Temperature and Ca\(^{2+}\) dependence of \(^{3}\text{H}\)ryanodine binding in the burbot (\textit{Lota lota} L.) heart

Matti Vornanen

Department of Biology, University of Joensuu, Joensuu, Finland

Submitted 23 June 2005; accepted in final form 15 September 2005

\textbf{Vornanen, Matti.} Temperature and Ca\(^{2+}\) dependence of \(^{3}\text{H}\)ryanodine binding in the burbot (\textit{Lota lota} L.) heart. \textit{Am J Physiol Regul Integr Comp Physiol} 290: R345–R351, 2006. First published September 22, 2005; doi:10.1152/ajpregu.00443.2005.—Opening and closing of the cardiac ryanodine (Ry) receptor (RyR) are coordinated by the free intracellular Ca\(^{2+}\) concentration, thus making the Ca\(^{2+}\) binding properties of the RyR important for excitation-contraction coupling. Unlike mammalian cardiac RyRs, which lose their normal function at low temperatures, RyRs of ectothermic vertebrates remain operative at \(-2–4^\circ\text{C}\), as indicated by Ry sensitivity of contractile force. To investigate the mechanisms of low temperature adaptation of ectothermic RyRs, we compared Ca\(^{2+}\)-dependent kinetics of \(^{3}\text{H}\)ryanodine binding in cardiac preparations of a fish (burbot, \textit{Lota lota}) and a mammal (rat). The number of ventricular \(^{3}\text{H}\)ryanodine binding sites determined at 20°C was 1.54 times higher in rat than burbot heart (0.401 ± 0.039 and 0.264 ± 0.019 pmol/mg protein, respectively) \((P < 0.02)\), while the binding affinity \((K_d)\) for \(^{3}\text{H}\)ryanodine was similar (3.38 ± 0.63 and 4.38 ± 1.14 nM for rat and burbot, respectively) \((P = 0.47)\). The high-affinity \(^{3}\text{H}\)ryanodine binding to burbot and rat cardiac preparations was tightly coordinated by the free Ca\(^{2+}\) concentration at both 20°C and 2°C and did not differ between the two species. Half-maximal \(^{3}\text{H}\)ryanodine binding occurred at 0.191 ± 0.027 μM and 0.164 ± 0.034 μM Ca\(^{2+}\) for rat and at 0.212 ± 0.035 μM and 0.188 ± 0.039 μM Ca\(^{2+}\) for burbot \((P = 0.65)\), at 2°C and 20°C, respectively. In two other fish species, rainbow trout (\textit{Oncorhynchus mykiss}) and crucian carp (\textit{Carassius carassius}), the Ca\(^{2+}\)-binding affinity at 20°C was 4.4 and 5.9 times lower, respectively, than in the burbot. At 20°C, the rate of \(^{3}\text{H}\)ryanodine binding to the high-affinity binding site was similar in rat and burbot but was drastically slowed in rat at 2°C. At 2°C, \(^{3}\text{H}\)ryanodine failed to dissociate from rat cardiac RyRs, and at 10°C and 20°C, the rate of dissociation was two to three times slower in rat than burbot preparations. The latter finding is compatible with a channel gating mechanism, where the closing of the Ca\(^{2+}\) release channel is impaired or severely retarded by low temperature in rat but less so in burbot preparations. The stronger effect of low temperature on association and dissociation rate of \(^{3}\text{H}\)ryanodine binding in rat compared with burbot suggests that RyRs of the ectothermic fish, unlike those of endothermic rat, are better able to open and close at low temperatures.

