

## Ca<sup>2+</sup>-ATPase ACTIVITY AND Ca<sup>2+</sup> UPTAKE BY SARCOPLASMIC RETICULUM IN FISH HEART: EFFECTS OF THERMAL ACCLIMATION

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### Summary

This study was designed to compare the activities of sarcoplasmic (SR) Ca<sup>2+</sup>-ATPase and Ca<sup>2+</sup> uptake in fish and mammalian hearts and to determine whether thermal acclimation has any effect on the function of the cardiac SR in fish. To this end, we measured thapsigargin-sensitive Ca<sup>2+</sup>-ATPase activity and thapsigargin-inhibitable Ca<sup>2+</sup> uptake velocity in crude cardiac homogenates of newborn and adult rats and of two teleost fish (crucian carp and rainbow trout) acclimated to low (4 °C) and high (17 °C and 24 °C for trout and carp, respectively) ambient temperatures.

The TG-sensitive Ca<sup>2+</sup>-ATPase activity was highest in adult rat, and the corresponding activities of cold-acclimated trout, warm-acclimated trout, warm-acclimated carp, cold-acclimated carp and newborn rat were 76, 58, 43, 28 and 23%, respectively, of that of the adult rat at 25 °C. SR Ca<sup>2+</sup> uptake velocity, measured using Fura-2 at room temperature (approximately 22 °C), was highest in cold-acclimated trout, and the values for adult rat, warm-acclimated trout, newborn rat, warm-acclimated carp and cold-acclimated carp were 93, 56, 24,

21 and 14% of the uptake velocity of cold-acclimated trout, respectively. When corrected to the body temperature of the animal, the relative rates of SR Ca<sup>2+</sup> uptake 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 show that SR Ca<sup>2+</sup> uptake is slower in fish than in mammalian hearts and that marked species-specific differences exist among teleost fish in this respect. Furthermore, acclimation to cold increases the Ca<sup>2+</sup> uptake rate of trout cardiac SR (complete thermal compensation) but decreases the SR Ca<sup>2+</sup> uptake rate of crucian carp heart. This difference in acclimation response probably reflects the different activity patterns of the two species in their natural habitat during the cold season.

Key words: temperature acclimation, fish heart, sarcoplasmic reticulum, Ca<sup>2+</sup> uptake, excitation–contraction coupling, thapsigargin, Fura-2, rainbow trout, crucian carp, *Carassius carassius*, *Oncorhynchus mykiss*.

### Introduction

Sarcoplasmic reticulum (SR) participates in the contraction and relaxation of cardiac muscle by a Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release mechanism and an ATP-dependent resequestration process, respectively. Ryanodine-sensitive Ca<sup>2+</sup> efflux channels are confined to the junctional and corbular SR, while thapsigargin- and cyclopiazonic-acid-inhibitable Ca<sup>2+</sup>-pumping ATPase molecules are localized both in junctional and in nonjunctional SR (Feher *et al.* 1988; Jorgensen *et al.* 1993). The significance of SR in the contraction–relaxation cycle of cardiac muscle varies greatly among different vertebrate classes, among different species within the same phylogenetic group and during the ontogenetic development of an individual. The SR is well developed in mammalian and avian cardiac myocytes, where it is thought to be the major source and sink of the activator Ca<sup>2+</sup>. In ectothermic vertebrates, such as lizards, frogs and fishes, and in newborn mammals, cardiac SR is relatively

sparse, and contraction and relaxation are more directly dependent on sarcolemmal Ca<sup>2+</sup> fluxes (Fabiato, 1982; Bossen and Sommer, 1984; Vornanen, 1996a,b).

Electron microscopic findings suggest that there are marked differences in the amount of cardiac SR among fish species (Santer, 1985), although rigorous morphometric analyses of SR in teleost species are still lacking. Species-specific differences are also evident in the inhibition of contraction by ryanodine: in crucian carp heart, ryanodine has no effect on ventricular contraction (Vornanen 1996a); in rainbow trout ventricle, ryanodine slightly reduces the force of contraction, especially at high experimental temperatures and at low contraction frequencies (Keen *et al.* 1994); in the atrium of tuna heart, ryanodine exerts a clear negative inotropic effect (Keen *et al.* 1992). These findings suggest that the more active fish have a higher Ca<sup>2+</sup>-handling capacity in the cardiac SR than the less active species. Furthermore, recent studies suggest

that thermal acclimation may alter the excitation–contraction coupling process of the fish heart. Morphometric analyses of perch heart suggest that SR is better developed in cold-acclimated than in warm-acclimated fish (Bowler and Tirri, 1990), and functional studies in thermally acclimated trout have shown that contraction is more sensitive to ryanodine inhibition in cold-acclimated than in warm-acclimated animals (Keen *et al.* 1994). However, to our knowledge, the effects of thermal acclimation on the activity of Ca<sup>2+</sup>-ATPase and the Ca<sup>2+</sup> uptake velocity of cardiac SR have not been directly determined in the fish heart. Therefore, the aim of the present study was to measure the Ca<sup>2+</sup>-ATPase activity and Ca<sup>2+</sup> uptake velocity of cardiac SR in two fish species, crucian carp and rainbow trout, species that differ in activity pattern and in acclimation response to changing ambient temperature. Furthermore, the function of fish cardiac SR was compared with that of newborn and adult rats to give a more comprehensive picture of the Ca<sup>2+</sup>-handling capacity of fish heart SR. The results show that Ca<sup>2+</sup> uptake by cardiac SR is slower in fish than in mammalian ventricle and that it is significantly faster in trout than in crucian carp heart. Acclimation to cold increases SR Ca<sup>2+</sup> uptake activity in trout, but decreases it in crucian carp.

## Materials and methods

### Animals

Crucian carp, *Carassius carassius* L. ( $N=120$ ), were collected in May and June (1995, 1996, 1997) from local ponds within 7 km of the campus area of the University of Joensuu in eastern Finland. The fish were randomly divided in two groups and were acclimated at 4 °C or 24 °C in the laboratory for more than 4 weeks. The body masses of warm-acclimated and cold-acclimated carp were  $34.4 \pm 2.4$  g ( $N=68$ ) and  $29.8 \pm 1.4$  g ( $N=52$ ) (mean  $\pm$  S.E.M.,  $P=0.12$ ), respectively. Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] ( $N=33$ ), were obtained from a local fish farm. The trout were acclimated at 4 or 17 °C for a minimum of 4 weeks. The mean body masses of warm-acclimated and cold-acclimated trout were  $227.9 \pm 31.5$  g ( $N=13$ ) and  $210.4 \pm 36.7$  g ( $N=15$ ) ( $P=0.72$ ), respectively. During the acclimation, the fish were held in 500 l stainless-steel aquaria with a continuous circulation of aerated tap (ground) water. The fish were fed five times a week with commercial fish pellets (Ewos, Sweden). Photoperiod was a constant 12 h:12 h light:dark cycle. The rats were Sprague-Dawley strain. Adult rats were 3–6 months in age, neonates were used within 12 h of birth. Fish were stunned by a blow on the head and killed by cutting the spine. Rats were killed by cervical dislocation under light anaesthesia before the hearts were removed.

