Comparison of sarcoplasmic reticulum calcium content in atrial and ventricular myocytes of three fish species

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Submitted 3 March 2009; accepted in final form 13 August 2009

Haverinen J, Vornanen M. Comparison of sarcoplasmic reticulum calcium content in atrial and ventricular myocytes of three fish species. Am J Physiol Regul Integr Comp Physiol 297: R1180–R1187, 2009. First published August 19, 2009; doi:10.1152/ajpregu.00022.2009.—Ryanodine (Ry) sensitivity of cardiac contraction differs between teleost species, between atrium and ventricle, and according to the thermal history of the fish. The hypothesis that variability in Ry sensitivity of contraction is due to species-specific, chamber-specific, and temperature-related differences in the sarcoplasmic reticulum (SR) Ca\(^{2+}\) content, was tested by comparing steady-state (SS) and maximal (Max) Ca\(^{2+}\) loads of the SR in three teleost fish, rainbow trout (Oncorhynchus mykiss), burbot (Lota lota), and crucian carp (Carassius carassius), which differ in the extent of SR contribution to excitation-contraction coupling. Fish were acclimated at 4°C (cold-acclimation, CA) or 18°C (warm-acclimation, WA), and SR Ca\(^{2+}\) content was released by a rapid application of 10 mM caffeine to single cardiac myocytes; its amount was determined from the Na\(^+/\)Ca\(^{2+}\) exchange current at 18°C. SS Ca\(^{2+}\) load was larger in atrial (304–915 \(\mu\)mol/l) than ventricular (224–540 \(\mu\)mol/l) myocytes in all fish species (\(P < 0.05\)), and the same was true for Max SR Ca\(^{2+}\) content: 550–1,522 \(\mu\)mol/l and 438–840 \(\mu\)mol/l for atrial and ventricular myocytes, respectively (\(P < 0.05\)). Consistent with the hypothesis, acclimation to cold increased Ca\(^{2+}\) load of the cardiac SR in the burbot heart, but contrary to the hypothesis, temperature acclimation did not affect SR Ca\(^{2+}\) content in rainbow trout and crucian carp hearts. Furthermore, there was an inverse relation between SR Ca\(^{2+}\) content and Ry sensitivity of contraction force: the species with the smallest SR Ca\(^{2+}\) content (burbot) is most sensitive to Ry. Collectively, these findings show that SR Ca\(^{2+}\) content of fish cardiac myocytes is several times larger than that in mammalian cardiac SR.

Excitation-contraction coupling; sarcoplasmic reticulum calcium load; caffeine; sodium-calcium exchange current

The process by which electrical excitation of the myocyte leads to cardiac contraction via transient rise in cytosolic Ca\(^{2+}\) concentration is known as excitation-contraction (e-c) coupling (16). It is well established that in the hearts of endothermic vertebrates, a small amount of Ca\(^{2+}\) influx through sarcolemmal (SL) L-type Ca\(^{2+}\) channels and Na\(^+/\)Ca\(^{2+}\) exchange (NCX) triggers a much larger release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) through Ca\(^{2+}\) release channels or ryanodine receptors (RyRs) of the SR membrane (6, 16, 57). In mammals, contribution of SR Ca\(^{2+}\) stores to systolic Ca\(^{2+}\) transient varies from 60 to 95%, mainly according to allometric scaling of the heart rate; small animals with high heart rates and short action potentials (AP) rely more on SR Ca\(^{2+}\) stores than larger species with lower heart rates and longer AP plateaus (6, 53). Species-specific differences in e-c coupling appear in ryanodine (Ry) sensitivity of cardiac contraction, e.g., in rabbit and rat hearts, Ry inhibits force of ventricular contraction by 77% and 95%, respectively (39, 43), which correspond well the estimates of SR Ca\(^{2+}\) transport (70% and 92–95%, respectively) obtained with more direct methods in ventricular myocytes (3, 59).

E-c coupling is a complex sequence of events produced by overlapping activities of several subcellular structures and molecular entities, and it is often difficult to assess the relative importance of SL Ca\(^{2+}\) entry and SR Ca\(^{2+}\) release, especially in ectothermic hearts where SL Ca\(^{2+}\) entry is larger and potentially able to compensate for the SR Ca\(^{2+}\) release, when the latter is blocked with Ry. Although our knowledge on e-c coupling of ectothermic hearts is scanty, it is quite generally accepted that in ectotherms the contribution of SR Ca\(^{2+}\) stores to contractile activation is significantly less than in endothermic hearts (15, 16, 48, 58). In particular, experiments on multicellular cardiac preparations strongly suggest that contribution of SR Ca\(^{2+}\) release to e-c coupling of the fish heart is significantly less than SL Ca\(^{2+}\) entry, because in the majority of fish species examined, the force of cardiac contraction is weakly responsive to Ry. At physiological heart rates and temperatures, Ry inhibition of cardiac contraction varies from 0 to 32% (2, 12, 14, 33, 49), the most notable exceptions being tuna and an eurythermic neotropical teleost, Synbranchus marinus (28, 37), species in which Ry inhibition of force generation ranges from 30 to 53%.