\textbf{IN CARDIAC EXCITATION–CONTRACTION (E-C) COUPLING, sarcoplasmal action potential induces a transient increase in free intracellular Ca\(^{2+}\) concentration, which sets contraction in motion. In endothermic animals, a small influx of extracellular Ca\(^{2+}\) triggers a much larger Ca\(^{2+}\) release from intracellular stores of the sarcoplasmic reticulum (SR), which is the main source of Ca\(^{2+}\) for the cytosolic Ca\(^{2+}\) transient. In the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) process, the high-affinity Ca\(^{2+}\) binding site of the SR Ca\(^{2+}\) release channel or ryanodine receptor (RyR) acts as an amplifier by opening the release channel in proportion to the size of the trigger signal. The sensitivity of RyR to Ca\(^{2+}\) activation is physiologically important because it determines the efficacy of sarcolemmal Ca\(^{2+}\) influx as a trigger for Ca\(^{2+}\) release. In ectothermic animals, the mechanism of cardiac e-c coupling seems to be more heterogeneous with marked species-specific differences. In many ectotherms the influx of extracellular Ca\(^{2+}\) through sarcolemmal (SL) L-type Ca\(^{2+}\) channels and/or Na\(^{+}\)-Ca\(^{2+}\) exchange seems to be the sole source for cytosolic Ca\(^{2+}\) transient, with negligible contribution by the SR Ca\(^{2+}\) stores (7, 11, 26). On the other hand, more recent findings suggest that in some ectotherms Ca\(^{2+}\) release from the SR makes a sizeable, sometimes, perhaps even, major contribution to Ca\(^{2+}\) transient (2, 9, 16, 18, 19). Clearly, the significance of SR Ca\(^{2+}\) stores in e-c coupling strongly varies between ectothermic species. In addition to species-specific differences, the extent of SR participation to Ca\(^{2+}\) transient is modified by acute and chronic temperature changes (for a recent review, see Ref. 27). For example, in rainbow trout (\textit{Oncorhynchus mykiss}), an acute drop in temperature diminishes or completely abolishes the contribution of ryanodine-sensitive Ca\(^{2+}\) stores to e-c coupling (17), as if the function of RyRs were impaired at near-freezing temperatures. As the mammalian RyRs are known to be locked in an open state, draining the SR Ca\(^{2+}\) content when suddenly cooled to 0°C (21), temperature-dependent failure of Ca\(^{2+}\) release channels is a plausible explanation for the minor role of the SR in e-c coupling in ectothermic hearts (23). From an evolutionary perspective, it is, however, difficult to explain why a property that makes RyRs functionally incapable had been preserved in vertebrates, which are otherwise physiologically adapted to low temperatures. Indeed, it was recently shown that in the cold stenothermal fish, the burbot (\textit{Lota lota}), a sizeable ryanodine (Ry)-sensitive component contraction was present at 2°C (24), thus providing strong evidence that cardiac RyRs of ectothermic animals retain some functionality in the cold. The finding that chronic exposure of fish to low temperatures increases the contribution of cardiac SR Ca\(^{2+}\) stores to contractile activation (1, 10) contradicts the concept that RyRs completely fail in the cold. Therefore, the objective of the present study was to test the hypothesis that the gating of cardiac RyRs has different thermal sensitivity in ectothermic and endothermic animals, which accounts for the divergent behavior of the SR at low temperatures. Ca\(^{2+}\) release channel gating was probed by \(^{3}\text{H}\)ryanodine binding in burbot hearts.

Address for reprint requests and other correspondence: M. Vornanen, Dept. of Biology, Univ. of Joensuu, P.O. Box 111, 80101 Joensuu, Finland (e-mail: matti.vornanen@joensuu.fi).

http://www.ajpregu.org

\textbf{The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.}
that retain SR function at 2°C (24) and compared with that of the endothermic rat.

MATERIALS AND METHODS

Animals. Burbot, 200–400 g in body mass, were captured in January and February (Lake Pielinen, Finland) and reared in the laboratory in 500-liter thermostated aquaria at 2°C with the continuous circulation of aerated groundwater. Burbot were fed dead vendace (Coregonus albus) three times a week. Rainbow trout (O. mykiss; 200–400 g) were obtained from a local fish farm (Kontiolahti, Finland) and maintained at 4°C for a minimum of 4 wk before the experiments. Crucian carp (Carassius carassius) (30–150 g) were captured from local ponds with weirs in early summer and were maintained at 23°C in the laboratory. Trout and crucian carp were fed five times per week with commercial fish fodder (Biomar, Brande, Denmark) and aquarium fish food (Tetra, Melle, Germany), respectively. All fish species were maintained on a 9:15-h light-dark cycle. Adult (4–6 mo) male rats (Rattus norvegicus) of Wistar strain (350–500 g) were used as a mammalian reference. All procedures with animals were done with consent of the local committee for animal experimentation.

