The Sarcoplasmic Reticulum and the Evolution of the Vertebrate Heart

The sarcoplasmic reticulum (SR) is crucial for contraction and relaxation of the mammalian cardiomyocyte, but its role in other vertebrate classes is equivocal. Recent evidence suggests differences in SR function across species may have an underlying structural basis. Here, we discuss how SR recruitment relates to the structural organization of the cardiomyocyte to provide new insight into the evolution of cardiac design and function in vertebrates.

Contraction and relaxation of cardiac muscle is initiated by the rise and fall in intracellular Ca\(^{2+}\). Ca\(^{2+}\) can enter the cardiac cell (cardiomyocyte) across the sarcolemmal membrane or it can be released from intracellular stores. In the mammalian myocardium, the majority of Ca\(^{2+}\) that activates contraction is released from the intracellular stores of the sarcoplasmic reticulum (SR) (13). The SR is a specialized endoplasmic reticulum that surrounds the myofilaments and mitochondria and acts as a rapid Ca\(^{2+}\)-storage and Ca\(^{2+}\)-release site. Changes in the rate of SR Ca\(^{2+}\) cycling and the magnitude of SR Ca\(^{2+}\) release are key mechanisms by which the rate and force of cardiac contraction are altered. The indispensable role of the SR in mammalian species can be appreciated by the failure or reduction in cardiomyocyte contraction and relaxation after SR Ca\(^{2+}\) cycling is inhibited (13).

The SR is present in all vertebrate classes studied to date including fishes, amphibians, reptiles, birds, and mammals, but the contribution of SR Ca\(^{2+}\) release to cardiac contraction varies considerably among species (47). Briefly, SR Ca\(^{2+}\) cycling is more prevalent in atrial vs. ventricular tissue, active vs. sedentary species, adults vs. neonates, and endothermic species (those whose body temperature is dependent on internally generated metabolic heat: mammals and birds) vs. ectothermic species (those whose body temperature is dependent on external sources of heat: fishes, amphibians, and reptiles). The data supporting these general observations has been discussed in detail in earlier reviews (47, 144, 156) and can be appreciated by the work compiled in FIGURES 1 AND 2. An obvious interpretation is that the SR is critical for the strong and/or fast contractions characteristic of adult endothermic vertebrate hearts and is not routinely required for the slower, less powerful contractions of most ectothermic vertebrate hearts.

All vertebrates have the capacity to store sizeable quantities of Ca\(^{2+}\) in their SR, but, paradoxically, ectothermic species, whose contractility is less dependent on SR Ca\(^{2+}\) cycling, have greater steady-state and maximal SR Ca\(^{2+}\) contents than their mammalian counterparts (47, 48, 60) (see FIGURE 4). If all vertebrates can store significant quantities of Ca\(^{2+}\) in the SR, why is it released in some species and not others? In other words, what factors limit SR Ca\(^{2+}\) release in ectotherms and what is the significance of these stores if they are not being utilized for excitation-contraction (E-C) coupling? Answers to these questions could provide insight into the evolution of cardiac design and function. In this review, we build on the excellent and extensive work of others (18, 41, 45, 156) to suggest that SR spatial organization and SR regulation in ectotherms limits both the magnitude and the rate of Ca\(^{2+}\) cycling. We discuss correlations between SR organization, SR Ca\(^{2+}\) cycling, heart rate, and power generation across vertebrates. We begin with a brief overview of the role of the SR in mammalian ventricular cardiomyocyte E-C coupling. We discuss the organizational and regulatory prerequisites for SR Ca\(^{2+}\) release in mammals and relate this to what is known for ectothermic cardiomyocytes. We include a limited discussion of mammalian atrial, neonatal, and avian cardiomyocytes, which appear to represent a structural and functional intermediate between ectotherm and mammalian ventricular myocytes. Last, we discuss the potential significance of the large but “static” SR Ca\(^{2+}\) stores in ectotherms.

The Role of the SR in Mammalian Ventricular E-C Coupling

Functional Role of the SR

In the mammalian ventricle, the SR is central to E-C coupling. E-C coupling begins with an action potential (AP), which depolarizes the sarcolemmal membrane and drives extracellular Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels [LTC\(_{\text{Ca}}\) or dihydropyridine receptors (DHPR\(_{\text{L}}\))] (DHPRs)]. This small Ca\(^{2+}\) entry activates clusters of SR Ca\(^{2+}\) release channels [ryanodine receptors (RyR\(_{\text{S}}\)], causing them to open and release a larger amount of Ca\(^{2+}\) from the SR. This
process is called Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) and is shown diagrammatically in **FIGURE 2** (red lines). The close apposition of LTCCs and RyRs is referred to as the couplon (137) and enables local control of Ca$^{2+}$ release from the SR (136). CICR is assisted by invaginations of the surface sarcolemma, the transverse (t)-tubular system, which transmits excitation deep within the cell (see Table 1). In this way, the t-tubular system activates CICR nearly simultaneously throughout the large volume of the mammalian ventricular myocyte and ensures strong spatially and temporally coordinated contractions. Recent imaging in both large (sheep) and small (rat) mammalian ventricular myocytes show adjacent t-tubules are linked via the SR membrane, ensuring synchronicity of ventricular E-C coupling (112). Relaxation of the mammalian ventricular myocyte occurs when Ca$^{2+}$ is pumped back into the SR via the SR Ca$^{2+}$ ATPase (SERCA) or transported across the sarcolemma by the Na$^+$/Ca$^{2+}$ exchanger (NCX); the sarcomemal Ca$^{2+}$ pump plays a minor role (40). Thus, in the mammalian ventricular myocyte, the SR underpins the large and rapid changes in intracellular Ca$^{2+}$ that facilitates the strong and fast heart beat of mammals.

