BI-DIRECTIONAL COUPLING BETWEEN DIHYDROPYRIDINE RECEPTORS AND RYANODINE RECEPTORS

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1. ABSTRACT

The control of calcium signaling between plasma membrane dihydropyridine receptors (DHPRs or L-type calcium channels) and ryanodine receptors (RyRs or calcium release channels) located in the endoplasmic/sarcoplasmic reticulum (ER/SR) underlies a broad array of functions including skeletal muscle contraction, cardiac performance, arteriole tone, neurosecretion, synaptic plasticity, and gene regulation. It has long been appreciated that DHPR activation of RyRs and subsequent calcium release from intracellular stores represents a key event in the control of these processes. In excitable cells, DHPRs trigger the release of intracellular calcium by promoting the opening of nearby RyRs (termed *orthograde coupling*). Interestingly, the signaling interaction between DHPRs and RyRs is often bi-directional such that the calcium-conducting activity of DHPR channels is also regulated by its interaction with the RyR (termed *retrograde* coupling). Recent data indicate that skeletal, cardiac, and neuronal cells utilize fundamentally distinct DHPR/RyR bi-directional coupling mechanisms (chemical and mechanical) in order to control the efficiency, fidelity, and activity of each of these two essential calcium channels. This review will focus on evaluating the nature and molecular determinants of these coupling mechanisms, as well as the extent to which excitable cell function is influenced by bi-directional DHPR/RyR calcium signaling.

2. INTRODUCTION

In muscle cells, the process whereby depolarization of the muscle plasma membrane (i.e. an action potential) leads to an increase in intracellular Ca^{2+}, ultimately resulting in muscle contraction is referred to as excitation-contraction (EC) coupling. In both cardiac and skeletal muscle, dihydropyridine receptors (DHPRs) (also known as L-type calcium channels or L-channels) link sarcolemmal depolarization to the rapid release of calcium ions through calcium release channels (also termed ryanodine receptors or RyRs) located in the terminal cisternae of the sarcoplasmic reticulum (SR). Over the past quarter century, considerable effort has focused on the nature of the signal transmission by which cardiac and skeletal muscle DHPRs trigger the opening of SR calcium release channels (DHPR-to-RyR signaling or orthograde coupling). In cardiac muscle, the prolonged ventricular action potential duration results in significant calcium influx through cardiac L-type calcium channels (1-3). This calcium influx subsequently activates nearby SR calcium release channels via a calcium-induced-calcium-release (CICR) mechanism (1,2,4). Thus, the mechanism of signal transmission in cardiac muscle is believed to predominantly involve a chemical signal (i.e. calcium). On the other hand, calcium influx through L-type calcium channels is not required for skeletal muscle contraction (5) and muscle contraction persists even in the complete absence of extracellular calcium (6). Rather, skeletal muscle DHPRs function as voltage sensors for EC coupling by providing a physical, possibly direct, link between sarcolemmal depolarization and the release of calcium from the sarcoplasmic reticulum (7). Thus, the triggering mechanism of skeletal muscle EC coupling is thought to be primarily mechanical in nature (8,9).

In the process of investigating the molecular mechanisms of orthograde coupling, recent results have revealed that signaling between DHPR and RyR proteins is bi-directional in cardiac muscle, skeletal muscle, and
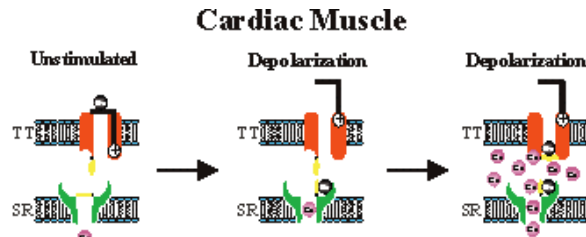
Figure 1. Bi-directional DHPR/RyR2 signaling in cardiac muscle. (Left). In unstimulated cardiac ventricular myocytes, L-type calcium channels (DHPR, red) and SR calcium release channels (RyR2, green) are in a closed state. For clarity, calcium ions originating from extracellular (black) and intracellular release sites (magenta) in Figures 1-3 are depicted using different colors. (*Middle*) Sarcolemmal depolarization during the ventricular action potential causes conformational changes in the DHPR that result in L-channel opening and the rapid influx of extracellular calcium. Influx of extracellular calcium triggers the opening of nearby SR calcium release via calcium-induced-calcium-release (orthograde coupling). Calcium released from the SR promotes both activation of the myofilaments, calcium-induced L-channel inactivation, and a subsequent reduction in further calcium influx and CICR (negative retrograde coupling).

neurons. Accordingly, the calcium conducting activity attributable to the DHPR is strongly influenced by a functional association with its respective RyR. The ability of the presence/activity of the RyR to alter DHPR channel behavior (RyR-to-DHPR signaling) is referred to as retrograde coupling (10). The balance of this review will focus on recent advances made in understanding the molecular and mechanistic nature of bi-directional coupling between DHPRs and RyRs in cardiac muscle, skeletal muscle, and neurons. Wherever possible, the following sections will discuss the current state of knowledge with regard to three central questions: 1) What is the nature of the bi-directional signal (chemical and/or mechanical)? 2) What regions of the DHPR and RyR proteins are required for bi-directional coupling? 3) What role does retrograde coupling play in regulating excitable cell function?

3. CHEMICAL CROSS-TALK

3.1 Bi-directional calcium signaling in cardiac muscle
3.1.1. Orthograde coupling in cardiac muscle
It has now been more than 15 years since the classic work by Fabiato (1,4,11) demonstrated that a rapid rise in intracellular free calcium is able to trigger a concentration-dependent release of calcium from the SR and that this release could be blocked by ryanodine. As a result of these experiments, Fabiato surmised that rapid and local calcium influx through voltage-gated L-type calcium channels triggers a larger release of calcium from the SR via a calcium-induced-calcium release (CICR) mechanism. Since the pioneering work of Fabiato, numerous other groups have confirmed that CICR represents the primary mechanism of orthograde coupling in cardiac muscle. Measurements of spontaneous and localized calcium release events (calcium sparks), first recorded by Cheng and colleagues (1993), demonstrated that CICR in cardiac

myocytes occurs within discrete calcium microdomains (12). More recently, elegant experiments conducted by Wang *et al.* (2001) demonstrated that calcium influx through a single L-channel opening is sufficient to trigger the opening of ~4-6 ryanodine receptors, and thereby, lead to the generation of a localized calcium spark (13). Thus, orthograde coupling in cardiac muscle is widely believed (but see section 6 below) to involve a chemical signal (i.e. calcium ions) transmitted from sarcolemmal DHPRs to the cardiac isoform of the RyR (RyR2) that occurs within a limited structural and functional calcium microdomain (Figure 1).

Although calcium activation of RyR2 is thought to result from the binding of calcium to a specific site(s) within the RyR homotetramere (14), the precise molecular determinants of the high affinity calcium activation site(s) has yet to be determined. Recent site-directed mutagenesis studies indicate that a highly conserved glutamate residue within the putative transmembrane segment M2 (according to the topological model of 15) of the neuronal (RyR3) (16) and cardiac (RyR2) RyR isoforms (17) contributes to a well-conserved high affinity calcium sensor. Accordingly, substitution of an alanine residue for the conserved glutamate residue at this location in rabbit RyR3 (E3885A) reduces the sensitivity for calcium activation of the release channel by ~10,000-fold (16). Alanine substitution for the conserved glutamate residue in mouse RyR2 (E3987A) caused >1,000-fold reduction in release channel sensitivity to activation by calcium (17). Although alanine substitution of the corresponding glutamate residue in the skeletal muscle isoform of the RyR (RyR1) also reduces release channel sensitivity to activation by calcium (18), this effect may arise from alterations in the proper folding of the RyR transmembrane assembly in a manner that results in a global insensitivity of the release channel to activation by various agonists, including calcium (19,20). Clearly, further work will be required to more completely characterize the structural determinants for calcium activation of the RyR and to what degree the mechanism for calcium activation is conserved across the three different RyR isoforms.

