Molecular tools to elucidate problems in excitation-contraction coupling*

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ABSTRACT In this review, constituting the 1990 International Lecture of the Biophysical Society, research is described in two areas in which molecular genetic techniques were used to dissect problems related to sarcoplasmic reticulum proteins: the use of site-directed mutagenesis to gain insight into the mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase; and the use of cloning and genetic linkage analysis to identify the Ca^{2+} release channel (RYR1) gene as a candidate gene for the predisposition to malignant hyperthermia, a neuromuscular disease of humans and domestic animals.

INTRODUCTION

The sarcoplasmic reticulum is an extensive muscle membrane system surrounding each myofibril within muscle cells. It lies at the junction between the transverse tubule, the intracellular extension of the electrically excitable sarcolemmal membrane, and the contractile apparatus of the muscle cell, forming a part of the pathway of excitation-contraction coupling. Depolarizing currents in the transverse tubule culminate in a signal for Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} released from the sarcoplasmic reticulum regulates muscle contraction. The sarcoplasmic reticulum has two additional functions essential to excitation-contraction coupling, Ca^{2+} reuptake to initiate muscle relaxation and Ca^{2+} storage to maintain relaxed muscle in a quiescent state. This concept was well understood in 1969, when I began to work on the system, and was summarized in a superb review by Dr. S. Ebashi and his colleagues in that year (Ebashi et al., 1969).

My goal in studies of the sarcoplasmic reticulum, after 6 years of training with the pioneer of membrane protein fractionation and isolation, Dr. David E. Green, was to isolate and characterize the major proteins of the system to understand how they related to Ca^{2+} transport, sequestration, and release. The protein of most immediate interest was the Ca^{2+} transport ATPase that Dr. Ebashi had identified earlier (Ebashi and Lipmann, 1962), but, on the basis of farsighted advice from Dr. Green, I chose to work on the system of membrane proteins rather than confining my work to a single protein. Our work led to the isolation and partial characterization of the Ca^{2+}-ATPase (MacLennan, 1970; MacLennan et al., 1985), calsequestrin (MacLennan and Wong, 1971; Fliegel et al., 1987), a proteolipid and a high affinity Ca^{2+} binding protein (calreticulin) (MacLennan et al., 1972; Fliegel et al., 1989), a 53,000 glycoprotein (Campbell and MacLennan, 1981; Leberer et al., 1989*a*) a 160,000 glycoprotein (sarcalumenin) (Leberer et al., 1989*b*) and a calmodulin-dependent protein kinase (Campbell and MacLennan, 1982; Tuana and MacLennan, 1988). With the advent of recombinant DNA technology in the early 1980s, Chris Brandl, then a graduate student, and I began to clone cDNAs encoding these proteins. Thus we were not involved with the isolation and identification of the Ca^{2+} release channel protein, or ryanodine receptor, which was so ably executed in the laboratories of Tony Caswell, Sidney Fleischer, Gerhard Meissner, and Kevin Campbell (Cadwell and Caswell, 1982; Kawamato et al., 1986; Smith et al., 1985; Inui et al., 1987; Lai et al., 1987, 1988; Campbell et al., 1987). We were, however, able to contribute to knowledge of this protein through cloning of cDNA encoding skeletal (Zorzato et al., 1990) and cardiac (Otsu et al., 1990) muscle isoforms of the protein and through identification of the ryanodine receptor gene as a candidate gene for the predisposition to malignant hyperthermia (MacLennan et al., 1990).

In this review I will describe aspects of our work on two different problems relating to sarcoplasmic reticulum structure and function. In the first section I will describe our use of cloning, expression, and site-directed mutagenesis to gain insight into the mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase. In the second, I will describe our use of cloning and genetic analysis to gain insight into the structure, function, and involvement in disease of the Ca^{2+} release channel.

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THE Ca^{2+}-ATPASE

Structure

The Ca^{2+} ATPase is comprised of cytoplasmic headpiece and stalk sectors and a transmembrane basepiece, making up a tripartite structure (MacLennan and Reithmeier, 1985). The enzyme is asymmetrically oriented in the membrane with virtually all of its extramembranous mass in the cytoplasm. Thus our first task on obtaining the deduced amino acid sequence of the protein from analysis of a cDNA was prediction of the topology and the secondary structure of the Ca^{2+}-ATPase that would be consistent with a headpiece, stalk and basepiece structure. These predictions were carried out in collaboration with Drs. Michael Green and Willie Taylor. We were guided by the fact that NH_2 and COOH-termini, as well as tryptic cleavage and ATP binding sites are located in the cytoplasm and that disulfide bonds in the protein must exist in the luminal region of the protein. Analysis of hydrophobic sequences led to assignment of 10 transmembrane helices, 4 in the NH_2-terminal quarter and 6 in the COOH-terminal quarter, to make up a basepiece with very little protrusion into the luminal space (MacLennan et al., 1985; Brandl et al., 1986). The stalk sector was proposed to be made up of five predicted alpha helices that are contiguous with transmembrane helices. We predicted that the headpiece would be made up of three globular domains, the first a seven membered beta strand domain lying between stalk sectors 2 and 3 and the second and third occurring in alternating alpha-beta sequences lying between stalk sectors 4 and 5. The second and third cytoplasmic domains would be rejoined by a long helix at the COOH-terminal end of the third domain and this would form a hinge between the two. We have called these two predicted domains the phosphorylation domain and the nucleotide binding domain.

