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Review

Personal recollections on the discovery of the ryanodine receptors of muscle

Sidney Fleischer *

Department of Biological Sciences, Vanderbilt University, 465 21st Avenue South, MRBIII Room 1210, Nashville, TN 37235, USA

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Abstract

The intracellular Ca^{2+} release channels are indispensable molecular machinery in practically all eukaryotic cells of multicellular animals. They serve a key role in cell signaling by way of Ca^{2+} as a second messenger. In response to a signaling event, the channels release Ca^{2+} from intracellular stores. The resulting rise in cytoplasmic Ca^{2+} concentration triggers the cell to carry out its specialized role, after which the intracellular Ca^{2+} concentration must be reduced so that the signaling event can again be repeated. There are two types of intracellular Ca^{2+} release channels, i.e., the ryanodine receptors and the inositol triphosphate receptors. My focus in this minireview is to present a personal account, from the vantage point our laboratory, of the discovery, isolation, and characterization of the ryanodine receptors from mammalian muscle. There are three isoforms: ryanodine receptor 1 (RyR1), first isolated from rabbit fast twitch skeletal muscle; ryanodine receptor 2 (RyR2), first isolated from dog heart; and ryanodine receptor 3 (RyR3), first isolated from bovine diaphragm muscle. The ryanodine receptors are the largest channel structures known. The RyR isoforms are very similar albeit with important differences. Natural mutations in humans in these receptors have already been associated with a number of muscle diseases.

Keywords: Ryanodine receptor; Calcium release channel; Skeletal muscle; Heart muscle; Sarcoplasmic reticulum; Terminal cisternae; Junctional face membrane; Excitation–contraction coupling; Muscle; Triads; Smooth muscle

By the passing of Professor Setsuro Ebashi, we lost one of the towering figures in muscle research. Dr. Ebashi was responsible for a number of seminal concepts that we now take for granted. I will cite two of these.

One major discovery was elucidating the role of sarcoplasmic reticulum in Ca^{2+} uptake, responsible for muscle relaxation. He described an isolated skeletal muscle fraction, ''relaxing factor'', referable to a membrane fraction (fragmented sarcoplasmic reticulum) capable of ATP-driven uptake of Ca^{2+}. This uptake causes the muscle to relax. He also demonstrated that the energized sequestering of the calcium ions was by means of a Mg^{2+}- activated ATPase in the sarcoplasmic reticulum membrane. Since a rise in Ca^{2+} concentration had already been known to be responsible for muscle to contract, lowering the Ca^{2+} concentration enables muscle relaxation.

A second major discovery was the description of troponin/tropomyosin as the switch within the thin filaments of the sarcomere, which responds to Ca^{2+} causing muscle to contract.

Prof. Ebashi was chairman of the prestigious Department of Pharmacology at the University of Tokyo for nearly a quarter of a century (1959–1983) and thereby was responsible for training a number of outstanding leaders in the field of muscle research. His intellect was widely felt through his leadership in learned societies. He served as President of the International Union of Pure and Applied Biophysics (1978–1981), and as President of the International Union of Basic and Clinical Pharmacology (1981). He organized the Eighth World Congress of Pharmacology held in Tokyo in 1981. Prof. Ebashi was the recipient of numerous awards in recognition of his profound scientific achievements.

Abbreviations: RyR, ryanodine receptor; CRC, calcium release channel; SR, sarcoplasmic reticulum; LT, longitudinal tubules; TT, transverse tubules; TC, terminal cisternae; JFM, junctional face membrane; JTC, junctional terminal cisternae; FKBP, FK506 Binding Protein; FS, foot structures; CPM, calcium pump membrane.

* Fax: +1 615 343 6707.
E-mail address: Sidney.fleischer@vanderbilt.edu

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My own personal remembrance of Tetsuro was that he was brilliant and creative, albeit modest in nature. He was reserved, but could be surprising in a delightful way. For example, after an international conference on muscle in Poland, we sat around chatting after dinner when Tetsuro burst into song. A fond personal memory that I will treasure is the presentation to me of the Medallion of the Red Gate of the University of Tokyo by Prof. Ebashi, on behalf of the Tokyo Society of Medical Sciences and the Faculty of Medicine of the University of Tokyo. The last Christmas card that I received from him was in 2005. His cards were copies of lovely Japanese woodblock prints, which I happen to be fond of. Tetsuro wrote me that there would be a symposium celebrating the 40th anniversary of his finding of troponin in Okazaki, Japan. He had suffered a stroke five years earlier and was still suffering from its complications. He had hoped to be well enough to attend this symposium, but sadly passed away before the symposium was held in 2006. We shall miss our dear friend and colleague.

Personal recollections of the discovery and characterization of the calcium release channels (ryanodine receptors) of sarcoplasmic reticulum

In the early 1980s very little was known about the Ca^{2+} release machinery at the molecular level. My focus in this mini-review is to present a personal account of the discovery of the intracellular Ca^{2+} release channels, i.e., how we went about to isolate and characterize the Ca^{2+} release machinery, which mediates Ca^{2+} release from intracellular stores. My aim is not to cover the literature, but to provide a personal account.

My approach to identify the Ca^{2+} release machinery on a molecular level was to first isolate highly purified membrane fractions of rabbit fast twitch skeletal muscle [1–3]. With such membrane fractions in hand, we would aim to deduce where the molecular machinery for Ca^{2+} release is localized. We would then have to develop an in vitro assay to measure calcium release on a subcellular level which can be modulated by ligands. Modulation by high affinity ligands could then be used to follow the purification. The isolation of the molecular machinery would then be feasible. After some success with skeletal muscle, similar studies were carried out on heart muscle.

Background information

The mammalian skeletal muscle fiber (myofiber) is a huge multinucleate cell resulting from the fusion of many myoblasts [4]. The dimensions of the mammalian myofiber can be 100 μm in diameter and many centimeters long. In one myofiber there can be hundreds of myofibrils extending the full length of the myofiber. Myofibrils consist of linear arrays of sarcomeres, the fundamental units of muscle contraction. The sliding of the thick myosin filaments between the thin filaments, consisting mainly of actin and a number of regulatory components including troponin/tropomyosin, results in the shortening of the length of the sarcomere. This shortened length of the sarcomere is amplified many-fold to macroscopic dimensions in the length of the myofiber, since the myofibrils consist of linear arrays of hundreds of sarcomeres. Despite the large size of the myofiber, the response time is rapid, in the millisecond time-scale. This is because the myofiber is electrically excitable. The depolarization of the sarcolemma of the myofiber is rapidly transferred longitudinally along the full length of the fiber and transversely to within the fiber by way of the transverse tubules (TT), invaginations from the plasma membrane. The transverse tubules are associated with the sarcoplasmic reticulum (SR) to form the sarcotubular network system. The macroscopic phenomenology of muscle contraction in skeletal muscle is referred to as "depolarization induced Ca^{2+} release". That is to say, excitation at the plasma membrane results in contraction of the muscle fiber, by way of release of Ca^{2+} release from the sarcoplasmic reticulum compartment into the myoplasm. The rise in myoplasmic Ca^{2+} concentration causes muscle to contract. No external Ca^{2+} is necessary. The reuptake of Ca^{2+} by the ATP-energized calcium pump protein (Ca^{2+} stimulated ATPase) of the calcium pump membrane of SR enables muscle to relax again. Each and every sarcomere of the myofibril is surrounded by the sarcotubular membrane system which surrounds the myofibrils like a tight sleeve (Fig. 1). Ca^{2+} uptake and release by the sarcotubular mem-

