be a direct analogue of the substrate inhibition effects observed with the chicken enzyme, the differences being ascribed to rate effects associated with conformational changes related to the occupancy of the putative regulatory (inhibitory) site.

Inability of the Chicken Mg^{2+}-ATPase to Catalyze partial Reactions

The comparative evaluation of membrane ATPases by assessment of their abilities to hydrolyze alternative, unnatural, nonnucleotide substrates is directly complemented by probing of their abilities to catalyze "partial" exchange reactions; the latter reactions are normally examined by means of isotropic probes at equilibrium that can involve both radioactive and non-radioactive isotopes. ^{18}O exchanges, in particular, can provide vital information on many aspects of catalysis and energy coupling, including assessment of discrete sections of the overall catalytic sequence of the ATP hydrolysis reaction, of subunit interactions during catalysis, and of sidedness (Boyer, 1967, 1977; Mitchell, 1984). Significantly, a number of ion-translocating ATPases with M_r 100,000 catalytic units, the E_1E_2 or "P"-type enzymes (Pedersen and Carafoli, 1986), including the (Na^+, K^+)-ATPase and the SR Ca^{2+}-ATPase, have been demonstrated to catalyze a rapid exchange of the oxygens of P_i by reversal of E-P formation at the level of P_i (Dahms and Miara, 1983), and these exchange rates often exceed the ATP hydrolytic rate by 20 to 50-fold, thus providing a potent probe of a number of

Table II. Properties of Chicken Muscle Membrane ATPases

	Mg^{2+}-ATPase	Ca^{2+}-ATPase	(Na^+, K^+)-ATPase
Inhibition by			
Oligomycin (50 μg/ml)	−	−	+
Azide (5 mM)	−	−	−
Ouabain (1 mM)	−	−	+
Vanadate (50 μM)	−	+	+
NBD-F (0.1 mM, 3 min, 25°C)	+	+	+
NBD-F (0.1 mM, 3 min, 25°C) + Con-A (10 μg/μg)	−	+	+
Fluorosulfonylbenzoyl adenosine (500 μM, 5 min, 25°C)	−	−	+
Fluorescein isothiocyanate (5 μM, 15 min, 25°C)	−	+	+
Tetraiodofluorescein isothiocyanate (5 μM, 15 min, 25°C)	−	+	+
Quercetin (50 μM, 25°C)	+	+	+
N-Ethylmaleimide (5 mM, 15 min, 25°C)	−	+	+
ADP (5 mM, 25°C)	−	+	+
P_i (50 mM, 25°C)	−	+	+
Other properties			
Con-A activation (3 μg/μg T-tubule vesicle, 25°C)	+	−	−
p-Nitrophenylphosphatase (5 mM, 25°C)	−	+	+
$P_i \leftrightarrow$ ATP exchange	−	+	−
ADP $\leftrightarrow$ ATP exchange	−	+	+

transport and enzymatic properties (Shaffer *et al.*, 1977). Oxygen exchange reactions can also be catalyzed by F_1F_0("F")-type H^+-ATPases of chloroplasts, mitochondria, and bacteria as well as by myosin and dynein-type ATPases through a bound ATP molecule and without participation of a phosphoenzyme.

In marked contrast to the E_1E_2 or "P"-type, the vacuolar or "V"-type, or the F_1F_0 or "F"-type ATPases, the T-tubule Mg^{2+}-ATPase in highly purified chicken T-tubule membrane vesicles not only is incapable of catalyzing the hydrolysis of alternate substrates and of mediating $P_i \leftrightarrow$ ATP and ADP $\leftrightarrow$ ATP exchanges [$< 10^{-4}$ of ATP hydrolytic rates (Norton *et al.*, 1986) and Table II], but the Mg^{2+}-ATPase is incapable of mediating a medium ^{18}O (water–phosphate, $P_i \leftrightarrow$ HOH) exchange (Table I). The data show the presence of a very low level $P_i \leftrightarrow$ HOH exchange (< 0.05 the rate of ATP hydrolysis) that is unaffected by 100 μM vanadate, 50 μg/ml oligomycin, 1 mM fluorescein isothiocyanate (FITC), 50 μM free Ca^{2+}, 1 mM ouabain, Con-A (10 μg/μg protein), 7-fluoro-4-nitrobenz-2-oxa-1,3-diazole (NBD-F) (500 μM), incorporation of ATP, ADP, GTP, or GDP at substrate

levels, or by raising P_i to 200 mM. The exchange activity that survives the above inhibitors is clearly unrelated to the Mg^{2+}-ATPase; the residual exchange could arise from minor levels of several other soluble or membrane-bound phosphotransferases known to mediate low-level $P_i \leftrightarrow$ HOH exchange.

The enzymatic hydrolysis of ATP by the Mg^{2+}-ATPase appears to be fully irreversible at all stages of the reaction (microscopic reversibility, of course, must apply, but it is not detectable under the conditions employed). Clearly, there is little evidence for the existence of a T-tubule Mg^{2+}-ATPase phosphoenzyme derived from the level of P_i, based upon the absence of any P_i product inhibition and the lack of medium $P_i \leftrightarrow$ HOH and $P_i \leftrightarrow$ ATP exchanges, in marked contrast to classic E_1E_2-type ion-translocating ATPases. The absence of $P_i \leftrightarrow$ HOH, ADP $\leftrightarrow$ ATP, and $P_i \leftrightarrow$ ATP exchanges catalyzed by the chicken T-tubule Mg^{2+}-ATPase also distinguish the Mg^{2+}-ATPase from the (Na^+, K^+)-ATPase and the SR Ca^{2+}-ATPase from the same tissue (Table II) (Dahms and Miara, 1983). Even though the lack of vanadate sensitivity (q.v., below) implicates the absence of an E-P in the catalytic process, an unsuccessful search for a T-tubule Mg^{2+}-ATPase E-P from ATP has been conducted by several laboratories; one study in particular showed that Ca^{2+}- and ATP-dependent E-P was < 0.05 pmol/mg vesicle protein (Hidalgo, 1983); E-P formation under other conditions has not been assessed, except in a recent study by Horgan and Kuypers (1987), who have demonstrated that the M_r 104,000 component of detergent-extracted rabbit T-tubules was phosphorylated under (Na^+, K^+)-ATPase E-P formation conditions.