Crystallization of the Ca^{2+}-ATPase is currently being carried out (Taylor et al., 1988; Stokes and Green, 1990) and analysis of x-ray diffraction patterns will ultimately solve the structure of the protein. In the meantime, other approaches provide useful information. In our model of the Ca^{2+} ATPase (Fig. 1) the sequence between residues 758 and 994 makes up 6 transmembrane helices which form three hairpin loops in the membrane with residues 783–789, 859–896, and 950–962 lying in the lumen (Brandl et al., 1986). This folding model has been supported by the work of Matthews et al. (1989) who used monoclonal antibodies to show that the NH_2- and COOH-termini are cytoplasmic. Shull and Greeb (1988) also showed that the plasma membrane Ca^{2+} ATPase has six distinct hydrophobic sequences in this region and that the calmodulin binding site, which must be cytoplasmic, is located near the COOH-terminus, following the tenth proposed transmembrane sequence.

We have used monoclonal antibodies (mAbs) to provide further evidence for this model (Clarke et al., 1990*d*). Antibody A52 (Zubrzycka et al., 1984) reacts with the sequence 657–672 which we predict to be cytoplasmic. We have found that A52 binds equally to the ATPase in sarcoplasmic reticulum preparations in the presence and absence of the detergent $C_{12}E_8$, confirming this prediction. A second monoclonal, A20, reacts with the sequence 870–890. MAb A20 binding to intact sarcoplasmic reticulum vesicles was virtually at background level, but was increased 20-fold in the presence of the detergent, $C_{12}E_8$ or when vesicles were opened by EGTA at elevated pH. These observations demonstrated the localization of the sequence 870–890 on the luminal surface of the sarcoplasmic reticulum and are consistent with 2, 4, or 6 transmembrane sequences in the COOH terminal quarter.

Function

The Ca^{2+}-ATPases are members of a class of asymmetrically oriented, transmembrane proteins of ~110 kDa which form a phosphoprotein intermediate during the course of ATP hydrolysis, which alternate between E_1 and E_2 conformations, and which transport ions either uni- or bidirectionally. The Ca^{2+}-ATPase of rabbit fast-twitch muscle sarcoplasmic reticulum binds 2 mol of Ca^{2+} with high affinity and 1 mol of ATP per mole of protein (Inesi, 1985). The two Ca^{2+} ions are bound sequentially in two sites. The first Ca^{2+} bound is the first to be released to the lumen in the forward reaction; the second Ca^{2+} bound is the first to be released to the cytoplasm in the reverse reaction (Inesi, 1987). As ATP is hydrolyzed, a phosphorylated intermediate is formed which is initially of high energy and capable of carrying out ATP/ADP exchange (E_1P state), but which is transformed to a low energy conformation (E_2P) during the translocation cycle. Ca^{2+}, which is bound initially with high affinity and is readily exchangeable, is rapidly occluded and no longer displaceable from the external binding sites by EGTA (Takisawa and Makinose, 1983). As the reaction cycle proceeds, the affinity of the Ca^{2+} binding site is reduced by three orders of magnitude and Ca^{2+} is released to the luminal surface as the phosphoprotein decays to a low energy form. These events can be interpreted as being due to a change in orientation of helical strands and a disruption of the Ca^{2+} binding sites formed from them, resulting in a vectorial transfer of Ca^{2+} from the cytoplasm into the lumen of the sarcoplasmic reticulum (Tanford, 1982).

The final steps in the process consist of hydrolytic cleavage of the phosphoenzyme and relaxation of the

