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Review

# Ca channels in cardiac myocytes: structure and function in Ca influx and intracellular Ca release

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**Abstract**

There are Ca channels in the plasma membrane and also the sarcoplasmic reticulum (SR) membrane in cardiac myocytes. The relationship between channel structure, associated proteins and function of these Ca channels is discussed. The sarcolemmal Ca channels are crucial both to the basic cellular electrophysiological properties and control of cardiac contractility (via excitation–contraction coupling). The intracellular Ca release channels (or ryanodine receptors) respond to triggering events mediated by sarcolemmal ion currents and are largely responsible for releasing Ca which activates the myofilaments to produce contraction. Several possible mechanisms of excitation–contraction coupling are discussed. The Ca released from the SR can also feedback on several sarcolemmal ion currents and alter action potential configuration as well as contribute to arrhythmogenesis. 

## 1. Introduction

There are sarcolemmal Ca channels in cardiac myocytes which serve to bring Ca into the cell (L- and T-type Ca channels). This Ca influx contributes an inward current tending to make (or keep) the membrane potential more positive, but this Ca also serves as an important second messenger in the excitation–contraction (E–C) coupling process which leads to activation of contraction. There are also intracellular Ca-release channels which are responsible for releasing Ca from the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER; ryanodine and $IP_3$ receptors). The Ca released from the SR greatly amplifies the Ca which enters via the sarcolemma in the activation of contraction and can also feed back on sarcolemmal ion currents. Structural and functional components of the Ca channel types which occur in cardiac myocytes will be addressed in the following sections.

## 2. Sarcolemmal Ca currents in cardiac myocytes

### *2.1. L-type vs. T-type Ca current*

There are two main classes of Ca current ($I_{Ca}$) in cardiac myocytes [1–3]. The L-type $I_{Ca}$ ($I_{Ca,L}$) both activates and inactivates at more positive membrane potential ($E_m$), inactivates more slowly (particularly when Ba is used as the charge carrier), is sensitive to dihydropyridines and has a larger single-channel Ba conductance [4]. By contrast, T-type $I_{Ca}$ ($I_{Ca,T}$) activates and inactivates at more negative $E_m$, is more transient (with Ba), has a smaller single-channel Ba conductance and is less sensitive to block by dihydropyridines [5]. $I_{Ca,T}$ is also more sensitive to block by Ni or mibefradil than is L-type $I_{Ca}$ [6–9].

A common method to separate L- and T-type $I_{Ca}$ makes use of $I_{Ca,T}$ inactivation at a holding potential ($E_h$) of about −50 mV (which does not inactivate $I_{Ca,L}$). Fig. 1A shows current–voltage relationships for $I_{Ca}$ in a canine Purkinje fiber activated from $E_m = -50$ ($I_{Ca,L}$ only) and −90 mV ($I_{Ca,L}+I_{Ca,T}$). Thus the difference gives an assessment of $I_{Ca,T}$ [6].

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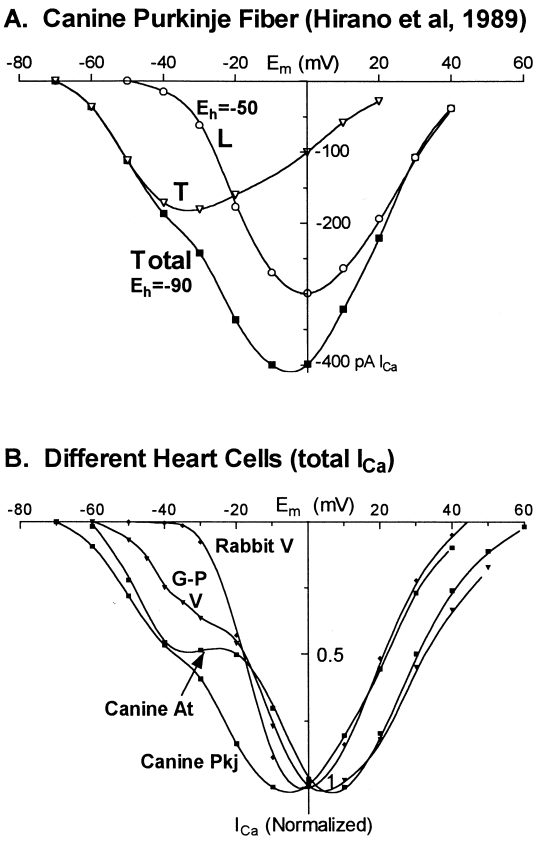


Fig. 1. L- and T-type Ca current in cardiac myocytes. (A) voltage clamp data from Hirano et al. [6] in a canine Purkinje fiber in 2 mM $[Ca]_o$ showing peak $I_{Ca}$ for pulses from either −90 mV (■) or −50 mV (○). Current activated from −50 mV is taken as $I_{Ca,L}$ and the difference between the values from −50 and −90 mV is taken as $I_{Ca,T}$ (▽). (B) Total $I_{Ca}$ from several species (from holding potentials of −80 to −100 mV), where the hump near −40 mV is due to $I_{Ca,T}$ and differs among the tissues studied. Canine Purkinje from panel A (from Hirano et al., [6]) and rabbit ventricle are in 2 mM Ca and dog atrium (from Bean, [1]) and guinea-pig ventricle (from Mitra and Morad, [11]) were recorded with 5 mM Ca, but are shifted by −10 mV to compensate for surface potential differences.

The relative amounts of $I_{Ca,L}$ and $I_{Ca,T}$ vary among cardiac myocytes. In the canine Purkinje fiber there is a relatively large component of $I_{Ca,T}$. At a test $E_m$ of −20 mV the two types of $I_{Ca}$ in Fig. 1A are about equal, whereas at 0 mV peak $I_{Ca,L}$ is three times larger than $I_{Ca,T}$. L-type $I_{Ca}$ appears to be a prominent feature in all cardiac myocytes, whereas T-type $I_{Ca}$ is much more variable. Fig. 1B shows total $I_{Ca}$ current–voltage relationships in four different cell types. The extent of the hump at negative $E_m$ (~−40 mV) reflects the relative amount of $I_{Ca,T}$. Purkinje cells seem to have the most [6], pacemaker cells, and some atrial myocytes also have significant amounts [2,10], but the amount of $I_{Ca,T}$ in ventricular myocytes is either modest in guinea-pig [11] or undetectable in bullfrog, calf, cat, rabbit, rat and ferret [2,12–14]. Interestingly, Nuss and Houser [12] reported that $I_{Ca,T}$ in cat ventricular myocytes became substantial during the development of ventricular hypertrophy and we typically find significant $I_{Ca,T}$ in neonatal rat ventricular myocytes in culture.

Thus the T-type current is typically small or absent in ventricular myocytes, but may be more prominent during development and hypertrophy. The relative prominence of $I_{Ca,T}$ in pacemaker and conducting cells and its activation at $E_m$ in the range of diastolic depolarization has led to speculation and some evidence supporting a role of $I_{Ca,T}$ in atrial pacemaking [7,10]. Because $I_{Ca,T}$ is relatively small and inactivates very rapidly, the total amount of Ca flux via $I_{Ca,T}$ is probably small compared to that via $I_{Ca,L}$ and negligible in most ventricular myocytes. This may reflect different functional roles, where $I_{Ca,L}$ is more involved in triggering SR Ca release and refilling SR Ca stores (see below), rather than pacemaking.

### 2.2. Ca-dependent inactivation

$I_{Ca,T}$ inactivation is very fast, steeply voltage-dependent and independent of the charge carrying ion. This contrasts strikingly with $I_{Ca,L}$, where inactivation is both voltage- and Ca-dependent [15–17]. Fig. 2 shows the time course of inactivation of L-type Ca channel current in ventricular myocytes under several different conditions. In the absence of divalent cations the Ca channel is highly permeant to monovalent cations and is sometimes referred to as $I_{ns}$ (for nonspecific, [18–20]).

Inactivation of $I_{ns}$ in Fig. 2 is very slow ($t_{1/2}$>500 ms) and probably reflects purely $E_m$-dependent inactivation

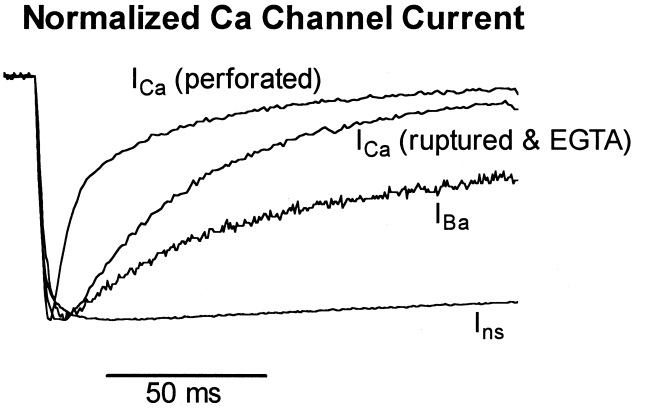


Fig. 2. Inactivation of Ca channel currents with different charge carriers. Normalized current amplitudes measured at 0 mV (except $I_{ns}$ at −30 mV to obtain comparable activation state). $I_{Ca}$ was recorded under both perforated patch conditions (where normal SR Ca release and Ca transients occur) and in ruptured patch with cells dialyzed with 10 mM EGTA (to prevent global Ca transients). $I_{Ba}$ was also recorded with ruptured patch (with 10 mM EGTA in the pipette). Extracellular [Ca] and [Ba] were both 2 mM and $I_{ns}$ was measured in divalent-free conditions (10 mM EDTA inside and out) and $[Na]_o$ at 20 mM and $[Na]_i$ at 10 mM. Peak currents were 1370, 808, 780 and 5200 pA and were attained at 5, 7, 10 and 14 ms for $I_{Ca}$ (perforated), $I_{Ca}$ (ruptured), $I_{Ba}$ and $I_{ns}$ respectively. Halftimes of current decline were 17, 37, 161 and >500 ms respectively (modified from Bers, [26]).