On the other hand, it has been found that in cardiac myocytes of rainbow trout, caffeine-releasable Ca\(^{2+}\) stores are massive, almost an order of magnitude larger than in mammalian cardiac myocytes and approaching to the values of the mammalian skeletal muscle fibers (26, 35, 45). Thus, there is a striking mismatch between massive SR Ca\(^{2+}\) stores and relatively weak effect of Ry on cardiac contraction at least for the trout heart (2, 22, 33, 46), which still lacks a mechanistic explanation and raises several questions. Is this feature special for the trout heart or is it a more general characteristic among the teleost fish? Is there any difference in SR Ca\(^{2+}\) content between atrial and ventricular myocytes or between warm-acclimated (WA) and cold-acclimated (CA) fish? What is the physiological significance of the cardiac SR Ca\(^{2+}\) stores in fish? We try to answer some of these questions by comparing SR Ca\(^{2+}\) content in three fish species, which differ in regard to Ry sensitivity of contraction force. Cardiac contraction in crucian carp (Carassius carassius) is quite resistant to Ry, since only atrial contraction of the WA fish is slightly (6%) inhibited by Ry (49). Rainbow trout (Oncorhynchus mykiss) heart is slightly more sensitive to Ry than crucian carp heart (2), while the burbot (Lota lota) heart is clearly most sensitive to Ry among the three species, with maximal inhibition of atrial and ventricular contraction by 32% and 16%, respectively (51). It was hypothesized that Ry sensitivity of contrac-

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tion force correlates positively with the SR Ca\textsuperscript{2+} content, the Ca\textsuperscript{2+} load being larger in burbot compared with trout and carp, larger in atrium than ventricle, and larger in CA than WA fish (for trout and burbot), but larger in WA than CA crucian carp, as this species shows inverse temperature compensation in SR function (49).

**MATERIALS AND METHODS**

*Fish.* Rainbow trout (336.5 ± 18.9 g, n = 12) were obtained from the local fish farm (Kontiolahti, Finland) around the year. Crucian carp (65.4 ± 12.6 g, n = 8) acclimatized to summer or winter conditions were caught in June and November, respectively, from a small local lake. Burbot (202.5 ± 18.0 g, n = 10) were captured during spawning time in February. In the laboratory, each fish species was separately reared in temperature-controlled 500- or 1,000-liter stainless-steel tanks, with a continuous supply of aerated ground water at the rate of about 0.2 l/min. Rainbow trout and burbot were randomly divided in two groups for acclimation at either 18°C (warm-acclimation, WA) or 4°C (cold-acclimation, CA). Acclimation to different temperatures began from the water temperature at the time of fish catching and proceeded at the rate of 3°C per day. After the desired temperature was reached, a minimum period of 4 wk was allowed for thermal acclimation under a 15:9-h light-dark photoperiod. During that time, trout were fed 5 times/wk with aquarium fish food (Tetra, Melle, Germany), while laboratory before experiments. WA crucian carp were fed 3 times/wk with dead vendace (*Coregonus albula*) or small live crucian carp. Burbot is the most stenothermal fish among the three species and less tolerant to high temperatures than crucian carp and rainbow trout. Burbot is the most stenothermal fish among the three species and less tolerant to high temperatures than crucian carp and rainbow trout. The same experimental temperature was used for both acclimation groups, as SR Ca\textsuperscript{2+} load is not dependent on temperature (25).

A small aliquot of myocyte suspension was placed in the recording chamber (RC-26, volume 150 μl; Warner Instruments Brunswick, Handen, CT), and the cells were superfused with the external saline solution containing (in mmol/l) 150 NaCl, 5.4 CaCl\textsubscript{2}, 1.8 MgCl\textsubscript{2}, 10 glucose, and 10 HEPES (pH 7.6 with CsOH). Tetrodotoxin (0.1 μM, Alomone Labs, Jerusalem, Israel) and E-4031 (1 μM, Alomone Labs) were added in the solution to inhibit Na\textsuperscript{+} channels and delayed rectifier K\textsuperscript{+} channels, respectively. The composition of the pipette solution was as follows (in mmol/l): 140 CsCl, 3.4 NaCl, 5 Na\textsubscript{2}ATP, 1 MgCl\textsubscript{2}, 0.025 EGTA, 0.01 Na\textsubscript{2}GTP, 10 HEPES (pH 7.2 with CsOH). The total Na\textsuperscript{+} concentration of the pipette solution (13.4 mM) is as determined for rainbow trout ventricular myocytes (7).