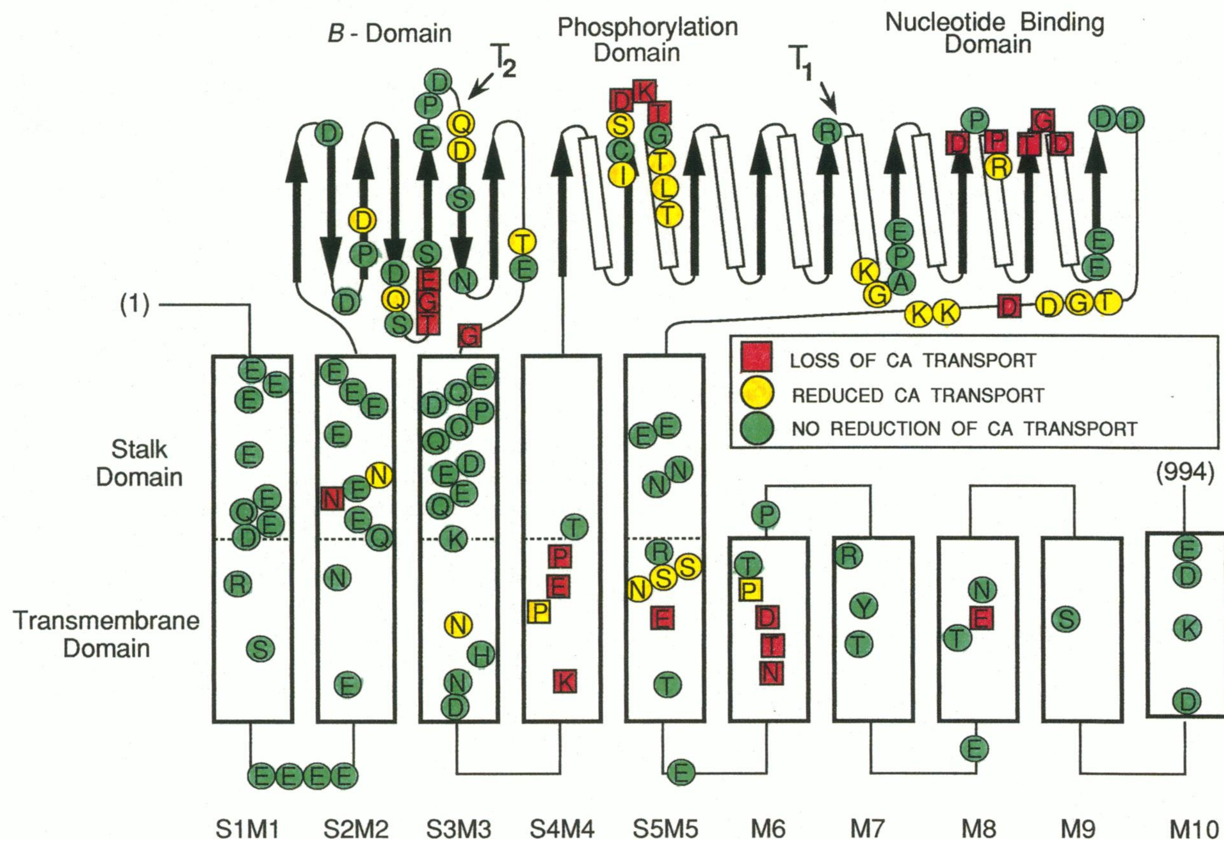

FIGURE 1 Structural diagram of the Ca^{2+}-ATPase molecule based on predicted structure and hydropathy plots. The locations of mutated residues are circled and the wild type residues are identified by a single letter code. The functional consequence of each mutation is indicated by a color code: green = no effect on Ca^{2+} transport; yellow = reduced rate of Ca^{2+} transport; red = background level of Ca^{2+} transport; white = no expression of mutant. Note the clustering of red and yellow mutants near the center of the transmembrane domain, where we predict the sites of Ca^{2+} binding to lie, and in the loops between alpha helices and beta strands in the cytoplasmic domain, where we believe the site of ATP binding to lie. Mutations in the stalk sector and in the periphery of the transmembrane domain had little effect on Ca^{2+} transport function.

enzyme from the E_2 to the E_1 conformation, with reformation of the high affinity Ca^{2+} binding sites. The entire process is reversible. The enzyme can be phosphorylated by inorganic phosphate (Pi), provided all Ca^{2+} is sequestered by EGTA (de Meis and Vianna, 1979). The presence of Ca^{2+} may drive the E_2 form of the enzyme to the E_1 conformation where it cannot be phosphorylated by inorganic phosphate. The phosphorylated intermediate can then be transferred to ADP to form ATP if Ca^{2+} and ADP are added simultaneously.

In early models of the Ca^{2+} ATPase we postulated that Ca^{2+} would bind with high affinity in the acidic stalk sector whereas ATP would bind on the surface of the headpiece (Brandl et al., 1986; MacLennan et al., 1986). Rotation of parts of the stalk sector, as a result of ATP hydrolysis and phosphorylation of the enzyme, would move Ca^{2+} into a channel formed by stalk and transmembrane sequences, at the same time breaking up the high affinity Ca^{2+} binding sites and releasing Ca^{2+} so that it could flow into the lumen through a channel formed from transmembrane sequences. In support of this model, studies of fluorescence energy transfer among various sites have indicated that the Ca^{2+} binding sites and the ATP binding site are widely separated in the molecule and that the ATP binding site is distant from membrane lipids (Scott, 1985; Teruel and Gomez-Fernandez, 1986; Joshi and Shamoo, 1988).

To test our ideas concerning ligand binding sites and the transport mechanism we decided to use site-directed mutagenesis to examine structure/function relationships. This method has provided a novel approach to further advancement of knowledge in this area.