### Determination of Ca<sup>2+</sup>-ATPase activities

The amount of ventricular tissue needed for the determination of SR Ca<sup>2+</sup>-ATPase was approximately 50 mg. A portion of rainbow trout ventricle was sufficient for one preparation, while whole ventricles from 3–4 crucian carp had to be pooled for one homogenate. A small piece from the apex

of an adult rat ventricle and a whole ventricle from a newborn rat were used for the preparation of one homogenate. The Ca<sup>2+</sup>-dependent ATPase activity of SR was determined from unfractionated ventricular homogenates using two different methods. The Ca<sup>2+</sup>-ATPase activity of SR can be selectively inhibited at high (28 mmol l<sup>-1</sup>) Ca<sup>2+</sup> concentration, which saturates the low-affinity inhibitory binding site on the enzyme. This feedback inhibition phenomenon was exploited to determine SR Ca<sup>2+</sup>-ATPase activity according to the procedure developed by Simonides and van Hardeveld (1990) for skeletal muscle homogenates (method 1). In the second assay procedure (method 2), SR Ca<sup>2+</sup>-ATPase was specifically inhibited with 20  $\mu$ mol l<sup>-1</sup> thapsigargin (TG) (Sagara and Inesi, 1991).

Ventricular muscle was minced with scissors and homogenised in 10 volumes of cold sucrose–histidine buffer containing (in mmol l<sup>-1</sup>): sucrose, 200; L-histidine, 40; EDTA, 1; NaN<sub>3</sub>, 10; pH 7.8) with a glass homogeniser (Heidolph) using three 10 s periods each separated by a 30 s interval. The final dilution of homogenates was 1:30. In method 1, the Ca<sup>2+</sup>-ATPase activity of SR was determined as the difference in the rate of ATP splitting in the presence of low (1 mmol l<sup>-1</sup>; pCa 5.0) and high (28 mmol l<sup>-1</sup>; pCa 1.6) CaCl<sub>2</sub> concentrations (Simonides and Van Hardeveld, 1990). The final incubation medium contained (in mmol l<sup>-1</sup>): Hepes, 20; KCl, 200; MgCl<sub>2</sub>, 15; NaN<sub>3</sub>, 10; EGTA, 1; Na<sub>2</sub>ATP, 5; and CaCl<sub>2</sub>, 1 or 28; at pH 7.5. Furthermore, Triton X-100 (0.005%) was included to make sealed membrane vesicles leaky. The enzyme reaction was initiated by adding 0.1 ml of the homogenate and terminated after 10 min of incubation at 25 °C with acid phosphomolybdate reagent. Samples were centrifuged at 1000g for 5 min to sediment the tissue, and liberated inorganic phosphate was determined according to Atkinson *et al.* (1973). In method 2, the activity of SR Ca<sup>2+</sup>-ATPase was determined as the TG-sensitive portion of the total Ca<sup>2+</sup>-activated ATPase activity in the low-Ca<sup>2+</sup> (1 mmol l<sup>-1</sup>) medium. SR homogenates were incubated for 10 min in 1 ml of low-Ca<sup>2+</sup> medium in the presence and absence of 20  $\mu$ mol l<sup>-1</sup> TG at 25 °C. The released inorganic phosphate was determined in the protein-free supernatant as described above. Homogenate protein concentration was determined using the method of Lowry (Lowry *et al.* 1951).

The effect of TG (20  $\mu$ mol l<sup>-1</sup>) on myofibrillar Ca<sup>2+</sup>-ATPase was also determined. Ventricular muscle (approximately 150 mg) was collected from 10–12 crucian carp, and myofibrils were purified as described previously (Vornanen, 1996a). Purified myofibrils were suspended in a buffer of low ionic strength containing 45 mmol l<sup>-1</sup> imidazole and 50 mmol l<sup>-1</sup> KCl at pH 7.0. Myofibrillar Ca<sup>2+</sup>/Mg<sup>2+</sup>-ATPase activity was determined in buffer solution containing (in mmol l<sup>-1</sup>): imidazole, 45; KCl, 50; EGTA, 5; MgCl<sub>2</sub>, 5; Na<sub>2</sub>ATP, 3; and CaCl<sub>2</sub>, 5.137; to give a free [Ca<sup>2+</sup>] of 100  $\mu$ mol l<sup>-1</sup> (calculated according to Fabiato, 1988) at pH 7.0. The difference in ATPase activity in the presence and absence of Ca<sup>2+</sup> was taken as myofibrillar Ca<sup>2+</sup>/Mg<sup>2+</sup>-ATPase activity.

### Ca<sup>2+</sup> uptake in the sarcoplasmic reticulum

ATP-dependent Ca<sup>2+</sup> uptake in the SR was measured

spectrophotometrically in crude ventricular homogenates using a Ca<sup>2+</sup>-selective fluorescent dye, Fura-2 (Hove-Madsen and Bers, 1993b; Kargacin and Kargacin, 1994). Ventricular homogenates were prepared in a medium containing (in mmol l<sup>-1</sup>): Hepes, 20; KCl, 100; and MgCl<sub>2</sub>, 4; pH 7.0. Ca<sup>2+</sup> uptake was measured in a buffer containing (in mmol l<sup>-1</sup>): KCl, 100; Hepes, 20; MgCl<sub>2</sub>, 4; oxalate, 10; Na<sub>2</sub>ATP, 1.25; and creatine phosphate, 1.25 (pH 7.0). Creatine phosphokinase (0.4 units ml<sup>-1</sup>) was included in the medium to regenerate ATP. Fura-2 was added to a final concentration of 2 μmol l<sup>-1</sup>. Ca<sup>2+</sup> uptake was initiated by the addition of 1 mmol l<sup>-1</sup> CaCl<sub>2</sub> stock solution to give a final concentration of 2.5 or 5 μmol l<sup>-1</sup>. The uptake medium and all other constituents were mixed by turning the capped cuvette (4 ml) quickly upside down. Fluorescence was measured using a Shimadzu RF-5000 fluorimeter at room temperature (22 °C). The excitation wavelength alternated between 340 and 380 nm at a frequency of 0.5 Hz. Emission was monitored at 510 nm. The emission signal was integrated for 2 s at various times following Ca<sup>2+</sup> addition. In all steps of fluorescence measurement, special care was taken to avoid Ca<sup>2+</sup> contamination from glassware and chemicals. Glassware was routinely soaked in 1 mmol l<sup>-1</sup> EGTA and thoroughly rinsed in Millipore water (resistance >17 MΩ) before use. Hepes (Sigma), oxalate (Fluka), MgCl<sub>2</sub> (Fluka), CaCl<sub>2</sub> and KCl (Merck) were of the highest quality available. Creatine phosphate, creatine phosphokinase and Na<sub>2</sub>ATP were obtained from Sigma. Fura-2 (Sigma) was dissolved in dimethyl sulphoxide at 1 mmol l<sup>-1</sup> and stored frozen in small samples.