which is very slow at this test $E_m$ (–30 mV). With respect to $E_m$-dependent channel gating properties, this test $E_m$, in the absence of divalent cations is comparable to $E_m = -10$ mV with normal $[Ca]_o$ (due to surface charge screening by Ca, [14]). The divalent cation currents were recorded during pulses to 0 mV. At much more positive potentials $I_{ns}$ inactivates increasingly rapidly (i.e. $E_m$-dependent inactivation becomes more prominent at large positive potentials). The Ba current ($I_{Ba}$) in Fig. 2 inactivates more rapidly ($t_{1/2}=161$ ms) than $I_{ns}$, and this might reflect a modest ability of Ba to mimic Ca-dependent inactivation [21]. When Ca is the charge carrier, with 10 mM EGTA in the pipette to abolish cellular Ca transients (and in this case SR Ca release), inactivation is faster still ($t_{1/2}=37$ ms). This probably reflects Ca-dependent inactivation due to Ca entering via the channel itself. EGTA is a slow Ca buffer and thus cannot prevent a rise in local $[Ca]_i$ near the mouth of the Ca channel. High concentrations of faster buffers (like BAPTA) can slow this Ca-dependent inactivation. During normal E–C coupling there is also Ca released from the SR and this can elevate local $[Ca]_i$ near the L-type Ca channel. The top $I_{Ca}$ trace in Fig. 2 is under conditions where a normal Ca transient occurs in the cell (clamped using the perforated patch mode). $I_{Ca}$ inactivates much faster ($t_{1/2}=17$ ms) and this emphasizes that SR Ca release also plays a major role in $I_{Ca}$ inactivation in a physiological setting. Indeed, based on action potential clamp experiments, Puglisi et al. [22] inferred that when normal SR Ca release occurs it reduces the integrated Ca influx via $I_{Ca,L}$ by 50%.

If voltage clamp pulses (or presumably action potentials) are very long, $I_{Ca}$ can be largely inactivated during the cytosolic Ca transient, but then recover as $[Ca]_i$ declines [23]. This sort of reactivation of L-type Ca channels during long action potentials may be the molecular basis of early after-depolarizations (EADs) and the arrhythmogenic consequences thereof [24,25].

### 2.3. Ca influx during the action potential

$I_{Ca}$ characterization has mostly been done using traditional square voltage clamp pulses. Fig. 3 shows the difference in $I_{Ca}$ waveform when rabbit ventricular myocytes are subjected to the usual square pulses vs a voltage clamp waveform taken from an action potential recorded from the same cell type (under physiological ionic conditions, [14]). Peak $I_{Ca}$ during the action potential clamp is lower and occurs later. This is because the peak of the action potential (~+50 mV) activates Ca channels rapidly, but the driving force for Ca is initially low because $E_m$ is close to the reversal potential for $I_{Ca}$ (~+60 mV). As $E_m$ falls the driving force apparently increases faster than the channels inactivate, producing a larger current at later times during the action potential. The current is also more sustained during the action potential than during a square pulse. The amount of Ca entry during this action potential

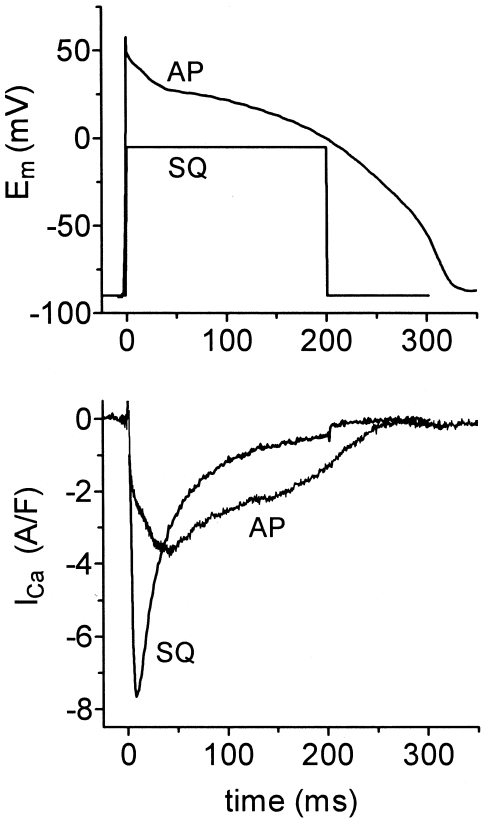


Fig. 3. $I_{Ca}$ measured during square pulse and action potential clamp in a rabbit ventricular myocyte. Command $E_m$ waveforms were either the traditional 200-ms square depolarization to 0 mV (SQ) or an action potential (AP) which was recorded from a rabbit ventricular myocyte under more physiological conditions (i.e. normal Ca Na and K concentrations, [14]). The current measurements were under conditions to isolate $I_{Ca}$ (e.g. Na-free and Cs-rich inside and out).

clamp is sufficient to bring in 21–25 μmol Ca/l cytosol in rabbit ventricular myocytes [14,26]. However, this is an overestimate of the physiological Ca influx because these measurements were in cells dialyzed with EGTA (such that there was less Ca-dependent inactivation of $I_{Ca}$). It should also be noted that a total Ca influx of 20 μmol/l of cytosol would only raise free $[Ca]_i$ by ~200 nM (due to the high, ~100:1 buffering of $[Ca]_i$, in the cell [26] When integrated $I_{Ca}$ was measured during action potential clamp in rabbit ventricular myocyte at 25 and 35°C where SR Ca release occurred, the value was 6–12 μmol/l cytosol at both temperatures [22].

### 2.4. Regulation of Ca current

The amplitude and kinetics of $I_{Ca}$ clearly depend on the intrinsic channel properties as well as ionic conditions and $E_m$. In addition $I_{Ca}$ can be modulated by both physiological and pharmacological effects. During physiological increases in heart rate catecholamines activate β-adrenergic receptors, adenylyl cyclase and cAMP-dependent protein

kinase (PKA) and this dramatically alters the behavior of $I_{Ca,L}$. Increased frequency may also have an intrinsic facilitatory effect. Dihydropyridines can either inhibit or increase $I_{Ca}$ (see below). Many other factors may modulate $I_{Ca}$ [27], but space dictates limited discussion of only a few of these factors below.

*2.4.1. β-adrenergic receptors and protein kinase A*

Activation of $I_{Ca}$ by β-adrenergic agonists in cardiac muscle is a classic observation (e.g. [28]). This occurs mainly through PKA which causes a two–four-fold increase in basal $I_{Ca}$ in ventricular myocytes and shifts the voltage-dependence of activation and inactivation to a more negative $E_m$ [27,29,30]. The shift in gating also means that the increase in $I_{Ca}$ is most prominent at more negative potentials and that the voltage for maximal $I_{Ca}$ shifts to more negative potentials. At the single channel level there is no effect of PKA on unitary conductance, rather there are fewer blank sweeps (without any openings) and an apparent increase in open times which may also reflect a different gating mode [31,32].

There is also interaction between the β-adrenergic cascade and other signaling pathways in modulating $I_{Ca}$. For example, acetylcholine (ACh) has no effect on basal $I_{Ca}$ in ventricular myocytes, but strongly antagonizes the catecholamine- or forskolin-stimulated $I_{Ca}$ [33,34]. Part of this effect of ACh may be mediated by the muscarinic receptor-mediated activation of $G_i$ which inhibits adenylyl cyclase and thus net cAMP production [35]. In addition, ACh increases cGMP which can either decrease cAMP by stimulating a cGMP-activated phosphodiesterase (PDE) in frog [36] or increase cAMP in mammalian heart by a cGMP-inhibited PDE [27,37]. Increased cGMP can also activate cGMP-dependent protein kinase (PKG), which does not appear to alter basal $I_{Ca}$, but can (like ACh and cGMP) antagonize cAMP-mediated increases in $I_{Ca}$ [37]. Nitric oxide also stimulates cGMP production in cardiac myocytes [38] and this can mediate $I_{Ca}$ regulation as above [39–41]. Indeed, there is evidence to suggest that nitric oxide is required for the ACh-mediated antagonism of cAMP-activated $I_{Ca}$ in cardiac myocytes [41,42]. ACh has also been shown to stimulate protein phosphatase activity, which would also reverse PKA-mediated phosphorylation [43].

*2.4.2. Dihydropyridines (DHPS)*

A pharmacological hallmark of L-type Ca channels is their sensitivity to DHPs (e.g. nifedipine, amlodipine, nitrendipine, nimodipine, nisoldipine, PN 200-110, Bay K 8644, azidopine and iodipine). Most of these DHPs inhibit $I_{Ca}$ and are known as Ca-channel blockers or Ca-channel antagonists. Several DHPs (e.g. (−) Bay K 8644, (+) S-202-791 and CGP 28392) and a novel benzoyl pyrrole (FPL-64176) are referred to as Ca-channel agonists, because they enhance $I_{Ca}$ dramatically, by increasing the duration of single Ca-channel openings, with only a slight increase in single-channel conductance [44–48]. DHP binding is voltage dependent such that the apparent affinity for $I_{Ca}$ block by nitrendipine shifts from a $K_d$ of 700 nM at −80 mV to one of 0.36 nM at −10 mV [49]. This is generally interpreted as DHPs binding preferentially to the inactivated state of the Ca channel [49,50].

In addition to DHPs there are two other classes of specific L-type Ca channel blockers: (a) phenylalkylamines (such as verapamil, D600, D888 and D890) and (b) benzothiazipines (e.g. diltiazem), and these agents can interact allosterically with the Ca channel [51,52]. Sanguinetti and Kass [50] also compared DHPs and verapamil effects as a function of net charge at pH 7.4. Charged compounds (e.g. verapamil) appeared to block the channel preferentially in the open state (i.e. requiring depolarizing pulses) and as such are use-dependent. The neutral ligands (nitrendipine and nisoldipine), however, could block $I_{Ca}$ whether the channel was either in the open or inactivated state (steady depolarization, without requiring pulses) and as such are considered more voltage-dependent than use-dependent. This distinction between voltage- and use-dependence may be why DHPs are more useful vasodilators than verapamil. That is, resting vascular smooth muscle is more depolarized and can undergo long sustained depolarizations, while cardiac myocytes are repetitively depolarized (with more negative diastolic $E_m$). This may also explain why the cardiac effects observed with Ca antagonists are more apparent in pacemaker cells which have a relatively depolarized diastolic $E_m$ level.