SITE SPECIFIC MUTAGENESIS OF THE Ca^{2+}-ATPASE

Expression and mutagenesis

In our expression-mutagenesis system, developed by Dr. Kei Maruyama, a postdoctoral fellow in my laboratory and a former student of Dr. S. Ebashi, full length cDNAs encoding both slow-twitch and fast-twitch muscle isoforms of the Ca^{2+}-ATPase of rabbit muscle sarcoplasmic reticulum were inserted into the expression vector p91023(b) and expressed in COS-1 cells (Maruyama and MacLennan, 1988). Microsomal fractions isolated from the transfected COS-1 cells transported Ca^{2+} at rates ~20–50-fold over background, proving that the single protein encoded by the Ca^{2+}-ATPase cDNA is capable of carrying out the complete Ca^{2+} transport function. Immunofluorescence microscopy showed that the expressed Ca^{2+}-ATPase was localized within organellar structures in the transfected COS-1 cells whereas Western blotting and functional analysis showed that the Ca^{2+} pump is associated with microsomal fractions from these cells.

Dr. Maruyama, and later Dr. David Clarke (Maruyama et al., 1989; Clarke et al., 1989*a*, *b* and 1990*a–c*; Vilsen et al., 1989; Anderson et al., 1989; Fujii et al., 1989) demonstrated the feasibility of making virtually any mutation at a rapid rate and carrying out a range of functional assays on the mutated product. Virtually all of our mutations lead to products that can be expressed. Moreover, a large percentage of them have little effect on function, suggesting that, where perturbation of the function of the enzyme does occur, it is a meaningful event. In those mutants where overall activity is lost, partial reactions usually remain, allowing us to gain insight into the particular steps in overall Ca^{2+} transport that are affected by the mutations. Dr. G. Inesi assisted us in the early analysis of the phosphorylation of mutant proteins.

With our expression/mutagenesis system the cells on five 10-cm plates synthesize ~2–3 μg of the ATPase protein. With 2–3 μg of ATPase protein we can establish a Ca^{2+} transport curve with up to 10 data points. With another 2–3 μg we can carry out studies of phosphorylation and dephosphorylation or we can measure the Ca^{2+}-dependency of Ca^{2+} transport. We can also analyze electrophoretic mobility changes brought about by protein modification or proteolysis using PAGE and Western blotting. We cannot measure Ca^{2+} or ATP binding or Ca^{2+}-dependent ATP hydrolysis directly because the microsomal fraction is crude and the background binding and nonspecific ATPase levels are high. Moreover, we do not have sufficient material for extensive kinetic analysis.

Mutational analysis of Ca^{2+} binding sites

In Fig. 1, we present a summary of our mutations in the Ca^{2+}-ATPase on the background of our predicted model for its structure. The function of each mutation is color coded like a stop light; red means "stop," green means "go," and yellow means "proceed slowly."

One of our major objectives in site-directed mutagenesis of the Ca^{2+}-ATPase was to define the Ca^{2+} binding sites in the molecule. Because we could find no evidence for any known high affinity Ca^{2+} binding sequence in our initial analysis of the primary structure of the protein, we proposed that Ca^{2+} might bind in amphipathic helices of the stalk sector (Brandl et al., 1986). Accordingly, in our first study, we mutated a total of 31 Glu, Gln, Asp, and Asn residues in this sector, usually to Ala, a small uncharged residue (Clarke et al., 1989*a*). In most cases, multiple mutations (clusters of 3–4 altered residues) introduced into stalk or luminal sectors 1, 2, 3, or 5 resulted in only partial reduction of Ca^{2+} transport function whereas subsequent mutation of single residues in the cluster had no effect on activity. Measurement of the Ca^{2+}-dependency of Ca^{2+} transport, an approximate measurement of Ca^{2+} binding affinity, showed that none of these mutations altered Ca^{2+} affinity. Following mutation of one cluster in stalk sector 2, Ca^{2+} transport was reduced to near zero. It was possible to assign the transport defect to alterations in Asn 111 in which Ca^{2+} transport was lost and Asn 114 in which it was reduced to 50%. Nevertheless, these two mutated proteins were phosphorylated as effectively as wild type by ATP in the presence of Ca^{2+} and no phosphorylation was observed in the absence of Ca^{2+}. Thus, in these mutants, as in all mutations in the stalk sector, the Ca^{2+} binding sites were intact. We concluded that none of the acidic or amidated residues in stalk or luminal sectors 1, 2, 3, or 5 are involved in a critical way in Ca^{2+} transport.

In a second series of experiments, we mutated all charged residues in those sequences of the protein which we had earlier predicted would lie in the transmembrane sector of the protein (Clarke et al., 1989*b*), then all polar residues (Clarke et al., 1990*a*) and, finally, three proline residues (Vilsen et al., 1989) located in this same sector. In all, 34 amino acid residues in the transmembrane domain were substituted, some with several alternate amino acids. Of these substitutions, 20 had no significant effect on Ca^{2+} transport, four reduced Ca^{2+} transport, and 10 resulted in loss of Ca^{2+} transport. For those four in which Ca^{2+} transport rates were reduced, each caused an alteration in Ca^{2+} affinity. We refer to these mutants as Ca^{2+} affinity mutations.