**Relationship Between SR Ultrastructure and CICR**

Ultrastructural organization of the sarcolemmal and SR membrane systems underlies both the requirement for, and efficacy of, CICR (**FIGURE 3**). Mammalian SR can be grouped into at least three functional and structural elements. Longitudinal/network or “free” SR (ISR) makes up the majority of the SR membrane and is where Ca$^{2+}$ is pumped into the SR via SERCA. The other two elements are the junctional SR (jSR) and the corbular (cSR) (also referred to as nonjunctional SR because it is not associated with t-tubules or surface sarcolemma). Both jSR and cSR contain functional clusters of RyRs or “Ca$^{2+}$ release units” (CRUs), defined here as clusters of RyRs as designated in Ref. 44. CRUs in jSR are in close proximity to LTCCs, forming couplings at the surface sarcolemmal (peripheral couplings) or along t-tubules (dyadic couplings). Here, the CRUs are activated by Ca$^{2+}$ influx from LTCCs, causing Ca$^{2+}$ sparks, which summate spatially and temporally to produce global Ca$^{2+}$ signals (30, 24). cSR is present in ventricular myocytes but it is less prominent than in atrial myocytes. cSR can act as a secondary Ca$^{2+}$ amplification system in response to Ca$^{2+}$ diffusion from jSR release events in both cell types (see **FIGURE 3**). Importantly, cSR CRUs can be “silent” until activated, for instance through sympathetic hormones, to coordinate and augment SR Ca$^{2+}$ release throughout the myocyte (92). Differences in the relative proportion of peripheral, dyadic, and corbular CRUs occur with developmental stage, tissue type, cell size, and adult body size. For example, adult sheep ventricular cardiac myocytes have extensive peripheral couplings, whereas adult rat ventricular myocytes possess extensive dyadic couplings (112); such differences have been correlated with myocyte size (77, 117) and maximum heart rate (111). The number of RyRs in mammalian CRUs varies from 14 to 100 tetrameres (a functional RyR is a homeotetramere) (6, 27, 44). The physical distance between CRUs also varies from between 50 nm (6) and 750 nm (27, 44) (see Table 2). Both of these factors influence spatial and temporal Ca$^{2+}$ signals and the prevalence of propagative CICR between CRUs. Because the distance between CRUs is small in mammalian ventricular myocytes, activation of CICR at the cell periphery, or throughout the t-tubular system, initiates opening of neighboring CRUs (2, 7), causing propagated CICR and the possibility of regenerative Ca$^{2+}$ waves (16, 29).

**FIGURE 3. The relative contribution of Ca$^{2+}$ release**

The relative contribution of Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) vs. sarcolemmal transport (LTCC and NCX) in the generation of contractile force in isolated cardiac muscle preparations from a number of endothermic (red bars) and ectothermic (blue bars) vertebrates. These data are compiled from early studies where ryanodine (often in combination with thapsigargin, an inhibitor of SERCA) was used to inhibit force generation in isometric muscle strip preparations. Under these conditions, sarcolemmal Ca$^{2+}$ entry may overcompensate, thus SR contribution to force may be underestimated for some species. Additionally, stimulation frequency and temperature (both acclimation and experimental) influence the effect of ryanodine on contractile force, which complicates interpretation. Nevertheless, SR dependence across vertebrates correlates well with ultrastructural differences (Table 1 and **FIGURE 3**), and more recent functional outputs of SR function. v, Ventricular muscle; a, atrial muscle. All preparations were paced at 0.5 Hz, except for human and chick, which were paced at 1.0 Hz. Rat (12, 13), rabbit (15, 58), frog (12), and all reptiles (50) were tested at 30°C. Human (115) and chick (142) were tested at 35–36°C. Trout were tested at 22°C (124), and yellowfin tuna at 25°C (125).
Regulation of CICR

The efficacy of CICR is modulated not only by the structural organization of CRUs but by their regulation through accessory proteins, numerous small molecules, kinases, and phosphorylation/redox state (3, 105). Ca^{2+} is the primary ligand, and RyR opening is tightly regulated by cytosolic free Ca^{2+} concentration. Thus RyR Ca^{2+} sensitivity is a critical factor in determining the gain of E-C coupling (119). The Ca^{2+} sensitivity of RyRs is modulated by PKA, FK506-binding proteins (FKBP12 and FKBP12.6), calmodulin (CaM), CaMKII, and sorcin acting via the cytoplasmic domains to alter RyR opening. The effect of PKA phosphorylation on mammalian RyRs can vary, but most suggest increased RyR2 activity (95). FKBP12 and FKBP12.6 also affect channel opening; FKBP12 is thought to activate the channel, whereas FKBP12.6 is thought to inhibit the channel (46, 55, 94, 95, 147). RyR modulation from the SR lumen is achieved via triadin and junctin, which form complexes with calsequestrin (14, 56, 57, 86). Calsequestrin is the most abundant Ca^{2+} binding protein in the SR (9, 160). CSQ2 (the mammalian cardiac isoform) binds Ca^{2+} with a high capacity (~60–80 mol Ca^{2+}/mol CSQ2) and a moderate affinity [dissociation constant (K_d) of ~1 mM] (109), thereby buffering SR Ca^{2+}. In addition to setting SR Ca^{2+} content, CSQ2 directly regulates RyR open probability (28). Recently, a luminal Ca^{2+}-sensing mechanism, independent of CASQ and dependent on residues in the proposed gate of the receptor, has been reported in mice and controls RyR opening by luminal Ca^{2+} (28a). Although this residue is highly conserved across RyR isoforms, its involvement has not been investigated in ectotherm hearts. The large SR Ca^{2+} content and reluctance of spontaneous SR Ca^{2+} release in ectotherm myocytes (discussed in the next section) may suggest this mechanism, if present, is differentially regulated.