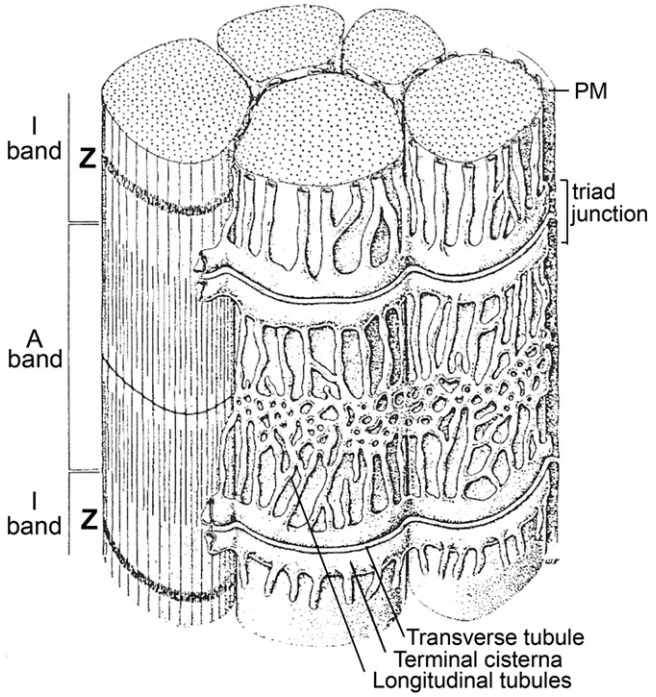

Fig. 1. Diagram of a portion of a mammalian striated muscle fiber. Two transverse tubules innervate one sarcomere. The transverse tubules are invaginations of the sarcolemma, close to the line where the A and I bands meet. Two terminal cisternae of SR are junctionally associated with one transverse tubule and connect with the longitudinal sarcotubules of SR located around the A band. The tripartite structure, seen in cross-section of two terminal cisternae (one from each adjacent sarcomere) flanking the transverse tubule, constitutes a triad (see Fig. 3). The sarcotubular network surrounds each sarcomere like a tight sleeve.

brane system is also rapid since the Ca^{2+} does not have far to diffuse because of the close proximity of the sarcotubular membrane system to the sarcomere. The sequence of information transfer is initiated by electrical depolarization at the sarcolemma membrane along the length of the fiber. The depolarization is transferred transversely to within the fiber by way of transverse tubules, which are invaginations from the sarcolemma. The transverse tubules are junctionally associated with the terminal cisternae (TC) of sarcoplasmic reticulum to form an intracellular junction, the triad junction (Figs. 1 and 2). There are two triad junctions per sarcomere. In mammalian skeletal muscle, the triads are at the region of the A/I band of the sarcomere. While the sarcomere is the fundamental unit of muscle contraction, the sarcotubular membrane system regulates the release and uptake of Ca^{2+} causing muscle to contract and relax, respectively.

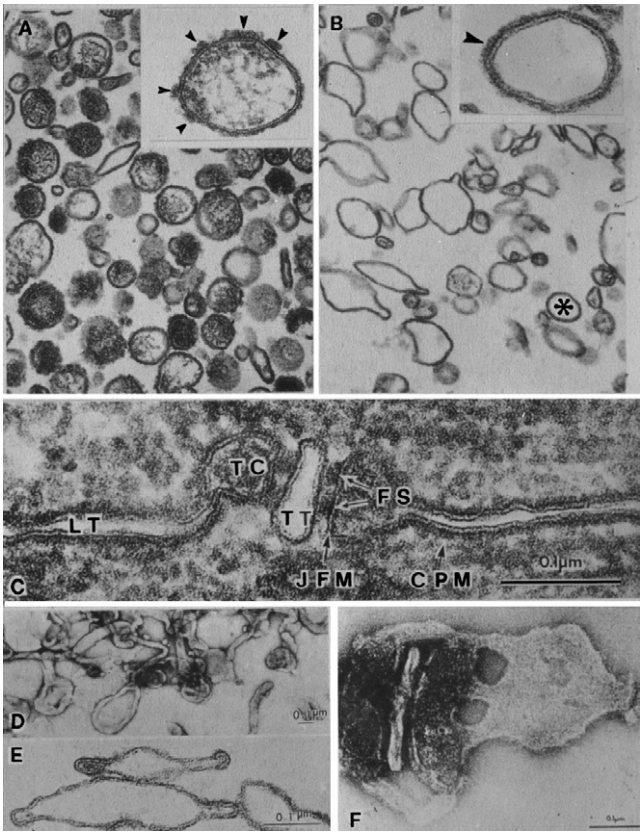

Fig. 2. Electron micrographs of purified subcellular fractions of fast-twitch skeletal muscle. Fractions are enriched in: (A) terminal cisternae of SR; (B) longitudinal tubules of SR, with the insets in (A) and (B) showing a selected vesicle of terminal cisternae and longitudinal tubules, respectively, at higher power; the arrowheads in inset (A) indicate the foot structures on the junctional face membrane (JFM); the arrowhead in *B* shows the asymmetric orientation of the membrane; (C) longitudinal section of intact fast-twitch skeletal muscle similarly fixed as in (A) and (B) with glutaraldehyde–tannic acid fixative, showing the triad junction. The association of two terminal cisternae (TC) with one transverse tubule (TT) is by way of the foot structures (FS), indicated by arrows. The longitudinal tubule (LT) of SR consists of the calcium pump membrane (CPM), and is highly asymmetric, as shown in the inset of panel (B); (D) plasmalemma; (E) transverse tubules; (F) triad junction, viewed by negative staining. (A–C) Adapted from [31] and [42].

By contrast with skeletal muscle, the macroscopic phenomenology in heart is referred to as Ca^{2+}-induced Ca^{2+} release [5]. That is to say, in order for heart muscle to contract, influx of a small amount of extracellular Ca^{2+} via the slow inward voltage-gated Ca^{2+} channel in the sarcolemma is essential to trigger the more massive release of Ca^{2+} from the SR into myoplasm thereby increasing the Ca^{2+} concentration to trigger muscle contraction. The cardiac myocyte is much smaller in size, about 10–20 μm in diameter and ~100 μm long. In heart tissue, the cardiomyocytes are connected to one another at their longitudinal ends by way of junctions referred to as intercalated discs. The signal for depolarization is transferred from one cardiomyocyte to another via gap junctions in the intercalated discs. This junctional association of cardiomyocytes with one another enables the action potential to travel from one cardiomyocyte to another, and hence the signaling in heart tissue is effective over long distances. The time response in heart is much slower (~0.1 s) than for skeletal muscle. In turn, the response time in heart is faster than that for smooth muscle, which has dimensions of 2–6 by 100–200 μm and has yet a much slower response time (seconds to minutes). The signaling in smooth muscle is hormonally stimulated and is referred to as pharmacomechanical coupling.

The electron micrograph in Fig. 2C focuses in on the triad junction in situ. The tripartite nature of the triad, consisting of two terminal cisternae of SR in junctional association with the transverse tubule membrane system can readily be seen. It is the junctional face membrane (JFM) of terminal cisternae that is involved in the junctional association with the transverse tubule. The triad junction appears to be held together by way of the large electron opaque "foot structures" [6] which can readily be observed using tannic acid fixation (Fig. 2C) [3]. A diagrammatic representation of the triad structure is shown in Fig. 3.

Isolation of purified membrane fractions of fast twitch skeletal muscle

After much painstaking effort, we succeeded in isolating purified membrane fractions referable to key membranes from rabbit skeletal muscle, which are shown in the electron micrographs in Fig. 2. These fractions include terminal cisternae (Figs. 2A and 4A) [3] and longitudinal tubules (LT) (Fig. 2B) of SR [7], sarcolemma (Fig. 2D) [8] and transverse tubules (Fig. 2E), as well as a fraction enriched in triads (Fig. 3F) [9].