Bay K 8644, S202-791 and FPL-64176 dramatically augment $I_{Ca}$ by prolonging the open times. In general, they also shift activation toward a more negative $E_m$. Because of the long open times, the Ca channels do not shut off as rapidly upon repolarization. This results in very long and large Ca tail currents attributable to the abrupt increase in electrochemical driving force upon repolarization while some channels are still open [29,53]. These could also contribute to EADs during the rapid repolarization phase of the action potential [54].

*2.4.3. Frequency-dependent facilitation of $I_{Ca}$*

Increasing the frequency of voltage clamp pulses from a holding potential of about −40 mV typically results in a progressive decline in $I_{Ca}$ amplitude [55,56]. This negative $I_{Ca}$ staircase probably reflects insufficient time for Ca channels to recover from the inactivated state between pulses (at −40 mV). In contrast at suitably physiological holding potentials (−80 mV) there is a pulse-dependent progressive increase in $I_{Ca}$ amplitude and a slowing of inactivation [11,55–63]. This positive $I_{Ca}$ staircase is Ca-dependent (not apparent with Ba as the charge carrier) and more recent work has clarified that this effect is mediated by CaMKII-dependent phosphorylation [13,64,65]. Since this Ca-dependent facilitation is still observed when cells are dialyzed with 10 mM EGTA (but abrogated by 20 mM BAPTA), it seems that the Ca-dependent activation of CaMKII must be highly localized near the L-type Ca channel [56]. The physiological impact of this Ca-depen-

dent facilitation is not entirely clear, but it could partly offset the more prominent direct Ca-dependent inactivation which increases at higher frequency due to the larger Ca transient.

A voltage-dependent facilitation of $I_{Ca}$ has also been described in chromaffin cells [66,67], skeletal [68] and cardiac Ca channels [65,69,70]. This appears to be mediated by cAMP-dependent protein kinase. This facilitation occurs with pulses to very positive potentials and dissipates extremely rapidly upon repolarization to physiological membrane potentials in ventricular myocytes. Thus the functional role of this PKA- and voltage-mediated $I_{Ca}$ facilitation in the normal cardiac myocyte is not clear.

## 3. Molecular structure–function relations of cardiac Ca channels

The foregoing section focused on the phenotype of $I_{Ca}$ recorded in the native cardiac myocyte environment. In this section we consider the molecular mechanisms involved.

### *3.1. Biochemical characterization of L-type Ca channels*

Biochemical characterization of L-type Ca channels was facilitated by the availability of both high affinity dihydropyridine (DHP) antagonists and a tissue rich in binding sites, the rabbit skeletal muscle. Purification of skeletal muscle DHP receptors led to the identification of five potential subunits, called $\alpha_1$, $\alpha_2$, $\beta$, $\gamma$, and $\delta$ (reviewed in Ref. [71]). The $\alpha_1$ subunit contains the ion-conducting pore, as well as the binding sites for the Ca channel blockers. Purification of cardiac Ca channels has been hampered by a lower number of DHP binding sites in sarcolemmal membranes [72]. Despite this limitation, the cardiac Ca channel was purified from various species using wheat-germ agglutinin chromatography (reviewed in Ref. [73]). The cardiac $\alpha_1$ protein could be identified by azidopine labeling, indicating it was larger than the skeletal muscle $\alpha_1$. Antibodies directed against the skeletal muscle $\alpha_2\delta$ cross-reacted with the cardiac $\alpha_2$ and $\delta$ subunits, suggesting structural homology. Antibodies have also been used to purify cardiac $\alpha_1$ subunits, either by immunoprecipitation or affinity-chromatography. A specific anti-peptide antibody directed against the $\beta_2$ sequence was used to immunoprecipitate cardiac DHP receptors [74]. These studies demonstrate that the cardiac L-type Ca channel is a multi-subunit complex, containing at least $\alpha_{1C}$, $\alpha_2\delta$, and $\beta_2$ subunits (Fig. 4).

### *3.2. Molecular structure of cardiovascular L-type Ca channels*

Numa and coworkers purified the skeletal muscle $\alpha_1$ subunit ($\alpha_{1S}$) to homogeneity, sequenced tryptic fragments from it, and cloned its cDNA [75]. Using this cDNA as

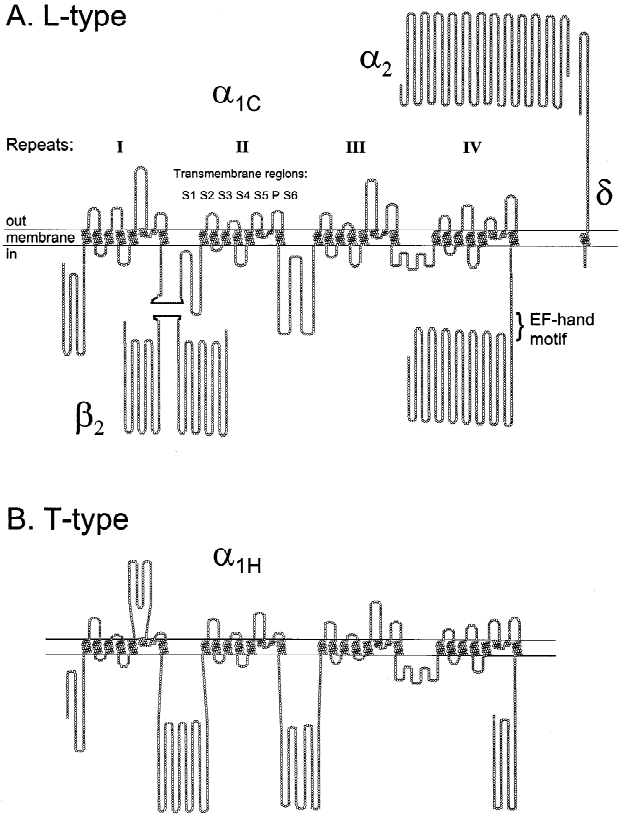


Fig. 4. Schematic representation of the structure of L- and T-type cardiac Ca channels. Each amino acid is represented by a circle. The protein is shown to snake its way in and out of the membrane. The $\alpha_1$ subunit for both channels contains four repeats, each of which contains six transmembrane regions and a pore loop. (A) The cardiac L-type channel contains at least four subunits: $\alpha_{1C}$, $\alpha_2\delta$, and $\beta_2$. The amino acids involved in the interaction between $\alpha_{1C}$ and $\beta_2$ are shown as filled circles. The location of the putative EF hand is also indicated. (B) The minimum structure of the cardiac T-type channel is shown as $\alpha_{1H}$, although it should be noted that heart also expresses $\alpha_{1G}$. In comparison to $\alpha_{1C}$, $\alpha_{1H}$ contains a larger extracellular loop before the pore loop in Repeat I and larger cytoplasmic loops linking Repeats I–II and II–III. In contrast, $\alpha_{1C}$ has a much larger carboxy terminus, which plays important roles in Ca-induced inactivation and possibly protein kinase regulation of the channel.

probe, a homologous $\alpha_1$ cDNA was cloned ($\alpha_{1C}$) using low stringency screening of heart cDNA libraries [76]. Injection of cRNA transcribed from a full-length $\alpha_{1C}$ cDNA into *Xenopus* oocytes led to the induction of dihydropyridine-sensitive Ba currents, thereby demonstrating that this $\alpha_1$ was part of the L-type channel. Molecular cloning studies have revealed the presence of at least ten genes encoding voltage-gated Ca channels. Four of these $\alpha_1$ subunits are L-type channels: $\alpha_{1S}$ is the skeletal muscle channel, $\alpha_{1C}$ the cardiac, $\alpha_{1D}$ is expressed in many of the same tissues as $\alpha_{1C}$, and the newly described $\alpha_{1F}$ appears to be retinal specific [77,78].

The human $\alpha_{1C}$ gene, *CACNA1C,* has been sequenced [79] and mapped to chromosome 12p13.3 [80]. The gene is also expressed in lung, aorta, ovary, fibroblasts, and brain [73]. Comparison of these cloned sequences reveals at least nine regions of sequence diversity (Table 1). Five of these

Table 1
Alternatively spliced regions of $\alpha_{1C}$ [a]

| Site | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Location | $NH_2$ | IS6 | I–II Loop | I–II Loop | IIIS2 | IVS3 | IVS3-S4 | COOH | COOH |
| Type | Alternate exons | Alternate exons | Deletion of exon | Alternate splice site | Alternate exons | Alternate exons | Deletion of exon | Alternate exons | Alt. exons and alt. splice |
| Size of region (aa) | A=60<br>B=45 | A=33<br>B=33 | 25 | 3 | A=20<br>B=20 | A=28<br>B=28 | 11 | A=35<br>B=71 | |
| Human genomic exon [139] | A, n.d.;<br>B=1 | A, n.d.;<br>B=8 | n.d. | 15 | A=21<br>B=22 | A=31<br>B=32 | 33 | A=45<br>B, n.d. | 40,40B,<br>41,42 |
| Rabbit heart [76] | A | A | − | − | A | A | + | − | |
| Human heart [80] | − | A | − | − | A | A/B | ± | B | |
| Rabbit lung [85] | B | B | + | − | A | B | + | − | |
| Rat aorta [87] | A | A | − | − | A | B | + | − | |
| Rat brain [82] | B | A | − | ± | B | A/B | + | − | |
| Mouse brain [138] | B | A | ± | ± | B | A/B | + | − | |
| Human fibroblast [139] | B | B | − | − | A/B | A/B | + | A | |