Of the 10 mutations which caused loss of Ca^{2+} transport, the three Pro and one Lys mutations permitted

phosphorylation of the protein from ATP in the presence of Ca^{2+}. Two of the pro mutations had altered affinities for Ca^{2+}, however. With each of these proline mutants, as with the Asn 111 to Ala mutant described earlier, the phosphorylated intermediate was found to be in the high energy E_1P form because each was dephosphorylated by ADP. When the forward reaction was stopped by sequestration of Ca^{2+} with EGTA, however, the phosphorylated intermediate was found to be stable. It could not go forward to form the E_2P intermediate and be dephosphorylated, but rather appeared to be blocked in the E_1P conformation. We refer to these mutants as conformational change mutants (Vilsen et al., 1989).

The remaining six mutant proteins, with substitutions for Glu309, Glu771, Asn796, Thr799, Asp800, and Gln908 in M_4, M_5, M_6, and M_8, which we refer to as Ca^{2+} binding mutants (Clarke et al., 1989*b*), were not phosphorylated by ATP in the presence of Ca^{2+}, but were phosphorylated by Pi in the absence of Ca^{2+}. In the wild type enzyme, Ca^{2+} inhibits phosphorylation by Pi, apparently by driving the enzyme towards the E_1 conformation. In these six mutants, Ca^{2+} did not inhibit phosphorylation by Pi. This could be due to destruction of the Ca^{2+} binding site or to inability of the mutants to assume the E_1 conformation. We believe that the mutations affect the Ca^{2+} binding site for two reasons. First, many of the mutations were isosteric (Glu for Gln or Asp for Asn) and would have minimal effects on residue size. Second, we have been able to make alternate substitutions in four of these sites and have observed that overall Ca^{2+} transport function (Ser for Thr799; Gln for Glu980) or partial reactions (Asp for Glu309; Ala for Asn796) could be preserved. In all cases, however, preservation of function occurred with altered affinity for Ca^{2+}. On the basis of our studies, we have put forth the hypothesis that the Ca^{2+} binding sites in the Ca^{2+} ATPase are located near the center of the transmembrane domain and are comprised of residues in transmembrane sequences M4, M5, M6, and M8.

Analysis of ATP binding sites

In a third study (Maruyama et al., 1989), we investigated the effects of mutation of residues in the phosphorylation and nucleotide binding domains to elucidate their role in ATP binding and phosphorylation (see Fig. 1). Mutations were carried out initially in the sequence ICSDKTGTLT357, which contains the phosphorylated residue Asp351 and is highly conserved in all P-type cation pumps. All mutations of Asp351, Lys352, or Thr353 disrupted Ca^{2+} transport function and, for Asp351 and Lys352, phosphoenzyme formation. Conservative substitutions in each of the residues flanking Asp351, Lys352, and Thr353, or the alteration of Cys349 to Ala, did not destroy Ca^{2+} transport activity or phosphoenzyme formation. Nonconservative substitutions for any of these residues, however, did disrupt function.

We have also examined the effect of mutating residues in several other conserved regions of the ATPase proposed to be involved in ATP binding (Clarke et al., 1990*c*). These are: KGAPE519; DPPR604; RDACGIRVIMITGDNK629; TGD703; and D707. In models presented by Taylor and Green (1989) and Serrano (1989) Lys515, Asp601, Asp627, Asp703, and Asp707 were predicted to be involved in ATP binding. Substitutions for some, but not all, of these residues led to virtually complete loss of Ca^{2+} transport. We have been able to show that ATPases with substitution of Glu for Asp601, Glu for Pro603, Ala or Pro for Gly626, and Asn for Asp707 did not form detectable phosphoenzyme intermediates in the presence of either ATP or Pi. These results are consistent with the involvement of these residues in ATP binding, but do not prove their involvement. All other substitutions did form phosphoenzyme intermediates in the presence of 2 μM ATP, indicating that the ATP binding site was probably intact. Thus further work is required to obtain a better understanding of the residues involved in formation of the Ca^{2+} binding site.

Model for Ca^{2+} transport

Our studies to date have allowed us to develop a very simple model for Ca^{2+} transport as follows: when the Ca^{2+} ATPase is in the E_1 conformation, cytoplasmic Ca^{2+} has free access to two binding sites near the center of the transmembrane domain where it binds with high affinity. ATP also has free access to an ATP binding site located in the headpiece domain. The Ca^{2+} binding sites and the ATP binding site are coupled at a distance through peptide strands in the stalk and headpiece, which form a syncytium capable of energy transfer between the two sites. When Ca^{2+} is bound in Ca^{2+} binding sites, ATP can phosphorylate Asp351; when Ca^{2+} is absent from the Ca^{2+} binding sites, Pi can phosphorylate Asp351. The level of energy in the phosphoryl group of Asp351 is related to the affinity for Ca^{2+} at the Ca^{2+} binding sites. All of these observations support the view that the state of the ATP binding site influences the state of the Ca^{2+} binding sites and that energy transferred from ATP to Asp351 is used to alter the conformation of the Ca^{2+} binding sites. In the E_1 and E_1P conformations, the Ca^{2+} binding sites are of high affinity. In the transition to the E_2 conformation the Ca^{2+} binding sites are disrupted, leading to low affinity for Ca^{2+}. In this transition, access of bound Ca^{2+} to the cytoplasm is lost, but Ca^{2+} released from the disrupted site gains access to the lumen. Thus, the overall "translocation" of Ca^{2+} simply involves diffu-

