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DIHYDROPYRIDINE AND RYANODINE BINDING IN VENTRICLES FROM RAT, TROUT, DOGFISH AND HAGFISH

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Summary

In the adult mammalian heart, the majority of Ca^{2+} required for contraction is released from the sarcoplasmic reticulum (SR) *via* the Ca^{2+}-release channel or ryanodine receptor (RyR). Such release is dependent upon a relatively small influx of Ca^{2+} entering the cell across the sarcolemma (SL) by means of the L-type Ca^{2+} channel or the dihydropyridine receptor (DHPR). In lower vertebrates, there is indirect evidence suggesting that Ca^{2+} influx across the SL may be sufficient to support contraction in the absence of Ca^{2+} release from the SR. This apparent difference in myocardial excitation–contraction (E–C) coupling was investigated further by determining DHPR and RyR densities in ventricular homogenate preparations from rat, trout, dogfish and hagfish. DHPR B_{max} values (means ± S.E.M.) were highest in rat (0.30±0.01 pmol mg^{-1}), lower in trout (0.16±0.01 pmol mg^{-1}) and dogfish (0.27±0.03 pmol mg^{-1}), and slightly above the level of detection in hagfish (0.03±0.01 pmol mg^{-1}). The DHPR dissociation constants (K_d) of 40–70 pmol l^{-1} in these three species were of similar magnitude. RyR binding revealed both high- and low-affinity sites in all species. RyR B_{max} for the high-affinity site was greatest in the rat (0.68 pmol mg^{-1}), lower in trout (0.19 pmol mg^{-1}) and dogfish (0.07 pmol mg^{-1}) and lowest in hagfish (0.01 pmol mg^{-1}). The RyR K_{d1} values for the high-affinity sites were comparable in all preparations (range 12–87 nmol l^{-1}). The quantitative expression of RyRs in these species is consistent with the relative amount of SR present as indicated in physiological experiments and electron micrographs. Taking into consideration myocyte morphology of teleost and elasmobranch species, the data are consistent with a greater reliance on Ca^{2+} influx across the SL during E–C coupling in lower vertebrates, although a functional role for Ca^{2+} release from the SR in the more active species awaits further investigation.

Key words: rainbow trout, *Oncorhynchus mykiss*, hagfish, *Eptatretus stoutii*, dogfish, *Squalus acanthias*, rat, myocardium, sarcolemma, sarcoplasmic reticulum, excitation–contraction coupling.

Introduction

Fishes have developed cardiovascular systems which must function under very different physiological conditions. Environmental factors, such as varying temperature, place unique demands on myocardial excitation–contraction (E–C) coupling mechanisms. In mammals, myocardial contraction is initiated with an influx of Ca^{2+} through the sarcolemmal (SL) L-type Ca^{2+} channels, otherwise known as dihydropyridine receptors (DHPRs). This influx of Ca^{2+} is believed to trigger the release of a greater amount of Ca^{2+} from the sarcoplasmic reticulum (SR) (Fabiato, 1983) *via* the SR Ca^{2+}-release channels or ryanodine receptors (RyRs) (Otsu *et al.* 1990). Although the magnitude of Ca^{2+} influx through the SL depends upon species (Bers, 1985), estimations taking into account the total amount of Ca^{2+} required for twitch generation, including intracellular Ca^{2+} buffering, suggest that it varies from 10 to 40 % of this total (Wier, 1990). Studies using ryanodine, an agent known to block the SR Ca^{2+}-release channel, provide evidence that Ca^{2+} released from the SR is the major source of Ca^{2+} for contraction in adult mammalian hearts (Sutko and Kenyon, 1983).

In lower vertebrates, a role for SR Ca^{2+} release in myocardial tension generation is generally dismissed for several reasons. Electron microscopy reveals a relative paucity of SR relative to the amount found in mammals, as well as a lack of organizational complexity in a number of poikilotherms (Santer, 1985). In addition, ryanodine does not reduce contractile force in ventricular strips from a number of ectotherms at physiological temperatures (10–15 °C) and at stimulation frequencies above 0.2 Hz (Driedzic and Gesser, 1988).

Experiments probing the mechanisms of E–C coupling in the hearts of lower vertebrates are also complicated by the varying temperatures to which poikilotherms are subject. Myofibrillar Ca^{2+} sensitivity in both endo- and ectotherms is reduced at lower temperatures (Churcott *et al.* 1994; Harrison and Bers, 1990). However, amphibians (*Rana pipiens*) and

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rainbow trout demonstrate a greater Ca^{2+}-sensitivity at lower temperatures than mammals, suggesting that poikilotherms have developed some degree of thermal adaptation. To complicate matters, the total amount of Ca^{2+} required by the contractile element, taking into account the degree of intracellular Ca^{2+} buffering, has yet to be determined in fish. In the assessment of Ca^{2+} buffering, the temperature- and pH-dependence of the affinity constant (K_a) values of the various intracellular Ca^{2+} binding sites would also have to be determined.

Despite the lack of information regarding temperature-dependencies of Ca^{2+} binding, there are a number of observations suggesting that the hearts of lower vertebrates place a greater reliance on SL Ca^{2+} influx to support tension generation than do the hearts of higher vertebrates (Tibbits *et al.* 1990). As with mammalian preparations, isometric force increases as extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is raised in agnathan, elasmobranch and teleost ventricular strips (Driedzic and Gesser, 1988). While the characteristics of the cardiac action potential may vary considerably among poikilotherms, the action potential is generally longer-lasting and has a more pronounced plateau phase than that of the mammalian ventricle (Tibbits *et al.* 1992*a*). In the frog and trout heart, a longer action potential duration is correlated with a greater time to peak tension. In addition, both amphibian (Bers, 1985) and teleost (sea raven and cod) ventricles (Driedzic and Gesser, 1988) are relatively insensitive to ryanodine at concentrations known to block SR Ca^{2+} release in mammals (Sutko and Kenyon, 1983). The smaller myocyte diameters observed in amphibians, teleosts and elasmobranchs would serve to reduce diffusional distances and substantially increase the SL surface area relative to cell volume (Santer, 1985). In addition to the absence of T-tubules and relatively sparse SR (Bossen and Sommer, 1984; Santer, 1985), the DHPR density has been reported to be significantly higher in trout hearts than in mammalian preparations (Tibbits *et al.* 1991). However, owing to variability among species with respect to the degree of SR development, environmental parameters and experimental conditions used, a physiological role for the SR cannot, as yet, be ruled out. Therefore, in this study, we have attempted to characterize the key components of E–C coupling by determining the relative densities of DHPRs and RyRs in phylogenetically distinct species.

Materials and methods

Animal care

Female Wistar rats (290–324 g) were obtained from the University of British Columbia and kept in a temperature- and light-controlled vivarium (23 °C, 12 h:12 h light:dark cycle). Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing 600–800 g were obtained from West Creek Trout Farm, Aldergrove, British Columbia, and maintained locally at approximately 12 °C for a minimum of 1 week outside during the summer months. Hagfish [*Eptatretus stoutii* (Lockington)] and dogfish [*Squalus acanthias* (Linnaeus)] were obtained and held in outdoor tanks (approximately 8 °C) during the summer at the Bamfield Marine Station, Bamfield, British Columbia. Dogfish and hagfish binding experiments were carried out in a laboratory at this marine research station. Rat and trout experiments were conducted at Simon Fraser University.