Effects of Inhibitors

The insensitivity of the T-tubule Mg^{2+}-ATPase from a variety of sources to a number of inhibitors known to affect other ATPases allows the Mg^{2+}-ATPase to be easily discriminated from other integral membrane enzymes such as the SR Ca^{2+}-ATPase and the (Na^+, K^+)-ATPase. The T-tubule Mg^{2+}-ATPase is not affected by aurovertin, rutamycin, oligomycin, arsenate, azide, cyanide, NEM, phenymethanesulfonyl fluoride, 5,5′-dithio-*bis*-(2-nitrobenzoic acid), mersalyl, vanadate, AMP, ADP, and P_i (Malouf and Meissner, 1979, 1980; Fernandez *et al.*, 1980; Rosemblatt *et al.*, 1981; Sabbadini and Okamoto, 1983; Moulton *et al.*, 1986). One of the most striking properties of the Mg^{2+}-ATPase is its lack of vanadate inhibition (Sabbadini and Okamoto, 1983); this is in contrast to prominent inhibition by vanadate of the E_1E_2 ion-translocating ATPases.

The enzyme is insensitive to NEM up to 20 mM under conditions that fully inhibit the SR Ca^{2+}-ATPase (Fernandez *et al.*, 1980; Malouf and Meissner, 1979). The enzyme is sensitive to *p*-hydroxymercuribenzoate

(Malouf and Meissner, 1979). The enzyme is relatively insensitive to aldehyde fixatives, in marked contrast to the effect on the SR Ca^{2+}-ATPase and the (Na^+, K^+)-ATPase (Malouf and Meissner, 1984); glutaraldehyde has been found to produce an intramolecular cross-linking of components in the active site of the Ca^{2+}-ATPase (Ross and McIntosh, 1987). The sensitivity of the SR Ca-ATPase to low Pb^{2+} and aldehyde fixatives and the relative insensitivity of the T-tubule Mg^{2+}-ATPase to the same reagents have enabled the facile definition of the separate subcellular localizations of the two enzymes. The rabbit enzyme is susceptible to the histidyl-selective reagent diethylpyrocarbonate (Kirley, 1988), a known histidyl-specific modifier of both SR Ca^{2+}-ATPase and (Na^+, K^+)-ATPase (Dahms and Miara, 1985).

A variety of nucleotide analogues have been employed in attempting to probe structural and functional aspects of the Mg^{2+}-ATPase. The chicken enzyme is irreversibly inhibited by NBD-Cl or NBD-F through modification of either a tyrosyl or lysyl moiety (Norton *et al.*, 1986; also see Table II); the inactivation kinetics are consistent with at least two residues participating in the inactivation; NBD-F modification is apparently occurring at the proposed regulatory site (Fig. 2) since catalytic site levels (10–500 μM) of adenine nucleotides and nucleosides do not protect, whereas Con-A fully protects against inactivation (Table II). The chicken enzyme is not susceptible to chemical modification by fluorosulfonylbenzoyladenosine [under conditions that markedly inhibit control enzymes (Table II)] or inhibition by $(A_p)_5A$ (Sabbadini and Okamoto, 1983). The rat enzyme is susceptible to irreversible photoinactivation in the presence of 8-azido-ATP (Beeler *et al.*, 1985).

In marked contrast to the (Na^+, K^+)-ATPase and Ca^{2+}-ATPase, the Mg^{2+}-ATPase is not inhibited by FITC in the sub-100 μM concentration range under conditions that profoundly inhibit the two former enzymes (Table II). Tetraiodofluorescein has been shown to be a potent nucleotide-mimetic inhibitor of a number of (di)nucleotide-dependent enzymes (Neslund *et al.*, 1984); although tetraiodofluorescein is a potent inhibitor of the Mg^{2+}-ATPase (K_i = 0.3 μM) and is competitive with MgATP, tetraiodofluorescein isothiocyanate under nearly saturing conditions does not covalently modify the enzyme, similar to the results seen with FITC; presumably this insensitivity is due to the absence of an appropriately positioned, reactive lysyl in the active center of the Mg^{2+}-ATPase (Norton *et al.*, 1986).

The Mg^{2+}-ATPase possesses a number of properties that easily distinguish it from the SL (Na^+, K^+)-ATPase and the SR Ca^{2+}-ATPases. These are summarized in Table II. With regard to the classification of ion-motive ATPases proposed by Pedersen and Carafoli (1986), the T-tubule Mg^{2+}-ATPase is clearly not of the E_1E_2 ("P") type due to its lack of vanadate sensitivity and medium oxygen exchange, not of the "V" or vacuolar-type

due to its lack of NEM and KNO_3 sensitivity, and not of the "F" type due to its lack of oligomycin sensitivity.

Effects of Detergents and Potential Regulatory Lipophilic or Amphipathic Substances

The Mg^{2+}-ATPase can be inhibited or activated by a variety of lipophilic and amphipathic substances, some of which are native cellular components, at levels well below their critical micelle concentrations (CMC's). The effects of physiological concentrations of these substances upon the Mg^{2+}-ATPase strongly suggest the presence of a highly sophisticated regulatory mechanism for control of Mg^{2+}-ATPase-mediated intracellular ATP hydrolysis. As discussed below, the responses to these native cellular components and variations of their levels in preparations of isolated T-tubules may also help to explain the widely varying specific activities of T-tubule Mg^{2+}-ATPases observed in some laboratories.

A number of laboratories have reported that the Mg^{2+}-ATPase is particularly sensitive to detergent inhibition at levels that do not substantially affect the SR Ca^{2+}-ATPase or the (Na^+, K^+)-ATPase (Hasselbach, 1974; MacLennan and Holland, 1975; Malouf and Meissner, 1979; Fernandez *et al.*, 1980; Beeler *et al.*, 1983; Hidalgo *et al.*, 1983; Martonosi, 1984; Okamoto *et al.*, 1985). Detergent inhibition of the rat enzyme has been studied by Beeler *et al.* (1983, 1985); detergents increased the rate of ATP inactivation of the enzyme under assay conditions and, at higher concentrations, totally inhibited the rat enzyme; detergents did not influence the initial rate of MgATP hydrolysis. A similar detergent effect on the apparent rate of ATP-dependent inactivation has been reported for the frog enzyme, although the phenomenon is less well studied than in the rat system (Hidalgo *et al.*, 1986*a*). Hidalgo *et al.*, (1983) have reported detergent inhibition of the rabbit enzyme and, as a result of LPC's lack of inhibition in the CMC range, LPC was used in the isolation of purified fractions of the rabbit Mg^{2+}-ATPase (Hidalgo *et al.*, 1983; Horgan and Kuypers, 1988; Kirley, 1988).