When a giga seal was obtained, the myocyte was lifted up from the bottom of the chamber and placed in front of two glass capillaries (0.5 mm in diameter) of the solution changer (RCS-200; Biologic, Claix, France) for a rapid exchange of the control external saline solution to the one containing 10 mM caffeine and back again. The charge transfer by the NCX was normalized to the cell surface area and is given as picocoulombs per picofarad (pC/pF) or transformed to μmol Ca\textsuperscript{2+}/l nonmitochondrial cell volume by using surface-to-volume-ratio of 1.15 μm\textsuperscript{2} and nonmitochondrial cell volume of 55% (54, 55).

**Protocols of Ca\textsuperscript{2+} loading.** For determination of SS Ca\textsuperscript{2+} load, myocytes were stimulated with 6, 12, 35, 70, and 140 square-wave voltage pulses from −80 to +10 mV (300 ms in duration) at the frequency of 1.12 Hz (corresponding a heart rate of 67 beats per minute) (Fig. 1). For Max Ca\textsuperscript{2+} loading via the reverse mode, NCX cells were depolarized to +70 mV for 6 s. Further increase in pulse length did not increase SR Ca\textsuperscript{2+} content, suggesting that one 6-s-long depolarizing pulse was sufficient to achieve Max Ca\textsuperscript{2+} load, i.e., the loading was not limited by time available for SR Ca\textsuperscript{2+} uptake. Ca\textsuperscript{2+} accumulated in the SR was released by a fast application of 10 mM caffeine on the myocyte for 3 s, and the amount of Ca\textsuperscript{2+} was determined from the integral of the inward NCX current due to the extrusion of the Ca\textsuperscript{2+} by the forward exchanger.

**Statistics.** After checking normality of distribution, a Student’s t-test for independent samples was used for evaluating the effect of temperature acclimation on SR Ca\textsuperscript{2+} load. Comparisons between mean values from three species were compared with one-way ANOVA using the Tukey post hoc test.

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**Fig. 1.** Voltage-clamp protocols used for determination of steady-state (A) and maximal (B) sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} load in atrial and ventricular myocytes of the fish heart. The upper traces show voltage-clamp and caffeine pulses used for SR Ca\textsuperscript{2+} loading and Ca\textsuperscript{2+} release, respectively. The middle traces show membrane currents during voltage-clamp and caffeine pulses. The bottom traces show integrals of the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange (NCX) current (Ca\textsuperscript{2+} influx and SR Ca\textsuperscript{2+} load) during the caffeine pulse as μmol Ca\textsuperscript{2+}/l nonmitochondrial cytosolic space. For steady-state Ca\textsuperscript{2+} loading, the number of voltage pulses varied between 6 and 140 (at 1.12 Hz), and maximal Ca\textsuperscript{2+} loading was achieved with one 6-s voltage pulse to +70 mV.
RESULTS

SR Ca\(^{2+}\) loading. The same loading protocol was used for all species and for both atrial and ventricular myocytes, which do not exactly match action potential duration or heart rate for any single species but enables direct comparisons of inherent abilities of the SR for Ca\(^{2+}\) uptake and storage between different species and tissues. SS Ca\(^{2+}\) loading was accomplished using 300-ms depolarizing pulses from the holding potential of \(-80\) mV to \(+10\) mV at the frequency of 1.12 Hz (duration of cardiac cycle = 900 ms). Under these conditions, SR Ca\(^{2+}\) uptake occurs during depolarization simultaneously with SL Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels and NCX, when Ca\(^{2+}\) efflux is largely inhibited (Fig. 1). At the diastolic phase (\(-80\) mV, 600 ms), SR and SL compete for the same Ca\(^{2+}\), and therefore, SS Ca\(^{2+}\) load of the SR depends on relative effectiveness of SR and SL Ca\(^{2+}\) transport mechanisms and negative feedback between SR Ca\(^{2+}\) release and SL Ca\(^{2+}\) influx. For Max Ca\(^{2+}\) loading of the SR, SL Ca\(^{2+}\) influx via the reverse NCX was activated with a strong depolarizing pulse (\(+70\) mV), which was maintained for 6 s to allow the SR Ca\(^{2+}\)-ATPase to fill the stores. Membrane current during the loading pulse is outward, mainly due to the reverse NCX (Ca\(^{2+}\) influx) (Fig. 1). Rapid application of caffeine induces a release of SR Ca\(^{2+}\), which is extruded by the forward NCX generating an inward current. Integral of this current allows estimation of SR Ca\(^{2+}\) content and its normalization to the cell volume (Fig. 1).