The isolation of the terminal cisternae turned out to be an important advance. The separation of the SR by density gradient centrifugation yielded heavy and light SR fractions [3]. Electron micrographs of a field of purified heavy SR fraction are shown in Figs. 2A and 4A. The heavy SR fraction as isolated consists of membrane vesicles with well-defined foot structures and containing electron opaque

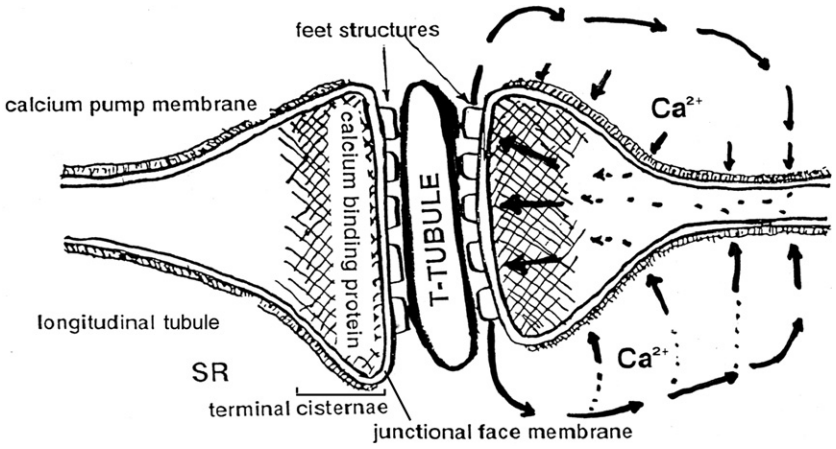

Fig. 3. Diagrammatic representation of the triad junction. The longitudinal tubules (LT) and terminal cisternae (TC) of SR are indicated. The TC consists of the junctional face membrane (JFM) and the calcium pump membrane (CPM), and the lumen contains calsequestrin. The intracellular Ca^{2+} circulation is shown by the arrows. Ca^{2+} is pumped into the SR lumen by the CPM of the LT and the TC, and stored within the lumen of the TC. When the TT is depolarized, Ca^{2+} release is triggered from TC by activation of the calcium release channels in the foot structures. Adapted from [43].

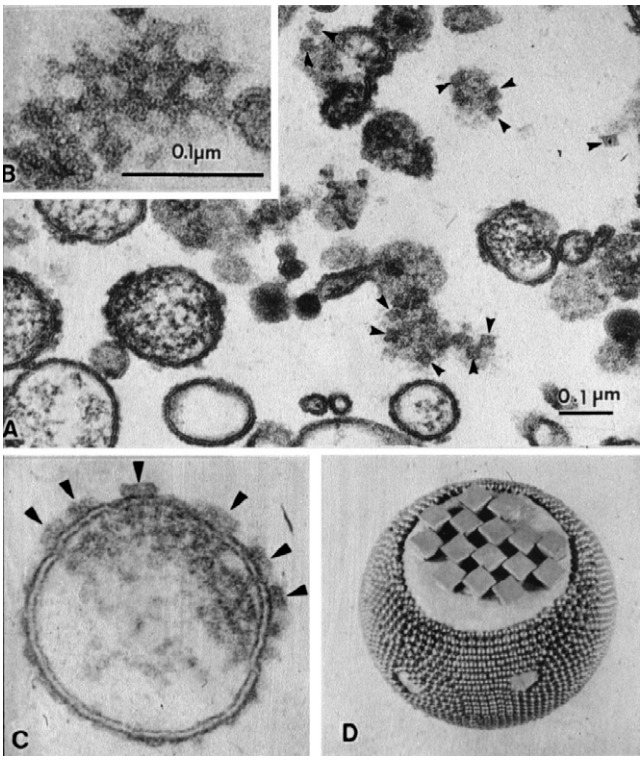

Fig. 4. Structure of isolated terminal cisternae. (A) Numerous foot structures (arrowheads) are observed in the cross-sections of the vesicles, and the tangential sections of the TC. The foot structure viewed tangentially is a square-like structure (see arrowheads). (B) In the tangential section, the arrays of alternating square-shaped foot structures (20 nm/side), and spaces, give a quasi-checkerboard-like lattice. (C) JTC showing two types of membranes, the JFM with foot structures (arrowheads) and the asymmetric CPM (see Fig. 2B). (D) Diagrammatic representation of JTC of SR. Reprinted with permission from [3].

material within the compartment. The junctional face membrane (JFM) which contains the foot structures is quantitatively a minor portion of the membrane of the terminal cisternae. Most of the membrane of terminal cisternae is referable to the Ca^{2+} pump membrane, the main membrane of the longitudinal tubules of SR.

The electron micrographs in Figs. 2A and 4A show that the fraction is highly enriched in vesicles containing foot structures [3,31,43]. They resemble the terminal cisternae of SR as observed in the tissue (Fig. 2C). The electron opaque material within the heavy SR fraction is observed in close proximity to the junctional face membrane. The terminal cisternae fraction clearly contains foot structures which appear to be similar in size to that seen in sections of the intact muscle (Fig. 2C). Tangential sections of the terminal cisternae fraction reveal that foot structures are square-like, about 200 Å/side. (Fig. 4A, see arrowheads). Selected tangential sections (Fig. 4B) reveal that the foot structures are organized in quasi-checkerboard array (Fig. 4B). A diagrammatic representation of a terminal cisterna is shown in Fig. 4D. Two types of membranes are depicted; the junctional face membrane containing the foot structures and the longitudinal tubule membrane enriched in Ca^{2+} pump membrane. By contrast the light SR fraction also consists of membrane vesicles [7], but is devoid of foot structures and electron opaque contents. It consists almost entirely of Ca^{2+} pump membrane, depicted by the beads. We concluded that the heavy SR fraction is referable to the terminal cisternae and that the light SR fraction derives from the longitudinal tubules of SR (Figs. 2B and 3), since the latter is devoid of both electron opaque contents and foot structures (Fig. 2B, inset). Further, tannic acid fixation for electron microscopy reveals that the light SR membranes are highly asymmetric (Fig. 2B, inset) similar to that observed for the longitudinal membrane in situ (Fig. 2C).

We were thus successful in isolating purified skeletal muscle membrane fractions which can then be characterized with regard to structure and function to provide a clue for the isolation of the Ca^{2+} release machinery.

Identification of the Ca^{2+}machinery of skeletal muscle and heart

Skeletal muscle

Energized Ca^{2+} uptake was studied in the fractions enriched in terminal cisternae and longitudinal tubules of SR. Actually, the assay that we used is referred to as "energized Ca^{2+} loading". The assay medium contains an anion (phosphate or oxalate) which readily diffuses across the membrane and precipitates with Ca^{2+} ions which are concentrated within the SR membrane compartment by the Ca^{2+} pump protein in the membrane. This is a convenient assay because linear rates are obtained for a relatively long time. The assay is shown in Fig. 5. The Ca^{2+} concentration, shown on the ordinate, is measured in the presence of an indicator for Ca^{2+}. The Ca^{2+} loading rate is obtained from the slope of the decrease in Ca^{2+} concentration as a function of time. The assay is initiated by adding ATP to the cuvette containing the SR fraction and Ca^{2+} [10].

The Ca^{2+} loading rate for the longitudinal tubule SR fraction was linear and quite rapid (~2.0 μmol Ca^{2+} uptake per minute per mg protein, not shown). The Ca^{2+} loading of the terminal cisternae fraction gave surprising results. The rate was very slow (0.10–0.15) compared with that of the longitudinal tubule fraction of SR, even when normalized for the amount of Ca^{2+} pump in the fraction. At this point we resorted to pharmacology. We found that the rate could be enhanced 7- to 10-fold by adding ruthenium red to the assay medium. The preincubation of the terminal cisternae fraction for several minutes with added ryanodine before adding the ATP, eliminated the enhancement of Ca^{2+} loading by ruthenium red. The inhibition by ryanodine of the rate enhancement with ruthenium red was found to be concentration-dependent. A double reciprocal plot gave an inhibition constant of ~ 20 nM ryanodine [10].