The mode of action of Triton X-100 upon the chicken enzyme has been studied, and the results have afforded considerable insights into possible mechanisms of regulation of ATP hydrolytic activity (Okamoto *et al.*, 1985; Moulton *et al.*, 1986). Sub-CMC levels of the detergent inhibited the ATP hydrolysis by 90% and, significantly, prior treatment with Con-A or the dimeric succinyl-Con-A protected the chicken Mg^{2+}-ATPase against Triton X-100 inactivation simultaneously with the abolition of the enzyme's complex substrate dependency, with the elimination of the negative energy of activation above the transition temperature of 27°C, and with the shift of the

transition temperature to 19.3°C. The ability of Con-A to preserve hydrolytic capacity in the presence of very low, sub-CMC levels of Triton X-100 has suggested that the detergent might be operating through another mechanism unrelated to solubilization or native annular lipid replacement; it has been proposed that the detergent might be interacting with the putative regulatory site (Fig. 2) and acting as a very high affinity, substrate homotropic-modifier-mimetic agent, i.e., like substrate inhibitory ATP (Moulton *et al.*, 1986). From this perspective, Con-A would thus impede either the interaction of the detergent at the regulatory site or the transmission of the Triton-binding event to the catalytic site. The ability of an amphiphile such as Triton X-100 to mimic the effect of a nucleotide is not without precedent; Askari (Huang *et al.*, 1985) demonstrated that Triton X-100 occupation of a hydrophobic site on extramembranous segments of the (Na^+, K^+)-ATPase mimicked the effect of ATP at a "low-affinity ATP regulatory site." Con-A protection of Triton X-100 inhibition is complete, allowing solubilization of the enzyme in the Con-A-activated state.

Even though Triton can mimic nucleotide effects with these cation-dependent ATPases, neither ATP nor Triton is the physiological ligand for that putative regulatory site of the Mg^{2+}-ATPase. We have considered the possibility that such a site may have been designed to recognize regulatory agents with combined adenine nucleotide and amphipathic properties, in particular fatty acyl-CoA's (Moulton *et al.*, 1986). Rather unexpectedly, as seen in Table III, several fatty acyl-CoA's were found to serve as potent activators in the physiologically significant submicromolar and sub-CMC concentration range. In contrast to the activation with palmitoyl- and linoleoyl-CoA, Con-A modulatable inhibition by several free fatty acids was observed (Table III). Free CoASH does not affect the enzyme; extensive competition studies examining the mode of interaction of these amphipathic agents with the chicken enzyme and the regulatory site have not been completed. It is intriguing to consider the possibility that metabolic variations in sarcoplasmic fatty acyl-CoA/free fatty acid ratio could regulate the expression of the activity state of the Mg^{2+}-ATPase. Of relevance, Askari has also shown that several CoA derivatives activate the (Na^+, K^+)-ATPase through interaction at the Triton-responsive, low-affinity ATP regulatory site with affinities in the submicromolar range.

The high-affinity and hydrophobic nature of the putative regulatory site for acyl-CoA's, free fatty acids, and Triton has led us to explore the effects of other significant regulatory lipids. As seen also in Table III, the chicken enzyme is inhibited by diacylglycerol and phorbol esters in the micromolar range; the 4-alpha-phorbol ester is nearly as inhibitory as TPA, thus distinguishing the Mg^{2+}-ATPase site from the TPA- and diacylglycerol-responsive activation site on protein kinase C. Prior addition of Con-A substantially

Table III. Response of the Chicken T-Tubule Mg^{2+}-ATPase to Amphiphilic and Lipophilic Modifiers

Substance	Activator	Inhibitor	Concentration (μM) for half-maximal effect
Palmitoyl-CoA	+		1.0
Linoleoyl CoA	+		1.1
Arachidonoyl-CoA (10 μM)	0[a]	0	–
Palmitic acid		+	5.0
Linoleic acid		+	2.0
Arachidonic acid		+	1.5
CoASH (15 μM)	0	0	–
Palmitic acid + Con-A[a]		+	> 20
Linoleic acid + Con-A		+	> 20
Octanoyl acetyl glycerol		+	1.7
Octanoyl acetyl glycerol + Con-A		+	> 15
Phorbol 12-myristate 13-acetate		+	0.5
Phorbol 12-myristate 13-acetate + Con-A		+	> 15
4-alpha-phorbol 12,13-didecanoate		+	0.5
4-alpha-phorbol 12,13-didecanoate + Con-A		+	> 15

[a]0, no effect
[b]Con-A at 3 μg/μg T-tubule protein.

reduces the inhibition by the above agents. The ability of the Mg^{2+}-ATPase to respond to physiological levels of important amphiphilic metabolic regulators and indicators of energy charge suggests the existence of highly elaborate and yet undiscovered regulatory mechanisms controlling the expression of the activity of this enzyme (Moulton *et al.*, 1986). The relationship of these lipid modifiers to the potential function of the enzyme is covered in a later section. Although there is not solid data as of yet, the regulation of ATPase activity by these lipophilic and amphipathic agents may also be synergistically modulated on a higher level by interaction of the Mg^{2+}-ATPase with potential endogenous lectins.

Temperature and pH Effects

The Mg^{2+}-ATPase has been generally characterized by a broad pH optimum that maximizes at near 7 (Hidalgo *et al.*, 1983; Malouf and Meissner, 1979; Sabbadini and Okamoto, 1983), which contrasts with the narrow optimum exhibited by the SR Ca^{2+}-ATPase (Hasselbach, 1974; MacLennan and Holland, 1975). With MgATP as substrate, the rabbit T-tubule enzyme Arrhenius plot exhibits a break at 26.4°C; the E_{act}'s below and above the transition temperature are 12.7 kcal/mol and 4.2 kcal/mol, respectively (Hidalgo *et al.*, 1983). Over the 22–45°C range, the rat enzyme Arrhenius plot has an E_{act} of 4 kcal/mol (Beeler *et al.*, 1983).