3.1.2. Retrograde coupling in cardiac muscle
Since the early work of Brehm and Eckert (1977) (21), it has become widely appreciated that L-type calcium channels, particularly those in cardiac muscle (22-24), exhibit prominent calcium dependent inactivation. Since a localized calcium microdomain between DHPR and RyR proteins mediates orthograde coupling (DHPR-to-RyR coupling) in cardiac muscle (12), it follows that this same calcium microdomain may in turn regulate calcium dependent inactivation of the cardiac L-channel during EC coupling. According to this idea, calcium released from the sarcoplasmic reticulum would act as a negative feedback mechanism for cardiac EC coupling by promoting L-channel inactivation, and thereby, reducing subsequent calcium influx and release (25) (Figure 1). Support for such a "negative retrograde feedback mechanism" in cardiac EC coupling was first provided by experiments conducted by Morad and colleagues (26-28). These experiments revealed that calcium released from the SR

promotes L-channel inactivation even when global calcium transients were eliminated by introduction of high concentrations of high affinity calcium buffers. Under these conditions, the majority of calcium ions would be bound before having diffused ~50 nm away from the site of release (27). Consequently, L-channel inactivation that occurs as a result of SR calcium release must arise from local, rather than global, calcium signaling.

Recent results clearly indicate that calmodulin acts as a Ca^{2+} sensor for calcium-dependent inactivation and facilitation of cardiac L-channels (29-31). The molecular determinants for Ca^{2+}-dependent inactivation and facilitation of cardiac L-channels involve the proximal region of the cytoplasmic C-terminus of α_{1C}, which contains a putative Ca^{2+} binding EF hand motif, an IQ-like domain, and a CaM-binding (CB) domain (29-32). In addition to effects on inactivation and facilitation, the C-terminal region of α_{1C} also regulates cardiac L-channel targeting (33-36), conductance, and open probability (35). Although calcium flux through one L-channel can facilitate the inactivation of an adjacent L-channel (37), ~70% of L-channel inactivation that occurs during EC coupling in rat ventricular myocytes arises from calcium released from the SR (27). The somewhat surprising preference of L-channel inactivation for calcium released from the SR rather than calcium flux through the L-channel itself presumably arises from the higher abundance and larger single channel conductance of RyRs compared to DHPRs (27). As a consequence, calcium ions entering the cell through cardiac L-channels activate ryanodine receptors to release an even greater amount of calcium from the SR such that the released calcium serves a more important role in inactivating sarcolemmal L-channels than calcium flux through the L-channels themselves. Alternatively, it is conceivable that the calmodulin-binding region of the α_{1C} C-terminus resides closer to RyR2 pore than the pore of the L-channel. In either case, retrograde calcium signaling in cardiac muscle serves to fine-tune the gain of EC coupling. Consequently, the close physical association between DHPRs and RyRs in cardiac muscle creates a bi-directional calcium signaling microdomain such that calcium flux through one channel modifies the functional behavior of the other channel (Figure 1, Figure 1.avi).

The functional components of the calcium signaling microdomain in cardiac muscle may extend beyond the reciprocal interaction between sarcolemmal DHPRs and RyRs of the SR. Electron microscopic studies of rat ventricular muscle indicate that junctions between t-tubules and individual SR calcium release channels are located in close proximity (from 37 to 270 nm) to mitochondria (38). These morphological observations suggest that temporal dynamics of the DHPR-RyR calcium microdomain may be influenced by nearby mitochondrial calcium transport mechanisms. Support for this hypothesis comes from the observation that blockade of mitochondrial calcium uptake mechanisms significantly delays the recovery from frequency-dependent inactivation of cardiac L-type calcium channels that occurs during EC coupling, particularly at high stimulation frequencies (39). These observations suggest that mitochondrial clearance of

calcium from the DHPR-RyR microdomain limits the accumulation of L-channel inactivation during EC coupling (39), and therefore influences the degree of retrograde negative feedback (RyR-to-DHPR signaling) in cardiac muscle. It will be important for future work to determine whether bi-directional coupling between cardiac DHPRs and RyRs are altered in response to regulatory factors (e.g. PKA) and under various pathophysiological conditions (e.g. hypertrophy and heart failure).

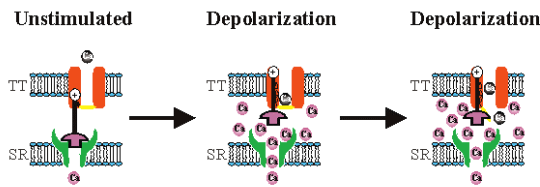### 3.2. Bi-directional calcium signaling in skeletal muscle

It has long been appreciated that influx of extracellular calcium through voltage-gated L-type calcium channels is not required for SR calcium release in skeletal muscle (5,6,40). Rather, depolarization of the skeletal muscle sarcolemma induces voltage-driven conformational changes in the DHPR that are recorded as intramembrane charge movement that precede L-channel opening (41,42). These voltage-driven charge movements presumably gate the opening of skeletal muscle RyRs (RyR1s) in the SR via a mechanical, possibly direct, link between the two proteins (7). Thus, under normal circumstances, bi-directional chemical signaling between DHPRs and RyRs does not occur in mammalian skeletal muscle. However, components of bi-directional chemical signaling in skeletal muscle may occur under some conditions. For example, calcium influx through DHP-sensitive L-channels may contribute to the force of contraction during tetanic stimulation (43), the regulation of gene expression (44,45), or metabolic stabilization of skeletal muscle acetylcholine receptors (46). In frog skeletal muscle, intramembrane charge movements attributable to the activation of sarcolemmal DHPRs exhibit two distinct kinetic components: 1) an early component (Q_β) that decays exponentially and 2) a late component (Q_γ) that appears as a "hump" at the base of Q_β (see ref. 8,47 for reviews). Rios and colleagues have suggested that Q_β reflects gating current of the voltage sensor induced by depolarization of the sarcolemma and Q_γ arises at least in part from an increase in sarcolemmal surface potential introduced by local changes in intracellular calcium that occur during SR calcium release (8). In this way, calcium release could promote further activation of the voltage sensor (positive feedback) and result in additional SR calcium release (48). However, while calcium released during EC coupling clearly influences voltage sensor activity (via a retrograde chemical signal) in amphibian muscle, similar effects are not observed in mammalian skeletal muscle.

4. MECHANICAL CROSS-TALK
4.1. Mechanism of bi-directional coupling in skeletal muscle

Over the years, several different mechanisms have been proposed to account for how changes in t-tubule membrane potential trigger calcium release from the SR in skeletal muscle (see 8,47 for excellent reviews). These mechanisms have included (among others) calcium influx dependent activation of RyR1, activation of calcium release by inositol trisphosphate, and mechanical coupling between the DHPR and RyR1. It is beyond the scope of this review to present a comprehensive evaluation of each of these

A. Normal Skeletal Muscle

Unstimulated   Depolarization   Depolarization

TT   TT   TT

SR   SR   SR

B. Dyspedic Skeletal Muscle
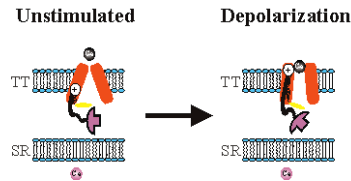
Unstimulated   Depolarization

TT   TT

SR   SR

Figure 2. Bi-directional DHPR/RyR1 signaling in skeletal muscle. (A) (*Left*) At resting membrane potentials, skeletal L-type calcium channels (DHPR, red) are in a closed state. A putative physical interaction between the II-III loop of the skeletal muscle DHPR is shown to inhibit release channel opening via a the "plunger model" proposed by Chandler *et al.* (1976) (7). (*Middle*) Sarcolemmal depolarization causes conformational changes in the DHPR that result in rapid DHPR gating charge movements and a mechanical activation of RyR1 that in independent of the influx of extracellular calcium (orthograde coupling) (5,6). (*Right*) The activation or opening of the skeletal L-channel and influx of extracellular calcium occurs very slowly (shown here to be mediated by a sluggish, spring-loaded activation gate), only after SR calcium release has already been maximally activated. (B) RyR1 promotes the calcium-conducting activity of the skeletal muscle DHPR (positive retrograde coupling). In dyspedic myotubes, which lack RyR1 proteins, the absence of an interaction between the DHPR II-III loop and RyR1 causes global conformational changes in the L-channel complex (shown as a tilting in the transmembrane segments) (57) (*left*). L-channels in dyspedic myotubes continue to undergo voltage-driven conformational changes that produce charge movements following depolarization, but are unable to activate SR calcium release and exhibit significant alterations in the functional properties of L-channel (conductance, kinetics, divalent permeation, and modulation by DHPs) (50,55).

hypotheses. However, the demonstration in skeletal muscle of a clear fixed stoichiometry and ordered alignment of sarcolemmal DHPR and RyR proteins of the SR in elegant morphological studies (49) and strong correlations between the presence and properties of charge movements and SR calcium release in physiological experiments (41,42) provides compelling support for the mechanical coupling hypothesis (Figure 2).