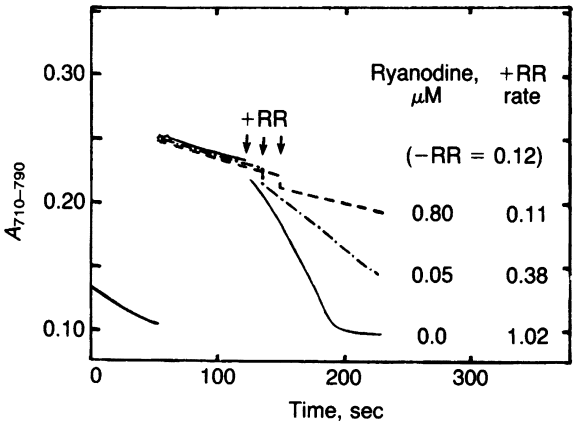

Fig. 5. Ryanodine inhibits Ruthenium Red (RR) stimulation of Ca^{2+} loading in isolated junctional terminal cisternae vesicles. The Ca^{2+} loading assay medium was admixed with terminal cisternae and ryanodine (when added) and incubated for 2 min. At zero time, Na_2ATP was added. The decline in absorbancy with time indicates uptake rate of calcium from the medium. Calcium ions (50 μM) were added at 60 s, resulting in an increase in absorbancy, and a slow "basal" Ca^{2+} loading rate (0.12 μmol/min mg of protein) was observed. RR (7 μM) stimulates this rate 8.5-fold. Preincubation with ryanodine in a concentration-dependent manner decreased the enhancement of Ca^{2+} loading with RR. Reprinted with permission from [10].

Ryanodine is a plant alkaloid which is used as an insecticide. The insects die in muscle contractures. Its mode of action was not known. We reasoned that a possible mode of action of the alkaloid was to make the SR compartment leaky to Ca^{2+}, so that even though the Ca^{2+} is pumped into the SR, it leaks out again. The concentration of Ca^{2+} in the myoplasm remains sufficient for muscle contraction. The muscles of the insects remain in a continual state of contracture, and the insects likely die from loss of their energy stores.

It appeared that the Ca^{2+} efflux machinery was localized to the terminal cisternae of SR and not in the longitudinal tubules of SR, since the rate of Ca^{2+} loading of the LT fraction was high and not enhanced by ruthenium red, whereas the TC fraction was modulated by these ligands. Ryanodine binding to TC was measured and was also found to be high affinity, in the nM concentration range. Hence, ryanodine was a ligand that could be used to identify and isolate the ryanodine receptor, the putative Ca^{2+} efflux machinery. The ryanodine receptor was thus localized to the terminal cisternae of SR and not in the longitudinal tubules of the SR. Ryanodine was a high affinity ligand for the receptor. In a laboratory like ours, with specialty in membrane biochemistry, it was a matter of time before the ryanodine receptor was isolated.

The ryanodine binding receptor was then purified from the terminal cisternae of SR by Makoto Inui (Fig. 6) [11]. Purification first requires solubilization with detergents. CHAPS and supplementation with phospholipid was found to be the detergent of choice. This was followed by purification using several modes of column chromatography of which heparin–agarose column chromatography was especially effective (see Fig. 6A for the SDS–PAGE of the purification for skeletal muscle). The purified receptor consists of a single high molecular weight band. Sometimes a proteolytic fragment of the ryanodine receptor is observed just below the high molecular weight band. The receptor binding of the purified receptor was enriched approximately 20-fold from the starting terminal cisternae. The ryanodine binding was high affinity in nature, and with a binding constant similar to that of the starting TC of SR.

The purified receptor was observed by electron microscopy, using negative staining with uranyl acetate. The field of the purified receptor from skeletal muscle was replete with square-like structures about 220 Å per side (Fig. 7C) which matched the shape and size of the foot structures observed in tangential sections at the junctional face of the terminal cisternae (Fig. 7E).

Is the receptor that we isolated truly a channel? This feature was studied together with Hansgeorg Schindler and his colleagues. The purified ryanodine receptor from skeletal muscle was incorporated into vesicle-derived planar bilayers. Indeed, the receptor displayed channel gating [12].

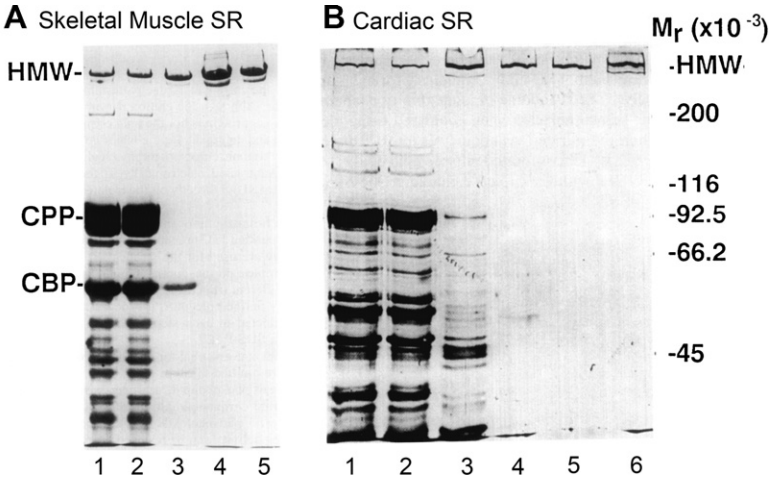

Fig. 6. Purification of RyRs from skeletal muscle and heart terminal cisternae. (A) The purification from JTC of rabbit fast-twitch skeletal muscle as viewed by SDS–PAGE. Lane 1, JTC; 2, solubilized JTC; 3, heparin–agarose chromatography; 4, hydroxylapatite chromatography; 5, gel permeation chromatography to yield purified skeletal muscle RyR, as the high M_r band on the gel. (B) The purification of RyR from cardiac microsomes as viewed by SDS–PAGE. Lane 1, cardiac microsomes; 2, solubilized microsomes; 3, heparin–agarose chromatography; 4, *p*-aminobenzamidine column chromatography; 5, gel permeation chromatography; 6, skeletal muscle RyR shown for comparison. The purified receptor from skeletal muscle sometimes has a small amount of proteolytic fragment of RyR1, just below the high molecular weight band referable to the RyR1. Reprinted with permission from [44].

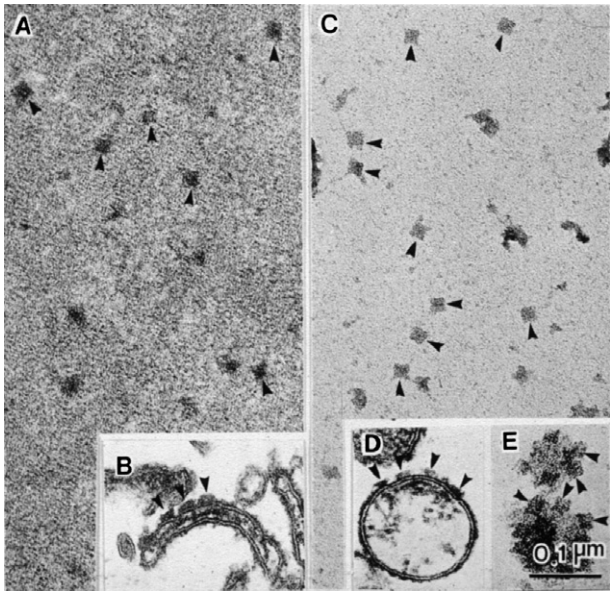

Fig. 7. Morphology of the purified RyR from heart and skeletal muscle. (A) An electron micrograph field of heart RyR. Inset (B) The heart TC fraction (B). (C) A field of skeletal muscle RyR. Inset (D) shows a cross-section of a TC vesicle, and (E) is a tangential section of the TC showing the square foot structures on the junctional face of the terminal cisternae. The purified RyRs from heart and skeletal muscle are similar square-shaped foot structures (indicated by arrowheads) (A, C and E) and are of similar size and shape, in purified form (A and C), and in TC (E). Reprinted with permission from [45].