Steady-state SR Ca\(^{2+}\) load. In all species, SS Ca\(^{2+}\) load was reached after 35 or 70 depolarizing pulses to \(+10\) mV, and this loading level was maintained if pacing continued for longer periods (Figs. 2 and 3). The values of SR Ca\(^{2+}\) load in fish cardiac myocytes varied between 224 (ventricular myocytes of WA burbot) and 915 (atrial myocytes of CA trout) \(\mu\)mol/l nonmitochondrial cytosolic space with some differences between tissues and species (Table 1). SS Ca\(^{2+}\) content of the SR was higher in atrial than ventricular myocytes in all other fish \((P < 0.05)\), except in WA burbot in which the SR Ca\(^{2+}\) load did not differ between atrial and ventricular cells (Fig. 3). The atrio-ventricular difference in SR Ca\(^{2+}\) load was particularly prominent in rainbow trout (2.18–2.69 fold) and much smaller in crucian carp (1.33–1.7 fold) and burbot (1.36–1.65 fold) (Fig. 3, Table 1). Atrial myocytes of WA rainbow trout and WA crucian carp hearts had a similar SR Ca\(^{2+}\) content, which was significantly higher than that in atrial myocytes of the WA burbot heart \((P < 0.05)\). In ventricular myocytes of WA fish, SR Ca\(^{2+}\) content was bigger in crucian carp than in rainbow trout and burbot \((P < 0.05)\). Differences between CA fish were smaller, but still Ca\(^{2+}\) stores of ventricular myocytes were

![Fig. 2. Steady-state SR Ca\(^{2+}\) content in atrial and ventricular myocytes of the fish heart. SR Ca\(^{2+}\) stores were loaded with 6–140 voltage-clamp pulses, and the SR Ca\(^{2+}\) load was released with a 3-s pulse of 10-mM caffeine. The results are expressed as \(\mu\)mol/l nonmitochondrial volume of the myocyte. *Statistically significant \((P < 0.05)\) difference between myocytes in cold-acclimated (CA) and warm-acclimated (WA) fish. Results are expressed as means ± SE of 10–28 cells from 4–6 fish.](http://www.ajpregu.org)
larger in crucian carp than trout. Temperature acclimation did not affect SS Ca\(^{2+}\) content in rainbow trout and crucian carp hearts, while in atrial and ventricular myocytes of the CA burbot, SR Ca\(^{2+}\) load was 106% and 70% larger, respectively, than in myocytes of the WA burbot. Furthermore, the atrio-ventricular differences in SS Ca\(^{2+}\) load were more pronounced in CA than WA fish.

**Maximal SR Ca\(^{2+}\) load.** In all teleost species, one 6-s long depolarizing pulse to +70 mV-loaded SR with more Ca\(^{2+}\) than 70 short and weakly depolarizing pulses (Figs. 2 and 3, Table 1). Further increase in pulse duration did not increase SR Ca\(^{2+}\) content, suggesting that Max Ca\(^{2+}\) load was obtained for these experimental conditions. The Max SR Ca\(^{2+}\) load in fish cardiac myocytes varied between 438 (ventricular myocytes of WA burbot) and 1,522 (atrial myocytes of WA trout) mol/l. These values of Max SR Ca\(^{2+}\) load are 1.6–2.4 times and 1.26–1.4 times larger than the SS Ca\(^{2+}\) load in atrial and ventricular myocytes, respectively. Thus, in atrial myocytes, SS Ca\(^{2+}\) load is closer to the maximum Ca\(^{2+}\) storing capacity of the SR compared with ventricular myocytes (Fig. 3, Table 1). Otherwise, the results are closely reminiscent to those of the SS Ca\(^{2+}\) load: atrial myocytes had larger Ca\(^{2+}\) stores than ventricular myocytes, and the species order in regard to the size of Max Ca\(^{2+}\) load was rainbow trout > crucian carp > burbot. Atrio-ventricular differences in Max Ca\(^{2+}\) load were smaller (1.26- to 1.87-fold) than the respective differences in SS Ca\(^{2+}\) load (Table 1).

**DISCUSSION**

The present results indicate that the SS Ca\(^{2+}\) content of the fish cardiac SR varies between 224 and 915 μmol/l, which is 1.9–10.5 times larger than the caffeine-releasable Ca\(^{2+}\) stores of rabbit or rat cardiac myocytes (87–120 μmol/l) (13, 52, 59). With a strong depolarizing pulse, Ca\(^{2+}\) load of the fish cardiac SR could be increased 1.3–1.6 times over the SS level, giving Max SR Ca\(^{2+}\) loads, which are 3.1–14.1 times larger than SS Ca\(^{2+}\) stores of the mammalian cardiac SR. Among the three fish species, burbot has the smallest SR Ca\(^{2+}\) stores, but even those are 1.9–6.3 times larger than the caffeine-releasable Ca\(^{2+}\) stores, which mammalian cardiac myocytes can retain under SS stimulation. Thus, the high SR Ca\(^{2+}\) content of the traut cardiac myocytes is not exceptional among teleost fishes, but a characteristic property of the SR for at least two other fish species, crucian carp and burbot. This suggests that fish cardiac myocytes are more generally able to store massive Ca\(^{2+}\) loads in the SR.