The concentration of free calcium in the cuvette and its first derivative were calculated from Fura-2 fluorescence after smoothing of the original fluorescence signal using moving window averaging (SigmaPlot, Jandel Scientific). The concentration of free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>free</sub>) was calculated from the Fura-2 fluorescence according to the equation (Gryniewicz *et al.* 1985):

$$[\text{Ca}^{2+}]_{\text{free}} = K_d[(R - R_{\text{min}})/(R_{\text{max}} - R)] \times \beta, \quad (1)$$

where  $R$  is the ratio of emission intensity at 340 and 380 nm excitation, and  $K_d$  is the dissociation constant for the Fura-2/Ca<sup>2+</sup> complex and was assumed to be 200 nmol l<sup>-1</sup>. Values for  $R_{\text{min}}$ ,  $R_{\text{max}}$  and  $\beta$  were determined for the buffer conditions of the experiments and were 0.67, 20.5 and 4.39, respectively. Ca<sup>2+</sup> bound to Fura-2, oxalate and protein was derived from [Ca<sup>2+</sup>]<sub>free</sub>, the concentrations of the various Ca<sup>2+</sup> buffers and their dissociation constant for Ca<sup>2+</sup>. Ca<sup>2+</sup> bound to Fura-2 was calculated as:

$$[\text{Ca}^{2+}/\text{Fura}] = [\text{Fura}]_{\text{total}} - [(K_d[\text{Fura}]_{\text{total}})/(K_d + [\text{Ca}^{2+}]_{\text{free}})]. \quad (2)$$

Ca<sup>2+</sup> bound to oxalate (10 mmol l<sup>-1</sup>) was calculated analogously using a  $K_d$  of 4.0 mmol l<sup>-1</sup> (Hove-Madsen and Bers, 1993a). Cardiac protein was assumed to have one low-affinity ( $K_d$  79 μmol l<sup>-1</sup>) and one high-affinity ( $K_d$  0.42 μmol l<sup>-1</sup>) binding site, as described by Hove-Madsen and Bers (1993a). The concentrations of the low-affinity and high-affinity binding sites were 4.13 and 1.27 nmol mg<sup>-1</sup> protein

(Hove-Madsen and Bers, 1993a), respectively, and were presumed to be the same for all cardiac preparations. The total [Ca<sup>2+</sup>] in the cuvette was then calculated as the sum of [Ca<sup>2+</sup>]<sub>free</sub> plus the Ca<sup>2+</sup> bound to oxalate (Ca<sup>2+</sup>/Ox), protein (Ca<sup>2+</sup>/Pr-ha and Ca<sup>2+</sup>/Pr-la, where ha signifies high affinity and la signifies low affinity) and Fura-2 (Ca<sup>2+</sup>/Fura) (see Fig. 3C). [Ca<sup>2+</sup>]<sub>free</sub> varied slightly from preparation to preparation owing to the differences in protein content of the different tissue samples. Therefore, the Ca<sup>2+</sup> uptake velocity was determined at a constant [Ca<sup>2+</sup>]<sub>free</sub> of 0.4 μmol l<sup>-1</sup>, which is close to the  $K_m$  of the SR Ca<sup>2+</sup>-ATPase. The velocity of SR Ca<sup>2+</sup> uptake is expressed as μmol Ca<sup>2+</sup> g<sup>-1</sup> wet tissue mass min<sup>-1</sup>.

The temperature coefficient ( $Q_{10}$ ) of TG-sensitive Ca<sup>2+</sup> uptake was determined for cold-acclimated trout, cold-acclimated crucian carp and adult rat. To this end, the Ca<sup>2+</sup> uptake velocity was first measured at room temperature, and the temperature of the thermostatted cuvette compartment of the fluorimeter was then either lowered to 12 °C (trout and carp) or raised to 32 °C (rat) for Ca<sup>2+</sup> uptake determinations at the second temperature.  $Q_{10}$  values were calculated and used to determine the Ca<sup>2+</sup> uptake velocity at the physiological body temperature of the animal. The body temperatures of warm-acclimated trout and warm-acclimated carp were close to the experimental temperature, and any small deviation was corrected using the  $Q_{10}$  values of the cold-acclimated fish.

### Statistics

All results are given as mean ± S.E.M. Differences between species, acclimation temperature and age groups were compared by one-way analysis of variance (ANOVA) and *post-hoc* Student–Newmann–Keuls tests. Before statistical analysis, the data were log<sub>e</sub>-transformed to fulfil the assumptions of the parametric test. The differences were considered to be significant at  $P < 0.05$ .