sional entry of Ca^{2+} to high affinity binding sites near the center of the transmembrane domain and diffusional movement away from disrupted low affinity sites. The Ca^{2+} translocation site can be compared with a turnstile in a subway system. Ca^{2+} enters and exits on its own diffusional energy, but can only pass the barrier through investment of a "token," in this case, activation of the site by a distant high energy source, the hydrolysis of ATP. This model is presented in Fig. 2.

THE CA^{2+} RELEASE CHANNEL PROTEIN

Structure and function

Each myofibril is segmented into sarcomeric structures and the sarcoplasmic reticulum surrounding it is also segmented into longitudinal reticulum with terminal cisternae at either end. The terminal cisternae from adjacent segments do not abut each other, but are separated by a transverse tubule, an invagination of the sarcolemma which carries a depolarizing current into the interior of the cell. In thin sections it can be seen that the transverse tubule and the terminal cisternae are connected by a series of proteinaceous "feet" structures (Franzini Armstrong, 1979; Ferguson et al., 1984; Block et al., 1988) which have recently been identified as the Ca^{2+} release channel of the sarcoplasmic reticulum (Fleischer and Inui, 1989).

Identification and isolation of the Ca^{2+} release channel were facilitated through the use of the plant alkaloid, ryanodine, which was shown to bind to the protein with high affinity and to modulate its function (Fleischer and Inui, 1989). Single channel recordings showed that purified "ryanodine receptor" preparations, comprised of homotetrameric complexes of a single polypeptide of 565,000, exhibit an intrinsic Ca^{2+} channel activity that is modulated by Ca^{2+}, ATP, and Mg^{2+} in a manner similar to native Ca^{2+} release channels (Imagawa et al., 1987; Hymel et al., 1988; Smith et al., 1988; Lai et al., 1988).

Studies of the morphology of the ryanodine receptor (Wagenknecht et al., 1989) have shown it to have a quatrefoil structure, with hydrophobic segments of the four identical subunits forming a putative membrane-spanning baseplate structure, and hydrophilic segments forming a cytoplasmic domain that surrounds and decorates the central baseplate. Three-dimensional image reconstruction suggests the presence of four internal channels which branch from a common origin above the baseplate and open into vestibules in the four quarters of the tetramer.

Drs. Francesco Zorzato, Junichi Fujii, and Kinya Otsu, postdoctoral fellows in my laboratory have cloned cDNAs encoding both skeletal and cardiac forms of the Ca^{2+} release channel (Zorzato et al., 1990; Otsu et al., 1990). The two proteins are essentially colinear, although there are many gaps or additions over the 5,000 or so amino acid residues making up their sequences. As is usual in protein isoforms, hydropathy plots, repeat sequences, predicted secondary structure, and predicted ligand binding sites are well conserved between the two proteins. Although very little hard evidence is yet available concerning structural features of the protein, predictions of its

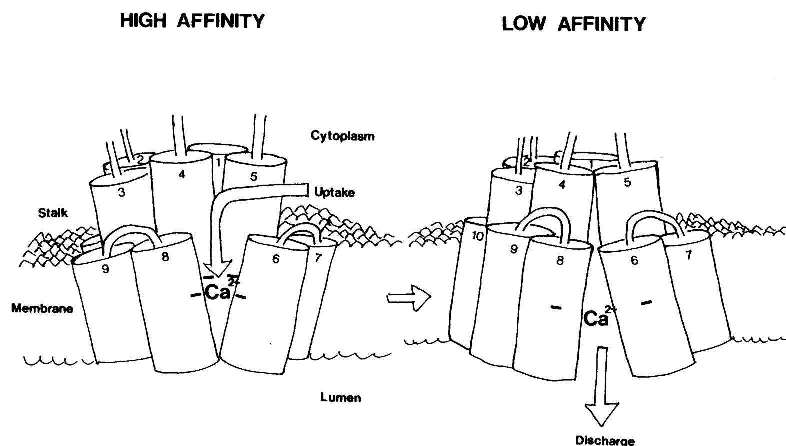

FIGURE 2 Model illustrating the mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase. In the E_1 conformation, high affinity Ca^{2+} binding sites located near the center of the transmembrane domain are accessible to cytoplasmic Ca^{2+}, but not to luminal Ca^{2+} (high affinity state). The sites are made up from amino acid residues located in proposed transmembrane sequences M4, M5, M6, and M8. Conformational changes induced by ATP hydrolysis lead to the E_2 conformation in which the high affinity Ca^{2+} binding sites are disrupted, access to the sites by cytoplasmic Ca^{2+} is closed off and access to the sites by luminal Ca^{2+} is gained (low affinity state). The Ca^{2+} transport cycle thus involves binding of cytoplasmic Ca^{2+} to high affinity sites in one conformation and release of the same Ca^{2+} to the lumen when the high affinity sites are disrupted in the transition to the second conformation.