How can fish cardiac SR store large amounts of Ca\(^{2+}\)? The Max Ca\(^{2+}\) content of the SR depends at least on two factors, total SR volume and luminal Ca\(^{2+}\) buffering, while the SS

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### Table 1. SR Ca\(^{2+}\) load of fish cardiac myocytes

<table>
<thead>
<tr>
<th>Myocyte type</th>
<th>Steady-state load, μmol/l</th>
<th>Maximal load, μmol/l</th>
<th>Steady-state load/maximal load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atrial</td>
<td>Ventricular</td>
<td>Atrial</td>
</tr>
<tr>
<td>Cold acclimation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>915.2±120.9 (28)*</td>
<td>340.3±46.7 (22)*</td>
<td>1222.4±103.8 (17)*</td>
</tr>
<tr>
<td>Burbot</td>
<td>627.9±50.3 (19)*</td>
<td>380.6±20.1 (15)</td>
<td>866.0±85.2 (18)*</td>
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<td>Crucian carp</td>
<td>830.9±78.1 (20)*</td>
<td>488.2±54.9 (18)*</td>
<td>1044.1±137.0 (12)</td>
</tr>
<tr>
<td>Warm acclimation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>725.3±54.5 (23)*</td>
<td>324.5±45.3 (17)*</td>
<td>1521.9±189.4 (12)*</td>
</tr>
<tr>
<td>Burbot</td>
<td>304.3±16.6 (15)b</td>
<td>224.5±14.4 (17)*</td>
<td>550.3±73.1 (10)b</td>
</tr>
<tr>
<td>Crucian carp</td>
<td>718.3±84.3 (11)b*</td>
<td>540.5±51.2 (11)b</td>
<td>842.7±107.2 (10)b</td>
</tr>
</tbody>
</table>

*Statistically significant difference (P < 0.05) between atrial and ventricular myocytes within species and acclimation group; dissimilar letters (a, b) between different fish species. The results are from 4–6 fish for each group with a number of myocytes indicated in parenthesis.
Ca\(^{2+}\) load may involve a third factor, the activity of the SR Ca\(^{2+}\)-ATPase. Electron microscopic studies suggest that the cardiac SR is less extensive in fish than mammalian hearts (38). Estimates of the total SR volume in mammalian hearts vary between 1 and 12% of the myocyte volume (18, 40), and the value of 3.5% is often regarded as a representative estimate (34). Quantitative data about the fish cardiac SR exist only for the perch (Perca fluviatilis) ventricle, where the total SR volume was estimated to be 4.5% and 6.05% of the nonnuclear myocyte volume in WA (20°C) and CA (5°C) fish, respectively (8). Thus, in the light of current knowledge, at least in some fish species, the SR volume is similar to the volume of the mammalian cardiac SR and, therefore, adequate for relatively large Ca\(^{2+}\) storage. Most (about 50–90%) of the SR Ca\(^{2+}\) within mammalian cardiac myocytes is bound to calsequestrin (CASQ) (9), a Ca\(^{2+}\) binding protein that has been recently shown to be present also in fish hearts (31). Even though rigorous morphometric comparisons between fish and mammalian SR are missing, it seems that SR volume is not that much larger in fish cardiac myocytes that it would explain the 2–14 times larger Ca\(^{2+}\) stores of the fish cardiac SR compared with mammalian SR. Therefore, the large difference in SR Ca\(^{2+}\) storage capacity between fish and mammalian cardiac myocytes reflects functional differences in cardiac Ca\(^{2+}\) management, which will result in higher total Ca\(^{2+}\) concentration within the fish cardiac SR.

Functional differences between mammalian and fish SR. In native state, mammalian cardiac myocytes are unable to keep high SR Ca\(^{2+}\) loads, but when Ca\(^{2+}\) release channels of the digitonin-permeabilized myocytes are blocked with ruthenium red, SR can retain similar amounts of Ca\(^{2+}\) (up to 1,000 \(\mu\)mol/l) as fish cardiac myocytes and much higher Ca\(^{2+}\) loads than intact rat cardiac myocytes in a physiological state (41). This strongly suggests differences in the properties or regulation of SR Ca\(^{2+}\) release channels between fish and mammalian hearts. Ca\(^{2+}\) release from the cardiac SR is triggered by Ca\(^{2+}\) binding to the cytosolic site of the RyR, which induces opening of the Ca\(^{2+}\) release channel (17). In fish hearts, Ca\(^{2+}\) binding affinity of RyRs on the cytosolic side is often much lower than in mammalian hearts (11, 56), which may stabilize SR Ca\(^{2+}\) and thereby enable much higher SR Ca\(^{2+}\) loads. Consistent with this, SS and Max Ca\(^{2+}\) loads of the SR in the three fish species correlate negatively with Ca\(^{2+}\) sensitivity of their RyRs to cytosolic Ca\(^{2+}\). RyRs of the burbot heart have the highest Ca\(^{2+}\) affinity (\(K_d\), half-maximal binding at 0.188 \(\mu\)M) and the smallest SR Ca\(^{2+}\) loads, while RyRs of rainbow trout and crucian carp heart require 4.4 (\(K_d\), 0.834 \(\mu\)M) and 5.9 (\(K_d\), 1.104 \(\mu\)M) times more Ca\(^{2+}\) for half-maximal occupation of the Ca\(^{2+}\)-binding site, respectively, than burbot RyRs (56), while they have significantly larger SR Ca\(^{2+}\) stores than the burbot SR. The inverse relationship between Ca\(^{2+}\) binding affinity of RyRs and SR Ca\(^{2+}\) load suggests that one of the mechanisms that contributes to the high Ca\(^{2+}\) loads of the fish heart is the low Ca\(^{2+}\) sensitivity of the release channels; spontaneous opening of a single RyR cannot elicit regenerative Ca\(^{2+}\) release if the trigger threshold is high.