## Results

### *Thapsigargin-inhibitable Ca<sup>2+</sup>-ATPase activity*

The crude homogenate contains a number of cation-activated ATPases including those of the mitochondria, myofibrils and sarcoplasmic reticulum. Although TG is a highly specific inhibitor of SR Ca<sup>2+</sup>-ATPase, without effect on plasma membrane Ca<sup>2+</sup>-ATPase or Na<sup>+</sup>/K<sup>+</sup>-ATPase (Lytton *et al.* 1991), the experimental conditions should be selected to optimize the expression of SR Ca<sup>2+</sup>-ATPase and to minimize background activities. Under the experimental conditions used in this study, approximately 80 % of the total ATPase was due to the background ATPase activities. Since mitochondrial ATPase activity was abolished by 10 mmol l<sup>-1</sup> azide, the background activity probably originates from the myofibrils. Therefore, it was necessary to ascertain that actomyosin ATPase does not contribute to the TG-sensitive activity. At 20 μmol l<sup>-1</sup>, TG neither inhibited nor activated the Ca<sup>2+</sup>/Mg<sup>2+</sup>-ATPase of purified myofibrils, suggesting that the TG-sensitive ATPase is not contaminated by actomyosin ATPase (Fig. 1A). The effectiveness of TG as an inhibitor of SR Ca<sup>2+</sup>-ATPase in

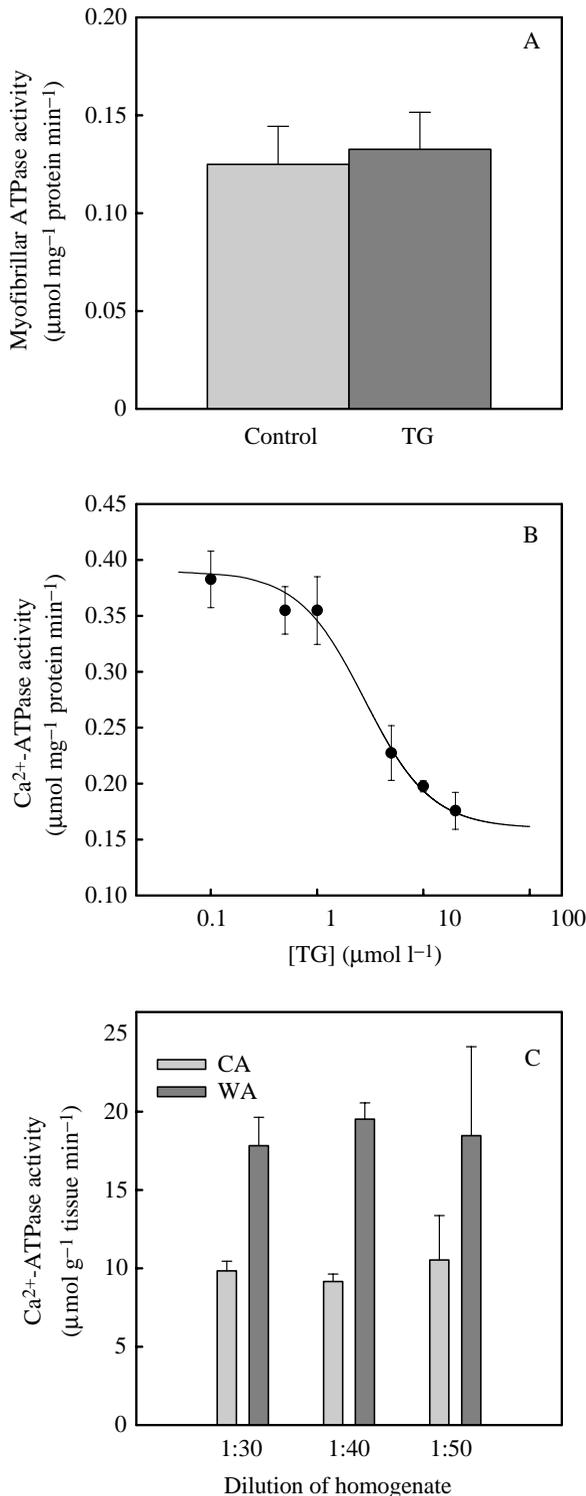


Fig. 1. Effects of thapsigargin (TG) on  $\text{Ca}^{2+}$ -ATPase activities of crude cardiac homogenates and purified myofibrils from crucian carp heart. (A) TG ( $20 \mu\text{mol l}^{-1}$ ) has no effect on  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity of purified cardiac myofibrils. (B) TG inhibits the  $\text{Ca}^{2+}$ -activated ATPase of crude cardiac homogenates in a dose-dependent manner. (C) Inhibition of the  $\text{Ca}^{2+}$ -ATPase of cardiac homogenates by TG ( $20 \mu\text{mol l}^{-1}$ ) is independent of the dilution of the homogenate. Results are means  $\pm$  S.E.M. of four preparations. CA, cold-acclimated; WA, warm-acclimated.

cardiac homogenates was examined by incubating homogenates in the presence of different TG concentrations. Half-maximal inhibition occurred at  $1.6 \mu\text{mol l}^{-1}$  and maximal inhibition was obtained at a TG concentration of  $20 \mu\text{mol l}^{-1}$  (Fig. 1B). As a lipophilic compound, TG shows a high level of nonspecific binding, which may reduce its effectiveness as an inhibitor of SR  $\text{Ca}^{2+}$ -ATPase. Therefore, the inhibition of total  $\text{Ca}^{2+}$ -dependent ATPase activity by  $20 \mu\text{mol l}^{-1}$  TG was tested at different homogenate dilutions. The inhibition of SR  $\text{Ca}^{2+}$ -ATPase was independent of the dilution of the homogenate within the range used in the present experiments (Fig. 1C). The above findings suggest that SR  $\text{Ca}^{2+}$ -ATPase can be confidently assayed in crude cardiac homogenates as the TG-sensitive ATPase activity.

Fig. 2 shows  $\text{Ca}^{2+}$ -ATPase activities of SR from trout, carp and rat determined as ATPase activity inhibitable by  $20 \mu\text{mol l}^{-1}$  TG (Fig. 2A) or high concentration of  $\text{Ca}^{2+}$  (Fig. 2B). The TG-sensitive  $\text{Ca}^{2+}$ -ATPase activity was greatest in adult rat, and the corresponding activities of cold-acclimated trout, warm-acclimated trout, warm-acclimated carp, cold-acclimated carp and newborn rat were 76, 58, 43, 28 and 23 %,