[a] The approximate locations of the splice variant regions are noted in row 2. The number of amino acid residues in the spliced region is given in row 4. If the splicing involves alternate exons, then each variant exon is arbitrarily assigned the letter A or B. Some of the human genomic sequences were not determined, and this is indicated by the abbreviation n.d. If it was sequenced then the apparent exon number is reported. Rows 6–12 indicate which one of the alternate exons, or presence (+) or absence (−) of an exon, was reportedly cloned.

divergent regions are due to mutually exclusive exon splicing, such that the mature mRNA transcript contains sequences from either one (A) or another (B) exon. Splicing in two regions is due to exon skipping events, leading to the deletion of a small stretch of amino acids. A third type of splicing is the use of alternative 5′ splice sites, which also leads to the deletion of a small stretch. One of the most studied regions is the alternate splicing of two exons that each encode a version of the third membrane spanning region of the fourth domain (IVS3) [81]. It has been suggested that splicing of IVS3 exons is regulated in both a tissue-specific manner [82] and during development [83,84]. In any case both isoforms are present in mammalian heart [81]. Perhaps the only splicing event that is cardiac specific is the amino terminus [85]. Two different $\alpha_{1C}$ sequences have been cloned from heart [76]. Surprisingly these isoforms differ in at least three regions (sites 1,6, and 8; Table 1). Similarly, two different $\alpha_{1C}$ sequences have been cloned from smooth muscle [86,87], yet these isoforms differ in at least three regions (sites 1,2, and 3; Table 1). A major problem in deducing the structure from cDNA cloning is that often only a single clone is isolated for a particular region, therefore rare splicing events can be incorporated into the full-length sequence. More studies (PCR, RNase protection assays) are required to determine the consensus structure of $\alpha_{1C}$ in any tissue.

In addition to $\alpha_{1C}$, RNA transcripts for $\alpha_{1A}$ [88] and $\alpha_{1D}$ [89] have been detected in heart. Expression of neuronal $\alpha_1$ HVA subunits correlates with the presence of P-($\alpha_{1A}$), N-type ($\alpha_{1B}$), and R-type ($\alpha_{1E}$) currents in intracardiac neurons [90]. Although it is generally accepted that $\alpha_{1C}$ is the predominant L-type $\alpha_1$ in cardiac myocytes, $\alpha_{1D}$ has also been detected at both the mRNA and protein level [91,92].

### *3.3. Structure–function relationships of $\alpha_{1C}$*

Cloning of voltage-gated ion channels has led to large advances in our understanding of their structure–function relationships. One concept to emerge from these studies was that there was a superfamily of voltage-gated channels that included not only the Ca and Na channels, but also some K channels. These channels all contain the fundamental unit or repeat that includes six transmembrane regions (S1–S6) and a pore loop. Since Na and Ca channels contain four of these repeats (I–IV; Fig. 4) it is thought that they are evolutionary descents of K channels, produced by two rounds of gene duplication. Because K channels are simpler, more is known about their structure (reviewed in Ref. [93]). The voltage sensor is contained in the S4 region, which contains an interesting series of positively-charged residues every third residue This region is thought to move outward with membrane depolarization, but how this leads to channel opening has not been elucidated. S3 regions contain a few charged residues that are highly conserved, and may play a role in charge–charge interactions with S4. The amino acid sequence of the S1 and S2 segments are poorly conserved, suggesting

that these residues may face the lipid. The S5 and S6 regions appear to form the inner walls of the channel, forming a hydrophobic pocket for numerous classes of drugs.

A second emerging theme in ion channel structure is that ion selectivity is produced by pore loops that dangle into the outer mouth of a larger channel [94]. The structure of the pore loop of an inwardly rectifying K channel was recently determined by X-ray crystallography [95]. The descending loop was formed by an α helix, which dips into the channel mouth angling directly at the selectivity filter. The ascending loop is less structured, but forms the selectivity filter with main chain carbonyl oxygen atoms. Calcium channels may have a similar structure, but important differences are already known. One notable difference is that Ca ions are bound at the selectivity filter. This Ca binding site is thought to be formed by glutamic acid side chains on the ascending pore loop of each of the four repeats [96]. This Ca binding site also distinguishes Ca from Na channels. Although Na channels have similar pore loops, this ring of negative charges is no longer conserved and the channel no longer binds Ca. In the absence of Ca both channels conduct Na. In the presence of Ca this site becomes occupied by Ca channels, blocking the passage of Na. These glutamate residues are also involved in proton block [97].

Numerous studies have also attempted to elucidate the structures involved in Ca-dependent inactivation. Based on sequence homology of the Ca-binding motifs of EF hands, it was proposed that Ca channels may bind Ca at a site just after the last transmembrane region of the fourth repeat, IVS6 [98]. Preliminary studies with chimeric channels supported this notion [99]. However, site-directed mutagenesis experiments aimed at disrupting the binding site were not successful [100]. There are multiple regions involved in Ca-dependent inactivation, most, but not all, of these regions are located in the carboxy terminus [101,102]. Clearly more work will be required to determine how Ca controls this process.

Significant progress has also been made towards understanding the molecular determinants of L-type pharmacology (reviewed in Ref. [103]). The binding sites for the three major classes of Ca channel blockers have been identified. Important residues involved in dihydropyridine binding have been mapped in IIIS5, IIIS6, and IVS6. Residues involved in phenylalkylamine binding have also been mapped to the S6 regions of repeats III and IV. This overlap is somewhat surprising since these drugs modulate each other's binding in an allosteric manner, rather than simple competition. That the binding site includes determinants from more than one repeat also puts constraints on the arrangement of the repeats. A domain interface model has been proposed where the repeats are organized in a clockwise manner [104]. Ancillary subunits are also important in the formation of high-affinity drug binding sites and their interactions [105].

### *3.4. Ancillary subunits: structure, function, and interaction sites*

The $\alpha_2\delta$, β, and γ subunits of skeletal muscle have also been cloned (reviewed in Ref. [73]). Purification and sequencing of the δ subunit demonstrated that it is encoded in the $\alpha_2$ gene, indicating that it is a proteolytic fragment [106,107]. The $\alpha_2$ subunit is thought to be located on the extracellular surface of the membrane, being tethered to the δ protein through a disulfide bond. The δ protein contains a single hydrophobic segment which is thought to anchor the complex [108]. Despite the fact that Northern blot analysis detected the presence of two hybridizing mRNA species, it was concluded that there was only one gene encoding $\alpha_2\delta$, and that this gene was expressed in most tissues that have Ca channels [109]. This notion is probably incorrect, as related sequences have appeared in the GenBank (E. Perez-Reyes, unpublished observations).

Alternative splicing of $\alpha_2\delta$ mRNA transcripts have been reported to occur in a tissue specific manner [110–112]. Additional variants were detected using PCR and their relative expression was quantitated by RNase protection assays [112]. One form, called $\alpha_{2a}$, is predominantly expressed in skeletal muscle, $\alpha_{2b}$ in brain, while $\alpha_{2c}$ and $\alpha_{2d}$ are more abundant in heart. These isoforms differ by deletion of two small exons, one encoding 24 and the other seven amino acids. The functional significance of these variations has yet to be demonstrated.

Coexpression of $\alpha_2\delta$ with $\alpha_{1C}$ causes an approximate two-fold increase in expression of dihydropyridine binding sites, gating currents, and ionic currents [105,113,114]. These studies indicate that $\alpha_2\delta$ plays a role in the formation of functional channels at the plasma membrane surface. It also causes a four-fold increase in the apparent affinity of those channels for dihydropyridines [105]. In addition, it appears to accelerate channel opening and closing [114]. The $\alpha_2\delta$ subunit may also bind drugs directly, as has been suggested for the anticonvulsant, gabapentin [115]. However, the role of such binding in the therapeutic action of this drug has not been established.

Northern analysis suggested that expression of the γ gene is restricted to skeletal muscle [116,117]. Coexpression with $\alpha_{1S}$ led to a small increase in the number of dihydropyridine binding sites detected, and altered the allosteric regulation of binding by a phenylalkylamine analog [118]. Coexpression of γ with $\alpha_{1C}$ also affects dihydropyridine binding [105], and may have a small effect on inactivation of its currents [113]. Although there is no evidence for a cardiac γ subunit, recent evidence suggests that there are additional γ genes expressed in brain [119].

The cardiac Ca channel also contains a β subunit, called $\beta_2$. Alternative splicing of $\beta_2$ exons occurs in at least two regions, the amino terminus and a central region right beside the α interaction domain [73,120]. In both these regions there appears to be three exons that are spliced in a

mutually exclusive manner. Recent data suggests that the $\beta_{2a}$ isoform is only expressed in brain [121]. $\beta_2$ is not the only β cDNA that has been cloned from heart; $\beta_3$ and $\beta_1$ variants have also been detected [122,123]. Northern analysis of rat and rabbit tissues indicates that $\beta_2$ mRNA is the most abundant β subunit in heart, while $\beta_3$ is the most abundant in lung. Immunoprecipitation using a $\beta_2$ specific antibody was able to pull down 80% of the dihydropyridine binding sites from rabbit heart. Therefore $\beta_2$ is the predominant cardiac β subunit.

The β subunits of Ca channels play several roles, including direct effects on the biophysical properties of the channel, effects on the assembly of the channel complex, and possibly a role in the regulation of channel activity by protein kinases. Coexpression of $\beta_2$ with $\alpha_{1C}$ leads to a dramatic ten-fold increase in currents, acceleration of activation and inactivation kinetics, and effects on steady-state inactivation. In addition, coexpression leads to a large increase in the number of high-affinity DHP binding sites [124]. Phosphorylation of the $\beta_2$ subunit may be responsible for the increased activity of cardiac L-type Ca channels after β-adrenergic stimulation [125]. Numerous studies have tried to determine the mechanism by which β stimulates $\alpha_1$ activity [126,127]. One likely explanation is that β subunits act like chaperonins, helping nascent $\alpha_{1C}$ molecules to fold properly and reach the plasma membrane, thereby causing an increase in the detectable DHP binding sites. Studies from Campbell's group have elucidated the interaction sites between β and α subunits [120,128]. The β interaction site on α occurs in the loop between Repeats I and II. Splice variants of this loop have been reported, suggesting that β's may interact differently with these variants. The α interaction site on β occurs just after a region that is highly spliced in $\beta_2$, again, suggesting that splice variation may alter the interaction between α's and β's. Indeed, splice variants of both this site and the amino terminus ($\beta_{2a}$vs. $\beta_{2b}$) have been shown to cause different kinetic effects when coexpressed with $\alpha_{1E}$ [121,129]. One major difference between these splice variants is that only $\beta_{2a}$ is palmitoylated. Mutation of the residues that are palmitoylated modifies its interaction with the α subunit [121,130].