The receptor forms Ca^{2+} specific-channels which become highly activated at 50 nM Ca^{2+}. At suboptimal Ca^{2+} levels (100 nM), the channel was strongly activated by 1 mM ATP and was blocked by ruthenium red. Ryanodine was found to stabilize the open state and block the action of ruthenium red to close the channel. Thus, the purified ryanodine receptor incorporated into planar bilayers has the channel characteristics consistent with the Ca^{2+} release observed with isolated terminal cisternae vesicles (Fig. 5).

Heart muscle

Our next goal was to identify the Ca^{2+} release machinery of heart. As pointed out in the background material, heart myocytes are quite different from the skeletal muscle fiber in many respects. Heart myocytes are considerably smaller than the myofibers of skeletal muscle. Their macroscopic phenomenology is different, i.e., it is characterized as "Ca^{2+} induced Ca^{2+} release". Heart myocytes are junctionally linked to one another by intercalated discs. Cardiomyocytes obtain their energy mainly from mitochondrial oxidative phosphorylation. The mitochondria make up about 35% of the mass of the myocyte. The myocyte response time is much slower than for skeletal muscle. Cardiomyocytes are not innervated by nerves, and transmission is from cardiomyocyte to cardiomyocyte by way of gap junctions at the intercalated discs. Nonetheless, foot structures are observed in triad junctions as well as in dyad junction of terminal cisternae with sarcolemma. The morphology of the triad junctions in heart is also somewhat different. The terminal cisternae are flattened and the transverse tubules are larger in size.

A similar approach for was carried out for heart. We succeeded in isolating light and heavy SR from heart (Fig. 7A) [13]. The TC fraction responded similarly to the modulation of Ca^{2+} loading by ryanodine and ruthenium red (as in Fig. 5, not shown). The terminal cisternae enriched fraction displayed high affinity ryanodine binding (not shown). The LT fraction from heart was not modulated by these ligands. The heart ryanodine receptor was purified by similar procedures as for skeletal muscle

(Fig. 6B) [14]. The purified receptor from heart was morphologically similar in shape and size to that of skeletal muscle (Fig. 7A and C). The purified cardiac receptors were incorporated into planar bilayers and found to be Ca^{2+}-activated channels.

To summarize the work described above: our laboratory identified and purified the Ca^{2+} release machinery from both heart and skeletal muscle. The ryanodine receptors from skeletal muscle and heart were purified to near homogeneity. For both, skeletal muscle and heart, the ryanodine receptors are localized to the terminal cisternae of SR. They consist of a single high molecular weight polypeptide. The ryanodine receptors have been identified morphologically as the foot structures observed in situ in the triad (or dyad) junction. The purified ryanodine receptors incorporated into planar phospholipid bilayers possess Ca^{2+} channel activity, characteristic of Ca^{2+} release in SR. That is, the receptors from both heart and skeletal muscle are similarly modulated by ryanodine and ruthenium red and other ligands. *We concluded that the calcium release channels have been identified both morphologically and in molecular terms.*

Molecular mass of the ryanodine receptor and oligomeric structure

The molecular mass of the ryanodine receptor from skeletal muscle was measured by scanning transmission electron microscopy (STEM) of unstained receptors. These studies were carried out together with Joseph Wall of the Brookhaven National laboratory. Images were obtained by dark field of the unstained samples using a large annular detector. The mass of the receptor was found to be 2.3 ± 0.3 megadaltons [15]. The monomeric size of the ryanodine receptor was established by cloning technology. At that time, we were collaborating with Andrew Marks and Bernardo Nadal-Ginard to clone the ryanodine receptor. The size of the polypeptide was estimated by northern blot analysis to be about 550 kDa [16]. Soon thereafter the ryanodine receptor was cloned, in the laboratory of Soshaku Numa [17] and in the laboratory of Dave MacLennan. The full amino acid sequence became available for skeletal muscle RyR. The size obtained from the amino acid sequence was found to be 565,233 Da. Dividing 565,000 into 2.3 million, gives a value of 4. Therefore, the ryanodine receptor appeared to be a homotetramer. That is, the receptor consists of four identical subunits. The RyR receptor from heart was likewise cloned. The mass of the protomer, also obtained from cloning, was nearly the same (564,711) [18] as for skeletal muscle. Considering the 4-fold symmetry of the receptor, it was reasonable to postulate that the RyR from skeletal muscle and heart are homotetramers.

There are three different isoforms of the ryanodine receptor in mammals, each is encoded by a different gene [19]. They have been designated as skeletal (RyR1), cardiac (RyR2) and brain (RyR3). Actually, those are the sources where the receptors are in higher amount. However, these receptors are present in small amounts in many tissues. RyR3 was also first purified in my laboratory by Loice Jeyakumar from bovine diaphragm SR using site specific affinity purified antibody [20].

FK506 binding protein (FKBP) is associated with the ryanodine receptors

The finding that FK506 binding protein is associated with the ryanodine receptor derived from our studies with Andrew Marks and Bernardo Nadal-Ginard. Proteolytic digestion was carried out to obtain insight into the surface topography of the native receptor [21]. Endopeptidases were used to cleave peptide bonds on the surface of the receptor. The peptides thereby obtained were sequenced and identified within the known primary sequence of the RyR1. Twenty-four cleavage sites were identified and equated to surface-exposed peptides. A 25th peptide was also obtained which was not in the primary sequence, nor could we find the sequence in the gene data bank. Later a match was reported for the 25th peptide. It was identified as the N-terminal sequence of FK506 binding protein. The significance of FK506 Binding Protein is that it is a small soluble receptor protein, (also referred to as immunophilin), first reported in T-cell lymphocytes that bind the potent immunosuppressive drug FK506. The complex of immunophilin and FK506 blocks activation of transcription factors for lymphokines and thereby inhibits graft rejection [22].

We therefore looked for the presence of FK binding protein in the purified RyR1 and found that the FKBP was indeed present and tightly associated with the purified ryanodine receptor [23]. The stoichiometry of FKBP to ryanodine receptor was 4. Since the RyR is a homotetramer of four identical RyR subunits, there is one FKBP bound per RyR protomer. Thus, RyR is a hetero-oligomer with the structural formula $(\text{RyRprotomer})_4(\text{FKBP})_4$. FKBP is localized only to the TC and not to the LT of SR [24]. The Stokes radius of the FKBP is 12,000. The FKBP is therefore referred to as FKBP12.

How general is the association of FKPB in different vertebrate classes? Does FKBP modulate the function of the ryanodine receptor? The FKBP was found to be conserved in species from each of the 5 vertebrate classes [25]. The stoichiometry is similar to that of mammalian (rabbit) skeletal muscle. For each vertebrate class, the stoichiometry is four FKBP per ryanodine receptor, i.e. one FKBP per RyR protomer. Methodology was developed to release the FKBP from the RyR by treatment with FK506. The FKBP is readily released with this ligand. This is a gentle treatment. We could thereby carry out reconstitution experiments to measure the effect of FKBP on channel activity [26]. When FKBP is removed by this treatment, the RyR1 channel becomes activated. FKBP rebinds when added back to the RyR1 devoid of FKBP. The channel becomes quiescent again. Thus, the FKBP appears to modulate the channel activity of RyR. The FPBP helps to maintain the channel in the closed state.

Heart TC containing RyR2 was also found to be associated with a small polypeptide which has a stoichiometry of

four FKBP/RyR2, similar to that found with RyR1. The FKBP-like polypeptide is likewise tightly bound, and can be released by treatment with FK506. The polypeptide associated with dog heart RyR2 was found to have a somewhat slower mobility than the FKBP by SDS–PAGE. It is referred to as FKBP12.6 [27–29]. Cloning and sequencing shows that FKBP12.6 is a different isoform of FKBP [52]. Purified RyR3 was also found to be associated with tightly bound FKBP12, also with a stoichiometry of 4. FK506 also releases the tightly bound FKBP12 from RyR3.