Ventricular homogenate preparation

Animals were killed using standard protocols including lethal injection of sodium pentabarbitol (rat), a sharp blow to the head (trout), pithing (dogfish) or decapitation (hagfish). The hearts were rapidly excised and placed into a beaker of ice-cold homogenization medium (HM) containing 250 mmol l^{-1} sucrose, 20 mmol l^{-1} Mops, pH 7.4 at 37 °C. After rinsing, the ventricle(s) were isolated, blotted, weighed, and further rinsed in ice-cold HM. After mincing 0.5–1.7 g of tissue in approximately 5 ml of HM, the tissue was homogenized using a Tekmar Tissumizer (five times 3 s on setting 40) and filtered through two layers of wire (nos 30 and 40 stainless steel) mesh. The homogenate was then brought to a final volume of 15–20 ml g^{-1} wet mass. Protein was determined using the Bradford method with bovine serum albumin as a standard.

Sarcolemma isolation

In two additional studies, SL was isolated from rat and trout hearts using techniques described previously (Tibbits *et al.* 1981, 1990). Isolating trout SL involves several important modifications to existing purification steps employed for the rat. Owing to the relative fragility of the trout heart, reductions in both the duration and speed of homogenization as well as omission of the deoxyribonuclease incubation were required to enhance the recovery of SL. Briefly, pooled trout ventricles (at least 5.3 g) were minced with scissors in approximately 10 ml of ice-cold HM containing 280 mmol l^{-1} sucrose and 20 mmol l^{-1} *N*-tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid (Tes), pH 7.8 at 21 °C. Homogenization and filtration through two layers of stainless-steel mesh (nos 30 and 40) were followed by the addition of 1.0 mol l^{-1} KCl and 250 mmol l^{-1} sodium pyrophosphate to solubilize contractile proteins. Subsequent steps involved differential centrifugation and discontinuous sucrose gradient fractionation. The resultant pellets containing various membrane fractions were resuspended in 140 mmol l^{-1} NaCl and 10 mmol l^{-1} Tes, pH 7.8 at 21 °C, and frozen in liquid N_2. Protein was determined from a small sample taken prior to freezing. The sarcolemmal marker enzyme K^+-stimulated *p*NPPase was measured as described previously (Tibbits *et al.* 1981, 1990).

[^{3}H]DHP binding assay

All binding experiments were performed using [^{3}H](+)PN200-110 (PN) as the stereospecific DHP ligand and the same ventricular homogenate preparations used for the [^{3}H]ryanodine binding experiments (see below). Specific binding was determined by varying the amount of PN (0.025–1.6 nmol l^{-1}) added to tubes containing 150–160 μg of protein (diluted in 25 mmol l^{-1} Mops, pH 7.4 at 23 °C) and

2.5 mmol l^{-1} $CaCl_2$ in a final volume of 5 ml. Non-specific binding was determined in separate tubes containing the same solution as that for specific binding plus 1 μmol l^{-1} nifedipine. After incubation at room temperature in the dark for 90 min, the separation of free from bound ligands was achieved by rapid filtration of a 2.25 ml sample using Whatman GF/C glass-fibre filters. Each filter was washed three times with 4.5 ml of ice-cold 25 mmol l^{-1} Mops buffer. The filters were then dried in liquid scintillation vials, after which 5 ml of Beckman Ready Protein liquid scintillant was added followed by 2 h of orbital shaking at 200 revs min^{-1} to solubilize the protein. Vials were then counted (Beckman LS 7000) using standard liquid scintillation procedures.

[^{3}H]Ryanodine binding assay

To determine specific ryanodine binding, varying amounts (0–495 nmol l^{-1}) of unlabelled ryanodine (Calbiochem) plus 5 nmol l^{-1} [^{3}H]ryanodine were added to tubes containing 150 mmol l^{-1} KCl, 20 mmol l^{-1} Mops (pH 7.4 at 23 °C) and 100 μmol l^{-1} $CaCl_2$ in a final volume of 2 ml. Non-specific binding was measured by adding a 100-fold excess of unlabelled ryanodine to separate tubes. After the addition of 500 μg of ventricular homogenate protein, tubes were incubated at room temperature for 2 h to allow the binding to reach a maximum. Duplicates were taken for each of the 12 points. Separation of bound and free ligand was achieved by rapid filtration using Whatman GF/C filters, followed by rinsing twice with 4 ml of ice-cold buffer (containing 150 mmol l^{-1} KCl, 20 mmol l^{-1} Mops, 100 μmol l^{-1} $CaCl_2$) and once with 4 ml of ice-cold 10 % ethanol. After drying filters overnight in scintillation vials, 5 ml of Beckman Ready Protein liquid scintillant was added followed by 2 h of orbital shaking at 200 revs min^{-1} to solubilize the protein. Vials were then counted using standard liquid scintillation procedures.

Data and statistical analyses

DHPR binding data were analyzed by iterative non-linear regression and Scatchard analysis using LIGAND (Munson and Rodbart, NIH) to determine the maximum receptor density (B_{max}) and the dissociation constant (K_d) for each preparation. RyR binding data were analyzed using an iterative non-linear regression method using GRAFIT (Erithacus Software Ltd, UK). The goodness-of-fit of the predicted value from several different equations to the data set was determined by a χ^2 analysis.

Materials

All reagents used were of the highest purity and purchased from Sigma unless specified otherwise. Radioisotopes were purchased from New England Nuclear (Du Pont, St Laurent, Quebec, Canada).

Results

The wet mass of the ventricles used in preparing homogenates of the four species and the protein yields are given in Table 1. The protein content in both dogfish and hagfish was considerably lower than typically observed in either mammals or teleosts. Specific binding of DHP at different concentrations of PN200-110 to ventricular homogenate is shown in Fig. 1. At 0.10 nmol l^{-1} [^{3}H](+)PN200-110, the specific binding accounted for more than 70 % of the total binding for all species. Over the more than two orders of magnitude of [^{3}H](+)PN200-110 concentrations used in this study (0.025–1.6 nmol l^{-1}), the data fitted a single class of high-affinity binding sites as exemplified by linear Scatchard plots and low values of χ^2 in the appropriate non-linear regression equation. Scatchard analyses of the fitted data are shown in Fig. 2 and yielded the B_{max} and K_d values listed in Table 2. Both the B_{max} and K_d values for the trout and dogfish were comparable to those for mammals. The calculated densities of receptors (sites per μm^3 cell

Table 1. *Ventricular characteristics in the different species*

	Rat	Trout	Dogfish	Hagfish
Ventricular mass (g)	0.80±0.02	0.48±0.03	1.70±0.17	0.46±0.04
Protein content (mg g^{-1})	107.7±2.5	116.8±9.9	49.6±3.3*	66.3±6.3*
N	10	6	6	6

Values are displayed as means ± S.E.M.

Ventricular mass, wet mass of trimmed ventricle in grams; protein content, milligrams of protein per gram wet mass of ventricle; *N*, number of assays performed (with one assay on each ventricle for rat, trout and dogfish, and 2–6 ventricles pooled for each assay for the hagfish).

*Values for dogfish and hagfish are significantly (P<0.05) different from values for both rat and trout.

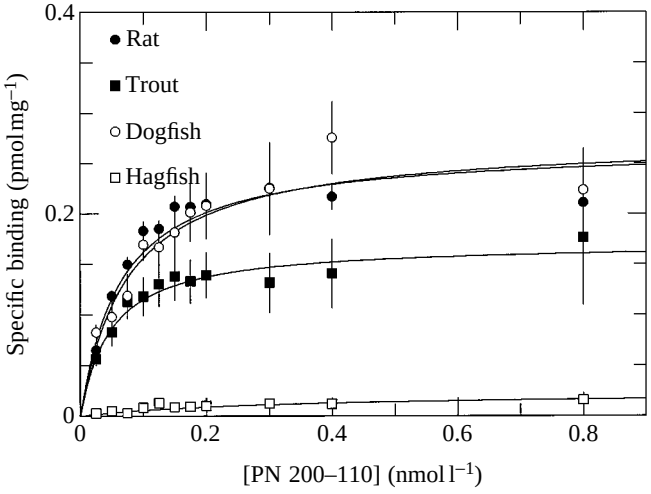

Fig. 1. Specific [^{3}H](+)PN200-110 (PN) binding as a function of total PN200-110 concentration to ventricular homogenates in rat (filled circles, N=10), trout (filled squares, N=6) dogfish (open circles, N=6) and hagfish (open squares, N=5). PN binding was determined as outlined in Materials and methods. Values are means ± S.E.M.