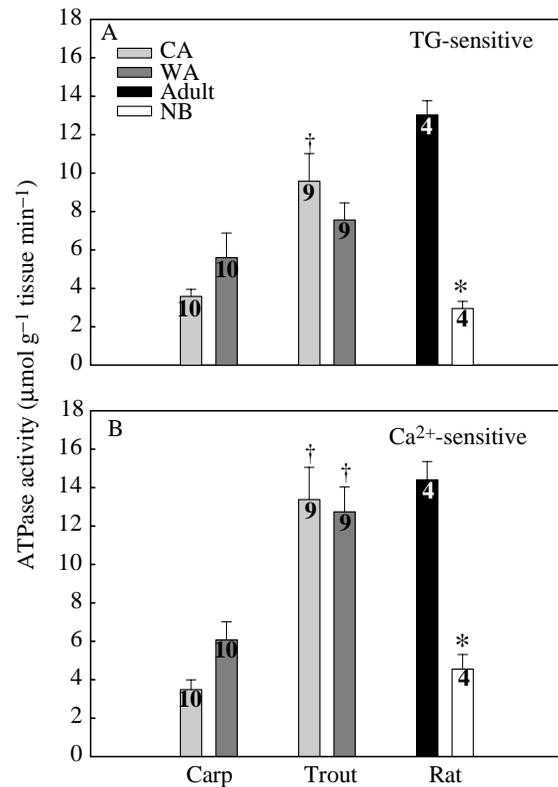


Fig. 2. Sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -ATPase activities of crude cardiac homogenates from rainbow trout, crucian carp and rat.  $\text{Ca}^{2+}$ -ATPase activity was determined at  $25^\circ\text{C}$  as the portion of the total ATPase activity inhibited by  $20 \mu\text{mol l}^{-1}$  thapsigargin (TG) (A) or by high  $[\text{Ca}^{2+}]$  ( $28 \text{ mmol l}^{-1}$ ) (B). The results are means  $\pm$  S.E.M. of 4–10 preparations as indicated. An asterisk indicates a statistically significant difference ( $P < 0.05$ ) between adult and newborn (NB) rat. A dagger indicates a statistically significant difference ( $P < 0.05$ ) between trout and carp. CA, cold-acclimated; WA, warm-acclimated.

respectively, of that of the adult rat at 25 °C. Similar activities and species order were obtained when SR Ca<sup>2+</sup>-ATPase was determined as the Ca<sup>2+</sup>-inhibitable portion of the total ATPase activity. However, unlike Ca<sup>2+</sup> uptake rates, the differences in ATPase activities between acclimation groups were not statistically significant (see below).

*Ca<sup>2+</sup> uptake in the sarcoplasmic reticulum*

Fura-2 was used to monitor ATP-dependent and oxalate-supported Ca<sup>2+</sup> uptake of the SR continuously in crude cardiac

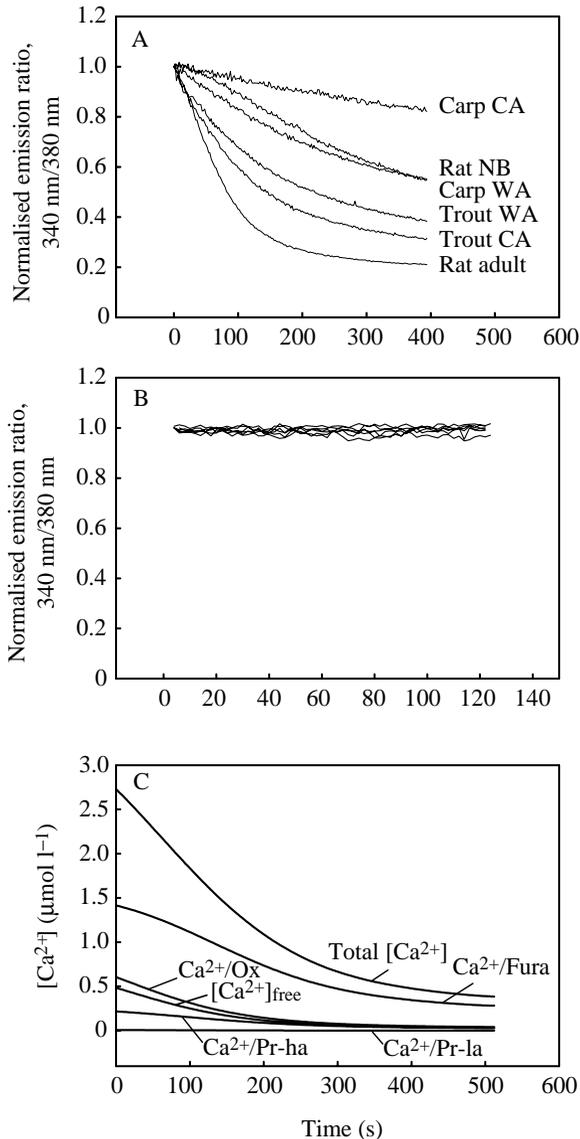


Fig. 3. (A) Recordings of sarcoplasmic reticulum (SR) Ca<sup>2+</sup> uptake by crude cardiac homogenates from trout, carp and rat monitored by the fluorescence emission ratio at 340 nm/380 nm. (B) Ca<sup>2+</sup> uptake was completely inhibited by a 3 min preincubation of homogenates with 20 µmol l<sup>-1</sup> thapsigargin. (C) The total [Ca<sup>2+</sup>] in the cuvette was calculated as the sum of [Ca<sup>2+</sup>]<sub>free</sub> plus Ca<sup>2+</sup> bound to oxalate (Ca<sup>2+</sup>/Ox), protein (Ca<sup>2+</sup>/Pr-ha, Ca<sup>2+</sup>/Pr-la) and Fura-2 (Ca<sup>2+</sup>/Fura). CA, cold-acclimated; WA, warm-acclimated; NB, newborn; ha, high affinity; la, low affinity.

homogenates. As Ca<sup>2+</sup> is transported into the SR, the cytosolic [Ca<sup>2+</sup>]<sub>free</sub> declines and the total Ca<sup>2+</sup> uptake by the SR can be calculated from [Ca<sup>2+</sup>]<sub>free</sub> and the known concentrations of Ca<sup>2+</sup> buffers in the uptake medium (Fig. 3). Accumulation of Ca<sup>2+</sup> within SR results in the formation of calcium oxalate crystals, which effectively block Ca<sup>2+</sup> efflux (Palade, 1987). Ruthenium Red was not used to block Ca<sup>2+</sup> leakage since, at 30 µmol l<sup>-1</sup>, it caused variable inhibition of Ca<sup>2+</sup> uptake both in fish and in mammalian preparations. The contribution of mitochondrial Ca<sup>2+</sup> uptake to Fura-2 fluorescence is excluded since [Ca<sup>2+</sup>]<sub>free</sub> in the uptake medium was less than 3 µmol l<sup>-1</sup>, which is below the threshold concentration for mitochondrial Ca<sup>2+</sup> uptake (Hove-Madsen and Bers, 1993a). Fig. 3A shows changes in Fura-2 fluorescence due to Ca<sup>2+</sup> uptake by various cardiac preparations. It is evident that the rate of Ca<sup>2+</sup> uptake is fastest in adult rat, intermediate in trout and relatively slow in carp and newborn rat. The oxalate-supported uptake of Ca<sup>2+</sup> was completely inhibited when homogenates were pretreated with 20 µmol l<sup>-1</sup> TG for 3 min (Fig. 3B).