The recent observation that the calcium conducting activity of skeletal muscle DHPRs is strongly dependent upon the presence of RyR1 provides additional support for a direct interaction between these two proteins (10,50). This finding was originally based on the

observation that skeletal myotubes derived from mice homozygous for a disrupted RyR1 gene (RyR1 knockout or dyspedic mice) lack both voltage-gated SR calcium release (DHPR-to-RyR1 or orthograde coupling) and robust voltage-gated L-type calcium currents (RyR1-to-DHPR or retrograde coupling) (10) (Figure 2b). The low level of L-current in dyspedic myotubes does not arise from an absence of DHPR expression since dyspedic myotubes exhibit both significant DHP binding (51,52) and voltage-driven intramembrane charge movements (53). Moreover, freeze fracture studies reveal that surface membranes of dyspedic myotubes exhibit clusters of unordered (i.e. non-tetradic) DHPRs within sarcolemmal/SR junctions (54). Finally, expression of RyR1 proteins in dyspedic myotubes is sufficient to restore both voltage-gated SR calcium release and high density L-type calcium current (55) in the absence of a change in the magnitude of intramembrane charge movement (but see ref. 53). As a result, RyR1 expression increases the ratio between maximal L-channel conductance and charge movement (G_{max}/Q_{max}), indicating that uncoupled DHPRs function poorly as calcium-permeable L-channels. Thus, RyR1 functions not only as an effector that is activated by the DHPR, but also as a transducer that delivers a retrograde signal that enhances L-channel activity (Figure 2).

The precise mechanism (chemical or mechanical) for the retrograde signal from RyR1 to the DHPR in skeletal muscle is still unclear. Feldmeyer *et al.* (1993) found that calcium release from the SR augments L-type calcium channel activity in frog skeletal muscle (56). However, the lack of SR calcium release in dyspedic myotubes is unlikely to account for the observed reduction in L-current magnitude since: 1) robust L-currents persist even under recording conditions (40 mM BAPTA and no ATP) in which voltage-gated calcium transients are abolished (57), 2) L-currents are augmented by agents that inhibit SR calcium release and attenuated by agents that activate calcium release (58) in murine skeletal myotubes, and 3) retrograde coupling is restored following expression of RyR1 mutants that lack depolarization-induced SR calcium release (e.g. I4897T [59] or C4958S [K. G. Beam, personal communication]). Thus, in contrast to cardiac muscle, retrograde coupling in skeletal muscle involves a positive feedback signal that is not conveyed by calcium released from the SR. Rather, the mechanism of retrograde coupling in skeletal muscle is likely to involve an allosteric modulation of L-channel activity that results from the DHPR/RyR1 mechanical interaction that underlies orthograde coupling (50,55,57) (Figure 2b).

There is considerable precedence for bi-directional coupling mediated by a mechanical interaction between two signaling partners. One needs only look at well-established paradigms for receptor/G-protein signaling for analogies with regard to how direct interactions between signaling partners result in bi-directional control of the efficiency, fidelity, and functional activity of each molecule. For example, the binding of Gαq to PLC-β1 following agonist stimulation leads to both activation of PLC-β1 and an increase in the intrinsic GTPase activity of Gαq (60). Consequently, the catalytic activities of both

PLC-β1 and Gαq are reciprocally controlled by a direct interaction between these two signaling proteins. In an analogous manner, the "catalytic activity" of skeletal muscle DHPRs and RyR1s (i.e. their ability to promote the movement of calcium ions from one compartment to another) may similarly be promoted by a physical interaction between the two proteins. One way in which such a mechanical interaction could promote DHPR calcium conductance is if RyR1 acts analogous to that of a conventional calcium channel accessory subunit that stabilizes global conformations of the L-channel that result in increased channel activity upon activation (50). For example, β-subunits augment calcium influx through activated L-channels by increasing L-channel surface expression (61,62), enhancing channel open probability (63,64), promoting the coupling between charge movements and channel opening (63), and by accelerating channel gating kinetics (62). The recent observations that RyR1 not only influences L-channel current magnitude (50,55), but also DHPR expression (50,53), activation kinetics, modulation by DHP agonists, and divalent conductance (50) supports the notion that RyR1 is an important allosteric modulator of the skeletal L-channel.

Although a direct DHPR/RyR1 physical interaction represents a parsimonious mechanism for both orthograde and retrograde coupling in skeletal muscle, other mechanisms for retrograde coupling in skeletal muscle have yet to be excluded. For example, mechanical DHPR/RyR1 coupling may depend on or be facilitated by an intermediary protein(s). Alternatively, the presence of RyR1 in the junction may produce a messenger other than calcium or alter the local chemical environment (e.g. redox state) in a manner that influences DHPR function. Finally, the expression of RyR1 proteins in the SR could conceivably promote α1 association with accessory subunits (e.g. β_{1a}), which serves to enhance L-channel open probability (55). Clearly, more work will be required before more definitive conclusions can be made with regard to the molecular mechanism of retrograde coupling in skeletal muscle.

4.2. Structural determinants of orthograde coupling in skeletal muscle

Following a series of classic experiments conducted almost thirty years ago, Chandler and colleagues (7,65,66) proposed that charge movements associated with the t-tubule voltage sensor directly control calcium release from the SR via a mechanical linkage. Ever since this pioneering work, numerous laboratories have attempted to characterize the molecular determinants of this linkage. The identification of specific regions within the DHPR and RyR1 proteins that mediate orthograde and retrograde coupling is essential because such information would provide: 1) compelling confirmation for the mechanical coupling hypothesis, 2) insight into possible mechanisms of mechanical coupling between other surface membrane and intracellular ion channels (e.g. store-operated Ca^{2+} channels and IP_3 receptors), and 3) critical insight into how mutations within specific regions of the DHPR and RyR1 proteins results in altered EC coupling efficiency and

muscle dysfunction (e.g. malignant hyperthermia and central core disease).

A major breakthrough in identifying the structural determinants of bi-directional coupling came 15 years ago with the cloning of the α1-subunit of the skeletal muscle DHPR by Tanabe et al. (1987) (67) and the observation by Beam et al. (1986) (68) that myotubes derived from dysgenic mice lack slowly-activating L-type calcium currents. Subsequent collaborations between these two groups lead to the demonstration that: 1) introduction of the skeletal muscle DHPR α1-subunit into dysgenic myotubes restores skeletal-type (calcium influx independent) EC coupling, slowly-activating L-currents (69), and intramembrane charge movements (41) 2) introduction of the cardiac muscle DHPR α1-subunit into dysgenic myotubes reconstitutes both cardiac-type (calcium influx-dependent) EC coupling and cardiac-like, rapidly-activating L-currents (70), and 3) a chimeric DHPR of cardiac origin except for skeletal sequence for the intracellular loop connecting the second and third transmembrane repeats (II-III loop) exhibits skeletal-type EC coupling and rapidly-activating L-currents (71). These results demonstrated that the intracellular II-III loop plays an obligatory role in orthograde coupling between the skeletal muscle DHPR to RyR1 and that differences in skeletal and cardiac L-channel activation kinetics are determined by the identity of the DHPR transmembrane repeats (72).

Subsequent work focused on narrowing down regions within the II-III loop of the skeletal muscle DHPR that are responsible for orthograde coupling. Chimeric dissection of the II-III loop and expression in dysgenic myotubes identified a minimal region in the middle of the II-III loop (CSk48; skeletal residues 725-742) that supported weak skeletal type EC coupling (i.e. calcium influx-independent calcium release was observed in some cases) (73). A slightly larger region of skeletal identity (CSk53; skeletal residues 720-765) produced strong skeletal type EC coupling (i.e. calcium influx-independent calcium release was observed in all cases) (73).