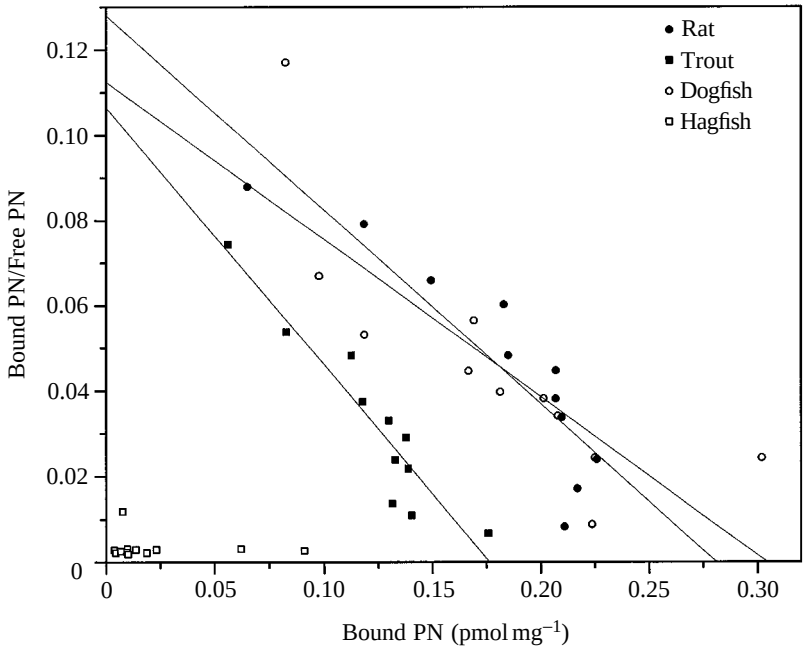

Fig. 2. Scatchard analysis of specific [^{3}H](+)PN200-110 (PN) binding in each species. Specific binding for rat (filled circles, N=10), trout (filled squares, N=6), dogfish (open circles, N=6) and hagfish (open squares, N=5) are shown as a function of free ligand concentration.

volume) are given in Table 4. In all species, except the hagfish, the DHPR density was within the range (0.08–0.3 pmol mg^{-1}) observed in mammals. The derived B_{max} for the DHPR in hagfish was 0.03 pmol mg^{-1} protein and was about an order of magnitude lower than that observed in the other species. Because the hagfish values were so low and barely above the limit of detection, one cannot be confident of the derived K_d.

Homogenates were used in this study because this preparation represents the entire pool of channels without the loss that occurs in membrane isolation. An example of the problems that can arise from membrane isolation is shown in Fig. 3. This demonstrates the differential loss of DHPRs relative to marker enzyme activity in rat sarcolemmal fractions compared with those of trout. In rat, the increase in specific DHP binding over the levels found in homogenates is markedly lower compared with the increase in SL marker enzyme activity. In contrast, the trout demonstrates comparable purification of both DHPRs and SL marker enzyme activity.

Table 2. *Comparison of DHP binding in different species*

	Rat	Trout	Dogfish	Hagfish
B_{max} (pmol mg^{-1})	0.30±0.02	0.16±0.01*	0.27±0.03	0.03±0.01*
K_d (nmol l^{-1})	0.07±0.01	0.04±0.01	0.07±0.01	0.42±0.01
N	10	6	6	5

Values are means ± S.E.M.

B_{max} is the maximum density of dihydropyridine (DHP) sites determined by Scatchard analysis; K_d is the dissociation constant determined by Scatchard analysis; N is the number of assays performed (with one assay on each ventricle for rat, trout and dogfish, and 2–6 ventricles pooled for each assay for hagfish).

*Value significantly different from those of the rat (P<0.05).

Fig. 4 shows specific [^{3}H]ryanodine binding to the same preparations used for the DHPR binding assays. When the RyR data from all lower vertebrate species were linearized in a Scatchard plot, they generated neither straight nor concave lines. Consistent with this result is the fact that neither a one- nor a two-site ligand binding equation fitted the data well. Because the RyR-specific binding as a function of ryanodine concentration (over the range 0–75 nmol l^{-1}) consistently demonstrated sigmoidicity, a two-site Adair equation was also used to fit the data. This resulted in clearly the best fit both by eye and in the χ^2 analysis. In order to observe the high-affinity site better, data are shown for concentrations ranging from 0 to 75 nmol l^{-1} ryanodine (Fig. 4). Over the full range of ryanodine concentrations used, the presence of at least two separate independent binding sites were observed (Fig. 5). At

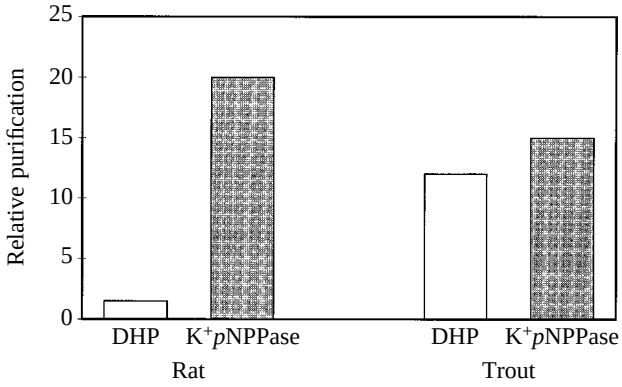

Fig. 3. Specific [^{3}H](+)PN200-110 (PN) binding and sarcolemmal marker enzyme activity (K^+-stimulated pNPPase, K^+pNPPase) from rat and trout heart. Maximum PN binding and K^+-stimulated pNPPase activity are normalized to the respective homogenate values. PN binding was determined as outlined in Materials and methods.

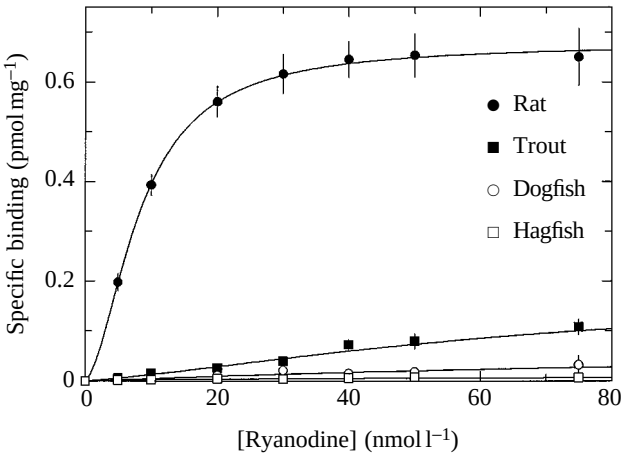

Fig. 4. Specific [^{3}H]ryanodine binding as a function of total ryanodine concentration to ventricular homogenates from rat, trout, dogfish and hagfish hearts. Only the high-affinity sites (0–75 nmol l^{-1}) are shown for simplicity. Each data set represent the mean values ± S.E.M. of separate rat (filled circles, *N*=10), trout (filled squares, *N*=6), dogfish (open circles, *N*=6) and hagfish (open squares, *N*=6) binding assays, with each experiment consisting of individual (rat, trout, dogfish) or pooled (hagfish) ventricles. Ryanodine binding was determined as outlined in Materials and methods.