Since [Ca<sup>2+</sup>]<sub>free</sub> varied slightly from experiment to experiment, the Ca<sup>2+</sup> uptake rate was calculated at a constant [Ca<sup>2+</sup>]<sub>free</sub> of 0.4 µmol l<sup>-1</sup>. SR Ca<sup>2+</sup> uptake velocity, measured using Fura-2 at room temperature (22 °C), was greatest in cold-acclimated trout, and the values for adult rat, warm-acclimated trout, newborn rat, warm-acclimated carp and cold-acclimated carp were 93, 56, 24, 21 and 14 %, respectively, of the uptake rate of cold-acclimated trout (Fig. 4). Therefore, the species order for SR Ca<sup>2+</sup> uptake was: cold-acclimated trout ≈ adult rat > warm-acclimated trout > newborn rat ≈ warm-acclimated carp > cold-acclimated carp (where > indicates a statistically significant difference). Furthermore, there was good correlation between Ca<sup>2+</sup>-ATPase activity and Ca<sup>2+</sup> uptake rate (Fig. 5), with a slope close to 1.0 for the percentage values (not shown). The major difference between ATPase activity and Ca<sup>2+</sup> uptake velocity in absolute terms is partly because Ca<sup>2+</sup>-

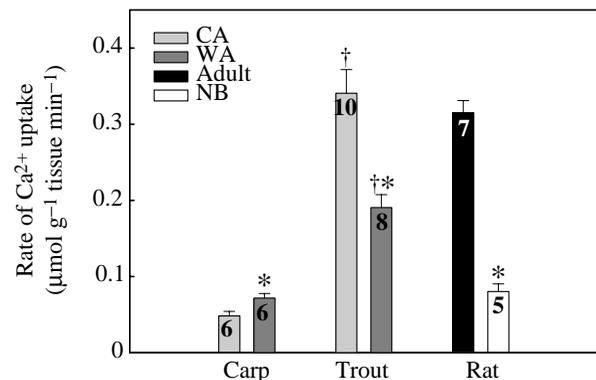


Fig. 4. Sarcoplasmic reticulum (SR) Ca<sup>2+</sup> uptake rate of crude cardiac homogenates from crucian carp, rainbow trout and rat. The results are means + S.E.M. of 5–10 preparations as indicated. An asterisk indicates a statistically significant difference (*P*<0.05) between age groups or acclimation groups. A dagger indicates a statistically significant difference (*P*<0.05) between trout and carp. CA, cold-acclimated; WA, warm-acclimated; NB, newborn.

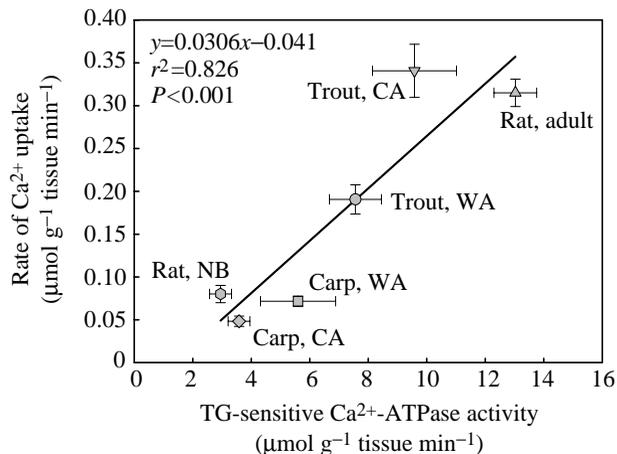


Fig. 5. Correlation between thapsigargin (TG)-sensitive ATPase activity and TG-sensitive  $\text{Ca}^{2+}$  uptake rate of crude cardiac homogenates from rainbow trout, crucian carp and rat. The results are means  $\pm$  S.E.M. of 4–10 preparations. CA, cold-acclimated; WA, warm-acclimated; NB, newborn.

ATPase activity was determined under conditions that allow maximal enzyme activity, while the rate of  $\text{Ca}^{2+}$  uptake was measured at a  $\text{Ca}^{2+}$  concentration close to the  $K_m$  of the SR  $\text{Ca}^{2+}$  pump.

Since the physiological body temperatures of the animals were markedly different, the measured  $\text{Ca}^{2+}$  uptake velocities were transformed to the respective body temperature of the species.  $Q_{10}$  values were 1.75, 1.64 and 1.79 for cold-acclimated carp, cold-acclimated trout and adult rat ( $N=2$  for each), respectively. When corrected to the body temperature of the animal, the relative rates of SR  $\text{Ca}^{2+}$  uptake 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.

### Discussion

The small body mass of many fish species, the small relative size of fish hearts and the relatively low content of SR in fish cardiac myocytes make species comparisons with respect to SR  $\text{Ca}^{2+}$ -handling capacity difficult. Quantitative estimates of the  $\text{Ca}^{2+}$ -ATPase activity and  $\text{Ca}^{2+}$  uptake rate of SR between different animal species are further complicated by the unpredictable loss of SR membranes and the variable inactivation of the ATPase during the long isolation procedures (Feher and LeBolt, 1990). The recent discovery of specific inhibitors for SR  $\text{Ca}^{2+}$ -ATPase and the use of  $\text{Ca}^{2+}$ -specific fluorescent indicators have enabled  $\text{Ca}^{2+}$ -ATPase activity and SR  $\text{Ca}^{2+}$  uptake to be analysed directly in isolated cardiac myocytes or unfractionated skeletal muscle homogenates, thus avoiding many of these problems. We have used these novel tools to study the effects of thermal acclimation on fish cardiac SR and to compare  $\text{Ca}^{2+}$  handling in the SR of fish and mammalian hearts.