Investigations of the effects of synthetic peptides derived from regions of the DHPR II-III loop on RyR1 activity assessed using various in vitro approaches (e.g. [^{3}H] ryanodine binding, $^{45}Ca^{2+}$ flux measurements, open probability of RyR1 channels incorporated into planar lipid bilayers) have identified a different region of the II-III loop that activates RyR1. The series of studies conducted by Lu et al. (74,75) found that the N-terminal half of the skeletal muscle DHPR II-III loop specifically activates RyR1 activity. In a similar manner, the work of Ikemoto and colleagues identified an N-terminal portion of the skeletal muscle DHPR II-III loop (671-690, termed peptide A), but not the cardiac II-III loop, that strongly activates RyR1 as deduced from [^{3}H] ryanodine binding and $^{45}Ca^{2+}$ efflux assays in triad-enriched microsomes (76,77). Moreover, peptide A also activates single RyR1 channels incorporated into planar lipid bilayers (78-80). Finally, the skeletal II-III loop strongly interacts with a 37 amino acid segment of RyR1, and this interaction is disrupted following

replacement of K677 and K682 in peptide A with the corresponding residues found in the cardiac II-III loop (81). Interestingly, a different II-III loop peptide (peptide C, residues 724-760), that roughly corresponds to the region of the II-III loop that supports strong skeletal-type EC coupling identified by Nakai *et al.* (1998), failed to activate RyR1 and antagonized peptide A activation of RyR1 (76,82). These observations led to the proposal that peptide C binds to and inhibits RyR1 activity at rest. According to this hypothesis, voltage-driven alterations in the voltage sensor might modify the conformation of the II-III loop in a manner that relieves peptide C inhibition of RyR1 and permits access of peptide A to its activation site on RyR1 (79,82,83). However, recent work conducted by several groups seriously calls into question the validity of this hypothesis. For example, according to this hypothesis the observation that expression of CSk53 (skeletal DHPR sequence only for residues 720-765) in dysgenic myotubes restores strong skeletal-type EC coupling could only be explained by cardiac sequence within the peptide A region being as efficient in activating RyR1 as skeletal sequence for peptide A; a result that was not observed in the *in vitro* experiments of El Hayek et al., (1998). Secondly, the magnitude and voltage dependence of skeletal EC coupling is unaltered following expression of DHPR constructs in which the peptide A sequence was either scrambled (84), deleted (85), or replaced by highly divergent II-III loop sequence obtained from the DHPR of the housefly, *Musca domestica* (86). In addition, Stange *et al.* (2001) demonstrated that a 46 amino acid peptide identical to that of the region of the II-III loop that supports strong orthograde coupling (720-765) and completely overlaps with the peptide C region, activates submaximally calcium-activated RyR1 channels, even under pseudo-physiological recording conditions (150 mM symmetrical KCl, 0 mV holding potential, and 10 mM luminal calcium as charge carrier) (80). Together, these results indicate that peptide A is not required for RyR1 activation in intact cells and suggest that under appropriate conditions the subregion of the II-III loop identified by Nakai *et al.* (1998) is sufficient to activate RyR1 during EC coupling.

The combined strengths of studies conducted on purified DHPR peptides in isolated systems and the expression of chimeric DHPRs in intact dysgenic myotubes clearly indicate that the intracellular II-III loop of the skeletal muscle DHPR plays an essential role in mediating orthograde coupling in skeletal muscle. Other regions of the skeletal muscle DHPR also influence the function and/or targeting of junctional DHPR and RyR1 proteins. For example, a series of reports from Coronado and colleagues have demonstrated that binding of the DHPR β_{1a}-subunit to the I-II intracellular loop of the α_{1S}-subunit is required for the proper targeting of the α_{1S}-subunit to the membrane (87,88) and that a 35-amino acid region of the C-terminus of β_{1a} facilitates voltage-gated SR calcium release either by promoting the interaction between DHPR and RyR1 or by contributing to the orthograde signal that activates the release channel during depolarization (89). In addition, a region of the C-terminus of the α_{1S}-subunit (including amino acids 1607-1610) that is proximal to a putative truncation site mediates triad targeting of the skeletal muscle DHPR (36,90). Interestingly, this triad targeting domain resides immediately downstream of a region of the α_{1S}-subunit C-terminus that has been shown to interact strongly with a calmodulin binding domain in RyR1 (3609-3643) (91). However, a functional role for the interaction between the C-terminus of the α_{1S}-subunit and RyR1 has yet to be unequivocally established. Similarly, Leong and MacLennan (1998) reported that the III-IV linker of the DHPR α_{1S}-subunit interacts with a specific region of RyR1 (954-1112) (92), though the functional implications of this putative interaction remain unknown.

Much less information is available with regard to the regions of the RyR that mediate orthograde coupling. Functional expression of the cardiac isoform of the RyR (RyR2) in dyspedic myotubes restores neither orthograde nor retrograde coupling (93). Thus, specific regions within both the DHPR and RyR1 proteins encode bi-directional DHPR/RyR coupling in skeletal muscle. Expression of RyR1/RyR2 chimeras in dyspedic myotubes identified a region within RyR1 (R10; 1635-2636) that supports both orthograde (calcium influx-independent calcium release) and retrograde (robust slowly–activating calcium currents) coupling, while an adjacent region (R9; 2659-3720) of RyR1 supported only retrograde coupling (94). However, in a preliminary report, Protasi et al (2001) found that chimeras containing either the R9 region or a smaller region with R10 (R16; 1837-2154) were sufficient to restore weak skeletal-type EC coupling and DHPR tetrads following expression in 1B5 myotubes (a myogenic cell line that lacks RyR proteins) (95). These studies indicate that functional domains within RyRs may be comprised of residues derived from very different regions of the primary sequence that are brought together by appropriate protein folding of the tetramere (94,95). As a consequence, interpretations of data obtained using the chimeric approach applied to RyRs may not be as straightforward as has historically proven to be the case for DHPRs. Such limitations will provide exciting challenges for future work designed to more precisely determine the essential structural domains within different RyR isoforms.

4.3. Structural determinants of retrograde coupling in skeletal muscle

The identification of multiple regions within the skeletal muscle DHPR that potentially interact with RyR1 raises the question of whether or not the critical region within the DHPR II-III loop that is essential for transmitting an orthograde signal during EC coupling is also responsible for receiving a retrograde signal from RyR1. Grabner *et al.* (1999) addressed this question by expressing in dysgenic myotubes chimeric GFP-tagged DHPR constructs containing the entire (or portions of the) cardiac II-III loop in an otherwise skeletal DHPR background (57). Dysgenic myotubes expressing a skeletal DHPR with the entire cardiac II-III loop (SkLC) exhibited large DHPR-mediated intramembrane charge movements but lacked voltage-gated SR calcium release and large slowly-activating L-currents. Thus, SkLC-expressing dysgenic myotubes were similar to dyspedic myotubes in that both orthograde and retrograde coupling were absent under both conditions. Substitution of the 46 amino acid

segment of the skeletal muscle DHPR II-III loop shown by Nakai *et al.* (1998) to support strong orthograde coupling into SkLC (SkLCS$_{46}$) was sufficient to restore both robust voltage-gated SR calcium release and slowly-activating L-current in the absence of a change in the magnitude of intramembrane charge movement (G$_{max}$/Q$_{max}$ was 15 nS/pC and 39 nS/pC for the SkLC and SkLCS$_{46}$, respectively; ref. 57). Interestingly, retrograde coupling was not restored by insertion of the smaller region of the II-III loop (725-742) identified by Nakai *et al.* (1998) to support weak orthograde coupling, suggesting the possibility that orthograde and retrograde coupling may not be strictly encoded a single motif within the II-III loop. Nevertheless, the critical role of the skeletal DHPR II-III loop in receiving the retrograde signal from RyR1 is reinforced by the observation that L-currents of cardiac DHPRs expressed in dysgenic myotubes are also enhanced by replacement of the II-III loop with sequence from the skeletal muscle DHPR (G$_{max}$/Q$_{max}$ was 55 nS/pC and 157 nS/pC for the wild-type cardiac DHPR and CSk3, respectively; ref. 41). Although these results demonstrate that skeletal muscle II-III loop sequence is required for both orthograde and retrograde coupling with RyR1, contributions of other regions of the DHPR to retrograde coupling, particularly those that are conserved between cardiac and skeletal muscle DHPRs, cannot be excluded.

As is the case with orthograde coupling, much less is known with regard to the regions of RyR1 that are critical for transmitting the retrograde signal to the DHPR. As discussed in Section 4.2, expression of RyR2 into dyspedic myotubes restores neither orthograde (calcium influx-independent calcium release) nor retrograde (enhanced skeletal muscle L-channel activity) coupling (93). In addition, experiments in which RyR1/RyR2 chimeras were expressed in dyspedic myotubes indicates that two separate regions within RyR1 (R10; 1635 – 2636 and R9; 2659 – 3720) are sufficient to support retrograde coupling (94) and a 318 amino acid subregion within R10 (R16; 1837 – 2154) is also sufficient to restore weak DHPR/RyR coupling (95). Since the R9, R10, and R16 regions of RyR1 are each capable of at least partially restoring DHPR channel activity, no single region within RyR1 is obligatorily required for retrograde coupling. Rather, the results to date suggest that two or more regions from different parts of the RyR1 primary sequence may come together in the folded protein to form an interaction domain for the critical region of the DHPR II-III loop (94,95).