Ca\(^{2+}\) release from the SR is also modulated from the luminal side, in which high Ca\(^{2+}\) loads sensitize the release mechanism, possibly by removing the inhibitory effect of CASQ on RyRs (30, 32, 60). High sensitivity of cardiac RyRs to cytosolic Ca\(^{2+}\) and sensitizing effect of luminal Ca\(^{2+}\) on RyR opening cause spontaneous releases of Ca\(^{2+}\) from rabbit and rat cardiac SR and thereby limit the SR Ca\(^{2+}\) load to about 120 \(\mu\)mol/l (42, 52). Indeed, in ferret cardiac myocytes, the Max Ca\(^{2+}\) load is only a few percentages larger than SS Ca\(^{2+}\) load (4). The role of luminal Ca\(^{2+}\) on RyR function of the fish heart has not been studied, and electrophysiological experiments on single RyRs are needed to resolve this issue. However, atrio-ventricular differences in SR Ca\(^{2+}\) release (see Comparison of atrial and ventricular myocytes) and the presence of CASQ in the fish SR suggest that a similar sensitizing mechanism exists also in fish hearts.

**SR Ca\(^{2+}\) content and Ry sensitivity of cardiac contraction.** Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the mammalian cardiac SR is strongly dependent on SR Ca\(^{2+}\) load (17, 21, 27, 60). If the SR Ca\(^{2+}\) content is 60% or less of the Max load, Ca\(^{2+}\) release will not occur, whereas at Ca\(^{2+}\) loads above 60% Ca\(^{2+}\), release increases steeply as a function of the SR Ca\(^{2+}\) content (4). Considering the massive Ca\(^{2+}\) stores of the fish cardiac SR and the load dependence of Ca\(^{2+}\) release, a large Ry-sensitive component of contraction in the fish heart would be consistent with the mammalian type of CICR. Contrary to this, Ry sensitivity of force generation in the fish hearts is quite modest compared with mammalian cardiac preparations. However, the three teleost fishes slightly differ in regard to Ry sensitivity of cardiac contraction. Contraction of the burbot heart is relatively strongly inhibited by Ry; at 1°C, Ry reduces the force of atrial and ventricular contraction by 32% and 16%, respectively (51). In rainbow trout, Ry has a clear force-reducing effect (19%) on the atrial contraction, but no effect on the force of atrial contraction in the WA trout or on the force of ventricular contraction in either WA or CA rainbow trout (2, 33). Crucian carp heart is generally resistant to Ry, but atrial contraction of WA fish is slightly inhibited by Ry at 23°C (49). In all species, the effect of Ry increases with increasing temperature and with longer diastolic intervals (24, 33, 44, 51). Overall, cardiac Ry sensitivity decreases in the order of burbot > rainbow trout > crucian carp. Surprisingly, SR Ca\(^{2+}\) content in cardiac myocytes of the crucian carp, the least Ry-sensitive species, is as large as that of the rainbow trout myocytes, and on the other hand, the most Ry-sensitive species, burbot, has the smallest SR Ca\(^{2+}\) stores among the studied species. Clearly, the hypothesis that the absolute level of the SR Ca\(^{2+}\) load is positively correlated with Ry sensitivity of contractile force in fish hearts is falsified by these results. In contrast, there is a negative correlation between Ry sensitivity of contraction force and the absolute size of the SR Ca\(^{2+}\) stores.

**Effect of temperature acclimation.** Thermal acclimation modifies Ry sensitivity of cardiac contraction in fish hearts (2, 29, 49). Acclimation to cold increases Ry sensitivity of contraction in the trout heart and depresses it in crucian carp heart (crucian carp show inverse thermal compensation). Nevertheless, temperature acclimation had no effect on SS or Max SR Ca\(^{2+}\) load in either species. Therefore, some other factor(s) than the size of SR Ca\(^{2+}\) stores is probably behind the temperature-induced changes in Ry-sensitivity of cardiac contraction in these animals. Interestingly, in the burbot heart, WA strongly reduced SR Ca\(^{2+}\) content in both atrial and ventricular myocytes. We do not yet know how the reduced SR Ca\(^{2+}\) load affects Ry sensitivity of cardiac contraction of the burbot heart. Inverse relationship between SR Ca\(^{2+}\) load and Ry sensitivity (see...
above) and temperature dependence of Ry inhibition in other fish hearts suggest that contraction of the WA burbot heart should be strongly inhibited by Ry at physiological temperature. Taken together, the hypothesis that acclimation-related changes in Ry sensitivity are related to SR Ca$^{2+}$ load is not supported by the results from rainbow trout and crucian carp, but remains open in the case of burbot.