With MgATP as a substrate, the chicken Mg^{2+}-ATPase exhibits a highly unusual temperature dependence (Sabbadini and Okamoto, 1983; Moulton *et al.*, 1986). The enzyme manifests an optimal activity at 27°C with 50% activity at both 10 and 37°C. The activity decrease in the 27–45°C range is not due to irreversible enzyme inactivation or denaturation, since the enzyme is stable at 45°C, pH 7.3, for over 1 h and since lowering the assay reaction temperature from 45 toward 25°C results in an increase in hydrolytic activity to expected control levels (Moulton *et al.*, 1986; Sabbadini and Okamoto, 1983). The bell-shaped temperature profile yields a concave-down Arrhenius plot with an E_{act} of 4.5 kcal/mol and -11.5 kcal/mol, respectively, below and above the very sharp transition temperature at 27°C (Moulton *et al.*, 1986). Of relevance to the proposed homotropic substrate-modifier mechanism, the concave-down Arrhenius plot can arise if the standard enthalpy is negative for the (inhibitory) modifier reaction and positive for the substrate reaction (Han, 1972; Moulton *et al.*, 1986). In the absence of lectin, the temperature response of the Mg^{2+}-ATPase in isolated T-tubules indicates that the enzyme would be $\sim$10% active at the core temperature of the chicken, 43°C. Importantly, pretreatment with Con-A at a three- to fivefold excess over T-tubule protein completely abolishes the unique temperature response seen in the absence of the lectin and yields a Q_{10} of 2.1; the E_{act}'s in the presence of Con-A are 14.3 kcal/mol and 4.3 kcal/mol, respectively, below and above the new transition temperature of 19.3°C, thermodynamic data not different from the data obtained by Hidalgo *et al.* (1983) for the rabbit enzyme. Importantly, the putative phase transition temperature at 19.3°C obtained in the presence of Con-A is not seen in the absence of Con-A. As discussed in further detail below, the data appear to suggest that Con-A in the chicken system plays the role of an endogenous activator or regulatory substance that is lost or displaced during the isolation of the chicken T-tubule vesicles. Due to the unique bell-shaped temperature response of the chicken enzyme, the extent of lectin activation of the enzyme is, of course, dependent upon the temperature of the assay, i.e., at temperatures in the 45–50°C area, lectin activation could approach several hundredfold (q.v., Moulton *et al.*, 1986, Fig. 8).

Lectin Modulation of the Mg^{2+}-ATPase

Lectins have been shown to affect the kinetic behavior of the rat and chicken Mg^{2+}-ATPases, in particular the latter (Beeler *et al.*, 1983, 1985; Okamoto *et al.*, 1985; Moulton *et al.*, 1986; Damiani *et al.*, 1987*b*). Tetrameric or dimeric Con-A or the dimeric WGA, but not peanut agglutinin or soybean lectin, (1) abolishes the complex, high-order non-Michaelis–Menton

response to MgATP with elimination of substrate inhibition and negative cooperativity, producing thereby a simple Michaelis–Menton response with 12-fold increase in K_M; (2) abolishes the bell-shaped temperature response curve and concave-down Arrhenius plot yielding a typical temperature response with a Q_{10} of 2.1 and a typical biphasic Arrhenius plot; (3) stabilizes the enzyme completely toward inactivation by detergents; (4) protects against NBD-F inactivation; and (5) modulates the inhibition produced by metabolically significant amphiphilic and lipophilic agents.

Importantly, Con-A modulation of plasma membrane ATPase activity is not unique to the T-system, and several mechanisms have been proposed to account for the phenomenon (reviewed in Moulton *et al.* 1986). ATPases have also been described that exhibit both positive and negative cooperative responses toward the lectin (Hamlyn and Senior, 1983; Moulton *et al.*, 1986). In the T-system, Con-A may be envisioned as (1) promoting or stabilizing the oligomeric state of the Mg^{2+}-ATPase, a proposal originally made by Beeler *et al.* (1983); (2) destabilizing the existence of a preexisting oligomer; (3) interfering with the state of or accessibility to potentially requisite glycolipid(s); or (4) influencing the state of general lipid fluidity (Beeler *et al.*, 1983). Kinetically speaking, it is not possible to distinguish between the first two alternatives; however, the observation by Beeler *et al.* (1983, 1985) that cross-linking agents such as glutaraldehyde act similarly to Con-A in impeding ATP-induced inactivation of the rat enzyme would support the first alternative. The activation of the chicken enzyme by Con-A proceeds with a Hill slope of $n = 1.8$ (Moulton, 1985). Mitochondrial Mg^{2+}-ATPase, chicken SR Ca^{2+}-ATPase, chicken SL (Na^+, K^+)-ATPase, canine renal (Na^+, K^+)-ATPase, or T-tubule preparation-containing (Na^+, K^+)-ATPase are not affected by WGA or Con-A.

Currently, neither the rabbit nor the rat enzyme appears to be activated by lectins; the rat enzyme is protected against substrate inactivation and, at high levels, is partially inhibited by lectins (Beeler *et al.*, 1983, 1985), and the rabbit enzyme is modulatable by lectins only after detergent solubilization (Kirley, 1988). Both the chicken and rat enzymes share the following properties: (1) prior addition of Con-A can block either nucleoside triphosphate effects of the regulatory site; and (2) Con-A cannot relieve the nucleotide-induced inhibition when added subsequently to nucleoside triphosphate.

The interesting Con-A-dependent conversion of the chicken Mg^{2+}-ATPase from a low-activity, high-affinity (K_M 14 μM), catalytically and thermodynamically bizarre behavioral state to a high-activity, low-affinity (K_M 166 μM), typical Michaelis–Menton behavioral state with thermodynamic properties indistinct from the native rabbit Mg^{2+}-ATPase strongly supports the contention that a regulatory substance has been depleted from,

or distorted in, isolated chicken T-tubules, a substance that is replaced by, or recoupled with, the lectin Con-A. This regulatory substance may persist within the rabbit Mg^{2+}-ATPase complex during the isolation of rabbit T-tubules, explaining the lack of lectin reactivity in the native state. It would be of interest to isolate the regulatory agent or endogenous lectin that Con-A mimics.