Sample preparation. Crude ventricular membranes were prepared according to the procedure of Milnes and MacLeod (15) and used to determine [3H]ryanodine binding. Rats were killed by cervical dislocation under light diethyl ether anesthesia. Fish were stunned by a blow to the head and killed by cutting the spine. Hearts were carefully removed; ventricular muscle was separated, blotted dry, and weighted to the nearest 0.1 mg. Ventricle(s) were diced with scissors and then homogenized using Teflon-glass homogenizer (Heidolph) for four 20-s bursts at maximum speed (2,200 rpm) in 15 volumes of ice-cold buffer containing (in mM): 500 KCl, 25 HEPES, 0.005 phenylmethysulfonyl fluoride (PMSF); pH 7.2 at 20°C. Homogenate was centrifuged at 1,000 g for 15 min at 4°C; the pellet was discarded, and the supernatant was used in binding experiments. Protein concentration was determined using the Lowry method (14).

[3H]ryanodine binding. Ca2+-release channel gating was probed by [3H]ryanodine binding, as it is known that Ry preferentially binds to open channels and that dissociation of Ry from its receptor is associated with channel closing (see DISCUSSION). [3H]ryanodine (Amersham, Little Chalfont, UK) binding was performed in a total volume of 0.5 ml of solution containing about 100 μg of homogenate protein, in 1,000 mM KCl, 300 mM sucrose, 25 mM HEPES, 1 mM Na2ATP, 0.1 mM CaCl2, 0.005 mM PMSF, and 0.1 mg/ml bovine serum albumin at pH 7.2 (25). Experiments were conducted at different temperatures (2°, 10°, and 20°C), and the pH of the binding buffer was separately set to 7.2 at each temperature. Nonspecific binding was determined by measuring [3H]ryanodine binding in the presence of 10-μM unlabeled ryanodine (Sigma, Poole, UK). The reaction was terminated with 6 ml of ice-cold wash buffer containing (in mM): 1,000 KCl, 25 HEPES; pH 7.2 at 20°C, and the suspensions were immediately filtered through Whatman GF/B filters (Merck, Poole, UK) with three 6-ml washes of cold buffer. Filters were soaked in 10 ml of scintillant (Ready Protein+, Beckman Coulter, Buckinghamshire, UK), and [3H]ryanodine bound to the filter was quantified by liquid scintillation counting (Wallac 1414 WinSpectral). This basic procedure of [3H]ryanodine binding was modified for different determinations, as described below.

The saturation binding of [3H]ryanodine to cardiac membranes was determined by incubating cardiac membranes with 0.1–30 nM [3H]ryanodine for 3 h at 20°C so that binding equilibrium was reached. After background correction, specific binding was plotted as a function of [3H]ryanodine concentration, and maximum number binding sites (Bmax) and dissociation constant (Kd) were obtained from Michaelis-Menten fits to the data points. To assess Ca2+-dependence of high-affinity ryanodine binding, cardiac preparations were incubated with 10 nM [3H]ryanodine at different free Ca2+ concentrations, which were obtained by mixing 1 mM EGTA and various concentrations of CaCl2 calculated using the MaxChelator software (Chris W. Patton). Data plots were fitted with Hill equation for determination of Ca2+ concentration for half-maximal [3H]ryanodine binding.