30 nmol l^{-1} total ryanodine, the specific binding accounted for more than 70 % of total binding for all species. After fitting the data to one of several different equations, the two-site Adair equation gave the best results for the high-affinity site and the one-site equation gave the best results for the low-affinity site. The B_{max} and K_d values obtained are listed in Table 3. The number of high-affinity sites is significantly reduced in the phylogenetically lower species (dogfish and hagfish). However, the K_d values remained comparable to values for mammalian preparations. The calculated densities (sites per μm^3 cell volume) are given in Table 4. The rat, which relies most heavily on Ca^{2+} release from the SR, had by far the greatest density of RyRs. In contrast, the hagfish, which is the most primitive species studied, had the lowest density of RyRs. Table 4 shows the ratios of RyR/DHPR densities in the various species.

Table 3. *Comparison of ryanodine binding in different species*

	Rat	Trout	Dogfish	Hagfish
High-affinity site				
B_{max} (pmol mg^{-1})	0.68 (0.53–0.87)	0.19 (0.09–0.23)	0.073 (0.04–0.23)	0.011 (0–0.05)
K_{d1} (nmol l^{-1})	33	87	67	12
K_{d2} (nmol l^{-1})	2	57	240	155
Low-affinity site				
B_{max} (pmol mg^{-1})	0.92	1.61	0.42	0.12
K_d (nmol l^{-1})	43	779	130	474
N	10	6	6	5

Values for the high-affinity site were obtained through non-linear regression analysis using the two-site Adair equation provided in the computer program GRAFIT (Erithacus Software Ltd, UK) and for the binding data obtained over the range 0–75 nmol l^{-1} ryanodine. The range of values for B_{max} in each species is given in parentheses.

Values for the low-affinity site were obtained using the same method, a one-site ligand binding equation and binding data for 100–500 nmol l^{-1} ryanodine.

All analyses were performed on the composite data for each species.

N is the number of assays performed (with one assay on each ventricle for rat, trout and dogfish and 2–6 ventricles pooled for each assay for hagfish).

Table 4. *Calculations of DHPR and RyR densities*

	Rat	Trout	Dogfish	Hagfish
DHPR B_{max}[a] (pmol mg^{-1})	0.30	0.16	0.27	0.03
RyR B_{max}[b] (pmol mg^{-1})	0.68	0.19	0.073	0.011
Protein content[c] (mg cm^{-3})	114	124	53	70
ECS[d] (%)	28[e]	32[f]	32[f]	32[f]
DHPR density[g] (sites μm^{-3} cell volume)	29	18	13	2
RyR density[h] (sites μm^{-3} cell volume)	65	21	3	<1
RyR:DHPR	2.2	1.2	0.2	0.5

RyR, ryanodine receptor; DHPR, dihydropyridine receptor.

[a]Mean DHPR B_{max} values taken from Table 2.

[b]Mean RyR high-affinity site B_{max} values taken from Table 3.

[c]Homogenate protein in milligrams per gram wet muscle × muscle density (1.06 g cm^{-3}).

[d]Percentage of total tissue occupied by extracellular space (ECS).

[e]Taken from Frank and Langer (1974).

[f]Taken from Farrell and Milligan (1986).

[g]DHPR density = DHPR B_{max} × protein content × ECS correction × 6.02 × 10^{23}.

[h]RyR density = RyR (high-affinity) B_{max} × protein content × ECS correction × 6.02 × 10^{23}.

Discussion

DHPR and RyR densities were determined in ventricular preparations from phylogenetically distinct species to characterize further the key components in E–C coupling in lower vertebrates.

Protein content

The protein content of ventricular homogenates is significantly lower for dogfish and hagfish than for rat and trout. A value of approximately 100 mg g^{-1} wet mass is typically observed in mammals (Lachnit *et al.* 1994).

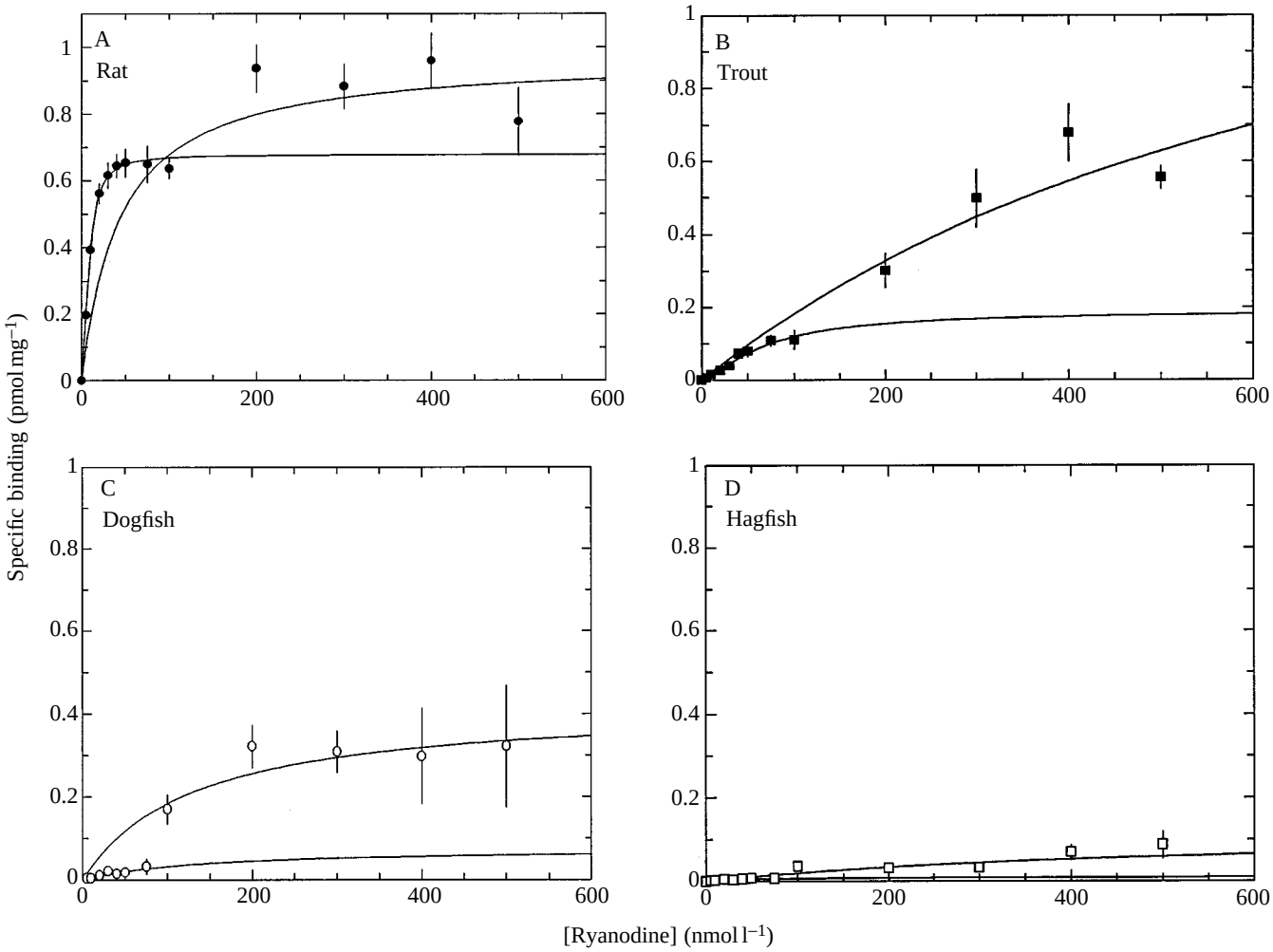

Fig. 5. Specific [^{3}H]ryanodine binding in ventricular homogenates from the four species as a function of total ryanodine concentration (0–500 nmol l^{-1}). The two curves represent best fits for the high- (0–75 nmol l^{-1}) and low- (100–500 nmol l^{-1}) affinity sites. Values for the high-affinity site were fitted using the two-site Adair equation while those for the low-affinity site were fitted using the one-site ligand binding equation, both from GRAFIT (Erithacus Software Ltd, UK). (A) Rat (filled circles, N=10 individual ventricles); (B) trout (filled squares, N=6 individual ventricles); (C) dogfish (open circles, N=6 individual ventricles); (D) hagfish (N=6 assays with 2–6 hearts pooled per assay). Values are means ± S.E.M.