Interestingly, Con-A binding to rabbit triadic complexes also alters the Ca^{2+} dependence of Ca^{2+} release produced by choline chloride replacement of potassium gluconate, suggesting the involvement of neuraminidase-sensitive glycoprotein(s) in the T-tubule-mediated Ca^{2+}-release from terminal cisternae (Ikemoto *et al.*, 1986; Cifuentes *et al.*, 1987). Con-A potentiation of Ca^{2+} plus caffeine-induced Ca^{2+}-release also suggested that oligosaccharide moieties in the SR were also involved in the regulation of SR release (Ikemoto *et al.*, 1986). Attempts at photoaffinity Con-A labeling of T-tubule glycoproteins were not successful although several SR components (M_r ~300,000 and ~160,000) were labeled (Ikemoto *et al.*, 1986). Due to some of the peculiarities of photoaffinity-labeling reagents, the data do not rule out the participation of T-tubule Con-A recognition components in the above Ca^{2+}-release process. There may or may not be a link between this Con-A effect and the rabbit Mg^{2+}-ATPase, but, in the native state, Con-A does not modulate the rabbit T-tubule Mg^{2+}-ATPase (Kirley, 1988). It is, of course, significant that Con-A recognizes intracellular-facing glycoproteins in the triad. As a general rule, glycoproteins are extracellularly facing, but an intracellularly facing glycoprotein has been detected associated with the nuclear pore complex (Holt *et al.*, 1987; Snow *et al.*, 1987).

Is the T-Tubule Mg^{2+}-ATPase an Ectoenzyme?

Cells from a variety of tissues have been demonstrated to possess intrinsic plasma membrane proteins whose active centers are externally rather than internally located (DePierre and Karnovsky, 1973; Trams and Lauter, 1974; Pearson, 1985). These enzymes have been termed "ectoenzymes" (in contrast to the term "endoenzymes"). The function of ectoenzymes is poorly understood; generally speaking, ectoenzymes are low-abundance, low-specific-activity entities that act on extracellular substrates and release products extracellularly (Pearson, 1985). A subset of the ectoenzymes exists, comprised of the "ectophosphohydrolases," some of which are active upon ADP and/or ATP; these have been found associated with Erhlich ascites cells (Wallach and Ullrey, 1962; Ronquist and Agren, 1975), rat mammary gland cells (Carraway *et al.*, 1980), human platelets (Chambers *et al.*, 1967), rat pancreas and liver (Hamlyn and Senior, 1983; Lin and Russell, 1988), guinea pig leukocytes (DePierre and Karnovsky, 1974), human granulocytes

(Smolen and Weissmann, 1978), and several other cell lines (Trams and Lauter, 1974).

One of the more interesting ectophosphohydrolases is that of the rat pancreas plasma membrane, which displays an unusual, very high specific activity ADP(ATP)ase (7–35 U/mg) (Hamlyn and Senior, 1983). The enzyme is NEM, vanadate, oligomycin, ouabain, and azide insensitive and is inhibited by *p*-hydroxymercuribenzoate; Con-A stimulates by up to twofold (with a pronounced positive cooperativity, Hill slope $n = 1.8$) associated with elimination of nonlinear initial rates, but is without effect upon initial ATP hydrolytic rate, interestingly similar to the effects reported for the rat Mg^{2+}-ATPase by Beeler *et al.* (1983, 1985). Typically, "ecto-ATPases" are inhibited by NEM and ADP (Pearson, 1985), although there are exceptions as exemplified by the above enzyme, which is NEM insensitive and hydrolyzes ADP. The rat liver plasma membrane "ecto-ATP(ADP)ase" hydrolyses GDP and ADP, is insensitive to NBD-Cl and *p*-hydroxybenzoate, and possesses micromolar affinities for the divalent cations and an apparent M_r of 70,000 (Lin and Fain, 1984; Lin and Russell, 1988).

Since some of these characteristics, particularly the lack of vanadate sensitivity and the prominent Con-A reactivity, resemble those of the T-tubule Mg^{2+}-ATPase, it might be concluded that this enzyme is also an ectoenzyme (Beeler *et al.*, 1983). The T-tubule Mg^{2+}-ATPase does not appear to satisfy the ectoenzyme criteria of Pearson (1985) in that the T-tubule enzyme does not deposit the reaction products extracellularly, but rather intracellularly; using *in situ* ATPase reaction product cytochemical analysis, Meissner has shown that the chicken skeletal muscle and canine ventricular Mg^{2+}-ATPase possesses a hydrolytic site that is exposed to the sarcoplasm (Malouf and Meissner, 1979, 1980, 1984). The question of whether the Mg^{2}-ATPase catalyzes the hydrolysis of ADP, similar to the ecto-ATPases described above, has not been sufficiently addressed. Malouf and Meissner (1979) and Hidalgo *et al.* (1983) have reported that T-tubule fractions possess an Mg^{2+}-ADPase activity of about one-sixth the rate of Mg^{2+}-ATPase; whether the ADPase activity detected in crude T-tubule fractions is due to a contaminating enzyme remains to be established.

Also relative to sidedness, it has been argued that the rabbit T-tubule vesicles are 80–90% sealed with inside-out orientation (Hidalgo *et al.*, 1986*b*) and that the prominent Mg^{2+}-ATPase observed for the rabbit vesicles must result from ATP binding to the cytoplasmic surfaces of the T-tubule membranes; this may also explain why Con-A does not affect the ATPase activity of intact rabbit T-tubule vesicles (Moulton *et al.*, 1986), but can only affect ATPase activity of permeabilized or detergent-treated rabbit T-tubule preparations (Kirley, 1988) in which case Con-A has access to the luminal surface of the membrane. The ability of chicken and rat T-tubule vesicles to

interact with Con-A might be explained by assuming that Con-A binds to right-side-out (and leaky) vesicles, which are more numerous in the chicken and rat preparations. Until more detailed information is available on the integrity of T-tubule preparations, the topological sidedness of the active site(s) relative to Con-A-binding site(s), and the oligomeric nature of the Mg^{2+}-ATPase, the question of whether the Mg^{2+}-ATPase is an ectoenzyme must remain incompletely resolved. In a similar vein, we cannot at this time disregard the possibility that Con-A binds to the cytoplasmic surface along with ATP. As seen above, there is a precedent for lectin recognition of cytoplasmically orientated glycoprotein.