5. BI-DIRECTIONAL CROSS-TALK IN NEURONS

The current state of knowledge regarding the functional role of bi-directional coupling between dihydropyridine receptors and ryanodine receptors in neuronal tissue is still in its infancy. However, many types of neurons express different isoforms of both L-type calcium channels and ryanodine receptors. Calcium influx through neuronal L-channels is known to promote activity-dependent gene expression through recruitment of specific calcium-dependent transcription factors, such as CREB (96,97) and NF-Atc4 (98). Knockout mice that lack the gene for the ubiquitously expressed isoform of the

ryanodine receptor (RyR3) exhibit enhanced long-term potentiation and spatial learning suggesting that neuronal ryanodine receptors may inhibit synaptic plasticity (99). Considering these observations, recent evidence demonstrating bi-directional chemical and mechanical coupling between DHPRs and RyRs in neurons (100-102) suggests that orthograde and retrograde DHPR/RyR signaling may regulate neuronal gene expression and/or synaptic plasticity.

Hashii *et al.* (2001) reported that intracellular cyclic ADP-ribose (cADPR) amplifies bi-directional calcium signaling between RyRs and DHP-sensitive calcium channels in NG108-15 cells neuroblastoma x glioma hybrid cells (101). In this study, depolarization-induced calcium release through RyRs (DHPR-to-RyR or orthograde signaling) was larger in cADPR-dialyzed cells. This potentiation was mimicked by β-NAD$^+$ (a cADPR precursor), blocked by 8-bromo cADPR (a cADPR competitive antagonist), and antagonized by ryanodine and FK-506. In addition, Mn^{2+} quench assays indicated that calcium influx through DHP-sensitive calcium channels were augmented in cADPR- and β-NAD$^+$-dialyzed cells (positive RyR-to-DHPR or retrograde signaling). The increase in DHP-sensitive Mn^{2+} influx was attributed to an effect of cADPR on RyRs rather than L-type calcium channels because the cADPR-induced enhancement in Mn^{2+} influx was inhibited by pretreatment with either ryanodine or the immunosuppressant FK506. The authors concluded that cADPR modulates neuronal calcium signaling by amplifying the degree of bi-directional coupling between DHPRs and RyRs (101,102) (Figure 3). It will be important for future work to confirm this hypothesis by more directly assessing the impact of cADPR on the magnitude, voltage-dependence, and kinetics of macroscopic L-type calcium currents (rather than Mn^{2+} quench) in the presence and absence releasable calcium stores in patch clamped NG108-15 cells.

Although the results obtained from NG108-15 cells are provocative, several essential questions remain to be resolved. For example, while cADPR may sensitize neuronal RyRs to activation by calcium, it is unclear whether cADPR modifies RyR activity by directly binding to the RyR, binding to a RyR-associated accessory protein, or altering the content of intracellular calcium stores (102,103). In addition, the nature (chemical and/or mechanical) of the bi-directional signaling interaction controlled by cADPR has yet to be rigorously evaluated. While the orthograde signal in NG108-15 cells is likely to involve calcium influx through voltage-gated L-channels, the role of mechanical DHPR/RyR coupling cannot be ruled out since similar experiments conducted in the absence of extracellular calcium have yet to be reported. The retrograde (RyR-to-DHPR) signal could be mediated by calcium released through RyRs, a depletion-operated mechanism, or a (direct or indirect) mechanical interaction between DHPR and RyR proteins (102). Interestingly, a tight functional, and ryanodine-sensitive signaling from RyRs-to-DHPRs (positive retrograde coupling), possibly arising from a direct physical interaction, has also been demonstrated in cerebellar granule cells (100). However,
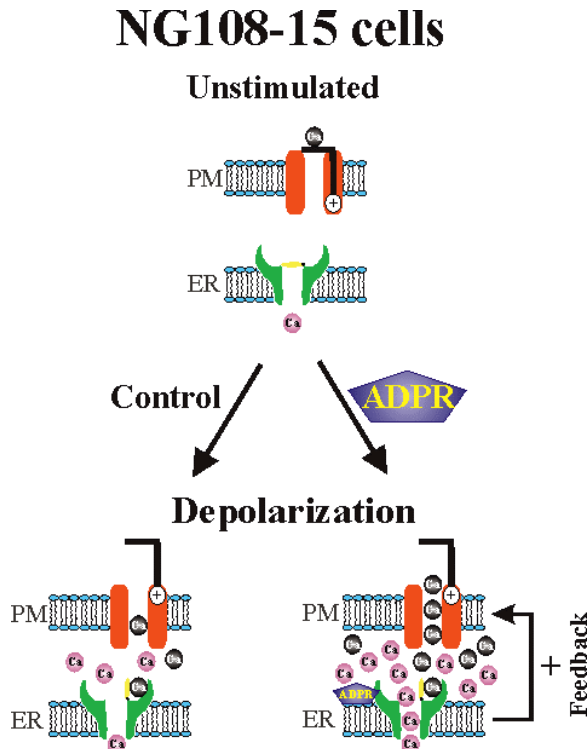
Figure 3. Bi-directional DHPR/RyR signaling in neuronal NG108-15 neuroblastoma x glioma hybrid cells. (Top) L-type calcium channel (red) and RyR (green) co-localization within NG108-15 cells results in the formation of a bi-directional calcium signaling microdomain that is modulated by cADPR . In unstimulated cells, significant calcium influx and calcium release from intracellular calcium stores does not occur. (*Bottom Left*) Depolarization that occurs in the absence of cADPR results in calcium influx through voltage-gated L-type calcium channels and minimal RyR-mediated calcium release (orthograde coupling). (*Bottom Right*) The presence of cADPR hypersensitizes RyR release channels such that depolarization-induced activation of the DHPR results in both calcium influx and a large increase in RyR-mediated calcium release (enhanced orthograde coupling). Enhanced calcium release though sensitized RyR channels induces promotes greater calcium entry though voltage-gated L-type calcium channels (positive retrograde coupling) and additional CICR. The precise mechanistic nature (chemical and/or mechanical) of the bi-directional DHPR/RyR signaling interaction in NG108-15 cells is unclear, but does not appear to involve activation of a conventional capacitative calcium-entry pathway (101).

the relationship between bi-directional DHPR/RyR coupling in NG108-15 and cerebellar granule cells is unclear since dialysis with cADPR was not required for retrograde coupling in cerebellar granule cells.

6. PERSPECTIVES

DHPRs or voltage-gated L-type calcium channels play pivotal roles in transducing extracellular stimuli into a variety of distinct cellular responses including muscle contraction, synaptic plasticity, neurosecretion, and gene regulation. This review has focused on the subset of these processes that are orchestrated by bi-directional coupling between DHPRs and RyRs that occurs within unique signaling microdomains present in cardiac muscle, skeletal muscle, and neurons. These signaling microdomains involve regions of close apposition between cell surface membranes and specialized portions of the ER/SR. The mechanisms and molecular components that coordinate the specialized arrangement of DHPR/RyR signaling microdomains, and how they are differentially regulated in cardiac muscle, skeletal muscle, and neurons is still not understood (104). Continued work in identifying the constituents of these microdomains and the processes that underlie their formation may lead to approaches that enable the assembly of artificial bi-directional DHPR/RyR1 signaling microdomains within heterologous expression systems. Such a breakthrough would enable the development of more straightforward and systematic dissections of both the molecular determinants of bi-directional DHPR/RyR coupling and the mechanisms that underlie the assembly of specialized DHPR/RyR microdomains found in excitable cells.

The degree to which a mechanism of bi-directional DHPR/RyR coupling originally defined within one system also applies to another system will likely represent another focus of future research. For example, an increase in cADPR levels and subsequent sensitization of RyR2 to activation by calcium following stimulation of cardiac β-adrenergic receptors has been suggested to contribute to enhanced SR calcium release during sympathetic stimulation (102). However, the putative role of cADPR in the modulation of RyR function in skeletal and cardiac muscle is controversial (105). Clearly, much work will be required in order to determine whether or not cADPR influences bi-directional chemical signaling between DHPRs and RyRs in cardiac muscle. Although CICR represents the dominant mechanism of SR calcium release in cardiac muscle, recent reports have suggested that a voltage-sensitive release mechanism may also operate under certain conditions (cAMP, physiologic temperatures, negative membrane holding potentials) in cardiac muscle (106-108). Unlike CICR, the voltage-sensitive release mechanism is characterized by calcium release that is graded by membrane voltage rather than the magnitude of L-type calcium current (see for 109 review). Although certain properties of the voltage-sensitive release mechanism in cardiac muscle are similar to those observed in skeletal muscle, the molecular details that underlie the voltage-gated SR Ca^{2+} release (e.g. a mechanical DHPR-RyR2 interaction) are still unknown. Given the complex requirements for its activation and inherent technical difficulties in separating the voltage-sensitive release from CICR (110), the putative role of the voltage-sensitive release mechanism in regulating cardiac muscle contraction is still a very contentious and controversial topic. Nevertheless, if a voltage-sensitive release mechanism does operate under some conditions in cardiac muscle, it will be important to determine if bi-directional DHPR/RYR mechanical coupling regulates calcium conductance through cardiac L-channels as has been documented in skeletal muscle.