The Role of the SR in Ectotherm Cardiomyocytes

Functional Role of the SR

The functional role of the SR is heterogeneous in ectotherms and has been reviewed recently (47). In the majority of ectothermic species, atrial and ventricular myocyte contractions do not require Ca^{2+} release from the SR. Contraction is supported exclusively by transsarcolemmal Ca^{2+} flux through LTCCs (144, 151, 152), and in some cases with contribution from reverse-mode NCX (62, 153). Ca^{2+} influx through LTCCs (I_{Ca}) has been estimated at 50–80 μM cytosol in fish myocytes (expressed per myofibril volume, which is ~40–55% of cell volume for carp and trout) and can initiate a full contraction (156). Sarcolemmal Ca^{2+} entry in mammalian cardiac myocytes is lower (<10 μM cytosol expressed per non-mitochondrial volume, 65% of cell volume) (113) and...
alone is insufficient for the activation of normal physiological contractions (156, 158). Nevertheless, there are ectothermic animals that rely more strongly on SR Ca\textsuperscript{2+} during E-C coupling. For the most part, these are species with elevated cardiac function or with the phenotypic capacity to elevate cardiac function when required. For example, varanid lizards are highly athletic reptiles with a functionally divided ventricle capable of developing high systemic blood pressures compared with other squamates (23). SR Ca\textsuperscript{2+} release in this lizard contributes to both contractile force (50) and the cellular Ca\textsuperscript{2+} transient (49). Similarly, bluefin tunas are apex oceanic predators with the highest heart rates and cardiac power output among fishes (42), and this species relies strongly on SR Ca\textsuperscript{2+} cycling during E-C coupling (123). These generalities in ectotherms correlate with data from endotherms where high activity and metabolism are coupled to high heart rates and SR involvement in E-C coupling (see FIGURE 1; Ref. 13). Thus the importance of SR Ca\textsuperscript{2+} cycling in the hearts of active species appears to be a general principle that can be applied to all vertebrates. SR Ca\textsuperscript{2+} cycling may also be recruited in some stenotherms.

### Table 1. Comparative morphometric data for vertebrate ventricular myocytes

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<th>Lamprey\textsuperscript{1}</th>
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<th>Frog</th>
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<td>No\textsuperscript{i}</td>
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Data are means; SE (when known) has been left out for clarity. \textsuperscript{a}Ref. 121; \textsuperscript{b}Ref. 152; \textsuperscript{c}Ref. 50; \textsuperscript{d}Ref. 50; \textsuperscript{e}Ref. 75; \textsuperscript{f}Ref. 54; \textsuperscript{g}Ref. 8; \textsuperscript{h}derived from Ref. 54, assuming an elliptical cross-sectional area; \textsuperscript{i}Ref. 131; \textsuperscript{j}Ref. 69 for finch and humming bird (not turkey); \textsuperscript{k}Ref. 22; \textsuperscript{l}Ref. 155.

**FIGURE 3. Ultrastructural organization of the SR**

Ultrastructural organization of the SR in a mammalian ventricular myocyte (A), an ectotherm atrial and/or ventricular myocyte (B), and mammalian atrial and/or avian myocytes (note that atrial myocytes from large mammals contain t-tubules; see Ref. 39) (C). Junctional SR (jSR) contains ryanodine receptors (RyRs), which cluster to form calcium release units (CRUs) that are brought in close proximity to L-type Ca\textsuperscript{2+} channels (LTCC), forming couplings (D), at the surface sarcolemma (peripheral couplings) or along t-tubules (dyadic couplings). CRUs can also exist in corbular or nonjunctional SR (i.e., SR not associated with t-tubules or surface sarcolemma). The CRU is shown as a single RyR for clarity but between 14 and 100 RyRs cluster together to form a CRU and are juxtaposed to many LTCC in the SL membrane (see text for detail). RyRs interact with several proteins, including the Ca\textsuperscript{2+} binding protein calsequestrin (CSQ) and two anchoring proteins, triadin and junctin. Note that differences exist between cell types in the relative proportion of each type of coupling, the distance between couplings, and the overall quantity of SR membrane. Figure created from data in Refs. 18 and 111.
mal fish [like burbot (126, 145)], which inhabit cold environments, and in eurythermal fish [like salmonids (128, 129) and tuna (123)], which undergo seasonal cold acclimation (i.e., chronic cold exposure in excess of 1 mo). Indeed, chronic cooling in both perch (21) and tuna (123) leads to a proliferation of total SR membrane. Increased SR function in the cold ectotherm heart is thought to augment sarcolemmal Ca\(^{2+}/\text{H}^+\) to overcome cold-related reductions in the Ca\(^{2+}/\text{H}^+\) sensitivity of myofilaments (78) and sarcolemmal Ca\(^{2+}\) influx (127). Similar strategies have been described in cold-hibernating mammals (11, 88).

Interestingly, regardless of the relative contribution of the SR to E-C coupling, all ectotherms studied to date possess sizable amounts of Ca\(^{2+}\) in the SR (FIGURE 4). Several possibilities exist, which may explain the limited SR Ca\(^{2+}\) release relative to the high Ca\(^{2+}\) storage capacity of ectotherm cardiomyocytes, including SR ultrastructure [density of RyRs, their organization into CRUs, the proximity of CRUs to LTCCs (i.e., formation of couplons)], and SR regulation (RyR sensitivity to opening).