However, the removal of FKBP12.6 from RyR2 or FKBP12 from RyR3 does not result in the activation of the channel. These channels do not appear to be modulated by FKBP. The rebinding of FKBP for RyR2 from dog heart is specific for FKBP12.6 [28]. However, hearts from other species were found to also bind FKBP12, although neither FKBP isoform modulates channel activity in RyR2 [28]. However, FKBP-depleted RyR1 can rebind either FKBP12 or FKBP12.6 to make the channel quiescent again. Thus, RyR1 channel activity is modulated by FKBP whereas RyR2 and RyR3 are not modulated by FKBP even though FKBP is bound with a similar stoichiometry [29].

In order to gain insight into the role of FKBP in heart, Hong-Bo Xin in my laboratory prepared a knockout mouse with respect to FKBP12.6 [30]. These studies with the knockout mouse are referred to in the last section.

Ultrastructural studies of the ryanodine receptors

The three isoforms of the ryanodine receptor, isolated in our laboratory were studied with regard to ultrastructure. These studies were carried out together with Terry Wagenknecht and Joachim Frank at the Wadsworth Center, New York State Dept of Health, Albany, NY. A field of purified RyR1, negatively stained with uranyl acetate is shown in Fig. 8 [31]. There is good detail of the image of the receptors, however, there is also considerable noise. A computer-averaged view of the receptor minimizes the noise. The enhanced image of the receptor displays 4-fold symmetry and ultrastructural detail. Correlation alignment analysis was used to enhance detail by decreasing the noise. In practice from these digitalized fields, 240 images were selected and subjected to correlation alignment with a typical particle as reference, the RyR is rotated one with respect to a typical particle by computer. Since the receptor displays 4-fold symmetry, a rotation of 360 degrees should give four peaks of roughly equal sizes. The computer-averaged top view of the RyR1 obtained by averaging 240 negatively stained images is shown in the inset in the lower right hand corner. A three-dimensional view of the receptor requires tilting the receptor to get the third dimension.

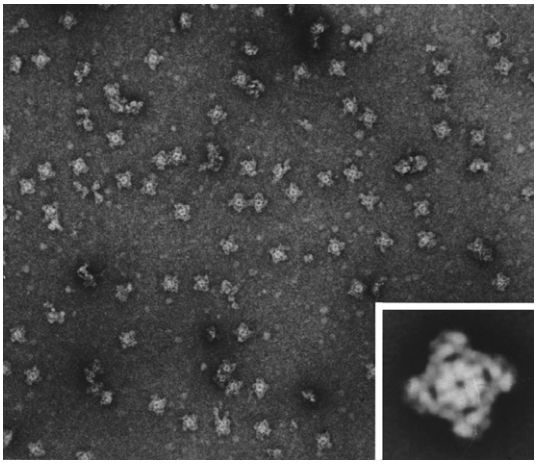

Fig. 8. Structure of the RyR as viewed by negative staining. Considerable detail can be observed. Image enhancement of 240 receptors was used to obtain a computer-averaged view of the receptor (inset). Reprinted with permission of [31].

The three-dimensional structure of the receptor was first obtained from the negatively stained image. More recently cryoelectron microscopy was used to obtain the three-dimensional structure of RyR1 [32]. Preparation of the samples for cryoelectron microscopy is by quick-freezing. The advantage with this newer procedure for sample preparation is (1) the sample is not contaminated with the reagent used for negative staining; and (2) the sample is not squashed by the drying process used in the negative staining.

The three-dimensional surface representation of the three isoforms of the RyR1 [32], RyR2 [33] and RyR3 [34] using cryoelectron microscopy and image enhancement is given in Fig. 9. In this study, channels in the "closed"

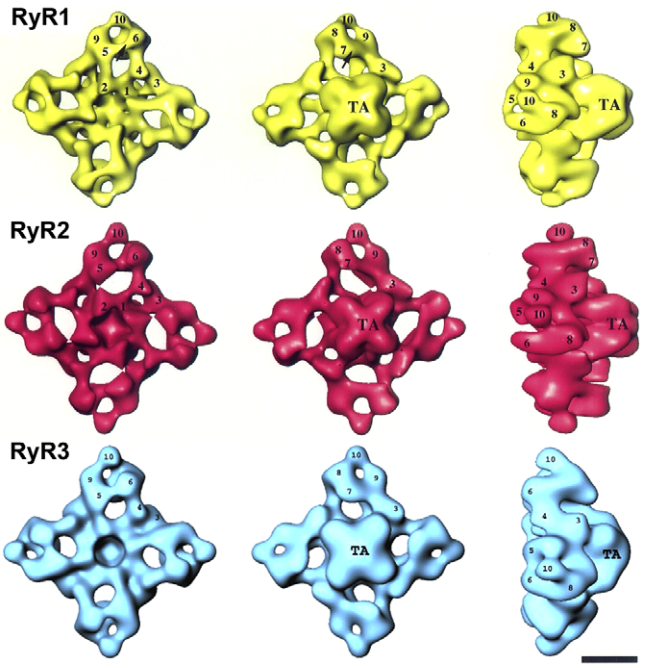

Fig. 9. Three-dimensional surface representation of RyR1, RyR2 and RyR3, using cryo-electron microscopy and image enhancement. The images are for the "closed state". *Left*, transverse tubule face; *middle*, terminal cisternae face indicating the transmembrane assembly (TA) portion; *right*, side view image. The TA is inserted into the JFM to form the triad junction. Adapted, and reprinted with permission from [32–34].

state'' are compared. Both ''open'' and ''closed'' states have been studied [35]. On the left is the transverse tubule face, the middle shows the terminal cisternae face. The transmembrane assembly from this face is inserted into the junctional face of the junctional face membrane of the TC. The side view is shown in the image on the right. The first decimal place message is that the receptors are very similar in structure. However, there are also significant differences. A discussion of these differences is detailed in the original papers [32–35].

The crystallization of the RyR would be the best way to get detailed structure. However, this has not yet been achieved. A possible direction is to identify specific components in the lower resolution structure obtained with the image enhancement technique. An example is given below.

It was possible to pinpoint the location of the two ligands, calmodulin (CaM) and FKBP that bind to the RyR1 receptor (Fig. 10, [36]). Both CaM and FKBP bind to the cytoplasmic assembly at sites that are 10 and 12 nm, respectively, from the putative entrance to the transmembrane ion channel. FKBP binds along the edge of the square-shaped cytoplasmic assembly near the face that interacts in vivo with the sarcolemma/transverse membrane system, whereas CaM binds within the cleft that faces the junctional face of the sarcoplasmic reticulum membrane at the triad junction. Studies are ongoing also in the laboratory of Susan Hamilton and Wah Chu to obtain high resolution imaging of the RyRs.

The dimensions of the receptor are $29 \times 29 \times 12$ nm and a smaller transmembrane assembly that protrudes 7 nm from the terminal cisternae face. A cylindrical low density region, 2–3 nm in diameter extends down into the center of the transmembrane assembly, which may correspond to the center of the transmembrane assembly, possibly corresponding to the Ca^{2+}-conducting pathway.

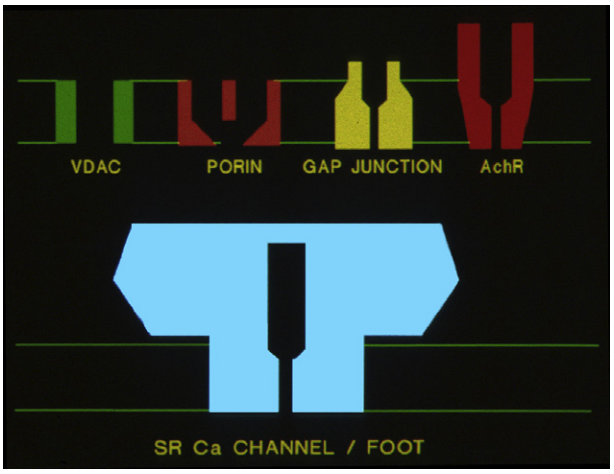

Fig. 11. Size comparison of various channels. The RyR is the largest channel known. It has dimensions of ~260 × 260 × 150 Å, and dwarfs even the next largest channel, the acetylcholine receptor, which is ~ 90 Å across and 140 Å perpendicular to the membrane. Image courtesy of Dr. Terry Wagenknecht.