The rate of [3H]ryanodine association to cardiac receptors was measured at 2° and 20°C by incubating membrane preparations with 10 nM [3H]ryanodine and quenching the reaction with 6 ml of the ice-cold wash buffer at times ranging from 10 to 1,600 min. Specific binding was plotted as a function of time, and the observed association rate constant (Kobs) was obtained from exponential fits to the data. The pseudo first-order association rate constant (k+1) was obtained as k+1 = kobs - k-1/[Ry] where k-1 is the dissociation rate constant and [Ry] the concentration of [3H]ryanodine.

To determine Ca2+-dependent dissociation of bound [3H]ryanodine, cardiac membranes were incubated with 10 nM [3H]ryanodine in a modified binding buffer [0.01 mM CaCl2, 0.5 M (rat) or 1.0 M (burbot) KCl and 25 mM HEPES at pH 7.2] for 3 h at 20°C to reach equilibrium. Then, different amounts of concentrated EGTA solution were added to produce a concentration of free Ca2+ between 0.003 and 10 μM, and the bound [3H]ryanodine was determined after 2 h incubation. The data were plotted as a function of free Ca2+ concentration and fitted with the Hill equation to obtain Ca2+ concentration for half-maximal [3H]ryanodine binding. The rate of [3H]ryanodine dissociation from its receptor sites was measured at 2°C, 10°C, and 20°C. First cardiac membranes were incubated with 10 nM [3H]ryanodine in a binding buffer containing 10 μM free Ca2+ for 3 h at 20°C, and one more hour at 10° or 2°C, when the dissociation rate was measured at 10° or 2°C. When binding equilibrium had been achieved, 36 μl of 10 mM EGTA solution were added to produce free Ca2+ concentration of 0.003 μM. At different time points from EGTA addition, the amount of residual [3H]ryanodine binding was counted as described above. The specifically bound [3H]ryanodine was plotted on logarithmic scale for determination of the dissociation rate constant (k-1).

Statistical analyses. Differences between rat and burbot hearts were compared by unpaired t-test. Comparisons between several species was accomplished by one-way ANOVA followed by Student-Newman-Keuls post hoc test. The differences were considered to be significant at P < 0.05.

RESULTS

Number and affinity of RyRs. Saturation binding experiments at 20°C showed that, within the range of 0.1–30 nM [3H]ryanodine, binding occurs at a single high-affinity binding site in both rat and burbot (Fig. 1). The number of [3H]ryanodine binding sites (Bmax) was 1.54 times higher in rat (0.401 ± 0.039 pmol/mg protein) than burbot (0.264 ± 0.019 pmol/mg protein) (P < 0.02), whereas ryanodine binding affinities (Kd) were similar in both species (3.38 ± 0.63 and 4.38 ± 1.14 nM for rat and burbot, respectively) (P = 0.47).

Ca2+ dependence of [3H]ryanodine binding. Ca2+-dependence of [3H]ryanodine binding was studied at Ca2+ concentrations between 0.01 and 30 μM to reveal the affinity of RyRs to trigger Ca2+. In both rat and burbot membranes, [3H]ryanodine binding was strongly dependent on Ca2+ concentration. At 20°C, the Ca2+ affinity of [3H]ryanodine binding was almost identical in rat (KCa: 0.164 ± 0.027 μM) and burbot (0.212 ± 0.035 μM) preparations (Fig. 2). Lower incubation temperature (2°C) did not alter the Ca2+-affinity of [3H]ryanodine binding in either rat (0.191 ± 0.027 μM) or burbot (0.212 ± 0.035 μM) preparations (Fig. 2). Thus [3H]ryanodine binding is tightly controlled by Ca2+ in both mammalian and fish cardiac preparations, even at 2°C.
Because there are marked species-specific differences among teleost fish to the extent in which ryanodine can inhibit force generation in intact tissue, we examined the Ca\(^{2+}\)/ryanodine sensitivity of \(^{3}H\)ryanodine binding in two other fish species at 20°C. The Ca\(^{2+}\)/ryanodine affinity of ryanodine binding was 4.4 and 5.9 times lower in the cardiac preparations of rainbow trout (0.834 ± 0.105 μM, n = 4) and crucian carp (1.104 ± 0.191 μM, n = 5), respectively, than in burbot cardiac membranes (Fig. 3). Thus among the three fish species, only burbot has comparable Ca\(^{2+}\)-affinity to those of mammals.