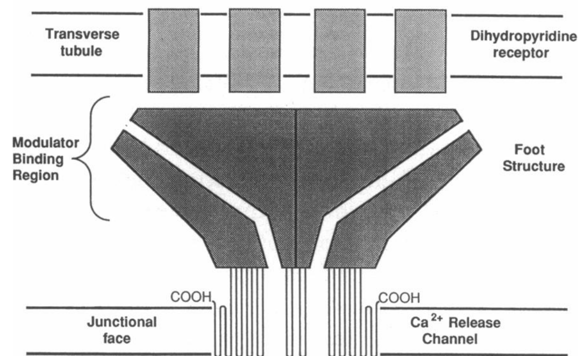

FIGURE 3 Structural diagram of the Ca^{2+} release channel of the sarcoplasmic reticulum (ryanodine receptor) based on predicted structure and hydrophathy plot. The morphology of the ryanodine receptor suggests that much of its cytoplasmic bulk lies in the gap between the sarcoplasmic reticulum and the transverse tubule, that this bulk is made up from a homotetramer and that radial channels lie within the structure (Wagenknecht et al., 1989). The ryanodine receptor appears to abut dihydropyridine receptors (slow Ca^{2+} channels) in the transverse tubule and may be functionally coupled to them (Block et al., 1988). The transmembrane domain is made up of 4 to 12 transmembrane sequences, 4 to 10 of which are in the COOH-terminal fifth of the molecule and 2 of which are more central. The postulated modulator binding domain is proposed to lie between amino acid residues 2,600 and 3,000, on an exposed surface of the cytoplasmic domain near cytoplasmic channel openings.

structure can be made. The hydropathy plots show four very hydrophobic sequences near the COOH-terminus of the protein (Fig. 3). Takeshima et al. (1989) suggested that these were the only transmembrane sequences in the protein. We identified eight additional sequences which are potential transmembrane sequences. In our analysis, 10 of these would reside in the COOH-terminal end of the molecule and two more would reside in a more central location. The remainder of the protein, ~95% of its mass, would make up the foot structure spanning the gap between the sarcoplasmic reticulum and the transverse tubule. We predicted that very little mass would lie in the lumen of the sarcoplasmic reticulum. We noted four repeat sequences, ~120 amino acids in length, in the NH_2-terminal sector of the protein, but we do not yet know how these relate to function. Finally, we noted a region between amino acid residues 2600 and 3000 that is surface exposed and very hydrophilic and which contains predicted ATP and calmodulin binding sites as well as a potential phosphorylation site in the cardiac isoform (Otsu et al., 1990). We believe that this represents a modulator binding region in the protein (Fig. 3).

Cloning of cDNA encoding the Ca^{2+} release channel protein has opened up new avenues of investigation of the protein, including its primary and predicted secondary structure, its expression and mutagenesis and its genomic structure, localization, and involvement in disease. Our cloning of cDNA encoding the human skeletal muscle ryanodine receptor allowed us to localize the ryanodine receptor (RYR1) gene to human chromosome 19q13.1 (Mackenzie et al., 1990). We have isolated most of the genomic DNA encoding the normal human ryanodine receptor and used cDNA probes to identify exon-containing sequences. We have sequenced genomic DNA containing ~90% of the exon sequences and their flanking intron sequences. Although we have not yet analyzed the entire sequence, we believe that the gene is between 200,000 and 300,000 bp long and will ultimately contain ~100 exons (M. Phillips, J. Fujii, and D. H. MacLennan, unpublished studies). A major objective in analyzing human RYR1 DNA was to determine whether the RYR1 gene is involved in human genetic disease, in particular malignant hyperthermia.

MALIGNANT HYPERTHERMIA

Manifestation

Malignant hyperthermia (MH) is manifested in humans and swine as an acute hyperthermic reaction, usually accompanied by skeletal muscle contracture, which is triggered by potent inhalation anaesthetics and muscle relaxants (Britt, 1985; O'Brien et al., 1990). Fever is a result of hypermetabolism in the muscle and not a cause of the biochemical abnormalities that occur in skeletal muscle in an MH reaction. As a result, it occurs late in onset but it may rise at a rapid rate, reaching 44–46° in a short period. Reactions can also be induced, rarely in humans but commonly in swine, by environmental stresses such as high temperature, infection, emotional excitement, muscle injury, or exercise. In the absence of triggering agents, susceptible individuals appear normal. Susceptibility to the disease is inherited in humans as an autosomal dominant trait, but in swine it is an autosomal recessive trait. The incidence of MH reactions is 1 in 15,000 anaesthesias in children's hospitals and 1 in 50,000 to 1 in 150,000 anaesthetics in adult hospitals.