A size comparison of some known receptors is compared in Fig. 11. The RyR is the largest channel structure known. It dwarfs the next largest channel, the acetylcholine receptor, which is ~90 Å wide and 140 Å perpendicular to the membrane.

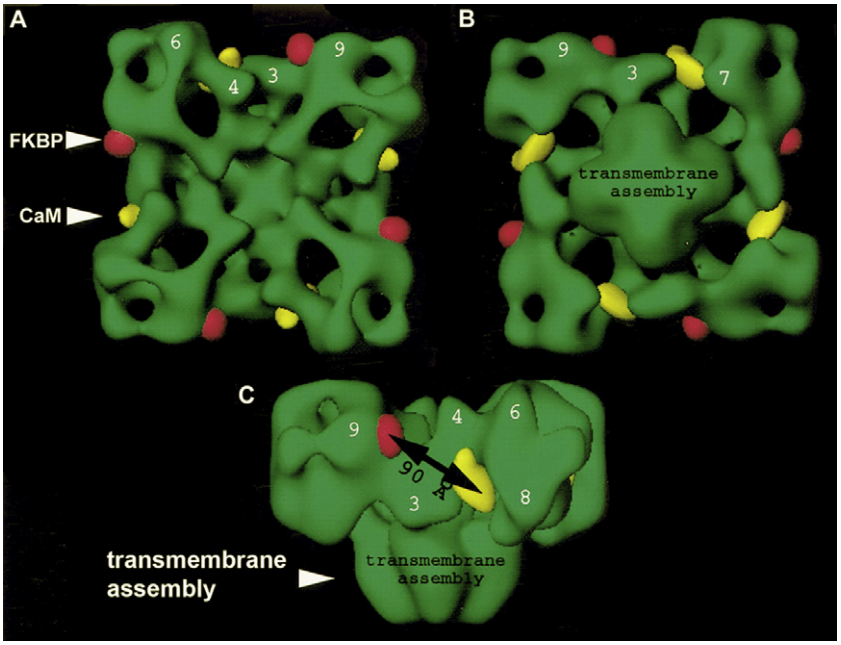

Fig. 10. Binding sites for FKBP and calmodulin (CaM) on the terminal cisternae from skeletal muscle. The three-dimensional reconstruction of RyR, together with the differences attributed to CaM (yellow) and FKBP (pink). (A) Transverse tubule face; (B) terminal cisternae face; (C) side view. Adapted, and reprinted with permission from [35,36].

Modulation of the ryanodine receptor channel activity by phosphorylation/dephosphorylation

Larry Jones' laboratory found that the heart ryanodine receptor was phosphorylated with Ca^{2+}/CaM protein kinase which activated the channel activity in the presence of ATP and calmodulin as measured by the open time probability (Po). The Po is a measure of the % of time that the channel stays open. The channel was phosphorylated at ser2809 [37]. They also reported that skeletal muscle RyR is not significantly activated under similar conditions.

The modulation of heart and skeletal muscle ryanodine receptors was studied in our laboratory together with Hansgeorg Schindler and his students Jurgen Hain and Martin Mayrleitner. Channel modulation was studied in planar bilayers. Terminal cisternae of SR were fused to planar bilayers for channel measurement. For this purpose, a microsyringe applicator was developed which permitted multiple sequential treatments with highly purified kinases and phosphatases. Single channel currents were measured at zero holding potential at 0.15 mM micromolar free Ca^{2+} , ±0.5 mM ATP, and ±2.6 mM Mg^{2+}. Sequential dephosphorylation and rephosphorylation rendered the channel sensitive and insensitive to block by Mg^{2+} (Fig. 12). Channel recovery from Mg^{2+} block was obtained by phosphorylation with exogenous PKA or Ca^{2+}/CaM-dependent protein kinase II. Channel block by Mg^{2+} was restored by dephosphorylation using protein phosphatase 1. Thus, the two states, sensitive and insensitive to Mg^{2+} block were interconvertible. These findings are consistent with a physiological role for phosphorylation/ dephosphorylation in the modulation of the RyR from skeletal muscle [38].

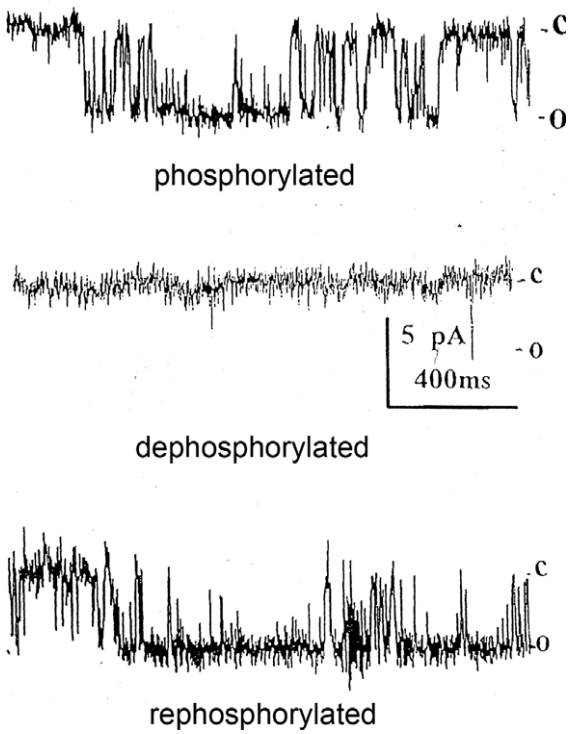

Fig. 12. Modulation of channel activity of the skeletal muscle RyR. Sequential dephosphorylation and phosphorylation rendered the calcium release channel sensitive and insensitive to block by Mg^{2+} ions, respectively. These studies were carried out by reconstituting the RyRs into planar bilayers and treating with protein kinase A or Ca^{2+} calmodulin protein kinase to activate the channels. Then sequential treatment with protein phosphatase leads to loss of channel activity that can again be activated by protein kinases [38–40] (S. Fleischer and H. Schindler, unpublished experimental traces).

Similar studies were carried out with the ryanodine receptor from heart, and similar results were obtained. Our studies indicate that the channel must be phosphorylated to be in the active state at conditions approximating physiological Mg^{2+} concentration [39].

What is the role of FKBP 12.6 in heart SR?

We were faced with the enigma that FKBP12.6 is tightly associated with the cardiac ryanodine receptor with a stoichiometry of 4 FKBP12.6/RyR2. This is the same stoichiometry as for FKBP12 in the skeletal muscle RyR The channel activity of RyR2 is not modulated by removal and re-addition of FKBP12.6. The FKBP12.6 is a different isoform of FKBP12 and the binding of the FKBP to heart RyR is specific for the FKBP12.6. The FKBP12 isoform does not bind to RyR2 from dog heart. Interestingly, both FKBP12 and FKBP12.6 isoforms can bind and reconstitute channel modulation in skeletal muscle RyR1.

We prepared a knockout mouse with respect to FKBP12.6 [30]. The mice come to term and appear normal in behavior. A knockout mouse was previously made with respect to FKBP12, but the animals died before coming to term [41].

Using echocardiography measurements, we find that the male mice have cardiac hypertrophy (Fig. 13). Interestingly, the female hearts are normal. Both male and female mice display similar dysregulation of release seen as increases in the amplitude and duration of Ca^{2+} sparks and Ca^{2+} induced Ca^{2+} release gain. Female null mice treated with tamoxifen, an estrogen receptor antagonist, also develop cardiac hypertrophy similar to that of the male mice. These studies suggest that FKBP12.6 modulates excitation–contraction coupling and that estrogen plays a protective role in the hypertrophic response of the heart to Ca^{2+} dysregulation [30].