Comparison of atrial and ventricular myocytes. Similar to the situation in mammals, atrial contraction of the fish heart is more strongly inhibited by Ry than ventricular contraction (2, 19). Furthermore, this chamber-specific difference is associated with larger SR Ca$^{2+}$ stores in atrial than ventricular myocytes both in fish (present study) and rat hearts (59), suggesting a possible causal relationship between SR Ca$^{2+}$ content and Ry sensitivity (i.e., opposite to what we found between fish species). In mammalian hearts, the SS SR Ca$^{2+}$ content must be about 60% of the Max load to enable CICR (42). In fish hearts, the critical limit of 60% is exceeded only in atrial myocytes of CA fish (all species) and in atrial and ventricular myocytes of WA crucian carp. If the load-dependence of Ca$^{2+}$ release, i.e., the sensitizing effect of intraluminal Ca$^{2+}$ on RyRs, is similar in mammalian and fish hearts, CICR would be possible in atrial myocytes of the fish heart and better so in CA than WA fish. An exception in this respect is crucian carp, in which the WA fish have a slightly higher relative SS Ca$^{2+}$ load than the CA fish, consistent with its inverse acclimation pattern in contrast to positive thermal compensation of burbot and trout hearts. Thus, the higher SS Ca$^{2+}$ loading relative to Max Ca$^{2+}$ load in atrial than ventricular SR is consistent with the higher Ry sensitivity of atrial contraction compared with ventricular contraction. Unlike Max Ca$^{2+}$ load, which is dependent on SR volume and Ca$^{2+}$ buffering by CASQ, SS Ca$^{2+}$ loading involves also the time domain and therefore the rate of Ca$^{2+}$ uptake by the SR Ca$^{2+}$-ATPase. Because atrial muscle of the fish heart has higher Ca$^{2+}$-ATPase activity than ventricular muscle (1), atrial myocytes are probably better able to load the SR with Ca$^{2+}$ within the limited period of time between voltage pulses compared with ventricular myocytes, especially in CA fish. In this scheme, it is the activity of SR Ca$^{2+}$-ATPase, not the Ca$^{2+}$-storing capacity of the SR, which correlates with Ry sensitivity of contraction.

Implications for e-c coupling. About 70 \(\mu\)mol/l of cytosolic Ca$^{2+}$ is needed for half-maximal activation of contraction in vertebrate cardiac myocytes (6), which is only 7.6% of the SS Ca$^{2+}$ content of the CA trout atrial myocytes and 31.1% of the SS Ca$^{2+}$ content of WA burbot ventricular myocytes, i.e., at the two extremes of SR Ca$^{2+}$ load in fish hearts. Thus, in regard to contractile requirements, there is large excess of Ca$^{2+}$ in the fish cardiac SR, which has some implications for the e-c coupling. In mammalian cardiac myocytes CICR occurs between L-type Ca$^{2+}$ channels and RyRs, which are closely apposed in the narrow junctional cleft between SL and SR. RyRs are clustered in dense arrays of several RyRs, which are elicited to open by a single L-type Ca$^{2+}$ channel, resulting in generation of a local Ca$^{2+}$ release event or a Ca$^{2+}$ spark (10). Because multiple RyRs are clustered together, opening of a single release channel is expected to result in regenerative opening of all other RyRs in the cluster. A major unanswered problem of the CICR mechanism is the gradation of Ca$^{2+}$ release in an inherently regenerative process, i.e., how Ca$^{2+}$ release can be terminated to produce a smoothly variable Ca$^{2+}$ transient under various physiological states (47). The suggestion that binding of Ca$^{2+}$ to a low-affinity cytosolic site causes inactivation or inhibition of RyRs (17) has got some experimental support but is not generally accepted (47). If cluster size is small and open probability of RyRs low, stochastic possibility for simultaneous closing of all RyRs in the cluster could theoretically cause termination of Ca$^{2+}$ release (47). However, the most plausible mechanism for termination of Ca$^{2+}$ release at the moment is depletion of Ca$^{2+}$ in the SR in association with depletion-dependent modulation RyR gating by luminal Ca$^{2+}$ (17, 36). High luminal Ca$^{2+}$ increases open probability of RyRs (30, 32, 60), and hence, a partial depletion of the SR Ca$^{2+}$ could terminate the release by substantially reducing probability for RyR opening. Depletion of SR Ca$^{2+}$ stores is a feasible mechanism of Ca$^{2+}$ release in mammalian SR due to the limited capacity of the SR Ca$^{2+}$ stores; with physiological Ca$^{2+}$ loads 40–60% of the SR Ca$^{2+}$ content is released (42). However, in fish cardiac myocytes, a fractional release of 60% would not only be more than sufficient for activation of contraction but even damaging to the cell. The low Ca$^{2+}$ sensitivity of the fish RyRs raises the threshold for CICR, but if the threshold level is exceeded, a Ca$^{2+}$ flux through RyRs should trigger a regenerative activation of all nearby receptors and massive increase in cytosolic Ca$^{2+}$ concentration. Obviously, an effective mechanism, other than store depletion (at least quantitatively different from the mammalian type), must exist in fish cardiac myocytes for termination of the Ca$^{2+}$ release. Massive SR Ca$^{2+}$ stores in association with limited contribution of SR Ca$^{2+}$ to e-c coupling makes fish cardiac myocytes interesting objects in elucidating the putative inactivation/deactivation mechanism of the Ca$^{2+}$ release.