Several key questions with regard to bi-directional DHPR/RYR coupling in skeletal muscle also remain unresolved. For example, is mechanical cross-talk in skeletal muscle mediated by a single, direct interaction between the DHPR and RyR1 or are other regions/proteins involved? What single channel properties (e.g. open probability, conductance behavior) of the skeletal L-channel are allosterically modulated by RyR1? How does a single critical domain in the DHPR II-III loop mediate both orthograde and retrograde coupling via distinct regions of the RyR1 primary sequence? Given that calcium influx through L-channels is not required for skeletal-type EC coupling (5,6), what is the functional role of retrograde coupling in skeletal muscle? These and other questions will provide substantial motivation for future work in defining the determinants and physiological roles of bi-directional coupling between DHPRs and RyRs.

7. ACKNOWLEDGEMENTS

8. REFERENCES

1. Fabiato A: Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J Gen Physiol* 85, 291-320 (1985)

2. Nabauer M, G. Callewaert, L. Cleemann & M. Morad: Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* 244, 800-803 (1989)

3. Beuckelmann D. J. & W. G. Wier: Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *J Physiol* 405, 233-255 (1988)

4. Fabiato A: Rapid ionic modifications during the aequorin-detected calcium transient in a skinned canine cardiac Purkinje cell. *J Gen Physiol* 85, 189-246 (1985)

5. Dirksen R. T. & K. G. Beam: Role of calcium permeation in dihydropyridine receptor function. Insights into channel gating and excitation-contraction coupling. *J Gen Physiol* 114, 393-403 (1999)

6. Armstrong C. M, F. M. Bezanilla & P. Horowicz: Twitches in the presence of ethylene glycol bis(-aminoethyl ether)-N,N'-tetracetic acid. *Biochimica et Biophysica Acta* 267, 605-608 (1972)

7. Chandler W. K, R. F. Rakowski & M. F. Schneider: Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J Physiol* 254, 285-316 (1976)

8. Rios E. & G. Pizarro: Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiological Reviews* 71, 849-908 (1991)

9. Melzer W, A. Herrmann-Frank & H. C. Luttgau: The role of Ca2+ ions in excitation-contraction coupling of skeletal muscle fibres. *Biochimica et Biophysica Acta* 1241, 59-116 (1995)

10. Nakai J, R. T. Dirksen , H. T. Nguyen, I. N. Pessah, K. G. Beam & P. D. Allen: Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature* 380, 72-75 (1996)

11. Fabiato A: Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J Gen Physiol* 85, 247-289 (1985)

12. Cheng H, W. J. Lederer & M. B. Cannell: Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* 262, 740-744 (1993)

13. Wang S. Q, L. S. Song, E. G. Lakatta & H. Cheng: Ca^{2+} signalling between single L-type Ca^{2+} channels and ryanodine receptors in heart cells. *Nature* 410, 592-596 (2001)

14. Meissner G, E. Darling & J. Eveleth: Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry* 25, 236-244 (1986)

15. Zorzato F, J. Fujii, K. Otsu, M. Phillips, N. M. Green, F. A. Lai, G. Meissner & D. H. MacLennan: Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 265, 2244-2256 (1990)

16. Chen S. R, K. Ebisawa, X. Li & L. Zhang: Molecular identification of the ryanodine receptor Ca^{2+} sensor. *J Biol Chem* 273, 14675-14678 (1998)

17. Li P & S. R. Chen: Molecular basis of Ca^{2+} activation of the mouse cardiac Ca^{2+} release channel (ryanodine receptor). *J Gen Physiol* 118, 33-44 (2001)

18. O'Brien J. J, P. D. Allen, K. G. Beam & S. R. Chen: Effects of mutation E4032A on the function of RyR1 expressed in HEK293 cells and in dyspedic myotubes. *Biophys J* 76, A302 (1999)

19. Du G. G. & D. H. MacLennan: Functional consequences of mutations of conserved, polar amino acids in transmembrane sequences of the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 273, 31867-31872 (1998)

20. Fessenden J. D, L. Chen, Y. Wang, C. Paolini, C. Franzini-Armstrong, P. D. Allen & I. N. Pessah: Ryanodine receptor point mutant E4032A reveals an allosteric interaction with ryanodine. *Proc Natl Acad Sci USA* 98, 2865-2870 (2001)

21. Brehm P. & R. Eckert: Calcium entry leads to inactivation of calcium channel in Paramecium. *Science* 202, 1203-1206 (1978)

22. Kass R. S. & M. C. Sanguinetti: Inactivation of calcium channel current in the calf cardiac Purkinje fiber. Evidence for voltage- and calcium-mediated mechanisms. *J Gen Physiol* 84, 705-726 (1984)

23. Lee K. S, E. Marban & R. W. Tsien: Inactivation of calcium channels in mammalian heart cells: joint

dependence on membrane potential and intracellular calcium. *J Physiol* 364, 395-411 (1985)

24. Chad J. E. & R. Eckert: An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones. *J Physiol* 378, 31-51 (1986)

25. Sham J. S, L. Cleemann & M. Morad: Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc Natl Acad Sci USA* 92, 121-125 (1995)

26. Adachi-Akahane S, L. Cleeman & M. Morad: Calcium cross-signaling between DHP and ryanodine receptors in cardiomyocytes. *Heart and Vessels* 9, 163-166 (1995)

27. Adachi-Akahane S, L. Cleemann & M. Morad: Cross-signaling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *J Gen Physiol* 108, 435-454 (1996)

28. Sham J. S, L. Cleemann & M. Morad: Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc Natl Acad Sci USA* 92, 121-125 (1995)

29. Peterson B. Z, C. D. DeMaria, J. P. Adelman & D. T. Yue: Calmodulin is the Ca^{2+} sensor for Ca^{2+} -dependent inactivation of L-type calcium channels. *Neuron* 22, 549-558 (1999)

30. Zuhlke R. D, G. S. Pitt , K. Deisseroth, R. W. Tsien & H. Reuter: Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature* 399, 159-162 (1999)

31. Zuhlke R. D, G. S. Pitt , R. W. Tsien & H. Reuter: Ca^{2+}-sensitive inactivation and facilitation of L-type Ca^{2+} channels both depend on specific amino acid residues in a consensus calmodulin-binding motif in the α1C subunit. *J Biol Chem* 275, 21121-21129 (2000)

32. Pate P, J. Mochca-Morales, Y. Wu, J. Z. Zhang, G. G. Rodney, I. I. Serysheva, B. Y. Williams, M. E. Anderson & S. L. Hamilton: Determinants for calmodulin binding on voltage-dependent Ca^{2+} channels. *J Biol Chem* 275, 39786-39792 (2000)

33. Flucher B. E, N. Kasielke, U. Gerster, B. Neuhuber & M. Grabner: Insertion of the full-length calcium channel α1S subunit into triads of skeletal muscle in vitro. *FEBS Lett* 474, 93-98 (2000)

34. Gao T, M. Bunemann, B. L. Gerhardstein, H. Ma & M. M. Hosey: Role of the C terminus of the α1C (CaV1.2) subunit in membrane targeting of cardiac L-type calcium channels. *J Biol Chem* 275, 25436-25444 (2000)

35. Kepplinger K. J, H. Kahr, G. Forstner, M. Sonnleitner, H. Schindler, T. Schmidt, K. Groschner, N. M. Soldatov & C. Romanin: A sequence in the carboxy-terminus of the α1C subunit important for targeting, conductance and open probability of L-type Ca^{2+} channels. *FEBS Lett* 477, 161-169 (2000)

36. Proenza C, C. Wilkens , N. M. Lorenzon & K. G. Beam: A carboxyl-terminal region important for the expression and targeting of the skeletal muscle dihydropyridine receptor. *J Biol Chem* 275, 23169-23174 (2000)

37. Imredy J. P & D. T. Yue: Mechanism of Ca^{2+}-sensitive inactivation of L-type Ca^{2+} channels. *Neuron* 12, 1301-1318 (1994)

38. Sharma V. K, V. Ramesh , C. Franzini-Armstrong & S.-S. Sheu: Transport of Ca^{2+} from Sarcoplasmic Reticulum to Mitochondria in Rat Ventricular Myocytes. *J Bioergetics and Biomembranes* 32, 97-104 (2000)