**Temperature dependence of association rate of \(^{3}H\)ryanodine binding.** At 20°C, maximal steady-state binding was achieved at about 2 h in both rat and burbot preparations (Fig. 4). The apparent association rate constant (k\(_{\text{obs}}\)) of the burbot heart (0.055 ± 0.015 min\(^{-1}\)) did not differ significantly from that of the rat heart (0.162 ± 0.061 min\(^{-1}\)) (P = 0.12). However, at 2°C, there was a prominent difference in time when binding equilibrium was achieved in the two species. In burbot preparations, the steady-state binding was achieved in 3 h, whereas in rat preparations, about 16 h were needed to reach the equilibrium state (Fig. 4). The k\(_{\text{obs}}\) of the burbot heart (0.020 ± 0.008 min\(^{-1}\)) was approximately five times larger than in the rat heart (0.0041 ± 0.060 min\(^{-1}\)) (P < 0.05). The rate at which the binding equilibrium is approached depends on both rate of \(^{3}H\)ryanodine binding to and unbinding from its receptor, and, therefore, k\(_{\text{obs}}\) will give incorrect association rates if there are differences in the rate of dissociation (see Dissociation of \(^{3}H\)ryanodine).

The pseudo first-order association rate constants (k\(_{1}\)) (burbot: 0.00210 min/nM and 0.01977 min/nM and rat: 0.00069 min/nM and 0.0162 min/nM at 2° and 20°C, respectively) indicate, however, that the rate of
association binding at 2°C is about three times slower in rat than burbot. Taken together, these experiments indicate that between 20°C and 2°C, association kinetics of [3H]ryanodine binding is much more drastically depressed in rat (Q10 = 5.77) than burbot heart (Q10 = 1.04).

Dissociation of [3H]ryanodine. [3H]ryanodine dissociates from its receptor site when free Ca²⁺ concentration of the incubation buffer is reduced (Fig. 5). At 20°C, the Ca²⁺-dependence of [3H]ryanodine dissociation was similar in burbot and rat cardiac preparations with EC50 values of 0.087 ± 0.019 and 0.056 ± 0.039 μM, respectively (P = 0.40).

The rate constant of [3H]ryanodine dissociation was determined by incubating the fully saturated receptors in low Ca²⁺ solution (0.003 μM) for different times. At 2°C, [3H]ryanodine did not dissociate at all from rat cardiac RyRs, and in burbot cardiac preparations, the rate of dissociation was very low (k₋₁ = 0.0003 min⁻¹). At 10°C and 20°C, the rate of [3H]ryanodine dissociation was two to three times faster in burbot (k₋₁ = 0.004 min⁻¹ and 0.034 min⁻¹ at 10°C and 20°C, respectively) than rat (k₋₁ = 0.0019 min⁻¹ and 0.011 min⁻¹ at 10°C and 20°C, respectively) cardiac preparations (Fig. 6). Thus, similar to the association, the rate of dissociation was more strongly affected by low temperature in the rat (Q10 = 8.5 between 10°C and 20°C) than burbot (Q10 = 5.7) heart. However, in both species, the depressive effect of low temperature was stronger on the dissociation kinetics than on the association kinetics.