Physiological, biochemical, and genetic basis for MH

The aetiology of MH is fully consistent with a lack of regulation of Ca^{2+} within muscle cells, either through an enhanced or chronic release of Ca^{2+} into the cell or through defective reuptake of Ca^{2+}. The continued presence of Ca^{2+} within muscle cells would account for

muscle contracture, whereas the continued hydrolysis of ATP would account for elevated temperature and for excess O_2 uptake and CO_2 release due to metabolic regeneration of ATP. Although the resting level of Ca^{2+} in MH susceptible swine was not found to be different using the dye Fura 2 as an indicator, intracellular Ca^{2+} was dramatically raised in these intact fibers by the infusion of caffeine or halothane (Iaizzo et al., 1988). Thus, the abnormal tension induced by caffeine and halothane in MH muscle is correlated with abnormal release of Ca^{2+} into the myoplasm.

Studies carried out on the response of sarcoplasmic reticulum isolated from MH susceptible (MHS) and MH normal (MHN) pigs to halothane and caffeine confirm that Ca^{2+} reuptake through the Ca^{2+} pump is not involved in MH (Nelson et al., 1986) but that the rate and extent of Ca^{2+} release from MHS sarcoplasmic reticulum exceeds the rate of Ca^{2+} release from MHN sarcoplasmic reticulum. Moreover, the threshold of activation of Ca^{2+} release by such agents as caffeine, halothane, and even Ca^{2+} itself, is lowered in MHS sarcoplasmic reticulum (Endo et al., 1983; O'Brien et al., 1990).

Such studies implicate defects in the Ca^{2+} release channel as causative of MH. In analysis of potential defects, Mickelson et al. (1988) have shown that the MHS ryanodine receptor exhibits an altered Ca^{2+} dependence of [^{3}H] ryanodine binding at the low affinity Ca^{2+} site, as well as a lower Kd for ryanodine, when compared with that from MHN pigs. Knudson et al. (1990) found that the tryptic digestion pattern of the ryanodine receptor differed between MHN and MHS pigs. These abnormalities of the channel may reflect an intrinsic defect in the MHS ryanodine receptor leading to malignant hyperthermia.

Genetic analysis of the inheritance of the halothane sensitive (HAL) gene in pigs, using a halothane challenge for diagnosis, has led to its assignment to a linkage group on pig chromosome 6 (Davies et al., 1988). Because the same linkage group is localized on the same region of the long arm of human chromosome 19 where we found the RYR1 gene, analysis of the linkage of the RYR1 and MH genes became a high priority in our studies.

Linkage between RYR1 and MH genes

After we had obtained the first human cDNA clones and, in collaboration with Drs. H. F. Willard, R. G. Korneluk, and B. Wieringa, localized the gene to human chromosome 19q13.1, we initiated a collaboration with Drs. R. G. Worton and B. A. Britt to carry out linkage analysis between the MH gene and the RYR1 gene in affected families (MacLennan et al., 1990). Catherine Duff in Dr. Worton's group was able to detect several useful restriction fragment length polymorphisms (RFLPs) in the human RYR1 gene which formed the basis for our linkage study between the MH and RYR1 genes.

Dr. Britt has carried out diagnosis of patients in MH families over the course of the past 19 years using the halothane and caffeine contracture test that she and Dr. Kalow originally developed in Toronto (Kalow et al., 1970). Thus several family trees were available for study of the cosegregation of the MH gene and the RFLPs in the RYR1 gene. Nine families were found in which at least one RFLP was informative. In each family, the affected parent was heterozygous for the RYR1 polymorphism, whereas the unaffected parent was homozygous for the marker, making all of the children informative. In each of the nine families, there was cosegregation of a single RYR1 allele with the MH phenotype and, in a study of 23 meioses, no recombination was found between the MH gene and the RYR1 gene. The log of the odds (lod score) that the MH and RYR1 genes are linked, is 4.2 with a theta max of 0.0 (MacLennan et al., 1990). Flanking markers also displayed tight linkage, although, as anticipated, recombination occurred with some markers. Linkage does not prove that the two genes are the same, but only demonstrates that they lie within a common region of ~1 million base pairs. Nevertheless, there is strong justification, from both physiological and genetic studies, for the view that the RYR1 gene and the MH gene are one and the same. Current studies in our laboratory are designed to discover whether mutant sequences, leading to MH, exist in both human and pig ryanodine receptor genes.

CONCLUSION

Our studies of the sarcoplasmic reticulum were begun with the hope of understanding the mechanism of Ca^{2+} transport and release. They have extended from protein chemistry through molecular cloning to expression, mutagenesis, and human genetics. They provide an example of the way that utilization of new technologies, as they develop, can provide new solutions to old problems, thereby bringing us ever closer to full understanding of complex biological mechanisms. In restrospect, it was always more fun to learn and apply new technologies to old problems than to struggle with the old technology or to discard the old problems.

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