Overview

Our studies began with the aim to identify the molecular machinery involved in release of Ca^{2+} from skeletal muscle. The ryanodine receptor was purified and identified morphologically as the foot structure, and found to be the Ca^{2+} release channel that releases Ca^{2+} from intracellular stores. Three isomers of the ryanodine receptor were isolated and found to be Ca^{2+} stimulated channels. The receptors have similar three-dimensional structures which were identified morphologically as the foot structures. At physiologic Mg^{2+} concentration, the receptors are modulated

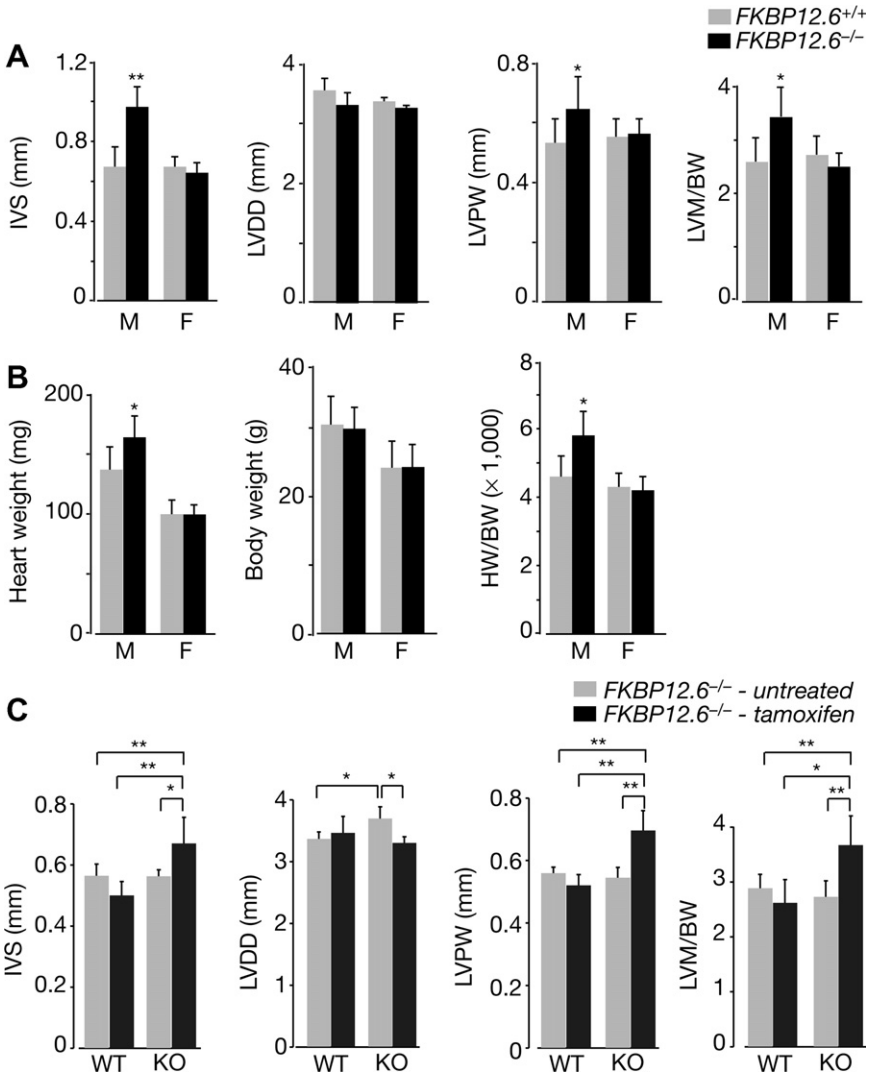

Fig. 13. Sex-specific cardiac hypertrophy in male FKBP12.6 knockout mice and cardiac hypertrophy in female knockout mice treated with tamoxifen. (A) Echocardiographic measurements in adult *FKBP12.6*$^{-/-}$ mice ($n = 8$ in male (M), $n = 11$ in female (F)) and wild type mice ($n = 8$ in male and female). IVS, interventricular septum; LVDD, left ventricular end-diastolic diameter; LVPW, left ventricular posterior wall; and LVM/BM, left ventricular mass/body weight ratio. (B) Comparison of heart weights in wild-type mice ($n = 6$ in male and $n = 10$ in female) and *FKBP12.6*$^{-/-}$ ($n = 6$ in male and $n = 30$ in female) mice. HW/BW, heart weight/body weight ratio. Data are presented as mean ± SE; within-sex significance was determined by Student's *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$). (C) Cardiac hypertrophy in *FKBP12.6* knockout female mice treated with tamoxifen. Female null mice treated with tamoxifen have significant hypertrophy relative to untreated knockout mice and treated and untreated wild-type mice. Each group contains 6–8 animals; statistical analysis was performed using ANOVA to compare the four groups (null and wild-type, treated and untreated; $^{*}P < 0.05$; $^{**}P < 0.01$). WT, wild-type; KO, knockout. Reprinted with permission from [30].

by phosphorylation with protein kinases (enhances channel activity) and dephosphorylation with protein phosphatases (inactivates channel activity). The ryanodine receptors from each of the three isomers have a small molecular weight protein associated with it. FKBP12 (Stokes radius of about 12,000) is associated with RyR1 and RyR3. Heart RyR is associated with FKBP12.6, a FKBP with a somewhat slower mobility. FKBP12 and FKBP12.6 were sequenced and found to be different isoforms.

The ryanodine receptors are the largest channel structures known. The RyR consist of four identical RyR protomers of about 565,000 MW each, and four identical FKBP of about 12,000 MW each. The size of the ryanodine receptor is 2.3 million MW. The ryanodine receptor is therefore a heterotetramer. The structural formula for the receptor is $(\text{RyRprotomer})_4\ (\text{FKBP})_4$.

RyR1 channel activity was found to be modulated by FKBP; the latter helps to keep channel activity in a quiescent state. However, FKBP does not seem to modulate RyR2 and RyR3 channel activity.

In order to study the role of FKBP 12.6 in heart, we prepared a knockout mouse with respect to FKBP12.6. Male mice, which are deficient in FKBP12.6, were found to have enlargement of the heart (cardiac hypertrophy), whereas the females were normal in this respect. Female null mice, when treated with tamoxifen, an estrogen receptor antago-

nist, developed cardiac hypertrophy similar to that of the male mice. Thus, estrogen plays a protective role in the hypertrophic response of the heart. There is no known cure for cardiac hypertrophy. Once the heart enlarges beyond an irreversible state, the patient dies within about 5 years. In this regard, our animal model of cardiac hypertrophy may reveal underlying pathways of signaling that lead to cardiac hypertrophy. If so, it may be possible to slow down the hypertrophy or even reverse it.

There are two categories of intracellular calcium release channels, the ryanodine receptors, the focus of this minireview, and the IP3 receptors [46,47]. They serve a critical role in signaling by way of Ca^{2+} as a second messenger. They are widely distributed in most tissues. Some cells have both channel types and even also several of the isoforms [48,49].

Defining the molecular aspects of the RyR Ca^{2+} release channels enabled scientists of different disciplines to make use of this system and by the same token contribute accordingly to the field. The discovery of the ryanodine receptors was soon followed by the recognition of natural mutations in the receptors and linked to human diseases. In skeletal muscle, RyR1 genetic mutations are responsible for malignant hyperthermia and central core disease [50]. In heart, mutations in the ryanodine receptor of RyR2 have been linked to two categories of sudden cardiac death [51].

There is hardly a research University that does not have someone working on some aspect of the intracellular Ca^{2+} release channels. We can anticipate profound new insights in the field of cell signaling.

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