**Relationship Between SR Ultrastructure and CICR**

The efficacy of transsarcolemmal Ca\(^{2+}\) flux is aided by the extended length-to-width ratio and high surface area-to-volume ratio of ectotherm myocytes (Table 1). This elongated or spindle-shaped morphology compensates for the lack of the transverse tubular system and is coincident with peripherally located myofilaments (120, 151, 152). For the majority of sedentary and/or sluggish species of ectotherms, this myocyte arrangement allows for normal contractions to occur without the SR. The lack of a functional role for the SR is also reflected by the low density of RyRs in ectothermic myocytes compared with endotherms. A series of \(^{3}H\)ryanodine-binding studies by Vornanen and colleagues has shown RyR density is considerably lower in carp (19%; Ref. 31), rainbow trout (28%; Ref. 143), burbot (65%; Ref. 154), and lamprey (68%; Ref. 155) when normalized to rat (100%; Ref. 154). A similar disparity in RyR density between species has been reported from protein expression studies (17, 20, 31, 32, 131) and broadly corresponds to the relative contribution of SR Ca\(^{2+}\) cycling during E-C coupling within each species. For example, frog ventricle shows no evidence of SR Ca\(^{2+}\) cycling during E-C coupling (79), which is confirmed by the absence of RyRs at peripheral couplings between the sarcolemmal and SR membranes (146). Similarly, carp demonstrate sparse immunohistochemical staining of RyRs (32), and functional studies show no effect of SR inhibition on contractility (150). The cold stenothermic burbot, on the other hand, shows strong SR depen-

<table>
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Values are means (SE, when known, has been left out for clarity). Data are compiled from diverse sources often employing different experimental techniques (e.g., EM vs. immunofluorescence), which complicate some comparisons. When temperature is given, it indicates acclimation temperature. CRU, calcium release unit.
dence of contractile force and structural evidence of peripheral couplings (145).

Clearly, the relatively low number of RyRs will be a contributing factor in the weak CICR typical of many ectotherms. At present, the number of RyRs that make up a CRU is not resolved for any ectothermic species. However, the spatial relationships between CRUs and the temporal/spatial properties of cellular Ca\(^{2+}\) cycling during E-C coupling have been measured. EM studies from the green lizard (Anolis carolinensis) ventricle show widely separated peripheral CRUs and a lack of cSR (111), but functional work is lacking for this species. EM studies confirmed the presence of peripheral couplings in the bluefin tuna, but the percentage of total SR that forms peripheral couplings was very low (0.98\% and 0.40\% in the atria and ventricle, respectively), and there was no evidence of cSR (38). Functional studies in both tuna (123) and trout (131, 89) show CICR occurs at the myocyte periphery but with limited propagated activation of neighboring peripheral CRUs or central CRUs under normal physiological conditions. In other words, the Ca\(^{2+}\) wave appears to be carried via diffusion between CRUs along the periphery and into the myocyte. For example, trout ventricular myocytes exhibit a small U-shaped delay (~6 ms) in the peak of the Ca\(^{2+}\) transient between the periphery and the center under routine conditions (131). However, increased Ca\(^{2+}\) influx at the periphery after application of BayK8644 (an agonist that increases the open probability of LTCCs) increased the rate and amplitude of centripetal Ca\(^{2+}\) transients, suggesting fish myocytes may possess “silent” CRUs that require inotropic stimuli to activate (131). Structural evidence is lacking, but support for this idea comes from recent work showing both \(\beta\)-adrenergic stimulation (500 nM isoprenaline) and low (~500 \(\mu\)M) levels of caffeine (which sensitizes the RyR to cytosolic Ca\(^{2+}\) and increases RyR opening) result in propagative CICR in trout ventricular myocytes (35) and in zebrafish ventricular myocytes (20). These functional studies strongly suggest that recruitment of CICR can occur in certain ectotherm myocytes. However, the spatial relationships between CRUs and the temporal/spatial properties of cellular Ca\(^{2+}\) cycling have not been measured together in the same ectothermic species (see Table 2). This information would help to clarify the limitations SR ultrastructure impose on ectothermic CICR.

### Regulation of CICR

Ryanodine receptors (RyRs) are very large (~2.2 MDa) homotetrameric assemblies with a large cytoplasmic head (originally described as “feet”; Ref. 44) that protrudes from the SR membrane into the cytosol, and a transmembrane stalk that forms the channel pore (140). Tissues can express multiple isoforms; however, RyR2 predominates in mammalian cardiac muscle, and a homologous RyR isoform has been identified in non-mammalian hearts (although the entire amino acid sequence of the non-mammalian cardiac RyR has not been completely determined). The Ca\(^{2+}\) sensitivity of RyR opening is an important regulator of CICR. In