DISCUSSION

In endothermic animals, cardiac e-c coupling is critically dependent on SR Ca²⁺ release through ryanodine-sensitive Ca²⁺ channels, which open and close under the coordination of sytosolic Ca²⁺. RyRs of the mammalian heart do not, however, function properly at freezing temperatures but are open and release SR Ca²⁺ stores, as dramatically demonstrated by rapid cooling contractures (12). In cold-bodied ectotherms, this kind of RyR failure would impair the cardiac SR as a functional Ca²⁺ store in cytosolic Ca²⁺ management and is clearly at variance with findings that demonstrate enhanced SR function in cold-acclimated or cold-adapted ectotherms (1, 10, 24). In burbot, a fish species that inhabits cold environments, ryanodine treatment at 2°C turns rest-potentiation of force into rest-decay and reduces steady-state force production at physiological heart rates by 40% and 8% in atrial and ventricular muscle, respectively (24). This suggests not only that the burbot cardiac SR retains its Ca²⁺ load but also that ryanodinesensitive Ca²⁺ channels must be able to close and open on a beat-to-beat basis in the cold. Obviously, freezing temperatures do not totally abolish the function of cardiac RyRs in cold-adapted ectotherms.

In this study, we have used [3H]ryanodine binding assays to probe the functional state of the Ca²⁺ release channel. Interpretation of the present findings is based on assumptions that [3H]ryanodine binds to the high-affinity RyRs only when the Ca²⁺ release channel is open and, conversely, that [3H]ryanodine dissociates from its receptor site when the channel closes. Both opening and closing of the channel are assumed to be coordinated by free [Ca²⁺].

There is ample evidence for these assumptions (4, 6). A similar Ca²⁺ dependence of [3H]ryanodine binding and single-channel activity has confirmed that [3H]ryanodine binding can

\[
\text{Ca}^{2+} \quad \downarrow \quad \text{Ca}^{2+} \\
C \quad \text{O} \quad \text{O} \quad \text{Ry} \quad \uparrow \quad \text{C} \\
\text{k}_{-1} \quad \uparrow \quad \text{k}_{+1} \\
\text{Ry} \quad \text{Ry}
\]

Fig. 5. Ca²⁺-dependence of [3H]ryanodine dissociation from the high-affinity binding site of ventricular preparations of burbot and rat hearts at 20°C. Preparations were incubated with 10 nM [3H]ryanodine in a binding buffer containing 10 μM free Ca²⁺ for 3 h at 20°C to reach equilibrium. At that point, different amounts of EGTA were added to induce dissociation, which was measured after 2 h incubation. The results are expressed as means ± SE of three and six preparations for rat and burbot, respectively.
be used to assess the Ca\(^{2+}\) sensitivity of the cardiac Ca\(^{2+}\) release channel opening (28). Similar to \(^{3}H\)ryanodine association, the dissociation of \(^{3}H\)ryanodine from its receptors is primarily Ca\(^{2+}\) dependent and closely associated with channel gating: removal of Ca\(^{2+}\) induces closing of the channel and dissociation of \(^{3}H\)ryanodine from the receptor when the channel closes (6). Therefore, Ca\(^{2+}\)-dependent dissociation of \(^{3}H\)ryanodine from its receptor should give useful information on channel closing.

Ca\(^{2+}\)-dependence of \(^{3}H\)ryanodine binding. Saturation binding experiments indicate, in agreement with earlier findings (25), that the number of RyRs is higher in rat than burbot heart, but the \(K_d\) values are similar in both species. Also the Ca\(^{2+}\)-dependence of \(^{3}H\)ryanodine binding was practically identical in burbot and rat cardiac preparations at both 2°C and 20°C. From these experiments, it is clear that \(^{3}H\)ryanodine binding to cardiac SR, and thus opening of the RyRs, is closely coordinated by intracellular free [Ca\(^{2+}\)] in both burbot and rat. Because ryanodine binds only to open channels, the smooth Ca\(^{2+}\)-dependence of \(^{3}H\)ryanodine binding at 2°C suggests that RyRs of the rat heart remain closed at the diastolic Ca\(^{2+}\) level (<0.1 \(\mu M\)) and open in increasing numbers when Ca\(^{2+}\) concentration rises. Thus the leakiness of the mammalian SR at freezing temperatures is not due to the loss of Ca\(^{2+}\) coordination of opening. This agrees well with single-channel studies on mammalian cardiac RyRs: at diastolic Ca\(^{2+}\) concentrations, cooling the channel does not increase its open probability, but at higher Ca\(^{2+}\) concentrations, open probability and open lifetime of the channel are increased because of impaired transitions from open to closed conformation (21). Thus, at freezing temperatures, Ca\(^{2+}\) release channels of the mammalian heart somehow fail to revert to the closed state. The failure of RyR channels to close would impair the SR as a functional store, suggesting that RyRs of the cold-adapted ectotherms must somehow differ from those of the rat heart to retain their normal working cycle with well-coordinated open and closed states at low body temperatures.