Reference values for ventricular protein content in elasmobranch or hagfish species are not available. This difference may reflect the fact that the myocardium of the dogfish *Squalus acanthias* contains a high percentage, approximately 80 %, of spongy trabeculae and the hagfish systemic heart is composed exclusively of spongiosa (Farrell and Jones, 1992). Previous studies indicated that spongy trabeculae from frog (Sommer *et al.* 1988), teleost (Santer and Walker, 1980), elasmobranch (Santer and Walker, 1980; Tota, 1989) and hagfish (Leak, 1969) ventricles have more extracellular space (ECS) than the compact myocardium of mammals (Frank and Langer, 1974).

DHPR binding

In mammalian hearts, the α_1 subunit of the SL Ca^{2+} channel contains both the DHP binding domain and Ca^{2+} conductance capabilities when expressed in oocytes (Mikami *et al.* 1989). The α_1 subunit from trout has a similar M_r of 185 and K_d for DHP binding to those of mammals (B. S. Tuana, B. J. Murphy, J. Wigle, C. Pratt and G. F. Tibbits, in preparation). The K_d values reported in Table 2 for both rat and trout are in agreement with these data. The K_d value in the dogfish preparation is comparable with the values for the rat and trout, suggesting that at least the DHP binding domain may be the same in this species. In contrast, the trout cardiac α_2 subunit appears to be quite different in both size and nucleotide sequence from the mammalian form (B. S. Tuana, B. J. Murphy, J. Wigle, C. Pratt and G. F. Tibbits, in preparation).

The densities of DHPRs were calculated for three of the four species and are recorded in Table 4. Values not measured were taken from the literature or estimated, as noted. The calculated densities rely on three assumptions. The first assumption is that all DHPRs are from myocyte sarcolemma. The finding that DHP binding in isolated myocytes from rabbit heart is nearly

twofold greater than in whole-muscle homogenates suggests that the number of DHPRs in non-myocyte tissue is minimal (Bers and Stiffel, 1993). The second assumption is that the relative contribution of ECS measured in the trout is the same for dogfish. Quantitative analysis using stereological measurements has indicated that the amount of total tissue occupied by ECS in rat heart is approximately 28 % including T-tubules (Frank and Langer, 1974). The only quantitative information available on the extent of the ECS in fish is a figure of approximately 32 %. This value was obtained by isotopic measurements of ECS in the *in situ* perfused trout heart (Farrell and Milligan, 1986) and was used to calculate DHPR density for both trout and dogfish. The third assumption is that all of the intracellular volume is made up of myocytes. While there are many cell types in the heart, the much larger myocytes account for approximately 75–90 % of the cell volume (Anversa *et al.* 1979). The calculated DHPR densities, in sites per μm^3 cell volume (Table 4), were 29 for rat, 18 for trout, 13 for dogfish and 2 for hagfish. The value for rat is high for mammalian cardiac muscle, but this is almost completely due to the observed difference in B_{max} rather than the assumptions made (Bers and Stiffel, 1993). A possible explanation may be that, for reasons unknown, fewer receptors were damaged or degraded during the experiments. DHPR densities for the trout and dogfish, while somewhat lower than the density for the rat, still remain well within the mammalian range (Bers and Stiffel, 1993; Lachnit *et al.* 1994). Using previously published data from DHP binding in homogenized rat cardiomyocytes, a density of approximately 12 sites per μm^3 is calculated (Bers and Stiffel, 1993), which is comparable to the densities calculated in this study for rat, trout and dogfish. The densities of DHPRs in the hagfish were almost an order of magnitude lower than those observed for the other species in this study. Previous experiments on ventricular strips from the Atlantic hagfish suggest that this species is relatively insensitive to changes in $[Ca^{2+}]_o$ (Poupa *et al.* 1984).

In a previous report (Tibbits *et al.* 1990), the B_{max} of purified SL preparations from the trout was determined to be 3.06 pmol mg^{-1} SL protein. This value is an order of magnitude higher than values reported for mammalian SL of similar purity (DePover *et al.* 1983; Lachnit *et al.* 1994). It was concluded from these observations that DHPR density is greatly increased in the trout compared with the mammalian heart (Tibbits *et al.* 1990). This conclusion turns out to be erroneous, as shown by the present data using ventricular homogenates (Table 2). The reason for this discrepancy may be explained by a differential DHPR purification during the SL isolation in the two species. Examination of Fig. 3 shows that, during SL purification, the percentage increases in DHP binding and of SL marker enzyme (K^+-stimulated *p*NPPase) are not proportional in the rat, suggesting that DHPRs are being lost during purification, presumably to denser fractions. Similar results have also been reported in canine heart (DePover *et al.* 1983) and, more recently, in cardiomyopathic hamsters (Lachnit *et al.* 1994). This apparent loss of DHPRs from the SL may be due to the tight coupling between the junctional SR RyR and the DHPR in the mammalian T-tubule (Franzini-Armstrong and Jorgensen, 1994). Since DHPRs are known to be clustered in the T-tubules in mammals (Wibo *et al.* 1991), a differential loss of T-tubule DHPRs over DHPRs not associated with RyRs would lead to an underestimation of mammalian B_{max} values. In contrast, the DHPR loss is not as apparent in the trout (Fig. 3). DHPR binding increases approximately 12-fold during trout SL purification, which is comparable to the approximately 15-fold increase in SL marker activity (Tibbits *et al.* 1990). The lack of T-tubules and the relatively sparse SR found in the trout suggest that the RyRs and DHPRs may be more loosely coupled, which may explain these results.

While DHPR densities in trout and dogfish are comparable to those of mammals, it is not known whether the total amount of Ca^{2+} entering the cell in each of these species is also of the same magnitude. Using a whole-cell patch-clamp technique, an L-type Ca^{2+} current (I_{Ca}) density of more than 3 $\mu A\,cm^{-2}$ (in 1.8 mmol l^{-1} $CaCl_2$ at 23 °C) has been recorded in frog ventricular myocytes (McDonald *et al.* 1994). This falls somewhat below the lower end of the 10–30 $\mu A\,cm^{-2}$ range of I_{Ca} densities typically observed in mammalian species under similar recording conditions (McDonald *et al.* 1994). However, I_{Ca} density does not take into account the extent of Ca^{2+}-dependent channel inactivation. Lower I_{Ca} densities are known to inactivate more slowly; thus, the integrated I_{Ca} could potentially be the same or even greater. In another study, $[Ca^{2+}]_i$ transients detected with aequorin recorded from salamander ventricular strips were prolonged relative to $[Ca^{2+}]_i$ transients from mammalian myocardium (Gwathmey and Morgan, 1991). If one assumes that the intracellular Ca^{2+}-buffering capacity is similar to that in mammals and that DHPRs represent functional L-type Ca^{2+} channels (Flockerzi *et al.* 1986), then Ca^{2+} entry of similar magnitude could provide a greater rise in cytosolic free $[Ca^{2+}]$ for the trout and dogfish myocytes simply because of the smaller myocyte diameters. In addition, the mammalian I_{Ca} amplitude is extremely temperature-dependent with a Q_{10} of approximately 3 (Cavalie *et al.* 1985). Without some sort of evolutionary selection with regard to the temperature-dependence of Ca^{2+} channel function, the contractility of the heart of these lower species would be profoundly impaired. The temperature-dependence of I_{Ca} amplitude in lower vertebrates is not known. Clearly, precise measurements of Ca^{2+} current density integrated over time and the effects of temperature on both the electrophysiological and Ca^{2+}-buffering processes are required to determine the extent of SL Ca^{2+} entry.