### *3.5. Protein kinase regulation of cloned L-type Ca channels*

Considerable evidence supports the hypothesis that the activity of L-type Ca channels is regulated by protein phosphorylation (reviewed in Refs. [27,131]). Therefore it is somewhat surprising that this regulation has been hard to demonstrate with cloned subunits [132]. Forskolin, which is expected to lead to increases in cAMP production and activation of protein kinase A, has little or no effect on the cloned channel [133]. Despite this lack of effect, the cloned subunits have been shown to be substrates for PKA and PKC [134]. One possible explanation is that a critical subunit has yet to be cloned. It was recently suggested that the PKA anchoring protein, AKAP-79, was such a missing link [135].

### *3.6. Molecular structure of cardiac T-type Ca channels*

Two $\alpha_1$ subunits of low voltage-activated, T-type, Ca channels were recently cloned, $\alpha_{1G}$ [136] and $\alpha_{1H}$ [137]. The location of these genes on human and mouse chromosomes were also mapped. The *CACNA1G* gene (encoding $\alpha_{1G}$) was localized to human chromosome 17q22 and the *CACNA1H* gene to 16p13.3. These channels are similar to high voltage-activated channels in terms of structure, each having four repeats of the six transmembrane regions and pore loop structure (Fig. 4). Sequence conservation is highest in the S4 and pore loop regions. However, the overall sequence identity between low and high voltage-activated channels is relatively low (<15%). Two motifs found in $\alpha_{1C}$ that are not found in $\alpha_{1H}$ are the β binding region and the putative EF hand.

Northern analysis indicated that both $\alpha_{1G}$ and $\alpha_{1H}$ are expressed in human heart. The $\alpha_{1G}$ cDNA was cloned from rat brain, which is the tissue with the highest expression of this gene [136]. Although $\alpha_{1H}$ was cloned from an adult human heart library, much more mRNA appears to be expressed in kidney and liver [137]. Future studies are required to determine which of these T channel subtypes is expressed in pacemaker tissue and which is expressed in vascular smooth muscle.

Expression of either $\alpha_{1G}$ or $\alpha_{1H}$ leads to the induction of classic T-type Ca channel currents. Distinguishing features of T-type currents are the criss-crossing pattern of the traces recorded during a IV protocol, activation near the resting membrane potential (−70 mV), slowly deactivating tail currents, and a small single channel conductance for Ba. These results were obtained after expression of the $\alpha_1$ subunit alone, suggesting that T channels do not require auxiliary subunits as observed for high voltage-activated channels.

## 4. Ryanodine receptors and $IP_3$ receptors

The other main Ca channel types present in cardiac myocytes are the intracellular Ca-release channels which are responsible for releasing Ca from the SR (or possibly also the ER). The ryanodine receptor (RyR) is known to be the key SR Ca-release channel involved in cardiac E–C coupling and RyR2 is the cardiac isoform (see reviews [140–143]). $IP_3$ receptor Ca-release channels have also been reported in the heart, although at this stage the function of these channels is less clear [144–148]. The main focus in this section will be on structural and functional properties of RyR2 in isolated systems.

### *4.1. Ryanodine receptor structure, calmodulin and FKBP interaction sites*

Ryanodine was used as a specific ligand in the purification of the RyR from skeletal muscle [149–153] and cardiac muscle [154,155]. The cardiac RyR was cloned by Otsu et al. [156] and Nakai et al. [157] and has a molecular weight of 564 711 Da. The protein appears to exist mainly as a homotetramer, based on its quatrefoil appearance [153,158,159], gel permeation chromatography [149] and stoichiometry of high affinity ryanodine binding [153,160]. The large size of this homotetramer (2260 000 Da) has helped to identify it ultrastructurally as the junctional foot process which spans the gap between the SR and sarcolemmal membranes at their junctions. Thus, it traverses the SR membrane providing a channel for SR Ca release and also extends toward the sarcolemmal membrane. This proximity is undoubtedly important in the process of triggering SR Ca release during E–C coupling (see below).

Wagenknecht and co-workers have continuously refined three-dimensional reconstructions of the RyR based on electron microscopic images [159,161–164]. Fig. 5 is a recent version and shows three different views of the RyR (from the T-tubule, from inside the SR and from the side; top to bottom panels respectively). Sites are also indicated where calmodulin and FK-506 binding proteins (FKBP, see Section 4.5) interact with the RyR. The complex is ~28 nm along each side and ~14 nm high above the SR membrane, which correspond reasonably well with the width and length of the junctional 'feet' observed ultrastructurally in electron micrographs of intact muscle [165,166]. The RyR reconstructions are intriguing because some have raised the possibility of a channel for Ca flux going through the center of the molecule from the SR lumen and coming out the sides of the RyR into the junctional space (see Fig. 6). The apparent location of FKBP on the RyR is indicated in Fig. 5 as the dark region at the left end of the double arrow (bottom panel). This is 9 nm away from where calmodulin is localized (right end of arrow). The FKBP location may relate to functional observations which suggest that FKBP is important in coupling monomers within the tetrameric array as well as between tetramers [167–169]. There is also some initial information about which sites on the skeletal RyR (RyR1) might interact with the skeletal L-type Ca channel, $\alpha_{1S}$ [164,170].

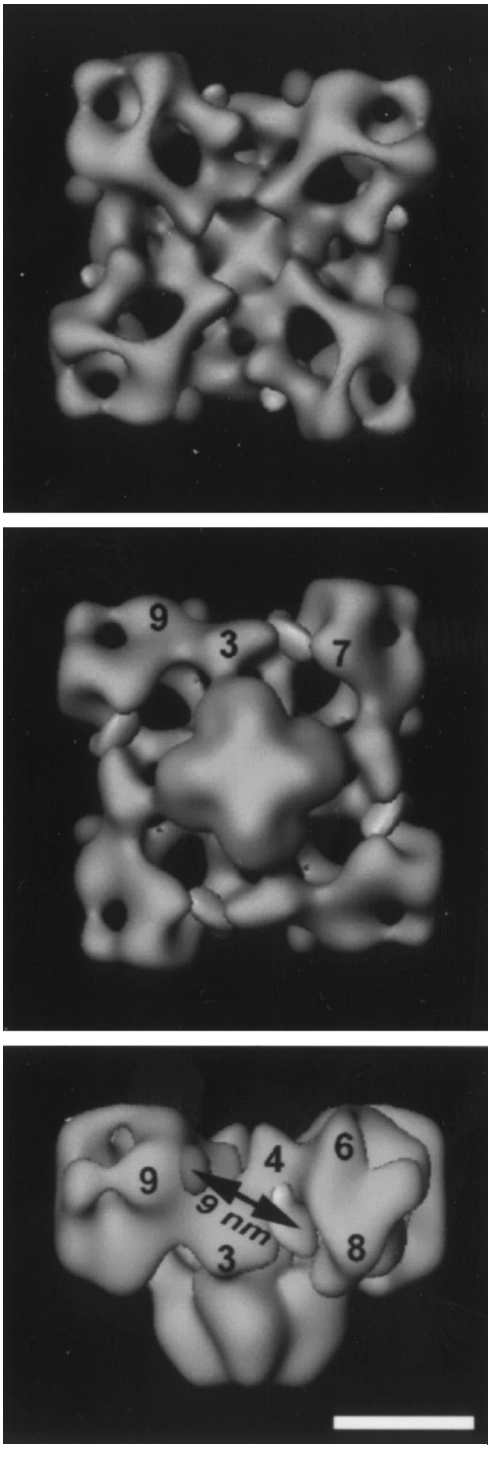


Fig. 5. Reconstructed image of the skeletal ryanodine receptor modified from Refs. [163,164] (kindly provided by T. Wagenknecht, Albany, NY). The views are from the T-tubule looking toward the top of the RyR (top), from inside the SR looking out at the RyR (middle) and from the side from along the SR membrane (bottom). Sites where calmodulin and FK-506 binding proteins (FKBP) interact with the RyR are also indicated. FKBP is the dark region at the left end of the double arrow in the bottom panel. This can also be seen in the top panel along the top edge near the right. Calmodulin is the light area 9 nm away from FKBP, at the right end of the arrow in the bottom panel. These images are of the channel in the closed configuration. In the open mode there is an apparent opening through the central axis of the structure [164].

### *4.2. Other RyR associated proteins (junctin, triadin, calsequestrin)*

In addition to calmodulin and FKBP there are other intrinsic proteins that may be physically associated with the RyR. These include triadin, junctin and calsequestrin (see Fig. 6). Calsequestrin is a low affinity, but high capacity Ca binding protein (~40 moles of Ca sites per mole of calsequestrin) which is localized to the junctional

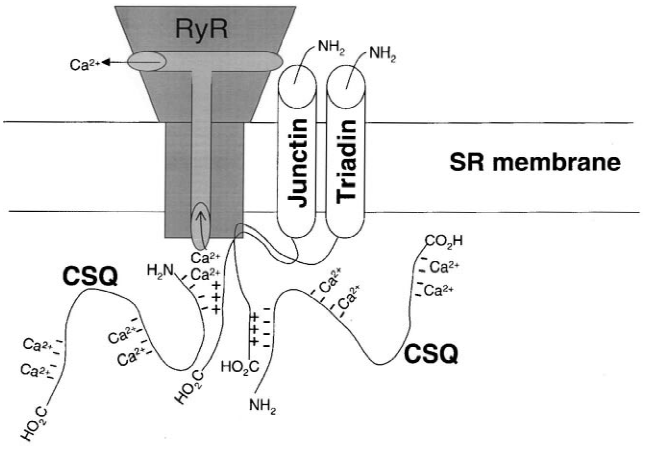


Fig. 6. Diagrammatic illustration of putative associations between SR junctional proteins in cardiac muscle. These include RyR2, calsequestrin (CSQ), triadin and junctin. Based on a diagram by Zhang et al. [176].