Kinetics of \(^{3}H\)ryanodine association and dissociation. In contrast to the Ca\(^{2+}\) sensitivity of \(^{3}H\)ryanodine binding, there were significant species-specific differences in association and dissociation kinetics of \(^{3}H\)ryanodine binding. The rates of association and dissociation of \(^{3}H\)ryanodine were depressed at low temperatures, but the effect of temperature on both processes was two to three times stronger in rat than burbot. In the light of the assumed relationship between \(^{3}H\)ryanodine binding and gating mechanism, the faster rates of \(^{3}H\)ryanodine association and dissociation of burbot cardiac preparations in the cold represent faster gating transitions of the Ca\(^{2+}\) release channel from closed to open state and return to closed state, respectively.

The effect of temperature was remarkably stronger on dissociation than association binding, suggesting that closing of the Ca\(^{2+}\) release channel is especially strongly affected and is, therefore, the limiting factor for Ca\(^{2+}\) release channel function at low temperatures. In fact, at 2°C, \(^{3}H\)ryanodine did not dissociate at all from the rat cardiac RyRs, suggesting a complete failure of mammalian Ca\(^{2+}\) release channels to close (21) and therefore an inability of the CICR to operate. Fabia-to’s (8) experiments indicate that CICR of the mammalian heart still works at 12°C, although not as well as at 20°C. It is conceivable from the \(^{3}H\)ryanodine binding kinetics that burbot cardiac RyRs function better at lower temperatures and are thus able to participate in e-c coupling even at freezing temperatures (24). However, it is evident that, with increasing temperature, the operation of burbot cardiac RyRs markedly improves, which is compatible with the findings that the contribution of ryanodine-sensitive Ca\(^{2+}\) stores to e-c coupling increases with increasing temperature in the hearts of burbot and other fish (24, 17).

Physiological implications. The cardiac CICR mechanism is generally much better developed in endothermic than ectothermic animals, although there are clear interspecies differences among both endotherms and ectotherms (7). CICR is modulated by 1) SR Ca\(^{2+}\) load, 2) the amount and rate of change of the trigger Ca\(^{2+}\), 3) coupling between RyRs, 4) sensitivity of RyRs to Ca\(^{2+}\) (3, 8, 22), and 5) in ectotherms by temperature-dependence of RyR function (21). Any of these factors could be involved in the large interspecies differences to the extent in which ryanodine-sensitive Ca\(^{2+}\) stores contribute to contractile activation (for a review on fish hearts, see Ref. 27).
As a Ca\(^{2+}\) amplifier, the RyR detects small local Ca\(^{2+}\) signals from L-type Ca\(^{2+}\) channel (and possibly Na\(^{+}\)-Ca\(^{2+}\) exchanger), which appears as smoothly graded and tightly controlled Ca\(^{2+}\) release in response to whole cell and single-channel Ca\(^{2+}\) currents (13). Mathematical modeling of Ca\(^{2+}\) release events suggests that the sensitivity of RyR to Ca\(^{2+}\) activation is an important qualification in determining the gain and stability of e-c coupling (22). Thus the striking differences in Ca\(^{2+}\) dependence of RyR activation between different fish species might have significant physiological consequences. The cardiac RyRs of the most Ry-resistant fish species, crucian carp, required 5.9 times more Ca\(^{2+}\) for half-maximal activation, and those of the trout heart required about 4.4 times more Ca\(^{2+}\) compared with RyRs of rat and burbot hearts. Similarly, the Ca\(^{2+}\) sensitivity of the cardiac RyRs in the common carp (Cyprinus carpio) has been found to be six times less sensitive than those in the rat (5). Only the RyRs of the burbot heart are equally sensitive to Ca\(^{2+}\) as the RyRs of the rat heart. Clearly, the relatively low Ca\(^{2+}\) sensitivity of the cardiac RyRs may be a contributing factor to weak CICR in many fish species.