Limitations. SR Ca$^{2+}$ load was determined as caffeine-releasable Ca$^{2+}$ extruded by the sarcolemmal NCX. This is an established method and is based on caffeine’s sensitizing effect on cytosolic Ca$^{2+}$ binding of RyRs, and hence it should be specific for the SR. It is, however, possible that some of the released Ca$^{2+}$ is buffered by mitochondria. Ca$^{2+}$ influx into mitochondria occurs via the mitochondrial Ca$^{2+}$ unipporter, a low-affinity (K_{0.5} about 0.5 \(\mu\)M) transporter, whose operation requires close connection between mitochondria and SR (20). During caffeine-induced Ca$^{2+}$ release, cytosolic Ca$^{2+}$ can momentarily be much higher than normal Ca$^{2+}$ transient and, therefore, sufficiently high to activate mitochondrial Ca$^{2+}$ influx. The peak of Ca$^{2+}$ efflux via the NCX in fish cardiac myocytes occurred within 150 ms and was over within 300–400 ms from the caffeine application leaving little time for mitochondrial Ca$^{2+}$ uptake. Since the mitochondrial unipporter is about 50 times slower than the NCX (5), it is unlikely it could effectively compete with the NCX, which has preferential access to the released Ca$^{2+}$ ion at the junctional cleft. Our results are based on this assumption.

SR Ca$^{2+}$ load was expressed relative to the nonmitochondrial space of the myocytes assuming a mitochondrial volume of 45%, according to the data from WA trout (54). Morphometric data on mitochondrial volumes are not available for atrial myocytes of rainbow trout and crucian carp, and no comparisons exist between CA and WA fish for any of the species. Therefore, the results do not take into account species-specific, chamber-dependent, or temperature-related differences in mitochondrial volume density. Mitochondrial densi-
ties in burbot and crucian carp myocytes are somewhat lower than in trout (50, 55), and therefore, SR Ca\(^{2+}\) loads might be slight overestimates in these species. However, the SR Ca\(^{2+}\) loads are directly comparable between species, between atrial and ventricular myocytes and between acclimation groups at the level of cell volume.

**Perspectives and Significance**

A major functional characteristic of the fish cardiac SR is the massive Ca\(^{2+}\) loading capacity of the SR, which is likely an outcome of low Ca\(^{2+}\) sensitivity of the release mechanism (11, 56) and high stability of SR Ca\(^{2+}\) stores. Sensitivity and stability are antagonistic properties of a system; hence, the high sensitivity of the Ca\(^{2+}\) release system of endothermic hearts has probably evolved from the low sensitivity of a Ca\(^{2+}\) release system of ectothermic animals with the trade-off being low stability of SR Ca\(^{2+}\) stores. High sensitivity of CICR is an effective system for recycling intracellular Ca\(^{2+}\) between SR and cytosol at relatively high heart rates in wide myocytes of the mammalian hearts. On the other hand, labile Ca\(^{2+}\) stores of the mammalian SR are prone to premature releases, which may generate life-threatening cardiac arrhythmias. Hearts of ectothermic fishes must function properly over a wide range of temperatures, i.e., under conditions in which a high-sensitivity system of CICR might be particularly vulnerable to arrhythmias. Therefore, large Ca\(^{2+}\) stores of the fish cardiac SR and low Ca\(^{2+}\) sensitivity of RyRs can be seen as adaptations that ensure stable SR function under widely variable temperatures and other environmental conditions. Comparison of SR function in fish inhabiting different environments and living under different environmental (hypoxic, anoxic, temperature, osmotic and oxidative) stresses might shed light on biological importance of the SR Ca\(^{2+}\) stores in different physiological situations. Future studies should also clarify what kind of molecular arrangements or innovations are involved in the evolution of c-c coupling from a high-capacity, low-sensitivity system of the fish heart to a high-sensitivity, low-capacity type of the endothermic hearts.

**ACKNOWLEDGMENTS**

We give special thanks to Anita Kervinen for technical assistance.

**GRANTS**

This research was supported by grants from The Academy of Finland (Grants 210400, 119583, and 127192) to M. Vornanen.

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