Finally, the potential contribution of mitochondrial Ca^{2+} has been ruled out in mammals by a variety of pharmacological and kinetic arguments (Fabiato, 1983). However, these experiments have not yet been performed in lower vertebrates, where Ca^{2+} cycling may be considerably slower.

RyR binding

The presence of both high- and low-affinity ryanodine binding sites in all of the species in this study is consistent with current knowledge of the mammalian cardiac RyR.

Conventional analysis, however, did not yield good results with respect to curve-fitting of the fish data. We were, therefore, forced to try several different approaches. The best results were obtained (lowest χ^2 and best fit by eye) when the high-affinity data were fitted with the two-site Adair equation. When microscopic dissociation constants are not equal, the Adair equation will describe the cooperative ligand binding that was observed in the different fish species. It also became apparent (see Fig. 5B–D) that in the poikiolotherm preparations, particularly the trout, this cooperativity was positive. A recently proposed sequential model of ryanodine binding in both mammalian skeletal and cardiac SR demonstrates negative cooperativity among the four putative binding sites (Pessah and Zimany, 1991). While further investigation involving kinetic and equilibrium measurements is needed, these preliminary results suggest that ryanodine binding in the lower vertebrates may reflect a functionally distinct cardiac RyR isoform.

To date there has been only one RyR isoform (RyR_2) identified in cardiac tissue (Meissner, 1994). The mammalian cardiac RyR is a polypeptide with a calculated M_r of approximately 560 as determined by cloning and sequencing of full-length cDNA (Otsu *et al.* 1990). Electron microscopy and biochemical studies have demonstrated that RyR *in vitro* exists as a homotetramer (Meissner, 1994). Single-channel recordings of isolated RyRs incorporated into lipid bilayers demonstrate an open subconductance state in the presence of nanomolar concentrations of ryanodine, while micromolar concentrations are known to close the channel completely (Sitsapesan *et al.* 1991). A negative cooperativity between the subunits is also demonstrated in this study by the presence of high- and low-affinity ryanodine binding. In each of the species examined, the K_{d1} values for the high-affinity site were within the range found in mammals under similar conditions and temperature (Meissner, 1994). The comparable ryanodine binding affinities observed in this study suggest that the ryanodine binding domain of the SR Ca^{2+}-release channel is similar to those found in mammalian myocardium. However, the presence of positive cooperativity within the high-affinity binding suggests that there may be some subtle differences in the fish RyR. While further investigation is required to elucidate the precise mechanisms underlying this positive cooperativity, the physiological implications may involve some sort of functional adaptation in the RyR, allowing the SR to participate in E–C coupling to a greater extent under certain conditions.

Maximum [^{3}H]ryanodine binding to the high-affinity site was greatest in the rat and decreased considerably in the order trout > dogfish > hagfish (Fig. 4). Overall, the maximum binding and calculated densities (Table 4) correlate with the phylogenetic development of SR in these species (Santer, 1985). The SR accounts for approximately 3.5 % of myocyte volume in rat (Page, 1978) and considerably less, less than 0.3 %, in amphibians (Bossen and Sommer, 1984). While limited information is available for fish, electron microscopic observations show that the SR tends to be better developed in teleosts than in either amphibians or agnathans, and is least developed in elasmobranchs (Helle and Storesund, 1975; Tota, 1989). A possible explanation as to why the elasmobranch has less total SR but more ryanodine binding than the hagfish may be related to the relative abundance of peripheral couplings or junctional SR, with which the RyR is known to be associated. More quantitative information is needed to resolve this issue. It is also not known whether there are differences between spongy and compact myocardium with respect to content or complexity of SR.

The calculated RyR receptor densities shown in Table 4 were 65 for rat, 21 for trout, 3 for dogfish, and <1 for hagfish. The value of 65 sites μm^{-3} for rat is comparable to a previous report of 0.83 pmol mg^{-1} myocyte protein, which corresponds to approximately 53 receptors μm^{-3} after correcting for 0.6 mg of myocyte protein per milligram of homogenate and 127 mg of homogenate protein per cm^3 (Bers and Stiffel, 1993). The lower number obtained from the myocyte study may be a reflection of greater receptor degradation due to the higher incubation temperatures used. The lower RyR densities for trout, dogfish and hagfish clearly reflect the reduced volume of SR and hence the contribution of SR Ca^{2+} in these species, as indicated by light and electron microscopy (Santer, 1985).

The presence of high-affinity RyRs binding at room temperature (Fig. 4) in trout, dogfish and hagfish, although in smaller numbers than in rat, suggests that functional RyRs may exist in the SR of these species. There is an abundance of evidence correlating the relative amount of SR with both the extent of [^{3}H]ryanodine binding and the ability of ryanodine to suppress tension development in mammals and amphibians (Bers, 1985; Wibo *et al.* 1991). Ryanodine is known to depress twitch tension by varying degrees depending upon species, tissue and development (Bers, 1985; Wibo *et al.* 1991). The frog heart is relatively insensitive to ryanodine and has considerably less total SR than mammalian heart (Bers, 1985). In the rat heart, a profound change in E–C coupling occurs during ontogeny (Wibo *et al.* 1991). Active tension generation in ventricular strips from the neonate is suppressed by the dihydropyridine nifedipine while being relatively insensitive to ryanodine. Conversely, the ability of ryanodine to suppress tension is greatly increased in the adult, while dihydropyridine-sensitivity is diminished. The amount of SR is known to increase during ontogeny and is associated with a concomitant increase in [^{3}H]ryanodine binding (Wibo *et al.* 1991).

In assessing the apparent effects of ryanodine, knowledge of the experimental conditions, especially incubation time and temperature, is of critical importance. Ryanodine binding is extremely slow and is both [Ca^{2+}]- and temperature-dependent with a Q_{10} of 4 (Carroll *et al.* 1991). Therefore, experiments using ryanodine at concentrations known to block SR Ca^{2+} release in mammals may not show an effect at the lower temperatures used in the fish preparations. In addition, cooling from 23 to 5–10 °C increases the open probability of sheep RyRs reconstituted into lipid bilayers from 0.1 to 0.7 (Sitsapesan *et al.* 1991). Rapid cooling to below 5 °C in isolated mammalian myocytes induces a rapid cooling contracture (RCC) which is caused, apparently, both by the

opening of the SR Ca^{2+}-release channel in response to the cold and by the inability of Ca^{2+} removal systems to operate at the lower temperatures. In the Amphibia, only very small RCCs can be elicited, partly because of the sparseness of SR and also because other Ca^{2+} transport mechanisms, such as the Na^+/Ca^{2+} exchanger, continue to function at lower temperatures (Bersohn *et al.* 1991). If the SR Ca^{2+}-release channel maintains a mammalian temperature-dependence, the SR may be rendered non-functional at the lower temperatures experienced by some species.