CICR is a summation of local Ca\(^{2+}\) release events or sparks, each of which consists of regenerative Ca\(^{2+}\) release among a cluster of several RyRs. Spark generation is a function of the single Ca\(^{2+}\) release channel conductance, the Ca\(^{2+}\) sensitivity of RyRs, and the number of channels in the cluster, such that, smaller unitary currents require higher RyR Ca\(^{2+}\) sensitivity and bigger cluster size to generate a spark (22). The low Ca\(^{2+}\) sensitivity of fish cardiac RyRs associated with reduced single-channel currents at low temperatures may not favor the generation of Ca\(^{2+}\) sparks. Indeed, Ca\(^{2+}\) sparks seem to occur very seldom in trout cardiac myocytes (20). Burbot seems to be an exception among the number of fish species studied, in that its cardiac RyRs are highly sensitive to Ca\(^{2+}\) and possibly better able to generate Ca\(^{2+}\) sparks, even at low temperatures.

**Limitations of the study.** In the present study, \[^{3}H\]ryanodine binding assays were used to probe temperature and Ca\(^{2+}\) dependence of SR Ca\(^{2+}\) release channel gating. Although the usefulness of the method has been validated by previous studies (4, 6), it is, however, an indirect procedure that uses optimized binding conditions to improve the resolution of responses. Single-channel studies in lipid bilayers under more optimized binding conditions to improve the resolution of single-channel studies (4, 6), it is, however, an indirect procedure that uses optimized binding conditions to improve the resolution of responses. Single-channel studies in lipid bilayers under more physiological ionic conditions will be needed to verify the physiological significance of the present findings and, especially, to provide clearer description of the underlying gating mechanisms. The present experiments were conducted at constant pH, which does not exactly correspond to the physiological condition in ectothermic animals. Because ectothermic animals are likely to experience relatively large temperature-related changes of intracellular pH and as RyRs are sensitive to alterations of H\(^{+}\) concentration (28), future studies should also address the effect of pH on RyR function. Irrespective of these limitations, the present results provide strong evidence that low temperature depresses the kinetics of association and dissociation binding of \[^{3}H\]ryanodine much less in cold stenothermic burbot than in endothermic rat. This suggests that the gating mechanism of the cardiac Ca\(^{2+}\) release channel of ectothermic animals is adapted to function at lower temperatures.

**ACKNOWLEDGMENTS**

Anita Kervinen is acknowledged for technical assistance.

**GRANTS**

This study was financed by the Academy of Finland Grant 78045.

**REFERENCES**


6. **Du GG, Guo X, Khanna VK, and MacLennan DH.** Ryanodine sensitizes the cardiac Ca\(^{2+}\) release channel (ryanodine receptor isof orm 2) to Ca\(^{2+}\) activation and dissociates as the channel is closed by Ca\(^{2+}\) depletion. *Proc Natl Acad Sci USA* 98: 13625–13630, 2001.

7. **Fabiato A.** Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am J Physiol Cell Physiol* 245: C1–C14, 1983.


15. **Milnes JT and MacLeod CT.** Reduced ryanodine receptor to dihydropyridine receptor ratio may underline slowed contraction in a rabbit model of left ventricular cardiac hypertrophy. *J Mol Cell Cardiol* 33: 473–485, 2001.


28. Xu L, Mann G, and Meissner G. Regulation of cardiac Ca²⁺ release channel (ryanodine receptor) by Ca²⁺, H⁺, Mg²⁺, and adenine nucleotides under normal and simulated ischemic conditions. Circ Res 79: 1100–1109, 1996.