### Phylogenetic and species differences

In the present study, SR  $\text{Ca}^{2+}$ -ATPase activity and  $\text{Ca}^{2+}$  uptake rate of fish and mammalian hearts were determined at the same experimental temperature, which allows direct species comparisons of SR  $\text{Ca}^{2+}$ -handling capacity. It is evident that, in crucian carp and newborn rat heart, SR  $\text{Ca}^{2+}$  uptake is relatively slow, while in rainbow trout and adult rat heart SR  $\text{Ca}^{2+}$  uptake is much faster. These findings agree well with previous observations on both mammalian and fish hearts. It is generally accepted that, in the newborn rat ventricle, the SR is both structurally and functionally weakly developed, while in the adult rat heart the SR is the major source and sink of the activator  $\text{Ca}^{2+}$  (Fabiato, 1982; Bers, 1985; Vornanen, 1996b). In our experiments using Fura-2, the SR  $\text{Ca}^{2+}$  uptake rate of newborn rat heart was approximately one-quarter of that in the adult rat, which is in good agreement with previous findings on the amount of SR  $\text{Ca}^{2+}$ -ATPase mRNA (Lompre *et al.* 1991) and the rate of  $^{45}\text{Ca}^{2+}$  uptake in the developing rat heart (Vetter *et al.* 1995). The SR  $\text{Ca}^{2+}$  uptake rates of trout and carp cardiac homogenates are also consistent with previous functional studies on intact trout and carp cardiac preparations. In agreement with the low  $\text{Ca}^{2+}$ -handling capacity of cardiac homogenates, the contraction of crucian carp ventricle is insensitive to the SR inhibitor ryanodine (Vornanen, 1996a). In contrast to the carp, the SR of trout ventricle seems to be involved in contractile regulation (Keen *et al.* 1994) as also suggested by the higher  $\text{Ca}^{2+}$  uptake rate of trout cardiac SR.

Since the body temperatures of fish and mammals are quite different, the real differences in SR  $\text{Ca}^{2+}$  uptake rate between ecto- and endothermic vertebrates might be greater than is evident from experiments performed at room temperature. When corrected to the physiological body temperatures, it is evident that the rate of  $\text{Ca}^{2+}$  uptake is much higher in mammalian than in teleost hearts. The  $\text{Ca}^{2+}$  uptake rate of trout SR would be approximately 20% of the activity of the SR in adult rat heart. The corresponding value for crucian carp heart would be only 2–11%. However, comparisons of  $\text{Ca}^{2+}$  uptake rates *in vitro* may exaggerate the true differences in the rates of SR  $\text{Ca}^{2+}$  accumulation during cardiac contraction *in vivo*. SR  $\text{Ca}^{2+}$  uptake will occur during sarcolemmal depolarization, when  $\text{Ca}^{2+}$  efflux through  $\text{Na}^+/\text{Ca}^{2+}$  exchanger is impeded. The duration of the action potential in adult rat heart is less than 100 ms, while it is closer to 500 ms in fish ventricles (Vornanen, 1989). Thus, despite its relatively slow  $\text{Ca}^{2+}$  uptake rate, the fish cardiac SR may accumulate significant amounts of  $\text{Ca}^{2+}$  during the long-lasting depolarization if the  $\text{Ca}^{2+}$ -storing capacity of SR is adequate. The true differences in  $\text{Ca}^{2+}$ -handling capacity of cardiac SR between vertebrate species can only be evaluated by experiments using intact cardiac myocytes conducted at physiological body temperatures. The present results support the hypothesis that efficient SR  $\text{Ca}^{2+}$  uptake is associated with high *in vivo* heart rates (mammals), which necessitate high rates of contraction and relaxation during the brief systolic period.

*Effects of thermal acclimation*

Temperature acclimation changed the activity of SR Ca<sup>2+</sup> handling in both crucian carp and rainbow trout. The temperature-induced changes in the activity of the Ca<sup>2+</sup>-sensitive ATPase, the TG-sensitive ATPase and TG-sensitive Ca<sup>2+</sup> uptake were qualitatively similar, although the differences were statistically significant only in the case of Ca<sup>2+</sup> uptake rate. It is possible that thermal acclimation changes not only the maximum rate of Ca<sup>2+</sup> sequestration but also the Ca<sup>2+</sup> affinity of the SR Ca<sup>2+</sup>-ATPase, which could explain the differences between Ca<sup>2+</sup>-ATPase activities (measured at optimal Ca<sup>2+</sup> concentration) and Ca<sup>2+</sup> uptake rates (measured at a Ca<sup>2+</sup> concentration close to the  $K_m$  of the enzyme). However, we cannot exclude the possibility that thermal acclimation also affects passive Ca<sup>2+</sup> leakage across SR membranes.

The effects of thermal acclimation in the two teleost species were opposite: an enhancement of SR Ca<sup>2+</sup> uptake in trout and a reduction in Ca<sup>2+</sup>-handling capacity of the SR in carp after acclimation to cold temperature. It is notable that, in the trout heart, there is complete thermal compensation of SR Ca<sup>2+</sup> uptake velocity. However, the physiological significance of this acclimation response is not clear. It has been argued that fish cardiac SR may be unable to store Ca<sup>2+</sup> (Keen *et al.* 1992), since Ca<sup>2+</sup> release channels tend to remain open at low ambient temperatures (Sitsapesan *et al.* 1991). The present findings agree with previous functional studies showing increased sensitivity of trout ventricle to ryanodine inhibition after acclimation to cold (Keen *et al.* 1994). Similarly, the low Ca<sup>2+</sup> uptake rate of cold-acclimated carp ventricle agrees with the reduced cardiac activity in cold-acclimated crucian carp, expressed as a long relaxation time, a slow heart rate and low myosin-ATPase activity of the heart (Matikainen and Vornanen, 1992; Vornanen, 1994, 1996a). Therefore, several kinds of evidence suggest that, in species that remain active at low ambient temperatures (rainbow trout, perch), adaptation to cold increases the volume and Ca<sup>2+</sup>-handling efficiency of the SR (Bowler and Tirri, 1990; Keen *et al.* 1994), while in species that become inactive or dormant in the cold (crucian carp), the limited Ca<sup>2+</sup>-handling capacity is depressed after acclimation to low ambient temperature.

In conclusion, the locomotor activity and cardiac performance of fish vary greatly. Over the entire spectrum of species, there are approximately tenfold differences in heart rate, cardiac output and maximum ventral aortic pressure (Farrell, 1996). The present study suggests that there are cellular differences in cardiac Ca<sup>2+</sup> regulation between teleost fish: 'athletic' fish species (such as trout) have a higher Ca<sup>2+</sup>-handling capacity in the cardiac SR than less active species (such as the crucian carp). Furthermore, acclimation to cold in active species is associated with an increased Ca<sup>2+</sup>-handling capacity of the SR, which may allow partial compensation for the detrimental effects of cold on cardiac contractility. The inverse thermal compensation in Ca<sup>2+</sup> uptake rate of crucian carp cardiac SR is probably associated with its unusual ecophysiological adaptations. Crucian carp are specialized to inhabit small shallow ponds which become anoxic in winter. Under these conditions positive thermal compensation with

associated increase in cardiac energy consumption would not be beneficial for the survival of the fish.