Owing to the relative paucity of SR and the general lack of ryanodine-sensitivity in lower vertebrates, the role of SR Ca^{2+} release in contractile activation has usually been dismissed. However, under certain conditions, it is possible to induce SR Ca^{2+} release in several poikilotherms (Driedzic and Gesser, 1988, 1994; El-Sayed and Gesser, 1989). Post-rest potentiation (PRP), a phenomenon ascribed to greater SR Ca^{2+} release after a prolonged rest interval, while nonphysiological and temperature-dependent, has been demonstrated in ventricular strips from skate at 10 °C and trout at 15 °C (Driedzic and Gesser, 1988; El-Sayed and Gesser, 1989). In addition, ryanodine at concentrations known to block SR Ca^{2+} release in mammals abolished the potentiated force in the trout. The inability of ryanodine to suppress force under more physiologically relevant conditions may have been due to the lower temperatures used. In a more recent study, contractility was substantially reduced by ryanodine in atrial strips from skipjack tuna at 25 °C (Keen *et al.* 1992). Further evidence of a role for SR in regulating contractility in trout myocardium comes from observations on the influence of temperature on both ryanodine-sensitivity and force–frequency relationships in trout heart (Hove-Madsen, 1992). In contrast to mammals and the frog, which show a decrease in force when temperatures are raised (Shattock and Bers, 1987), ventricular strips from trout are able to maintain twitch force (0.2 Hz) at temperatures between 15 and 25 °C (Hove-Madsen, 1992). This may be because there was a greater contribution of SR Ca^{2+} release at the higher temperature. This is supported by the finding that the ability of ryanodine to suppress both the twitch force and PRP was significantly greater at 25 °C than at 15 °C. In both cases, the reduction in force induced by ryanodine was due to a lower maximum rate of force generation while time-to-peak force was not altered. In agreement with a greater contribution of Ca^{2+} release from the SR is the observation that the concentration of ryanodine required to reduce contractile force by half ($K_{1/2}$) is raised substantially at lower temperatures. The $K_{1/2}$ at 25 °C agrees with the K_d values obtained in all the species compared in this study at the same temperature. The current evidence thus suggests that the RyR has not undergone any evolutionary selection for improved function at lower temperatures. Therefore, it seems possible that at higher temperatures, in some fish species, SR Ca^{2+} release may contribute to the regulation of contractility.

RyR/DHPR ratios

The RyR/DHPR ratios given in Table 4 reflect the relative density of receptors per μm^3 cell volume as assessed by binding of radiolabelled ligand. Optimal binding conditions, in terms of $[Ca^{2+}]$, time and temperature, were determined for both assays. The 2 h incubation time at room temperature for RyR represents a trade-off between maximum specific binding due to the slow binding kinetics of ryanodine and receptor degradation (Carroll *et al.* 1991). With these limitations in mind, the lower trout and dogfish ratios reflect primarily a reduction in numbers of RyRs. The rat RyR:DHPR ratio shows twice as many RyRs as DHPRs and reflects the large contribution of SR Ca^{2+} release to E–C coupling in this species. The range in heart rate from 300 to 600 beats min^{-1} requires an extremely rapid cycling of Ca^{2+}. In contrast, the heart rates of lower vertebrates are considerably slower; thus, the need for rapid cycling of Ca^{2+} is greatly reduced. A RyR:DHPR ratio of less than 1 may reflect an alteration in E–C coupling to one of reduced reliance on SR Ca^{2+} release and a greater reliance on Ca^{2+} entry across the SL in these species.

Implications for E–C coupling

In mammalian cardiac muscle, a small amount of Ca^{2+} enters the cell, triggering the release of a much larger amount of Ca^{2+} from the SR. While the relative roles of each Ca^{2+} source may vary among mammalian species, the SR remains the largest contributor to contractile activation. A greater involvement of SR in Ca^{2+} cycling would serve to increase the speed of contraction and relaxation. In the hagfish, a sedentary species in which heart rate rarely exceeds 35 beats min^{-1}, output pressures are low and myocardial tension development is slow (Farrell and Jones, 1992; Jensen, 1965). There is no apparent need to develop a rapid Ca^{2+} cycling system involving the SR. The long cardiac action potentials, often approximately 1 s in duration, support the idea of an almost exclusive reliance on SL Ca^{2+} influx (Jensen, 1965). However, because DHP binding was negligible in this species, SL Ca^{2+} influx may occur by another mechanism, such as Na^+/Ca^{2+} exchange. In contrast, both the dogfish (*Squalus acanthias*) and rainbow trout (*Oncorhynchus mykiss*) are more active and are capable of generating greater cardiac outputs and heart rates. As a consequence, there is a need to develop more rapid Ca^{2+} cycling, at least under certain conditions, in these species. Biochemical studies performed on compact and spongy myocardium support the idea that the development of a compact myocardium is a morphological adaptation related to the activity level of the fish species (Santer and Walker, 1980).

Finally, the reduced SR content in the lower vertebrates studied would necessitate a fundamentally different relative contribution to Ca^{2+} removal during relaxation. While little is known about the SL Ca^{2+}-ATPase in lower vertebrates, the SL Na^+/Ca^{2+} exchanger is known to be relatively active even at the lower physiological temperatures of both trout and amphibians (Bersohn *et al.* 1991; Tibbits *et al.* 1992*b*).

In conclusion, the results of this comparative study of DHPR and RyR binding in ventricular homogenates from mammalian, teleost, elasmobranch and agnathan species are consistent with

current evidence indicating that heart contraction in lower vertebrates is largely dependent upon trans-sarcolemmal Ca^{2+} entry, although Ca^{2+} release from the SR may also make a contribution, depending upon species and environmental factors such as temperature.

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References

ANVERSA, P., OLIVETTI, G., MELISSARI, M. AND LOUD, A. V. (1979). Morphometric study of myocardial hypertrophy induced by abdominal aortic stenosis. *Lab. Invest.* **40**, 341–349.

BERS, D. M. (1985). Calcium influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *Am. J. Physiol.* **248**, H366–H381.

BERS, D. M. AND STIFFEL, V. A. (1993). Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E–C coupling. *Am. J. Physiol.* **264**, C1587–C1593.

BERSOHN, M. M., VEMURI, R., SCHUIL, D. S. AND PHILIPSON, K. D. (1991). Effect of temperature on Na^+–Ca^{2+} exchange in sarcolemma from mammalian and amphibian. *Biochim. biophys. Acta* **1062**, 19–23.

BOSSEN, E. H. AND SOMMER, J. R. (1984). Comparative stereology of the lizard and frog myocardium. *Tissue & Cell* **16**, 173–178.

CARROLL, S., SKARMETA, J. G., YU, X., COLLINS, K. D. AND INESI, G. (1991). Interdependence of ryanodine binding, oligomeric receptor interactions and Ca^{2+} release regulation in junctional sarcoplasmic reticulum. *Archs Biochem. Biophys.* **290**, 239–247.

CAVALIE, A., MCDONALD, T. F., PELZER, D. AND TRAUTWEIN, W. (1985). Temperature-induced transitory and steady-state changes in the calcium current of guinea pig ventricular myocytes. *Pflügers Arch.* **405**, 294–296.

CHURCOTT, C. S., MOYES, C. D., BRESSLER, B. H., BALDWIN, K. M. AND TIBBITS, G. F. (1994). Temperature and pH effects on Ca^{2+} sensitivity of cardiac myofibrils: a comparison of trout and mammals. *Am. J. Physiol.* **267**, R62–R70.

DEPOVER, A., GRUPP, I. L., GRUPP, G. AND SCHWARTZ, A. (1983). Diltiazem potentiates the negative inotropic action of nimodipine in heart. *Biochem. biophys. Res. Commun.* **114**, 922–929.

DRIEDZIC, W. R. AND GESSER, H. (1988). Differences in force–frequency relationships and calcium dependency between elasmobranch and teleost hearts. *J. exp. Biol.* **140**, 227–241.

DRIEDZIC, W. R. AND GESSER, H. (1994). Energy metabolism and contractility in ectothermic hearts: hypoxia, acidosis and low temperature. *Physiol. Rev.* **74**, 221–258.

EL-SAYED, M. F. AND GESSER, H. (1989). Sarcoplasmic reticulum, potassium and cardiac force in rainbow trout and plaice. *Am. J. Physiol.* **257**, R599–R604.