Purification and Compositional Analyses of the T-tubule Mg^{2+}-ATPase

There is considerable disagreement as to the molecular composition of the Mg^{2+}-ATPase; the specific assignment of an individual polypeptide with a catalytic component of the Mg^{2+}-ATPase is not possible at this time due to a variety of problems unique to the T-tubule Mg^{2+}-ATPase, including its detergent sensitivity, the apparent change in turnover number of the enzyme with purification, and the presence of contaminating membranes containing other more stable ATPases. Hidalgo *et al.* (1983) obtained a LPC-solubilized rabbit Mg^{2+}-ATPase preparation that was enriched three- to fivefold and contained 3–4 proteins with the two main components of M_r 107,000 and 30,000 comprising at least 80% of the protein pool, neither of which was stained by periodic acid–Schiff reagent. Using a similar approach Horgan and Kuypers (1987, 1988) recently obtained a LPC-purified rabbit Mg^{2+}-ATPase preparation that contained proteins of M_r ($\times 10^3$) 104, 70, and 30 species (16, 16, and 31% of the protein pool, respectively) and claimed that the Mg^{2+}-ATPase activity was only associated with the M_r 30,000 polypeptide; additionally, SDS extraction resulted in a further enrichment of the M_r 104,000 and 30,000 proteins (28% each in the final pool). Since the M_r 104,000 protein was phosphorylated under (Na^+, K^+)-ATPase phosphorylation conditions, the M_r 104,000 protein was likely the alpha subunit of the (Na^+, K^+)-ATPase; the M_r 30,000 protein was not reactive with Stains-All [similar to the report by Hidalgo *et al.* (1983), who employed periodic acid–Schiff reagent], indicating that this protein was not the beta subunitit of the (Na^+, K^+)-ATPase (Horgan and Kuypers, 1988). Although Hidalgo *et al.* (1983) reported that the E-P level under SR Ca^{2+}-ATPase phosphorylation conditions was below the level of detection, they did not report whether they examined E-P levels under the sodium pump phosphorylation conditions with their purified Mg^{2+}-ATPase preparation.

Using LPC and digitonin, lectin-affinity and ion-exchange chromatographic separation, and gel electroelution, Kirley (1988) recently reported

that a M_r 105,000 glycoprotein species may comprise the only polypeptide associated with Mg^{2+}-ATPase-mediated hydrolytic capacity. These data confirm our earlier findings (Okamoto *et al.*, 1985; Moulton *et al.*, 1986; Damiani *et al.*, 1987*a*) showing that the chicken T-tubule Mg^{2+}-ATPase copurified with a Con-A reactive moiety at M_r ~ 100,000 and also that the Mg^{2+}-ATPase is strongly modulated by lectins, but these data also contrast with the claims of Hidalgo *et al.* (1983) and Horgan and Kuypers (1988) that a M_r 30,000 polypeptide is also associated with or identical to the Mg^{2+}-ATPase. Kirley (1988) also presented data suggesting that the rabbit Mg^{2+}-ATPase is not a major integral membrane component and that it was not structurally related to the SR Ca^{2+}-ATPase, as recently claimed by us (Damiani *et al.*, 1987*a*).

A common Con-A- and WGA-reactive protein is seen on Western-type lectin blots of SDS-PAGE gels of the chicken T-tubules; the lectin-reactive moiety is on a M_r 89,000 protein that also cross-reacts with rabbit anti-Mg^{2+}-ATPase IgG (Okamoto *et al.*, 1985; Damiani *et al.*, 1987*a*). Previously, the relative mass of this immunoreactive and lectin-reactive polypeptide was estimated to be ~ 102,000, but the development and availability of precise, prestained electrophoretic and fluorescent molecular-weight markers has enabled a more acurate determination of relative molecular weight. Rabbit antichicken Mg^{2+}-ATPase IgG inhibits the Mg^{2+}-ATPase, and the lectins and the antibody appear to compete for the same site (Damiani *et al.*, 1987*a*); the anti-Mg^{2+}-ATPase IgG also prevents the characteristic Con-A stimulation; conversely, Con-A prevents the inhibitory effect of the antibody, apparently by impeding antibody binding. Importantly, the M_r 89,000 polypeptide may not be the locus of the catalytic site, but may be a regulatory component of an oligomeric chicken Mg^{2+}-ATPase complex.

We have also presented data indicating that there was a structural relatedness between the T-tubule Mg^{2+}-ATPase and the SR Ca^{2+}-ATPase on the basis of a variety of criteria that employed strict attention to contamination by SR-derived Ca^{2+}-ATPase (Damiani *et al.*, 1987*a*). With the reassignment of the M_r of the lectin and antibody-reactive chicken Mg^{2+}-ATPase polypeptide to 89,000, it is unclear whether this 89,000 component is the sole catalytic component of the Mg^{2+}-ATPase or represents a regulatory subunit of a heteroligomer. The significance of the additional chicken polypeptides in the M_r 105,000 and 100,000 area of the LPC-purified preparation is a matter of current scrutiny, but they could be Mg^{2+}-ATPase-related, Na^{+}-pump-related, inactivated SR Ca^{2+}-ATPase, or even a T-tubule-specific low-activity isoform of the SR Ca^{2+}-ATPase. The concentration of the M_r 89,000 polypeptide relative to the other species is difficult to assess at this time due to the well-known inability of glycoproteins to bind Coomassie blue and its relatively poor silver-staining capability. Although the recent

report by Kirley (1988) on the rabbit T-tubules claimed a M_r of 105,000, close inspection of the SDS-PAGE (see Kirley, 1988, Fig. 4) shows that the putative rabbit Mg^{2+}-ATPase M_r 105,000 glycoprotein moves slightly slower than the phosphorylase standard, and that the protein actually is closer to, or identical with, the chicken M_r 89,000 species. To date, the only polypeptide that we can firmly assign to any T-tubule Mg^{2+}-ATPase is the Con-A-reactive M_r 89,000 chicken polypeptide.