SR lumen and serves to buffer the intra-SR Ca [171–176]. This allows the SR Ca content to be very high while SR free [Ca] ($[Ca]_{SR}$) is limited by the [Ca] gradient that can be established by the SR Ca–ATPase. This limit is a consequence of the amount of free energy from ATP available to build a [Ca] gradient ($\Delta G_{SR} = 2RT \log([Ca]_{SR}/[Ca]_i)$, [177]). Calsequestrin may also be involved in the modulation of RyR gating [178–181]. Recent information on the calsequestrin crystal structure [182] should hasten better understanding of the structure–function relationships for this protein.

Triadin and the structurally related protein junctin [174–176,183,184] are also co-localized to the junctional region and interact with each other, calsequestrin and RyR2. Thus there may be a physical interaction among these proteins as illustrated fancifully in Fig. 6 [176]. At this point, there is little clear functional information concerning triadin and junctin. However, Zhang et al. [176] suggested that triadin and junctin may be required for physically coupling calsequestrin to the RyR as a junctional complex (Fig. 6) and also showed that interactions between these proteins and calsequestrin are Ca-sensitive (possibly charge–charge interactions). Thus, as $[Ca]_{SR}$ falls during SR Ca release, binding between calsequestrin and triadin and/or junctin may be enhanced. The cooperative 'zipping up' of this association could, in principle, facilitate Ca unloading of calsequestrin and thereby facilitate SR Ca release. This functional model is speculative, but could help to explain the intriguing results of Ikemoto and colleagues with respect to calsequestrin modulating SR Ca release [178,179].

### *4.3. Gating by Ca, caffeine and ryanodine*

The purified RyR (or SR Ca release channel) has been incorporated into lipid bilayers allowing functional characteristics of the native SR Ca release channel to be studied in considerable detail [151,153,185,186]. These studies have been invaluable in understanding both the regulation of gating and the conductance of the SR Ca release channel. The effects of large numbers of agents on RyR gating have been examined, but we will focus on only a few which might be of most basic physiological relevance (for more extensive information see [141,142,187–190]).

Ca activates RyR2 over a range of [Ca] which are relevant for E–C coupling (1–100 μM) and make it likely that this is the normal physiological activator. This is consistent with the Ca-induced Ca-release hypothesis of cardiac E–C coupling [191,192]. ATP and other adenine nucleotides also can activate the release channel. Mg can also inhibit SR Ca release channel opening probability in the range of physiological [Mg]. However, the local concentrations of ATP and Mg are unlikely to change rapidly during E–C coupling and thus are not likely to be actively involved in the process of E–C coupling per se. Rather, the local concentrations of ATP and Mg are critical in establishing how the RyR responds to a given physiological Ca signal. This makes it especially valuable to know about Ca activation of the SR Ca release channel in the presence of physiological ATP and Mg concentrations. For example, while mM of Mg inhibits steady-state RyR2 open probability ($P_o$) for any given free [Ca], it also accelerates the decline in $P_o$ induced by a rapid increase in local [Ca] [193]. Free intracellular [Mg] can also increase three to four-fold during ischemia as ATP levels fall, presumably because ATP is a major buffer of intracellular Mg [194]. There could be secondary effects of altered ATP and Mg on the sensitivity of the RyR to Ca trigger signals.

Caffeine can strongly activate RyR2 in bilayers [195], in SR vesicles [196] and also in intact cardiac myocytes [197]. The action of caffeine is extremely rapid and is quickly reversible, even in intact myocytes (although mM concentrations are required). This makes caffeine a very useful experimental tool. For example, rapid caffeine application to isolated myocytes can activate SR Ca release almost as rapidly as during an action potential evoked twitch. Since 10 mM caffeine appears to release the entire SR Ca content, the amplitude of the caffeine-induced Ca transient (or contracture) can be used to assess SR Ca content [197–199]. Sustained caffeine application also causes most of the released Ca to be extruded by the Na/Ca exchange current, such that this current can be integrated to directly quantify SR Ca load [200,201].

Ryanodine binds to the RyR with high sensitivity and selectivity. In bilayer studies, ryanodine at low concentration (1 nM–10 μM) causes the RyR to open permanently to a subconductance level which is about half of that observed in control Ca activation [202]. At very high concentration (0.3–2 mM) ryanodine appears to bind to a second lower affinity site and completely block the RyR [160,202]. Lai et al. [160] demonstrated that there is one high affinity and three low affinity ryanodine binding sites per tetramer. Ryanodine also causes similar functional

effects on the RyR in isolated SR vesicles [203] and in intact cardiac muscle and myocytes [204,205]. The binding of ryanodine to the RyR is very slow and it is virtually irreversible. Consequently, the effects of ryanodine are slow to develop. In intact cells and tissues the result is that only a small fraction of RyR are typically activated. This creates a leak which is sufficient to drain the SR of Ca rapidly during rest. However, during repetitive stimulation the SR Ca-pump can exceed this leak rate and cause the SR to transiently accumulate Ca [204,205]. Thus caffeine or blocking the SR Ca-pump with thapsigargin are often more definitive ways to prevent net SR Ca uptake.

### *4.4. RyR adaptation or inactivation*

Györke and Fill [206] first described a higher Ca-sensitivity of RyR2 to rapid steps in [Ca] compared to steady state changes in [Ca]. The SR Ca release channel is activated by a much lower [Ca] during these fast steps in [Ca], but then the $P_o$ relaxes back to that predicted by the steady state [Ca] dependence (within ~2 s). Györke and Fill referred to this process as adaptation, because after this decline in open probability the individual Ca channel could be reactivated by a Ca pulse of higher amplitude. That is, it did not appear to reach an absorbing inactivation state. Very similar results were found by Valdivia et al. [193]. However, they found that inclusion of relatively physiological [Mg] accelerated the time course of adaptation so that it occurred over ~100 ms. This brings it closer to the time frame where it could be involved in the turn-off of SR Ca release during a single cardiac contraction.

Several other groups have found this same sort of increased Ca-sensitivity of RyR2 activation when the local [Ca] is raised rapidly [207–209]. The data from some of this work has been consistent with a more absorbing inactivation state than the adapted state described by Györke and Fill [206]. Whether the term adaptation or inactivation is used to describe this phenomenon, it is really strikingly similar to the observations of Fabiato [191] in mechanically skinned single ventricular myocytes, where the rate of Ca application was found to be a major modulator of the SR Ca release produced by a given [Ca] trigger.

### *4.5. Regulation by protein kinases, calmodulin and FKBP*

Gating of the isolated RyR2 in bilayers can be modulated by phosphorylation, calmodulin and FK-506 binding proteins (FKBP). Valdivia et al. [193] showed intriguing effects of RyR2 phosphorylation by cAMP-dependent protein kinase (PKA). The basal $P_o$ was decreased by PKA (at 100 nM Ca). However, PKA greatly increased peak $P_o$ (to nearly 1.0) during a rapid photolytic increase of local [Ca], but accelerated the subsequent decline in $P_o$. Thus, phosphorylation by PKA activates RyR2 gating in the same sort of dynamic way as it modifies cardiac contractile force and cellular Ca transients during a twitch (increasing amplitude and rate of decline).

There is also a specific RyR site (serine 2809) which is phosphorylated by Ca-Calmodulin dependent protein kinase (CaMKII, [210]). Several studies have evaluated CaMKII effects on RyR in bilayers, finding either increases or decreases in RyR channel openings [210–214]. Some results with RyR2 indicate a CaMKII-dependent increase in $P_o$ [210,214]. On the other hand, Lokuta et al. [213] reported that CaMKII decreased cardiac SR Ca channel open probability when Ca and calmodulin were both present (i.e. when the channel was phosphorylated). This discrepancy might be partly explained in terms of the dynamic changes of RyR gating, as discussed above for PKA (but similar data is not available for CaMKII).

An additional aspect which may complicate understanding of CaMKII effects on the RyR is that calmodulin also has independent effects on the RyR. Calmodulin inhibits Ca-induced, caffeine-induced, and ATP-induced Ca release from both cardiac and skeletal SR, with an $IC_{50}$~100–200 nM [196,203,215,216]. Inhibition of Ca release occurred only at free [Ca]>100 nM. This action was observed in the absence of ATP, showing that CaMKII was not involved. Tripathy et al. [217] described a biphasic action of calmodulin on RyR1. While calmodulin inhibited the channel at micromolar or millimolar [Ca], at <100 nM [Ca], ryanodine binding increased, Ca release was stimulated, and channel open probability ($P_o$) also increased. This dual mode of action was confirmed in skinned skeletal muscle fibers, where calmodulin enhanced Ca-induced Ca-release at low [Ca], but inhibited it at high [Ca] [218]. Although there is not this kind of detailed information about cardiac RyR, it is possible that cardiac RyR follows the same biphasic mode of action.

There are two to six calmodulin binding sites per RyR monomer, according to sequence analysis, electron microscopy imaging, and labeling studies [161,163,217,219–221]. The binding of calmodulin to the RyR is also Ca-dependent. At low [Ca] (<100 nM) four calmodulin molecules bind with high affinity to one RyR monomer, whereas at micromolar [Ca] only one calmodulin binds per RyR monomer [217]. Under conditions expected during contraction and relaxation, calmodulin dissociation is very slow so that during a contraction–relaxation cycle most calmodulin might remain bound [217]. Thus, there also may be a slower, stimulation rate-dependent changes, such that with increased pacing frequency (and average $[Ca]_i$) calmodulin gradually dissociates from the RyR. This could relieve a tonic sensitization of Ca-induced Ca-release (at low [Ca]) and result in gradual reduction of Ca-sensitivity of SR Ca release. This hypothetical mechanism has not been addressed in intact cells.