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**References**

- ATKINSON, A., GATENBY, A. D. AND LOWE, A. G. (1973). The determination of inorganic orthophosphate in biological systems. *Biochim. biophys. Acta* **320**, 195–204.
- BERS, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *Am. J. Physiol.* **248**, H366–H381.
- BOSSEN, E. H. AND SOMMER, J. R. (1984). Comparative stereology of the lizard and frog myocardium. *Tissue & Cell* **16**, 173–178.
- BOWLER, K. AND TIRRI, R. (1990). Temperature dependence of the heart isolated from the cold or warm acclimated perch (*Perca fluviatilis*). *Comp. Biochem. Physiol.* **96A**, 177–180.
- FABIATO, A. (1982). Calcium release in skinned cardiac cells: variation with species, tissues and development. *Fedn Proc. Fedn Am. Socs exp. Biol.* **41**, 2238–2244.
- FABIATO, A. (1988). Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Meth. Enzymol.* **157**, 378–417.
- FARRELL, A. P. (1996). From hagfish to tuna: A perspective on cardiac function in fish. *Physiol. Zool.* **64**, 1137–1164.
- FEHER, J. J. AND LEBOLT, W. R. (1990). Stabilization of rat cardiac sarcoplasmic reticulum Ca<sup>2+</sup> uptake activity and isolation of vesicles with improved calcium uptake activity. *Molec. cell. Biochem.* **99**, 41–52.
- FEHER, J. J., STEPHENS, M. J., ALDERSON, B. H. AND POLAND, J. L. (1988). Contribution of the ryanodine-sensitive fraction to capabilities of cardiac SR. *J. molec. cell. Cardiol.* **20**, 1107–1118.
- GRYNKIEWICZ, G., POENIE, M. AND TSIEN, R. Y. (1985). A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. biol. Chem.* **260**, 3440–3450.
- HOVE-MADSEN, L. AND BERS, D. M. (1993a). Passive Ca buffering and SR Ca uptake in permeabilized rabbit ventricular myocytes. *Am. J. Physiol.* **264**, C677–C686.
- HOVE-MADSEN, L. AND BERS, D. M. (1993b). Sarcoplasmic reticulum Ca<sup>2+</sup> uptake and thapsigargin sensitivity in permeabilized rabbit and rat ventricular myocytes. *Circulation Res.* **73**, 820–828.
- JORGENSEN, A. O., SHEN, A. C. Y., ARNOLD, W., MCPHERSON, P. S. AND CAMPBELL, K. P. (1993). The Ca<sup>2+</sup>-release channel/ryanodine receptor is localized in junctional and corbular sarcoplasmic reticulum in cardiac muscle. *J. Cell Biol.* **120**, 969–980.
- KARGACIN, M. E. AND KARGACIN, G. J. (1994). Methods for determining cardiac sarcoplasmic reticulum Ca<sup>2+</sup> pump kinetics from fura 2 measurements. *Am. J. Physiol.* **267**, C1145–C1151.
- KEEN, J. E., FARRELL, A. P., TIBBITS, G. F. AND BRILL, R. W. (1992). Cardiac physiology in tunas. II. Effect of ryanodine, calcium and adrenaline on force–frequency relationships in atrial strips from skipjack tuna, *Katsuwonus pelamis*. *Can. J. Zool.* **70**, 1211–1217.
- KEEN, J. E., VIANZON, D.-M., FARRELL, A. P. AND TIBBITS, G. F. (1994). Effect of temperature and temperature acclimation on the ryanodine sensitivity of the trout myocardium. *J. comp. Physiol. B* **164**, 438–443.
- LOMPRE, A. M., LAMBERT, F., LAKATTA, E. G. AND SCHWARTZ, K.

- (1991). Expression of sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase and calsequestrin genes in rat heart during ontogenetic development and aging. *Circulation Res.* **69**, 1380–1388.
- LOWRY, O. H., ROSENBOROUGH, N. J., FARR, A. L. AND RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.
- LYTTON, J., WESTLIN, M. AND HANLEY, M. R. (1991). Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. biol. Chem.* **266**, 17067–17071.
- MATIKAINEN, N. AND VORNANEN, M. (1992). Effect of season and temperature acclimation on the function of crucian carp (*Carassius carassius*) heart. *J. exp. Biol.* **167**, 203–220.
- PALADE, P. (1987). Drug-induced  $\text{Ca}^{2+}$  release from isolated sarcoplasmic reticulum. I. Use of pyrophosphate to study caffeine-induced  $\text{Ca}^{2+}$  release. *J. biol. Chem.* **262**, 6135–6141.
- SAGARA, Y. AND INESI, G. (1991). Inhibition of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  transport ATPase by thapsigargin at subnanomolar concentrations. *J. biol. Chem.* **266**, 13503–13506.
- SANTER, R. M. (1985). Morphology and innervation of the fish heart. *Adv. Anat. Embryol. Cell Biol.* **89**, 1–102.
- SIMONIDES, W. S. AND VAN HARDEVELD, C. (1990). An assay for sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase activity in muscle homogenates. *Analyt. Biochem.* **191**, 321–331.
- SITSAPESAN, R., MONTGOMERY, R. A. P., MACLEOD, K. T. AND WILLIAMS, A. J. (1991). Sheep cardiac sarcoplasmic reticulum calcium release channels: modifications of conductance and gating by temperature. *J. Physiol., Lond.* **434**, 469–488.
- VETTER, R., STUDER, R., REINECKE, M., KOLAR, F., OSTADALOVA, I. AND DREXLER, H. (1995). Reciprocal changes in the postnatal expression of the sarcolemmal  $\text{Na}^{+}$ - $\text{Ca}^{2+}$ -exchanger and SERCA2 in rat heart. *J. molec. cell. Cardiol.* **27**, 1689–1701.
- VORNANEN, M. (1989). Regulation of contractility of the fish (*Carassius carassius* L.) heart ventricle. *Comp. Biochem. Physiol.* **94C**, 477–483.
- VORNANEN, M. (1994). Seasonal and temperature-induced changes in myosin heavy chain composition of the crucian carp hearts. *Am. J. Physiol.* **267**, R1567–R1573.
- VORNANEN, M. (1996a). Effects of extracellular calcium on the contractility of warm- and cold-acclimated crucian carp heart. *J. comp. Physiol. B* **166**, 1–11.
- VORNANEN, M. (1996b). Excitation–contraction coupling of the developing rat heart. *Molec. cell. Biochem.* **163/164**, 5–11.