The unavailability of a specific inhibitor for the T-tubule Mg^{2+}-ATPase has clearly exacerbated the structural and functional characterization of the enzyme in a number of laboratories. On the positive side, the ability of the chicken enzyme to respond to Con-A is providing a unique and critical marker with which to monitor the enzyme in the purification process. Whether the M_r 89,000 polypeptide contains the catalytic site, the regulatory site, or neither site is likewise just as speculative at this time.

Possible Functions of the Mg^{2+}-ATPase

The initial trigger for the E-C coupling mechanism most likely involves Ca^{2+}-dependent charge movement associated with T-tubule membrane depolarization (Schneider and Chandler, 1973; Chandler *et al.*, 1976), possibly with the participation of trans-T-tubule Ca^{2+} fluxes. The junctional SR membrane contains a Ca^{2+}-gated Ca^{2+} release channel that has a high affinity for ryanodine and forms the "foot" protein spanning the T-tubule–SR junction (Lai *et al.*, 1988). Subsequent to the initial trigger, the nature of which is open to debate, the degree of opening or closure of the SR Ca^{2+} release channel may be modulated to varying extents by a variety of intracellular agents, including ATP, H^+, Mg^{2+}, sulfhydryl ligands, IP_3, and calmodulin. The action of these agents may be cumulative and restricted to a unique microenvironment presented by the 12-nm junctional gap. We suggest that their net effect may also depend upon the level of fatigue, energy charge of the cell, state of hormonal activation, or other metabolic conditions.

In light of the probable junctional or perijunctional location of the Mg^{2+}-ATPase and its likely cytoplasmic active site orientation and high hydrolytic capacity, it is tempting to speculate upon a role for the Mg^{2+}-ATPase in some stage of the contraction–relaxation cycle, in particular the control of the levels of several key Ca^{2+} channel regulatory agents in the junctional microenvironment. For example, it is possible that this high hydrolytic capacity enzyme could function in lowering the local ATP concentration and thus helping to maintain the Ca^{2+} channel in an *inactive*, closed state during the relaxation phase of muscle activity. The active Mg^{2+}-ATPase not only would lower the local ATP concentration, but would simultaneously lower the pH (due to scalar proton release)

and increase free Mg^{2+} levels (due a decrease in the level of the MgATP chelate). Since both of the latter two effects are known to individually facilitate Ca^{2+} channel closure and to reduce Ca^{2+} efflux (Meissner and Henderson, 1987), a cumulative effect or synergism may thus occur by the operation of the Mg^{2+}-ATPase. Ca^{2+} channel inactivation through the Mg^{2+}-ATPase would facilitate the relaxation phase by reducing SR Ca^{2+} efflux, thus augmenting net Ca^{2+} uptake rate. In the relaxation phase, due to the release or recruitment of either endogenous or exogenous lectins or other regulatory agents, the Mg^{2+}-ATPase could be reversibly activated to high levels of activity. With regard to the SR Ca^{2+}-ATPase, the T-tubule Mg^{2+}-ATPase could be in phase, e.g., both could be operative with relaxation, the former with concentrative Ca^{2+} uptake within the SR and the latter associated with Ca^{2+} release channel closing. During the excitation phase, the enzyme could be inactivated by potential inhibitory regulatory lipids such as diacylglycerol; indirect opening of the Ca^{2+} channel by diacylglycerol inhibition of the Mg^{2+}-ATPase would thus be complemented by the direct IP_3-induced channel opening. Subsequent to regulatory inhibition of hydrolytic activity, the repletion of microenvironmental ATP would allow the initiation of another cycle. The observation that the chicken Mg^{2+}-ATPase responds to the fatty acyl-CoA/free fatty acid ratio within the physiologically significant concentration ranges further suggests that the enzyme may also respond to cellular or microenvironmental energy charge. Thus, the full extent of Mg^{2+}-ATPase activation may be determined by a number of parameters, including lectin-like regulatory agents, phosphoinositide-cycle-derived lipids, and energy-charge-mediated regulatory lipids. Additionally, we are not dismissing the possibility that fluctuations in the Mg^{2+}-ATPase activity may not necessarily be in phase with each contraction–relaxation cycle; rather, the level of Mg^{2+}-ATPase activity as regulated by the above metabolic agents may serve to modify the responsiveness of the SR Ca^{2+} release channel during periods of fatigue or hormonal activation. It would be of interest to evaluate the effects of the above regulatory ligands on T-tubule depolarization-mediated Ca^{2+} release from triads.

From another perspective, the Mg^{2+}-ATPase could play a role in regulation of microenvironmental junctional pH, but in a direction opposite to that proposed above and by a mechanism involving the developmental of a trans-T-tubule pH gradient (forming a luminal acidic pH). It has been previously suggested that the release of Ca^{2+} from the SR may be controlled by a pH gradient localized across the T-tubule membrane. Nakamura and Schwartz (1970) observed that isolated SR vesicles preloaded with Ca^{2+} at pH 7 could be caused to release their Ca^{2+} rapidly if the pH of the extravesicular medium was raised to pH 8, an alkaline-induced Ca^{2+} release. Later work showed that Ca^{2+}-loaded skinned fibers treated with protonophores or