FABIATO, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am. J. Physiol.* **245**, C1–C14.

FARRELL, A. P. AND JONES, D. R. (1992). The heart. In *Fish Physiology*, vol. XII, part A (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 1–73. San Diego, CA: Academic Press Inc.

FARRELL, A. P. AND MILLIGAN, C. L. (1986). Myocardial intracellular pH in a perfused rainbow trout heart during extracellular acidosis in the presence and absence of adrenaline. *J. exp. Biol.* **125**, 347–359.

FLOCKERZI, V., OEKEN, H., HOFMANN, F., PELZER, D., CAVALIE, A. AND TRAUTWEIN, W. (1986). Purified dihydropyridine-binding site from skeletal muscle T-tubules is a functional calcium channel. *Nature* **323**, 66–68.

FRANK, J. S. AND LANGER, G. A. (1974). The myocardial interstitium: Its structure and its role in ionic exchange. *J. Cell Biol.* **60**, 586–601.

FRANZINI-ARMSTRONG, C. AND JORGENSEN, A. O. (1994). Structure and development of E–C coupling units in skeletal muscle. *A. Rev. Physiol.* **56**, 509–534.

GWATHMEY, J. K. AND MORGAN, J. P. (1991). Calcium handling in myocardium from amphibian, avian and mammalian species: the search for two components. *J. comp. Physiol.* **161**, 19–25.

HARRISON, S. M. AND BERS, D. M. (1990). Temperature dependence of myofilament Ca sensitivity of rat, guinea pig and frog ventricular muscle. *Am. J. Physiol.* **258**, C274–C281.

HELLE, K. B. AND STORESUND, A. (1975). Ultrastructural evidence for a direct connection between the myocardial granules and the sarcoplasmic reticulum in the cardiac ventricle of *Myxine glutinosa* (L.). *Cell Tissue Res.* **163**, 353–363.

HOVE-MADSEN, L. (1992). The influence of temperature on ryanodine sensitivity and the force–frequency relationship in the myocardium of rainbow trout. *J. exp. Biol.* **167**, 47–60.

JENSEN, D. (1965). The aneural heart of the hagfish. *Ann. N.Y. Acad. Sci.* **127**, 443–458.

KEEN, J. E., FARRELL, A. P., TIBBITS, G. F. AND BRILL, R. W. (1992). Cardiac dynamics in tunas. II. Effect of adrenaline, calcium and ryanodine on force–frequency relationships in atrial strips from skipjack tuna, *Katsuwonus pelamis. Can. J. Zool.* **70**, 1211–1217.

LACHNIT, W. G., PHILLIPS, M., GAYMAN, K. J. AND PESSAH, I. N. (1994). Ryanodine and dihydropyridine binding patterns and ryanodine receptor mRNA levels in myopathic hamster heart. *Am. J. Physiol.* **267**, H1205–H1213.

LEAK, L. V. (1969). Electron microscopy of cardiac tissue in a primitive vertebrate *Myxine glutinosa. J. Morph.* **128**, 131–158.

MCDONALD, T. F., PELZER, S., TRAUTWEIN, W. AND PELZER, D. J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal and smooth muscle cells. *Physiol. Rev.* **74**, 365–507.

MEISSNER, G. (1994). Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *A. Rev. Physiol.* **56**, 485–508.

MIKAMI, A., IMOTO, K., TANABE, T., NIIDOME, T., MORI, Y., TAKESHIMA, H., NARUMIYA, S. AND NUMA, S. (1989). Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature* **340**, 230–233.

OTSU, K., WILLARD, H. F., KHANNA, V. K., ZORZATO, F., GREEN, N. M. AND MACLENNAN, D. H. (1990). Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. biol. Chem.* **265**, 13472–13483.

PAGE, E. (1978). Quantitative ultrastructural analysis in cardiac membrane physiology. *Am. J. Physiol.* **235**, C147–C158.

PESSAH, I. N. AND ZIMANY, I. (1991). Characterization of multiple [^{3}H]ryanodine binding sites on the Ca^{2+} release channel of sarcoplasmic reticulum from skeletal and cardiac muscle: Evidence for a sequential mechanism in ryanodine action. *Molec. Pharmac.* **39**, 679–689.

POUPA, O., ASK, J. A. AND HELLE, K. B. (1984). Absence of calcium paradox in the cardiac ventricle of the Atlantic hagfish (*Myxine glutinosa*). *Comp. Biochem. Physiol.* **78**A, 181–183.

SANTER, R. M. (1985). Morphology and innervation of the fish heart. *Adv. Anat. Embryol. Cell Biol.* **89**, 1–102.

SANTER, R. M. AND WALKER, M. G. (1980). Morphological studies on the ventricle of teleost and elasmobranch hearts. *J. Zool., Lond.* **190**, 259–272.

SHATTOCK, M. J. AND BERS, D. M. (1987). Inotropic response to hypothermia and the temperature-dependence of ryanodine action in isolated rabbit and rat ventricular muscle: Implications for excitation–contraction coupling. *Circulation Res.* **61**, 761–771.

SITSAPESAN, R., MONTGOMERY, R. A. P., MACLEOD, K. T. AND WILLIAMS, A. J. (1991). Sheep cardiac sarcoplasmic reticulum calcium-release channels: Modification of conductance and gating by temperature. *J. Physiol., Lond.* **434**, 469–488.

SOMMER, J. R., JOHNSON, E. A., WALLACE, N. R. AND NASSAR, R. (1988). Cardiac muscle following quick-freezing: Preservation of *in vivo* ultrastructure and geometry with special emphasis on intercellular clefts in the intact frog heart. *J. molec. cell. Cardiol.* **20**, 285–302.

SUTKO, J. L. AND KENYON, J. L. (1983). Ryanodine modification of cardiac muscle responses to potassium-free solutions. *J. gen. Physiol.* **82**, 385–404.

TIBBITS, G. F., HOVE-MADSEN, L. AND BERS, D. M. (1991). Calcium transport and the regulation of cardiac contractility in teleosts: a comparison with higher vertebrates. *Can. J. Zool.* **69**, 2014–2019.

TIBBITS, G. F., KASHIHARA, H., THOMAS, M. J., KEEN, J. E. AND FARRELL, A. P. (1990). Ca^{2+} transport in myocardial sarcolemma from rainbow trout. *Am. J. Physiol.* **259**, R453–R460.

TIBBITS, G. F., MOYES, C. D. AND HOVE-MADSEN, L. (1992*a*). Excitation–contraction coupling in the teleost heart. In *Fish Physiology*, vol. XII, part A (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 267–304. San Diego, CA: Academic Press Inc.

TIBBITS, G. F., PHILIPSON, K. D. AND KASHIHARA, H. (1992*b*). Characterization of myocardial Na^{+}–Ca^{2+} exchange in rainbow trout. *Am. J. Physiol.* **262**, C411–C417.

TIBBITS, G. F., SASAKI, M., IKEDA, M., SHIMADA, K., TSURUHARA, T. AND NAGATOMO, T. (1981). Characterization of rat myocardial sarcolemma. *J. molec. cell. Cardiol.* **13**, 1051–1061.

TOTA, B. (1989). Myoarchitecture and vascularization of the elasmobranch heart ventricle. *J. exp. Zool*. (Suppl.) **2**, 122–135.

WIBO, M., BRAVO, G. AND GODFRAIND, T. (1991). Postnatal maturation of excitation–contraction coupling in rat ventricle in relation to the subcellular localization and surface density of 1,4-dihydropyridine and ryanodine receptors. *Circulation Res.* **68**, 662–673.

WIER, W. G. (1990). Cytoplasmic $[Ca^{2+}]_i$ in mammalian ventricle: Dynamic control by cellular processes. *A. Rev. Physiol.* **52**, 2440–2447.