![Figure 4](http://physiologyonline.physiology.org)
ectotherm cardiomyocytes, RyR Ca$^{2+}$ sensitivity correlates with the contribution of SR Ca$^{2+}$ to force production. Using [H$^3$]ryanodine binding, Vornanen (154) showed that the Ca$^{2+}$ sensitivity of the rat RyR ($K_d$ of $\sim 0.16$ $\mu$M) is similar to the cold stenothermic burbot ($\sim 0.19$ $\mu$M) but considerably greater than that of the lamprey ($\sim 0.35$ $\mu$M), trout ($\sim 0.83$ $\mu$M), and carp ($\sim 1.10$ $\mu$M). The reduced Ca$^{2+}$ sensitivity of RyRs means that a higher systolic Ca$^{2+}$ is required to open the ectothermic RyR, even if the structural organization for CICR exists. Clearly, the low Ca$^{2+}$ sensitivity of the RyR, coupled with a lower RyR density, is a major factor underlying low levels of CICR in most ectotherm hearts (154). The low RyR Ca$^{2+}$ sensitivity may also explain the lack of spontaneous SR Ca$^{2+}$ release events (i.e., Ca$^{2+}$ sparks) in ectothermic myocytes. Ca$^{2+}$ sparks are tiny Ca$^{2+}$ signals, which arise from the activation of a CRU (30), that summate in space and time to form the systolic Ca$^{2+}$ transient. Ca$^{2+}$ sparks are absent in quiescent ectotherm myocytes [trout ventricle, (131); zebrafish ventricle (20)] or in low abundance [trout atrium (89)] despite confirmation of large SR Ca$^{2+}$ stores by rapid application of high levels of caffeine (10 mM, which opens RyRs, causing Ca$^{2+}$ release). Moreover, strategies known to enhance spark frequency in mammals (>6 mM extracellular Ca$^{2+}$ or elevated intracellular free Ca$^{2+}$) fail to elicit Ca$^{2+}$ sparks or Ca$^{2+}$ waves in fish myocytes (20, 131). However, application of low levels of caffeine (200 $\mu$M to 1 mM) increased spark frequency in zebrafish myocytes, whereas higher concentrations (5 mM) synchronized SR Ca$^{2+}$ release (20). This suggests zebrafish RyRs are organized into CRUs that exhibit low Ca$^{2+}$ sensitivity with limited propagative coupling under routine conditions but can be recruited (i.e., via caffeine) to participate in E-C coupling (20). The zebrafish RyR also responds to PKA activation with a twofold increase in phosphorylation (20). These authors found that RyR phosphorylation increased SR Ca$^{2+}$ release, spark frequency, and Ca$^{2+}$ transient amplitude in half of the zebrafish cardiomyocytes studied. The other half of the myocytes responded to PKA activation via SERCA-dependent changes in SR Ca$^{2+}$ content (discussed below). Thus activation of RyRs after sympathetic stimulation is a mechanism through which ectotherms can recruit SR Ca$^{2+}$ cycling. Indeed, we have already discussed functional studies that show CICR during adrenergic stimulation in trout heart (35), and it seems highly probable that adrenergic activation of I$_{ca}$ and RyRs converge to coordinate and amplify Ca$^{2+}$ release.

Modulators known to affect RyR opening in mammals may increase the efficacy of CICR in ectotherms. FKB12 expression was recently investigated in fish heart [rainbow trout, crucian carp, and burbot (82)] and found to be greater in atrial compared with ventricular tissue in all three species studied and could be enhanced by cold acclimation in trout. If FKB12 sensitizes fish cardiac RyRs to cytosolic Ca$^{2+}$, then this finding may correlate with the greater SR involvement in E-C coupling apparent in atrial vs. ventricular tissue and after chronic cold exposure. Similar studies are lacking for other non-mammalian vertebrates; however, the association of FKBP12 with cardiac RyRs is evolutionally conserved, and both isoforms are found in most species (human, pig, rat, mouse, chicken, frog, and fish), but abundance varies greatly (70, 147, 161).

Regulation may also occur on the luminal side of the SR. As discussed earlier, in mammalian cardiomyocytes, RyR opening is regulated by luminal Ca$^{2+}$ via specific residues in the channel pore that provide a Ca$^{2+}$-sensing mechanism (28a). This is in addition to the regulation of RyR opening that is regulated by CSQ2 (10, 56). CSQ has been identified in the SR of fish (66, 80), amphibians (98), reptiles (111), aves (71, 111), and mammals (106). It has been cloned and sequenced in a range of vertebrates and appears to be relatively well conserved, suggesting a fundamental biological role (9, 66, 80). It is possible that CSQ inhibits RyRs to a greater extent in ectothermic myocytes and that a higher concentration of luminal Ca$^{2+}$ is necessary to relieve the inhibition. Although this possibility has not been directly measured, Korajoki and Vornanen (80) have examined the expression of the ectothermic isoform of CSQ in warm and cold-acclimated rainbow trout atrial and ventricular tissue. Based on known differences in SR Ca$^{2+}$ function (2), Korajoki and Vornanen (80) hypothesized that expression would be elevated in the atrium compared with the ventricle and would be increased after cold acclimation in both chambers. However, no differences were detected, suggesting CSQ is not involved in chamber or acclimation-specific enhanced SR function in trout. In agreement with this result, mammalian atrial muscle, which is known to be more SR dependent, does not express higher CSQ2 compared with ventricular muscle (157) and CSQ2 expression in the heart remains unchanged in mammalian cardiac pathologies that are known to alter RyR function (80, 87). Although these studies indicate the amount of CSQ does not correlate to SR function in vertebrates, the possibility of differential regulation (e.g., through phosphorylation), differential interaction with RyRs or other luminal proteins, and differential CSQ isoform expression (see Ref. 80) cannot be excluded. Indeed, altered CSQ isoform expression has been documented in cold-hibernating ground squirrels (100).
Collectively, the data from the limited number of ectotherms studied suggest two major E-C coupling schema: 1) that typified by frog and carp where there is no (or very limited) CICR during contraction and 2) that typified by ectotherms with enhanced cardiac function like salmonids where CICR occurs at CRUs in the cell periphery followed by Ca\(^{2+}\) diffusion through the cytosol without the requirement for propagative CICR. This peripheral initiation and centripetal diffusion appears to be the basal arrangement for cardiac E-C coupling among vertebrates (18, 47, 131) and is sufficient to drive the relatively slow, low-pressure hearts of most ectothermic species. Mobilizing silent CRUs through adrenergic stimulation, chronic cooling, or pharmacological intervention provides a mechanism to increase the rate and strength of ectotherm cardiac contractions in species with elevated cardiac function.