39. Sanchez J. A, M. C. Garcia, V. K. Sharma, K. C. Young, M. A. Matlib & S.-S. Sheu: Mitochondria regulate inactivation of L-type Ca^{2+} channels in rat heart. *J Physiol* 536, 387-396 (2001)

40. Garcia J. & K. G. Beam: Measurement of calcium transients and slow calcium current in myotubes. *J Gen Physiol* 103, 107-123 (1994)

41. Adams B. A, T. Tanabe, A. Mikami, S. Numa & K. G. Beam: Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. *Nature* 346, 569-572 (1990)

42. Garcia J, T. Tanabe & K. G. Beam: Relationship of calcium transients to calcium currents and charge movements in myotubes expressing skeletal and cardiac dihydropyridine receptors. *J Gen Physiol* 103, 125-147 (1994)

43. Sculptoreanu A, T. Scheuer & W. A. Catterall: Voltage-dependent potentiation of L-type Ca^{2+} channels due to phosphorylation by cAMP-dependent protein kinase. *Nature* 364, 240-243 (1993)

44. Shainberg A, S. A. Cohen & P. G. Nelson: Induction of acetylcholine receptors in muscle cultures. *Pflugers Archiv* 361, 255-261 (1976)

45. Klarsfeld A. & J. P. Changeux: Activity regulates the levels of acetylcholine receptor alpha-subunit mRNA in cultured chicken myotubes. *Proc Natl Acad Sci USA* 82, 4558-4562 (1985)

46. Rotzler S, H. Schramek & H. R. Brenner: Metabolic stabilization of endplate acetylcholine receptors regulated by Ca^{2+} influx associated with muscle activity. *Nature* 349, 337-339 (1991)

47. Melzer W, A. Herrmann-Frank & H. C. Luttgau: The role of Ca^{2+} ions in excitation-contraction coupling of skeletal muscle fibres. *Biochimica et Biophysica Acta* 1241, 59-116 (1995)

48. Pizarro G, L. Csernoch, I. Uribe, M. Rodriguez & E. Rios: The relationship between Q gamma and Ca release from the sarcoplasmic reticulum in skeletal muscle. *J Gen Physiol* 97, 913-947 (1991)

49. Block B. A, T. Imagawa , K. P. Campbell & C. Franzini-Armstrong: Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J Cell Biol* 107, 2587-2600 (1988)

50. Avila G. & R. T. Dirksen: Functional impact of the ryanodine receptor on the skeletal muscle L-type Ca^{2+} channel. *J Gen Physiol* 115, 467-480 (2000)

51. Buck E. D, H. T. Nguyen , I. N. Pessah & P. D. Allen: Dyspedic mouse skeletal muscle expresses major elements of the triadic junction but lacks detectable ryanodine receptor protein and function. *J Biol Chem* 272, 7360-7367 (1997)

52. Fleig A, H. Takeshima & R. Penner: Absence of Ca^{2+} current facilitation in skeletal muscle of transgenic mice lacking the type 1 ryanodine receptor. *J Physiol* 496, 339-345 (1996)

53. Avila G, K. M. S. O'Connell, L. A. Groom & R. T. Dirksen: Ca^{2+} release through ryanodine receptors regulates skeletal muscle L-type Ca^{2+} channel expression. *J Biol Chem* 276, 17732-17738 (2001)

54. Takekura H, M. Nishi, T. Noda, H. Takeshima & C. Franzini-Armstrong: Abnormal junctions between surface

membrane and sarcoplasmic reticulum in skeletal muscle with a mutation targeted to the ryanodine receptor. *Proc Natl Acad Sci USA* 92, 3381-3385 (1995)

55. Nakai J, R. T. Dirksen , H. T. Nguyen, I. N. Pessah, K. G. Beam & P. D. Allen: Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature* 380, 72-75 (1996)

56. Feldmeyer D, W. Melzer, B. Pohl & P. Zollner: A possible role of sarcoplasmic Ca^{2+} release in modulating the slow Ca^{2+} current of skeletal muscle. *Pflugers Archiv* 425, 54-61 (1993)

57. Grabner M, R T. Dirksen, N. Suda & K G. Beam: The II-III loop of the skeletal muscle dihydropyridine receptor is responsible for the Bi-directional coupling with the ryanodine receptor. *J Biol Chem* 274, 21913-21919 (1999)

58. Balog E. M & E. M. Gallant: Modulation of the sarcolemmal L-type current by alteration in SR Ca^{2+} release. *Am J Physiol* 276, C128-C135 (1999)

59. Avila G, J. J. O'Brien & R. T. Dirksen: Excitation-contraction uncoupling by a human central core disease mutation in the ryanodine receptor. *Proc Natl Acad Sci USA* 98, 4215-4220 (2001)

60. Berstein G, J. L. Blank, D. Y. Jhon, J. H. Exton, S. G. Rhee & E. M. Ross: Phospholipase C-beta 1 is a GTPase-activating protein for Gq/11, its physiologic regulator. *Cell* 70, 411-418 (1992)

61. Lacerda A. E, H. S. Kim , P. Ruth, E. Perez-Reyes, V. Flockerzi, F. Hofmann, L. Birnbaumer & A. M. Brown: Normalization of current kinetics by interaction between the alpha 1 and beta subunits of the skeletal muscle dihydropyridine-sensitive Ca^{2+} channel. *Nature* 352, 527-530 (1991)

62. Singer D, M. Biel, I. Lotan, V. Flockerzi, F. Hofmann & N. Dascal: The roles of the subunits in the function of the calcium channel. *Science* 253, 1553-1557 (1991)

63. Neely A, X. Wei, R. Olcese, L. Birnbaumer & E. Stefani: Potentiation by the beta subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* 262, 575-578 (1993)

64. Shistik E, T. Ivanina , T. Puri, M. Hosey & N. Dascal: Ca^{2+} current enhancement by alpha 2/delta and beta subunits in Xenopus oocytes: contribution of changes in channel gating and alpha 1 protein level. *J Physiol* 489, 55-62 (1995)

65. Chandler W. K, R. F. Rakowski & M. F. Schneider: A non-linear voltage dependent charge movement in frog skeletal muscle. *J Physiol* 254, 245-283 (1976)

66. Schneider M. F. & W. K. Chandler: Voltage dependent charge movement of skeletal muscle: a possible step in excitation-contraction coupling. *Nature* 242, 244-246 (1973)

67. Tanabe T, H. Takeshima, A. Mikami, V. Flockerzi, H. Takahashi, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose & S. Numa: Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* 328, 313-318 (1987)

68. Beam K. G, C. M. Knudson & J. A. Powell: A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells. *Nature* 320, 168-170 (1986)

69. Tanabe T, K. G. Beam, J. A. Powell & S. Numa: Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature* 336, 134-139 (1988)

70. Tanabe T, A. Mikami, S. Numa & K. G. Beam: Cardiac-type excitation-contraction coupling in dysgenic

skeletal muscle injected with cardiac dihydropyridine receptor cDNA. *Nature* 344, 451-453 (1990)

71. Tanabe T, K. G. Beam, B. A. Adams, T. Niidome & S. Numa: Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature* 346, 567-569 (1990)

72. Tanabe T, B. A. Adams, S. Numa & K. G. Beam: Repeat I of the dihydropyridine receptor is critical in determining calcium channel activation kinetics. *Nature* 352, 800-803 (1991)

73. Nakai J, T. Tanabe, T. Konno, B. Adams & K. G. Beam: Localization in the II-III loop of the dihydropyridine receptor of a sequence critical for excitation-contraction coupling. *J Biol Chem* 273, 24983-24986 (1998)

74. Lu X, L. Xu & G. Meissner: Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor. *J Biol Chem* 269, 6511-6516 (1994)

75. Lu X, L. Xu & G. Meissner: Phosphorylation of dihydropyridine receptor II-III loop peptide regulates skeletal muscle calcium release channel function. Evidence for an essential role of the beta-OH group of Ser687. *J Biol Chem* 270, 18459-18464 (1995)

76. el-Hayek R, B. Antoniu, J. Wang, S. L. Hamilton & N. Ikemoto: Identification of calcium release-triggering and blocking regions of the II-III loop of the skeletal muscle dihydropyridine receptor. *J Biol Chem* 270, 22116-22118 (1995)

77. el-Hayek R. & N. Ikemoto: Identification of the minimum essential region in the II-III loop of the dihydropyridine receptor alpha 1 subunit required for activation of skeletal muscle-type excitation-contraction coupling. *Biochemistry* 37, 7015-7020 (1998)

78. Dulhunty A. F, D. R. Laver, E. M. Gallant, M. G. Casarotto, S. M. Pace & S. Curtis: Activation and inhibition of skeletal RyR channels by a part of the skeletal DHPR II-III loop: effects of DHPR Ser687 and FKBP12. *Biophys J* 77, 189-203 (1999)