incubated at alkaline pH displayed transient tension responses resulting from the release of stored Ca^{2+} (Shosan *et al.*, 1981). Since Ca^{2+} release from preloaded SR was not enhanced by protonophores, MacLennan and co-workers further suggested that the pH-sensitive sites are components of the more complex skinned fiber system, but not of the more resolved fragmented SR, and thus might be localized in the T-system (MacLennan *et al.*, 1982). Theoretically, a T-tubule membrane H^+ gradient could be formed coincident with depolarization to produce a local increase in the alkalinity of the sarcoplasm in the immediate vicinity of the SR by activation of the Mg^{2+}-ATPase and increased proton movement to the T-system lumen. Ca^{2+} release would then occur in a fashion similar to that which occurs when Ca^{2+}-preloaded SR vesciles are presented with an alkaline medium. Subsequent relaxation of the transmembrane proton gradient could occur by transient opening of nucleotide-regulated channels allowing proton influx into the sarcoplasm. Our laboratories have conducted numerous studies in the past 2 years evaluating the presence of vanadate-, oligomycin-, and digoxin-insensitive ATP-driven trans-T-tubule membrane pH gradients and membrane potentials with a variety of pH-sensitive and membrane-potential-sensitive fluorescent and absorptimetric dyes and by distribution of isotopically labeled weak bases. Occasionally, preparations of vesicles can be isolated that exhibit ATP-driven pH gradients (Damiani *et al.*, 1986); however, the low frequency of its occurrence and the response to inhibitors, various ligands, and chemical-modifying agents show that this gradient formation occurs by a mechanism fully unrelated to the T-tubule Mg^{2+}-ATPase. These studies are particularly challenging to the investigator due to the small intravesicular volume, the very high internal buffering capacity presented by trapped proteins, and the exceptional ATP hydrolytic capacity of T-tubule vesicles. T-tubules are capable of retaining pH gradients for extended periods of time and in fact are isolated with a preestablished, stable gradient (unpublished observations) similar to those seen with pituitary secretory vesicles (Loh *et al.*, 1984). We have not fully abandoned this proposal from further consideration in our laboratories, but will continue only with the attainment of a definitively reconstituted enzyme system in which most of the above problems are overcome.

Other possibilities for Mg^{2+}-ATPase involvement in E–C coupling include (1) the possible ability of T-tubule membranes to support an ATP driven Ca^{2+} gradient, a topic covered in a previous section; and (2) the possible ability of the Mg^{2+}-ATPase to drive trans-T-tubule Mg^{2+} gradient formation. From this latter perspective, the ATPase would function to lower intrajunctional Mg^{2+}, thereby increasing Ca^{2+} release channel activity; currently, however, the negative results obtained in our membrane potential studies likewise have dampened somewhat the enthusiasm for exploring

Mg^{2+} distributions by sensitive, nonlinear, multiwave-mixing laser spectroscopic techniques.

Finally, the possibility that the Mg^{2+}-ATPase is an ectoenzyme raises certain functional questions. Many studies have shown that treatment of a variety of intact cells with extracellular ATP results in marked perturbation of both generalized and specialized cellular functions (reviewed in Dubyak and De Young, 1985). The changes specific to ATP that are ascribed to P_2-purinergic receptors include activation of cation conductance and ion fluxes, in particular intracellular Ca^{2+} mobilization, as well as the hydrolysis of phosphatidylinositiol 4,5-bisphosphate (Charest *et al.*, 1985*a*, *b*). Extracellular ATP has been reported to stimulate phosphatidylinositol turnover and tension development in smooth muscle (Watts and Morovay, 1983). The similarities of the hepatocyte P_2-purinergic receptor and the ecto-ATPase have led Lin and Russell (1988) to speculate that these proteins may have a common identity or that the ecto-ATPase may function in the regulation of P_2-purinergic receptor function. Extracellular ATP-induced mobilization of intracellular skeletal muscle Ca^{2+} via the Mg^{2+}-ATPase remains as an intriguing possibility and should be addressed in single cell studies.

Future Directions

The potential metabolic significance of the T-tubule membrane should not be underestimated, especially considering that its surface area is at least fourfold greater than that of the SL. There have been a number of recent discoveries of pharmacologically, hormonally and metabolically significant proteins within the T-system that emphasize the importance of the membrane, including Ca^{2+}-channel-blocker receptors (the richest source of this receptor of all tissues studied), hormonal receptors [80% of the insulin receptors in skeletal muscle (Burdett *et al.*, 1987) and perhaps 90% of total organismal insulin receptors are in the T-system; the T-system may also be a major site of adrenergic receptor action], and glucose transporters [50% of skeletal muscle cytochalasin-sensitive glucose transporters are in the T-system (Burdett *et al.*, 1987)]. The panoply of T-tubule proteins able to serve as substrates for endogenous and exogenous protein kinases suggests a heretofore unanticipated level of T-tubule involvement in muscle biochemistry. The presence and levels of these and other metabolically crucial membrane and membrane-associated proteins indicate that the T-system plays a far more important role than serving as a mere electrical conduit into the interior of the muscle fiber. It is anticipated that further scrutiny will uncover other receptors and transport proteins that have been more traditionally associated with the SL. The high levels of these transporters and receptors

indicate that there may be a far greater extent of T-tubule luminal solution interchange occurring with the external medium than would be intuitively estimated from a consideration of the tortuosity of the T-system, its narrow diameter, and its high length/diameter ratio; it is tempting to speculate that efficient luminal T-tubule–solvent exchange may occur by mechanical pulsations associated with muscle contraction.

Research into developmental aspects of the T-system has been lagging behind those of the SR (MacLennan *et al.*, 1985), undoubtedly due to previously discussed problems associated with isolation and identification of T-tubule membranes, the complexity of the T-tubule relative to the SR, and the fragmentary knowledge of true T-tubule components. It is anticipated that the isolation and characterization of these components will soon produce a variety of gene probes, mRNA probes, and immunological reagents to assess discrete steps in the biosynthesis of the T-tubule membrane and to probe possible defects relative to muscle pathophysiology.

The exact nature of the E–C-coupling mechanism remains obscure. Due to the complexity of isolated T-tubule membranes, it appears that selective and mixed reconstitution of integral membrane proteins and transport and charge movement devices into liposomal and bilayer systems with well-defined topologies will ultimately be required to elucidate the nature of primary and secondary E–C-coupling processes. The reconstitution of triads should be the focus of additional attention in the resolution–reconstitution heuristic analysis. Substantial focus should be applied to probing mechanisms of Ca^{2+}-release using biochemically defined and reconstituted triads with the application of appropriate antibodies and regulatory agents. The composition and structure of the T-tubule junctional face need to be elucidated, since the E–C-coupling mechanism ultimately will be understood only after a thorough knowledge of junctional T-tubule proteins is attained.

Acknowledgments

This work was supported by National Science Foundation grants DCB 8613881 and INT 8515846 and the California Metabolic Research Foundation.

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