The Mechanism and Significance of the Large SR Ca\(^{2+}\) Stores in Ectotherms

Because of the limited capacity to release SR Ca\(^{2+}\), one may expect that ectotherm SR would possess a modest Ca\(^{2+}\) content. However, quite to the contrary, the steady-state and maximal SR Ca\(^{2+}\) content of ectothermic vertebrates are considerably larger than those reported in mammalian cardiomyocytes (see FIGURE 4 and Ref. 47). This difference exists despite the fact that ectothermic SR density per cell volume (ranging between 0.38 and 6.1%) is generally less than that found in mammals (ranging between 3.5 and 12.3%) (47). The smaller SR Ca\(^{2+}\) content in mammalian myocytes may be partly explained by leakier RyRs, which limit the maximum amount of Ca\(^{2+}\) that can be sequestered at any given time. Indeed, spontaneous Ca\(^{2+}\) sparks are far more common in mammals than in ectotherms (130). Also, RyRs from fish do not show spontaneous opening in response to cooling; in fact, passive leak from trout SR vesicles decline at cold temperatures (61). However, when the RyR inhibitor tetracaine is applied to rat ventricular myocytes to abolish Ca\(^{2+}\) leak, maximum SR Ca\(^{2+}\) content is still at the lower end of those found in ectothermic myocytes (FIGURE 4 and Ref. 107). Thus the extent of RyR leakiness cannot fully explain the large SR Ca\(^{2+}\) contents found in ectotherms. Other possibilities, such as enhanced SR Ca\(^{2+}\) uptake and/or luminal Ca\(^{2+}\) buffering, are discussed below.

SR Ca\(^{2+}\) Uptake

SR Ca\(^{2+}\) uptake is achieved exclusively by SERCA (the cardiac isoform is SERCA2), which consumes ATP to actively pump Ca\(^{2+}\) into the SR lumen. The high Ca\(^{2+}\) binding efficiency of SERCA2 allows for rapid SR Ca\(^{2+}\) uptake and largely determines the rate of mammalian myocyte relaxation (4). Several isoforms of SERCA2 exist within the mammalian heart (SERCA2a, b, and c), which differ in their enzymatic turnover rate and affinity for Ca\(^{2+}\) (4), but SERCA2a is the dominant cardiac isoform. Few studies have analyzed the total number and sequence of SERCA2-encoding genes in non-mammalian vertebrates. Interestingly, as genome duplication occurred within the fish lineage (68), teleosts may possess paralogs of the SERCA2 gene, leading to greater diversification of SERCA2 isoforms. Indeed, melting curves of rainbow trout SERCA2 transcripts reveals three closely clustered peaks, suggesting the trout heart may possess various isoforms of SERCA2 (81), but the functional significance of these isoforms has not been clarified.

SERCA2 expression and activity is dependent on numerous factors, including region of the heart, developmental stage, species, environmental conditions, and pathological state (1, 81, 108). Nevertheless, SERCA2 expression and activity correlates well with the role of SR Ca\(^{2+}\) cycling during E-C coupling in vertebrates (see Ref. 47), showing a similar pattern to that discussed earlier for RyR density and structural organization. In mammals, SERCA protein/mRNA expression and SERCA activity are higher in adult vs. neonatal (5, 43, 59), larger vs. smaller animals (101, 139, 157), and atrial vs. ventricular cardiomyocytes (157). These differences correlate with SR Ca\(^{2+}\) content, which is greater in smaller animals and in tissues that are capable of generating high frequencies of contraction (139, 157). Mammalian knockout mouse models show reduced SERCA2 expression leads to lower SR Ca\(^{2+}\) content, whereas overexpression increases it (138, 149). Thus, if ectothermic animals have a relatively high expression of SERCA2, this may help to explain their large SR Ca\(^{2+}\) contents. Although direct comparison of ectothermic vs. endothermic SERCA2 expression has not been made, data regarding SERCA2 activity has shown that this theory is unlikely. In rat and rabbit ventricle, SERCA2 activity is four to sixfold higher at room temperature than a range of active/sedentary teleosts (25, 84, 85). When corrected for routine body temperature, Vornanen and colleagues (1) show relative SR Ca\(^{2+}\) uptake rates were 100, 26, 19, 18, 11, and 2% for adult rat, newborn rat, cold-acclimated trout, warm-acclimated trout, warm-acclimated carp, and cold-acclimated carp, respectively. These findings indicate that SR Ca\(^{2+}\) uptake is slower in fish compared with mammalian hearts but that activity can be regulated, in this case by temperature. Indeed, mammalian SERCA2 is regulated by a variety of molecules and hormones, including thyroid hor-
mone, Ca\(^{2+}\) concentration, ATP concentration, phospholamban (PLB), sarcoplgin, and various pathological states (110). It is possible that some of these factors may contribute to the superior ectothermic SR Ca\(^{2+}\) storage capacity. Although much is known about these regulatory processes in mammals (see reviews in Refs. 83, 110, 148, 160), similar information for birds and ectothermic vertebrates is scarce, but recent advances have been made with regard to PLB regulation of SERCA2.