The immunophillin targets for the immunosuppressant drug FK-506 (FKBPs) are peptide isomerases which also bind to and co-purify with the RyR [222–224]. FK-506

and rapamycin can cause dissociation of FKBP from the RyR. FK-506, rapamycin or direct removal of FKBP from the RyR has been shown to alter RyR1 and RyR2 channel gating in bilayer studies [167,168,225,226] although Barg et al. [227] found that removal of FKBP only modulated gating of RyR1. For the RyR2 Kaftan et al. [168] found that removal of FKBP destabilizes RyR2 gating such that overall $P_o$ is increased with the appearance of three additional subconductance states. Indeed, when exogenous recombinant FKBP was added to recombinant RyR in bilayers the normal channel gating properties with FKBP were restored. Recently Marx et al. [169] have also demonstrated that FKBP may be involved in a mechanical coupling between RyR tetramers that allow cooperative gating between adjacent SR Ca release channels. This may provide an additional redundant mechanism which, together with Ca-induced Ca-release, allow individual RyR channels to activate neighboring RyRs [228].

## 5. E–C coupling in cardiac myocytes

### *5.1. Discrete local SR Ca release events – Ca sparks*

Spontaneous localized SR Ca release events, known as Ca sparks, were first described in ventricular myocytes by Cheng et al. [229] using confocal fluorescence microscopy. These Ca sparks are due to SR Ca release via RyR channels and could, in principle, be due to single channel openings [229,230]. However, it seems more likely that Ca sparks are due to a cluster of RyR release channels working as a single functional unit [231–233]. Ca sparks can also be activated by Ca entry via $I_{Ca}$ [234,235]. Indeed, the global cell Ca transient during the normal twitch is probably composed of a very large number of Ca sparks occurring throughout the cell almost simultaneously [236]. Individual Ca sparks are generally not distinguishable during the normal twitch. This is because the Ca sparks are synchronized by $I_{Ca}$ during the action potential and overlap in both time and space. Opening of a single L-type Ca channel can trigger a Ca spark and spark probability may depend on binding of two Ca ions to the RyR [235,237]. Thus, spark frequency and SR Ca release clearly depend critically on the local cytosolic [Ca].

The frequency of Ca sparks also depends on SR Ca load [229,238]. Of course the increased SR Ca load can also increase the amplitude of Ca sparks. Indeed, as SR Ca load increases individual Ca sparks can merge into 'macrosparks' and also propagate in the form of Ca waves [238,239]. These Ca waves can activate inward ionic currents which contribute to transient inward currents and DADs. This SR Ca load effect may be related to increased open probability seen in bilayers when [Ca] is elevated on the luminal side of the RyR2 channel [240,241]. Thus, SR Ca load is also an important determinant of SR Ca release.

Immediately after SR Ca release, time is required for the recovery of the steady state Ca spark frequency despite unaltered SR Ca load [234,238]. This may reflect the recovery of the RyR from the adapted or inactivated state back to the normal level of sensitivity to activating Ca. This apparent refractoriness can be overcome when SR Ca load becomes very high [238], allowing wave initiation and propagation. Thus local $[Ca]_i$, $[Ca]_{SR}$ and time since the last activation all affect the probability of resting Ca sparks.

### *5.2. Ca-induced Ca-release during E–C coupling*

Fabiato and Fabiato [242] first demonstrated that Ca could induce SR Ca release in skinned ventricular myocytes. A tremendous amount of evidence since that time has made a compelling case that Ca influx via L-type Ca channels can trigger SR Ca release and is probably the main mode of E–C coupling in cardiac myocytes (for review see Refs. [192,243]). Key pieces of evidence include skinned fiber experiments [191] and voltage clamp studies which demonstrated close correlation between $I_{Ca}$ and Ca transients as a function of voltage [244–246]. Further support comes from the observation of $I_{Ca}$ 'tail transients' [244,245]. These occur when a cell which was voltage clamped near the reversal potential for Ca (where Ca channels are open, but $I_{Ca}$ does not flow) is then clamped back to a negative $E_m$ where Ca channels deactivate. Before the Ca channels close, a large but short-lived $I_{Ca}$ tail current induces a Ca transient and contraction. This seems most consistent with Ca-induced Ca-release, rather than any type of $E_m$-dependent Ca release [244]. Näbauer et al. [247] showed that Ca channel activation without Ca influx also could not induce SR Ca release. Abrupt photolytic release of caged Ca in intact cells can also activate SR Ca release [248,249] and this was not affected by either repolarization or depolarization.

There is also evidence that the SR Ca release channel can be activated by $I_{Ca}$ even when there is a high concentration of Ca buffer in the cell [250,251]. This is consistent with the RyR2 being close to the L-type Ca channel. There is extremely strong ultrastructural evidence for this sort of close physical association in skeletal muscle where alternate RyR tetramers in arrays are associated and aligned with four dihydropyridine receptors (tetrads) [252]. The structure–functional co-localization is also supported by work in dysgenic mouse myotubes, where the skeletal L-type Ca channel is missing, but transfection with this channel re-establishes both functional E–C coupling and physical localization of particles to sarcolemmal–SR junctions [253–255]. There is also strong support for a co-localization of RyR2 and the cardiac L-type Ca channel in heart [256]. However, the channel stoichiometry is much different. In cardiac muscle there are many more RyR than there are DHP receptors [257]. Thus, a single L-type Ca channel might be associated with four to ten RyRs (see Fig. 7).

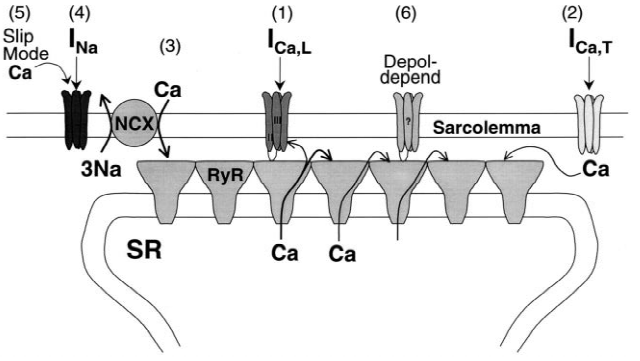


Fig. 7. Six possible mechanisms of cardiac E–C coupling. The most prominent current model is that Ca entry via $I_{Ca,L}$ activates the RyR Ca channel in the SR membrane and Ca-induced Ca-release can cause the activation to spread throughout the junction (1). Ca influx via $I_{Ca,T}$ could also trigger SR Ca release (2). Ca influx via Na/Ca exchange can be directly activated by depolarization (3), but could also occur secondary to Na entry via $I_{Na}$ and elevated [Na] (4). Ca might also enter via Na channels with altered selectivity (slip mode, 5). These are all variations on Ca-induced Ca-release. Depolarization might also directly activate SR Ca release without Ca entry (6). See Section 5.2 for additional details and references.

There is thus compelling evidence to support Ca-induced Ca-release mediated by $I_{Ca,L}$ in cardiac myocytes. This is a robust mechanism that can be observed under a very wide range of experimental conditions (e.g. temperature and ionic conditions) and preparations (e.g. purified RyR2 in bilayers, SR vesicles, skinned and intact myocytes). Manipulation of $I_{Ca,L}$ also produces generally predictable results on E–C coupling (including those secondary to altered SR Ca loading). There is consequently little doubt that this mechanism occurs physiologically, but there are several other candidate E–C coupling mechanisms which might coexist with this and provide valuable functional redundancy. These are described in the next section (and Fig. 7), but the physiological importance of these other mechanisms are largely open to debate at the present time.

### 5.3. Other candidate E–C coupling mechanisms

Most of the other candidate mechanisms still rely on Ca-induced Ca-release, but the mode of triggering Ca entry is different (T-type Ca channels, Na/Ca exchange or Na channels). The possibility of a more direct voltage-dependent mechanism like that in skeletal muscle has also been rekindled by new experimental data.

#### 5.3.1. Ca influx via $I_{Ca,T}$

As described above there is relatively little $I_{Ca,T}$ in ventricular myocytes although it can be significant in Purkinje and some atrial cells. There is also no information about whether T-type Ca channels are located at the sarcolemma–SR junctions. This mechanism could work essentially like $I_{Ca,L}$, but if T-type Ca channel were not preferentially localized at these junctions the effectiveness of $I_{Ca,T}$ as a trigger would be lessened. This is exactly the conclusion reached by Sipido et al. [258] in guinea-pig ventricular myocytes and Zhou and January [259] in canine Purkinje fibers where the effects of comparable $I_{Ca,L}$ and $I_{Ca,T}$ triggers were measured. Indeed, SR Ca release triggered by $I_{Ca,T}$ was delayed in onset and slower than that by a comparable $I_{Ca,L}$ trigger. Thus $I_{Ca,T}$ can function to trigger SR Ca release, but this is not physiologically important in E–C coupling in ventricular myocytes which lack $I_{Ca,T}$. Even in cells which exhibit $I_{Ca,T}$, it probably is only a slow, secondary E–C coupling system.

#### 5.3.3. Ca influx via Na/Ca exchange

Na/Ca exchange is a powerful Ca transport mechanism in cardiac muscle and is the main mechanism by which Ca is extruded from cardiac myocytes [205,260]. Ca can also enter in exchange for intracellular Na, producing an outward current and elevating $[Ca]_i$ [261]. Early experimental evidence suggested that Ca influx via Na/Ca exchange might be able to trigger SR Ca release [262]. Leblanc and Hume [263] demonstrated a tetrodotoxin-sensitive component of contraction. Their data gave credence to the hypothesis that Na influx via Na channels raised local $[Na]_i$ and that this caused Ca entry via Na/Ca exchange to trigger SR Ca release. Subsequent studies have supported these results [264–267], however, it remains unclear how much this mechanism contributes to the physiological activation of SR Ca release [267–269].