79. Gurrola G. B, C. Arevalo, R. Sreekumar, A. J. Lokuta, J. W. Walker & H. H. Valdivia: Activation of ryanodine receptors by imperatoxin A and a peptide segment of the II-III loop of the dihydropyridine receptor. *J Biol Chem* 274, 7879-7886 (1999)

80. Stange M, A. Tripathy & G. Meissner: Two domains in dihydropyridine receptor activate the skeletal muscle Ca^{2+} release channel. *Biophys J* 81, 1419-1429 (2001)

81. Leong P. & D. H. MacLennan: A 37-amino acid sequence in the skeletal muscle ryanodine receptor interacts with the cytoplasmic loop between domains II and III in the skeletal muscle dihydropyridine receptor. *J Biol Chem* 273, 7791-7794 (1998)

82. Saiki Y, R. el-Hayek & N. Ikemoto: Involvement of the Glu724-Pro760 region of the dihydropyridine receptor II-III loop in skeletal muscle-type excitation-contraction coupling. *J Biol Chem* 274, 7825-7832 (1999)

83. Ikemoto N. & R. el-Hayek: Signal transmission and transduction in excitation-contraction coupling. *Advances in Experimental Medicine & Biology* 453, 199-207 (1998)

84. Proenza C, C. M. Wilkens & K. G. Beam: Excitation-contraction coupling is not affected by scrambled sequence in residues 681-690 of the dihydropyridine receptor II-III loop. *J Biol Chem* 275, 29935-29937 (2000)

85. Ahern C. A, J. Arikkath, P. Vallejo, C. A. Gurnett, P. A. Powers, K. P. Campbell & R. Coronado: Intramembrane charge movements and excitation- contraction coupling expressed by two-domain fragments of the Ca^{2+} channel. *Proc Natl Acad Sci USA* 98, 6935-6940 (2001)

86. Wilkens C. M, N. Kasielke, B. E. Flucher, K. G. Beam & M. Grabner: Excitation-contraction coupling is unaffected by drastic alteration of the sequence surrounding residues L720-L764 of the alpha 1S II-III loop. *Proc Natl Acad Sci USA* 98, 5892-5897 (2001)

87. Strube C, M. Beurg, P. A. Powers, R. G. Gregg & R. Coronado: Reduced Ca2+ current, charge movement, and absence of Ca^{2+} transients in skeletal muscle deficient in dihydropyridine receptor beta 1 subunit. *Biophys J* 71, 2531-2543 (1996)

88. Strube C, M. Beurg, M. Sukhareva, C. A. Ahern, J. A. Powell, P. A. Powers, R. G. Gregg & R. Coronado: Molecular origin of the L-type Ca^{2+} current of skeletal muscle myotubes selectively deficient in dihydropyridine receptor beta1a subunit. *Biophys J* 75, 207-217 (1998)

89. Beurg M, C. A. Ahern, P. Vallejo, M. W. Conklin, P. A. Powers, R. G. Gregg & R. Coronado: Involvement of the carboxy-terminus region of the dihydropyridine receptor beta1a subunit in excitation-contraction coupling of skeletal muscle. *Biophys J* 77, 2953-2967 (1999)

90. Flucher B. E, N. Kasielke & M. Grabner: The triad targeting signal of the skeletal muscle calcium channel is localized in the COOH terminus of the α1S subunit. *J Cell Biol* 151, 467-478 (2000)

91. Sencer S, R. V. Papineni, D. B. Halling, P. Pate, J. Krol, J. Z. Zhang & S. L. Hamilton: Coupling of RYR1 and L-type Calcium Channels via Calmodulin Binding Domains. *J Biol Chem* 276, 38237-38241 (2001)

92. Leong P & D. H. MacLennan: The cytoplasmic loops between domains II and III and domains III and IV in the skeletal muscle dihydropyridine receptor bind to a contiguous site in the skeletal muscle ryanodine receptor. *J Biol Chem* 273, 29958-29964 (1998)

93. Nakai J, T. Ogura, F. Protasi, C. Franzini-Armstrong, P. D. Allen & K. G. Beam: Functional nonequality of the cardiac and skeletal ryanodine receptors. *Proc Natl Acad Sci USA* 94, 1019-1022 (1997)

94. Nakai J, N. Sekiguchi , T. A. Rando, P. D. Allen & K. G. Beam: Two regions of the ryanodine receptor involved in coupling with L-type Ca^{2+} channels. *J Biol Chem* 273, 13403-13406 (1998)

95. Protasi F, J. Nakai, K. G.Beam, H. Takekura, C. Franzini-Armstrong, & P. D. Allen: Two independent regions of RyR1 (1837-2154 and 2659-3720) restore skeletal type E-C coupling and DHPR junctional tetrads. *Biophys J* 80, 330a (2001)

96. Deisseroth K, E. K. Heist & R. W. Tsien: Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature* 392, 198-202 (1998)

97. Mermelstein P. G, H. Bito, K. Deisseroth & R. W. Tsien: Critical dependence of cAMP response element-binding protein phosphorylation on L-type calcium channels supports a selective response to EPSPs in preference to action potentials. *J Neurosci* 20, 266-273 (2000)

98. Graef I. A, P. G. Mermelstein, K. Stankunas, J. R. Neilson, K. Deisseroth, R. W. Tsien & G. R. Crabtree: L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* 401, 703-708 (1999)

99. Futatsugi A, K. Kato, H. Ogura, S. T. Li, E. Nagata, G. Kuwajima, K. Tanaka, S. Itohara & K. Mikoshiba: Facilitation of NMDAR-independent LTP and spatial learning in mutant mice lacking ryanodine receptor type 3. *Neuron* 24, 701-713 (1999)

100. Chavis P, L. Fagni, J. B. Lansman & J. Bockaert: Functional coupling between ryanodine receptors and L-type calcium channels in neurons. *Nature* 382, 719-722 (1996)

101. Hashii M, Y. Minabe & H. Higashida: cADP-ribose potentiates cytosolic Ca^{2+} elevation and Ca^{2+} entry via L-type voltage-activated Ca^{2+} channels in NG108-15 neuronal cells. *Biochem J* 345, 207-215 (2000)

102. Higashida H, M. Hashii, S. Yokoyama, N. Hoshi, X. Chen, A. Egorova, M. Noda & J. Zhang: Cyclic ADP-ribose as a second messenger revisited from a new aspect of signal transduction from receptors to ADP-ribosyl cyclase. *Pharmacol Ther* 90, 283-296 (2001)

103. Lukyanenko V, I. Gyorke, T. F. Wiesner & S. Gyorke: Potentiation of Ca^{2+} release by cADP-ribose in the heart is mediated by enhanced SR Ca^{2+} uptake into the sarcoplasmic reticulum. *Circ Res* 89, 614-622 (2001)

104. Flucher B. E. & C. Franzini-Armstrong: Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. *Proc Natl Acad Sci USA* 93, 8101-8106 (1996)

105. Copello J. A, Y. Qi, L. H. Jeyakumar, E. Ogunbunmi & S. Fleischer: Lack of effect of cADP-ribose and NAADP on the activity of skeletal muscle and heart ryanodine receptors. *Cell Calcium* 30, 269-284 (2001)

106. Ferrier G. R. & S. E. Howlett: Contractions in guinea-pig ventricular myocytes triggered by a calcium-release mechanism separate from Na$^+$ and L-currents. *J Physiol* 484, 107-122 (1995)

107. Hobai I. A, F. C. Howarth, V. K. Pabbathi, G. R. Dalton, J. C. Hancox, J. Q. Zhu, S. E. Howlett, G. R. Ferrier & A. J. Levi: "Voltage-activated Ca release" in rabbit, rat and guinea-pig cardiac myocytes, and modulation by internal cAMP. *Pflugers Archiv* 435, 164-173 (1997)

108. Howlett S. E, J. Q. Zhu & G. R. Ferrier: Contribution of a voltage-sensitive calcium release mechanism to contraction in cardiac ventricular myocytes. *Am J Physiol* 274, H155-H170 (1998)

109. Ferrier G. R. & S. E. Howlett: Cardiac excitation-contraction coupling: role of membrane potential in regulation of contraction. *Am J Physiol* 280, H1928-H1944 (2001)

110. Piacentino V, K. Dipla, J. P. Gaughan & S. R. Houser: Voltage-dependent Ca^{2+} release from the SR of feline ventricular myocytes is explained by Ca^{2+}-induced Ca^{2+} release. *J Physiol* 523, 533-548 (2000)

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