PLB is a regulatory phosphoprotein that is localized to the longitudinal “free” regions of the cardiac SR (72). In the dephosphorylated state, PLB acts as an inhibitor (brake) of SERCA2, whereas phosphorylation at Ser-16 and Thr-17 releases this inhibition and promotes Ca\(^{2+}\) uptake into the SR by increasing the Ca\(^{2+}\) affinity of SERCA2 (76). Therefore, SR Ca\(^{2+}\) content can be regulated by PLB by either altering PLB expression or phosphorylation status. Since PLB is an inhibitor of SERCA2, reduced expression of this protein is expected to increase SR Ca\(^{2+}\) content. Indeed, PLB knockout mice have a greater SR Ca\(^{2+}\) content (7, 102, 133), and the ratio of PLB/SERCA2 expression has been correlated with SR function in a number of mammals (90). Thus PLB expression may provide a mechanism to enhance SR Ca\(^{2+}\) content in the ectothermic heart. The presence of PLB has been confirmed in a wide range of vertebrates, and its molecular structure is well conserved. For example, the mammalian PLB shares a high sequence homology with chicken (85%), zebrafish (76%), and pufferfish (67%) (26), suggesting that PLB regulation of SERCA2 has early evolutionary origins. Early comparative analysis confirmed chick, frog, and carp PLB content are one to two orders of magnitude lower than that found in the dog, rat, or human heart (159). However, it is not known whether these differences reflect a mechanism for enhanced SR Ca\(^{2+}\) content or the lower SR density characteristic of these ectothermic species.

With respect to phosphorylation status, PLB can be phosphorylated by PKA and/or Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) (96). Phosphorylation most commonly occurs in response to \(\beta\)-adrenergic stimulation, which directly activates PKA and CAMKII by elevating cAMP and intracellular Ca\(^{2+}\), respectively (97). Together, this dual response leads to a potent acceleration of SR Ca\(^{2+}\) uptake and forms the Ca\(^{2+}\)-dependent basis of the adrenergically mediated quickening of cardiac relaxation. An increase in the phosphorylation status of PLB is therefore another possibility to explain the large SR Ca\(^{2+}\) stores of ectotherms. Recent work with zebrafish ventricular myocytes shows that stimulation with PKA increases SR Ca\(^{2+}\) content and leads to greater CICR (20), indicating that the SR is regulated via similar pathways in fish and mammals. Furthermore, Korajoki and Vorranen (81) found higher SERCA2 expression and a lower PLB-to-SERCA2 ratio in the atrium vs. the ventricle of the trout heart, which correlates with faster Ca\(^{2+}\) uptake in trout atrial muscle (1, 2).

The basal level of PLB activation in ectotherms has not been determined, and there is no evidence that PLB is phosphorylated to a different extent in ectotherms vs. endotherms. Furthermore, \(\beta\)-adrenergic stimulation has predominantly inotropic effects in the heart of the carp and frog, whereas acceleration of relaxation in these species is either absent (150) or minor (103, 135) compared with the mammalian adrenergic response. If an adrenergically mediated relaxation is observed in the frog heart, the mechanism appears to involve cAMP-dependent Na\(^{+}\)/Ca\(^{2+}\) exchange (114, 132) and not PLB regulation of SERCA2. Clearly, further studies are necessary to investigate the correlation between PLB expression/phosphorylation in ectotherm cardiomyocytes.

**Ectothermic SR Ca\(^{2+}\) Buffering**

The mammalian heart contains a range of luminal SR Ca\(^{2+}\) buffering molecules including sarcalumenin, calreticulin, calumenin, and CSQ2 (99, 118). Among these, CSQ2 is clearly the most predominant, and recent estimates suggest 75% of Ca\(^{2+}\) taken up by the SR is bound to CSQ2 (93). Similar to transgenic studies with PLB, overexpression of CSQ2 in mammalian cardiomyocytes, either acutely or chronically, leads to an increase in SR Ca\(^{2+}\) content (21, 43), whereas a reduction in the expression of CSQ2 by gene silencing results in a decrease in SR Ca\(^{2+}\) content (43). Coupled with the fact that CSQ2 interacts with RyR directly to inhibit Ca\(^{2+}\) release (discussed earlier), studies from mammals suggest differential expression or regulation of CSQ is a likely candidate to explain the large ectothermic SR Ca\(^{2+}\) contents. However, as discussed above, the limited studies conducted on ectotherm CSQ have not correlated with high SR Ca\(^{2+}\) content (80). However, these authors calculate the total buffering capacity of trout SR to be \(~15.8–21.12\) mM (396–528 \(\mu\)M myocyte volume), which is slightly larger than values from the mammalian heart (80). Clearly, there is a need to further quantify CSQ content in vertebrates to get a better understanding of species specific-buffering capacities in the SR and relate this to overall Ca\(^{2+}\) storage capacity.

**Significance of the Large Ectothermic SR Ca\(^{2+}\) Stores**

Ectothermic vertebrates are frequently challenged with a fluctuating environment that can alter body temperature, oxygen levels, and pH. Variation in these factors in mammalian vertebrates is known to
The Role of the SR in Avian, Neonatal, and Mammalian Atrial Cardiomyocytes

Myocytes from avian and neonatal hearts and the mammalian atria appear to represent a continuum of structural and functional SR intermediates between mammalian ventricular myocytes and ectothermic myocytes. In mammalian atrial and avian myocytes, the SR plays a central role in E-C coupling, and CICR occurs despite the reduced or complete lack of a t-tubular system. Atrial myocytes that possess a t-tubular network are from larger mammals that have larger myocytes (39, 117). Neonatal mammalian myocytes are devoid of t-tubules and have limited SR function (141), but both develop during ontogeny, coincident with myocyte growth (45, 63, 122).