In addition to this indirect mechanism which relies on Na current, Ca entry via Na/Ca exchange can also be activated directly by depolarization. This is a consequence of the 3:1 stoichiometry of Na/Ca exchange which means Ca entry will be an outward current. The reversal potential for this Na/Ca exchange current at rest is typically negative (−30 to −80 mV, [192]). Thus during the rapid upstroke of the action potential to +50 mV Ca influx via Na/Ca exchange is favored thermodynamically. Several labs have provided evidence that this Ca entry via Na/Ca exchange can trigger SR Ca release, especially at large positive $E_m$ and in the absence of $I_{Ca,L}$ [270–273]. It should be noted that when an L-type Ca channel opens (which also occurs early in the action potential) the high local $[Ca]_i$ would prevent further Ca influx via Na/Ca exchange. Thus Ca entry via Na/Ca exchange may provide a back-up or redundant system for activation of SR Ca release, or one that allows gradual rise of local $[Ca]_i$ that works synergistically with the L-type Ca channel opening.

While Frank et al. [274] indicated that much of Na/Ca exchangers are located in the T-tubule, Kieval et al. [275] found a more uniform distribution. In any event there is not as much compelling evidence for a junctional localization of Na/Ca exchange (compared to L-type Ca channels). In addition, the unitary flux though the Na/Ca exchanger is perhaps 1000 times lower than $I_{Ca,L}$. Thus, a comparable Ca influx trigger would require one L-type Ca channel or

~1000 Na/Ca exchanger molecules. This would place physical constraints on the ability of Na/Ca exchange to produce a comparably localized Ca trigger signal for E–C coupling. It is clear now that Ca influx via Na/Ca exchange can trigger SR Ca release, but again the physiological role remains to be definitively determined.

#### *5.3.4. Ca influx via Na channels*

Ca entry via tetrodotoxin-sensitive Na channels has also been reported [276,277]. This Ca influx could also mediate Ca-induced Ca-release. Santana et al. [278] recently showed provocative evidence for altered selectivity of cardiac Na channels induced by either β-adrenergic agonist activation or cardioactive steroids. These agents appeared to make Na channels relatively Ca selective (i.e. an altered selectivity mode or slip-mode conductance). This tetrodotoxin-sensitive Ca influx could then trigger SR Ca release. The authors proposed that this could be a novel mechanism explaining the inotropic effects of β-adrenergic agonists and cardiac glycosides. These effects have generally been thought to be mediated by increased $I_{Ca}$ and SR Ca-pump activity (for β-adrenergic agonists) or by inhibition of the (Na+K)ATPase and reduced Ca efflux via Na/Ca exchange (for glycosides) [192]. How this novel mechanism compares to these traditional mechanisms has yet to be clearly elucidated.

#### *5.3.5. Voltage-induced SR Ca release*

In skeletal muscle depolarization triggers SR Ca release even in the absence of Ca influx (see reviews, [192,279,280]). This is thought to be mediated by a conformational change in the skeletal L-type Ca channel linked to RyR1 via the loop between domain II and III of the skeletal L-type Ca channel [254]. Indeed, peptides from this II–III loop region of the DHP receptor can activate the isolated RyR [281–283]. There is also evidence that the DHP Bay K 8644 can alter resting RyR2 gating in intact ventricular myocytes, possibly mediated via some physical link between the L-type Ca channel and RyR2 [284,285].

New experimental E–C coupling data in ventricular myocytes has been reported suggesting a voltage-dependent mechanism of SR Ca release like that in skeletal muscle with an $E_m$-dependence negative to the activation of $I_{Ca,L}$ [286–288]. This $E_m$-dependent trigger appears to require intracellular cAMP, but not Ca influx to trigger SR Ca release. On the other hand, it still requires extracellular Ca and this Ca-dependence makes it somewhat less compelling as a truly Ca-influx independent E–C coupling mechanism. Thus there are several potential additional participants in addition to L-type $I_{Ca}$ in mediation of SR Ca release during cardiac E–C coupling.

### *5.4. Recovery of SR Ca release channels and rest potentiation*

Since the RyR2 in the bilayer either adapts or inactivates in response to an abrupt activation by Ca (see Section 4.4) there must also be a finite time and possibly other conditions required for recovery back to the initial higher Ca-sensitivity. Fabiato [191] demonstrated in skinned myocytes that this recovery took several seconds at [Ca]= 13 nM and longer at higher, more physiological [Ca]. The correlate in the intact cell is the common observation of rest potentiation of twitches and Ca transients after increasing rest intervals [192]. The potentiated SR Ca release takes several seconds to reach a maximum and can occur without any increase in either SR Ca content or $I_{Ca}$ trigger [289,290]. This sort of refractoriness of E–C coupling can also help to explain the negative force–frequency relationship commonly observed in rat ventricular myocytes where there may be accumulating refractoriness. This recovery from an adapted or inactivated state is also seen with respect to Ca sparks [238]. Thus, immediately after a twitch, there is a suppression of Ca spark frequency (despite unchanged SR Ca content and diastolic $[Ca]_i$) and this Ca spark frequency recovers with a time course almost identical to rest potentiation of global Ca transients.

### *5.5. Modulators of SR Ca release in the intact cell*

There are numerous factors involved in regulating SR Ca release in the intact cell. From the foregoing it is clear that the amount of Ca release will depend on the Ca trigger ($I_{Ca,L}$ plus other possible triggers), the SR Ca content and the history since the last activation. Furthermore, SR Ca load has a dual effect. Clearly one would expect greater SR Ca release simply as a consequence of increased SR Ca content (i.e. even for the same fractional SR Ca release). However, the increase in SR Ca content also sensitizes the SR Ca release process such that a larger fraction of SR Ca content is released [291]. Indeed, increasing SR Ca load seems able to overcome the refractoriness following a twitch (Section 5.4) and can be responsible for spontaneous SR Ca release propagating as Ca waves [238] which can produce DADs and aftercontractions. Furthermore, when the SR Ca content falls below a certain threshold (~50% of normal), SR Ca release cannot be activated by the normal trigger any longer [291]. Thus these intrinsic factors regulating SR Ca release interact in a dynamic manner in the intact cardiac myocyte.

FKBP, CaMKII, PKA and Mg can all be expected to modulate SR Ca release in intact cells as well as isolated systems. FK-506 enhances resting SR Ca release (as Ca sparks) as well as fractional SR Ca release during E–C coupling in intact cells [292,293]. This is consistent with bilayer results discussed above (Section 4.5) and a role for FKBP in optimizing cardiac E–C coupling. CaMKII produced complex effects in bilayers which are difficult to extrapolate directly to the intact cellular environment. However, in voltage clamped ventricular myocytes Li et al. [294] showed that blockade of CaMKII strongly depressed the SR Ca release for a given $I_{Ca}$ trigger and SR Ca load. Dialysis of ventricular myocytes with protein phosphatases

also depresses E–C coupling in a similar manner [295]. Thus, CaMKII can increase the efficacy of E–C coupling.

There are alterations in E–C coupling during ventricular hypertrophy and heart failure in the rat, even without changed $I_{Ca}$, SR Ca load or RyR2 density [296,297]. However, the SR Ca release activated by a given $I_{Ca}$ and SR Ca was depressed. This might be due to a progressive change as hypertrophy proceeds to failure. Cells from rats in more overt heart failure showed this depressed E–C coupling under normal conditions [296]. On the other hand, no E–C coupling depression was seen in pressure overload hypertrophy with less overt signs of failure, unless the trigger $I_{Ca}$ was reduced to a submaximal level [297].

## 6. Feedback between SR Ca release and ionic currents

Ionic currents during the action potential can trigger SR Ca release, but there is also feedback of SR Ca release onto sarcolemmal ionic currents. These can contribute to alterations of the action potential waveform and can also be arrhythmogenic.

### *6.1. SR Ca release causes $I_{Ca}$ inactivation, inward $I_{Na/Ca}$ and $I_{Cl(Ca)}$*

While Ca entry via $I_{Ca,L}$ contributes significantly to $I_{Ca}$ inactivation in cardiac muscle (see Section 2.2), the Ca released from the SR also contributes quite substantially to this [22,33,250,251,298]. Indeed, SR Ca release causes integrated Ca influx during the action potential to be reduced by ~50% compared to when the SR is empty. Thus, if for some reason there is less SR Ca release in the heart, there would be a consequentially higher Ca influx to support contraction and a greater total amount of inward $I_{Ca}$. This would tend to prolong action potential duration and increase SR Ca loading, potentially normalizing SR Ca release [299].

SR Ca release also activates the inward Na/Ca exchange current (three Na enter for each divalent Ca extruded; [200]). The Ca efflux produced is largely responsible for extruding the amount of Ca which entered via $I_{Ca}$ during the action potential. This inward Na/Ca exchange current will tend to prolong the action potential plateau and will work against repolarization. When spontaneous SR Ca release occurs, this is also a major contributor to the arrhythmogenic transient inward current ($I_{ti}$) that is responsible for delayed afterdepolarizations (DADs; [300]).

There is also a Ca-activated Cl current in ventricular myocytes [301–304]. This probably contributes significantly to early repolarization during the action potential. It may also contribute to $I_{ti}$ in response to spontaneous SR Ca release. However, since the reversal potential for Cl is typically −50 to −60 mV the extent of depolarization that this current can produce is somewhat limited. A Ca-activated nonspecific cation current has also been described in cardiac myocytes [305]. This could also contribute to $I_{ti}$, but the relative contribution with respect to Na/Ca exchange and Ca-activated Cl current is not known. Thus there are several currents sensitive to the intracellular Ca transient and this provides electrophysiological feedback between Ca release by the RyR2 in the SR membrane.

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