The organization of CRUs and the short diffusional distance for Ca\(^{2+}\) transport in narrow cells allows for strong and fast contractions in avian myocytes and mammalian atrial myocytes. In atrial cells from small mammals, the JSR couples predominantly with the surface sarcolemma, leading to extensive and propagative CICR between neighboring CRUs at the cell periphery (27, 91). The peripheral signal can propagate centripetally to activate the more extensive nonjunctional cSR in these cells and act as a Ca\(^{2+}\) signal accelerator and amplifier in the absence of, or a reduction in, the t-tubular system. However, the peripheral Ca\(^{2+}\) signal does not always spread to more central CRUs because the Ca\(^{2+}\) wave can be attenuated by distance and/or cellular constituents that buffer Ca\(^{2+}\), such as the mitochondria and the Ca\(^{2+}\) pumps of the FSR (65, 91). Activation of nonjunctional cSR RyRs deep inside the mammalian atrial myocyte, which in turn activate the more centrally located myofilaments, can be achieved in much the same way as in ectotherms (18) via pharmacological activators or sympathetic stimulation. When enhanced atrial contraction is required for ventricular filling, such as during exercise or stress, greater SR Ca\(^{2+}\) cycling is recruited and CICR propagates throughout the myocyte.

Birds are endothermic, with hearts rates and cardiac output pressures as high, if not higher, than mammals. However, bird cardiomyocytes are also thin and lack t-tubules (Table 1). [H\(^3\)]ryanodine binding studies in pigeon and finch heart show the density of RyRs and the Ca\(^{2+}\) sensitivity of RyRs are similar to mammals (74). EM studies of the avian myocardium (i.e., finch, hummingbird, and chick) show a high density of peripheral couplings containing CRUs (111, 134). However, the distance between these couplings may be too great for effective lateral transmission of CICR, suggesting peripheral RyR clusters are independently activated by LTCCs (111) in a manner similar to ectotherms. However, unlike ectotherms, and akin to mammalian atrial myocytes, bird hearts also contain large quantities of cSR (69, 111). Importantly, CRUs in the cSR of birds are in closer proximity than those found in the peripheral couplings (Table 2), which may enable propagated CICR release into the cell interior, facilitating strong and rapid contractions (111). Functional studies of temporal and spatial Ca\(^{2+}\) flux, which would support this idea, are still lacking for bird myocardium. However, birds with faster heart rates (finch) have a higher density of RyRs and possess CRUs that are in closer proximity to each other than birds with slower heart rates (chicken) (Table 2; Ref. 111). This reinforces the connection between structural organization of the myocyte, the SR CRUs, and the strength and rate of cardiac contraction across vertebrate classes.

Summary

All vertebrates regulate the strength and rate of cardiac contraction by altering cellular Ca\(^{2+}\) flux in their myocytes. However, the mechanisms by which Ca\(^{2+}\) flux is altered varies with species, developmental stage, and cardiac tissue (41). SR Ca\(^{2+}\) cycling predominates in endothermic animals, and

drastically alter myocyte ionic homeostasis (33, 34, 51). In particular, a rise in intracellular Ca\(^{2+}\) is a hallmark of environmental stress in the mammalian cardiomyocyte, and this leads to Ca\(^{2+}\) overload and the stimulation of cell death pathways (51). Interestingly, SR Ca\(^{2+}\) release is the major mechanism leading to Ca\(^{2+}\) overload, which suggests that ectothermic cardiomyocytes may be inherently protected from Ca\(^{2+}\) overload by possessing an SR whose Ca\(^{2+}\) stores are harder to release. If intracellular Ca\(^{2+}\) levels rise to unusual levels in ectothermic myocytes during environmental perturbation, the SR could act as a Ca\(^{2+}\) buffer with a vast storage capacity. These concepts, although attractive, have not been supported so far by experimental evidence. Early studies using pharmacological inhibition of the ectothermic SR during acidosis or anoxia suggested the SR does not contribute to cardiac function during these insults (52, 104). However, pharmacological inhibition can lead to compensation from other transporting mechanisms, such as the NCX, so these results do not exclude the possibility of a role for the SR. In this regard, future studies should elucidate the role of the SR during environmental stress in ectothermic cardiomyocytes where transport pathways can be isolated more accurately. This would be especially relevant for reptiles, such as freshwater turtles, which endure several months of anoxia and severe acidosis (67).
this facilitates high heart rates and robust cardiac pressure development. Ectotherms have thinner myocytes with higher SA-to-V ratios, which increases the efficacy of sarcolemmal Ca\(^{2+}\) flux in the absence of CSR and a T-tubular system. This arrangement contributes to the lower maximal rates and pressure development of many ectotherm species. CRUs at peripheral couplings are present in all myocytes but vary in size and frequency of distribution. The ability of a myocyte to release Ca\(^{2+}\) stored in the SR is related to the structural organization of CRUs rather than the quantity of Ca\(^{2+}\) in the SR stores. This can be appreciated by the large ectothermic SR Ca\(^{2+}\) stores that are not routinely utilized for E-C coupling. When comparing SR function across vertebrates, these large stores suggests that the basal role for the SR was Ca\(^{2+}\) storage rather than Ca\(^{2+}\) release. The mechanisms underlying the enhanced storage capacity of ectotherms are still being elucidated, but they are likely to involve different SR luminal regulation, enhanced luminal buffering, and RyRs that are less prone to activation. Correlative evidence suggests the structural organization required for CICR evolved in response to the demand for faster heart rates and/or stronger contractile force. This is true even of basal vertebrates, such as the cyclostome lamprey, which is a voracious predator during the parasitic life cycle phase and has the cyclostome lamprey, which is a voracious predator. This is true even of basal vertebrates, such as the cyclostome lamprey, which is a voracious predator. This is true even of basal vertebrates, such as the cyclostome lamprey, which is a voracious predator. This is true even of basal vertebrates, such as the cyclostome lamprey, which is a voracious predator. This is true even of basal vertebrates, such as the cyclostome lamprey, which is a voracious predator.

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