Myocardial sarcolemma isolated from skipjack tuna, *Katsuwonus pelamis*

GLEN F. TIBBITTS AND HARUYO KASHIHARA
Cardiac Membrane Research Laboratory, School of Kinesiology, Simon Fraser University, Burnaby, B.C.,
Canada V5A 1S6

AND

RICHARD W. BRILL
Southwest Fisheries Science Center, Honolulu Laboratory, National Marine Fisheries Service, National Oceanic and
Atmospheric Administration, 2570 Dole Street, Honolulu, HI, 96822-2396, U.S.A.

Received March 25, 1991
Accepted August 27, 1991


The high cardiac outputs observed in tuna compared with other teleosts may imply differences in the regulation of myocardial contractility. Sarcolemma is a critical organelle in the teleost heart in the regulation of intracellular calcium concentration and hence contractility. A procedure is described for the isolation of large quantities of highly purified sarcolemma from the skipjack tuna heart by using the sarcolemma marker K+ stimulated p-nitrophenylphosphatase (KpNPPase). From a starting ventricular wet weight of about 60 g, a yield of >20 mg sarcolemmal protein and a >43-fold purification of the enzyme were achieved. The percentage recovery of the total KpNPPase in the sarcolemma fraction was greater than 14%. Both the purification index and recovery were substantially greater than those achieved with a much smaller scale sarcolemma preparation recently described for the trout heart. The equilibrium binding constant of dihydropyridine PN200-110 and the dissociation constant were found to be 0.48 ± 0.07 pmol · mg protein−1 and 0.09 µM respectively, similar to that found in mammals. In crude homogenates, the dihydropyridine receptor binding density (Bmax) and dissociation constant (Kd) were 0.07 ± 0.01 pmol · mg protein−1 and 0.09 µM

**Introduction**

Cardiac output, normalized per unit body mass, is substantially higher in the tuna relative to other teleosts and is comparable to that observed in mammals (Brill and Bushnell 1991). This is a consequence of both a higher operating heart rate (HR) and higher normalized stroke volume (SV) compared with other lower vertebrates. The observation that SV is substantially higher than that of other teleosts when expressed per unit body mass may be related to the following: (i) a ventricular mass that is several times higher than that of other teleosts when normalized per unit body mass, (ii) high venous return, and (iii) enhanced contractility. Clearly, the higher heart (or more importantly, left ventricle) to body weight ratio is well documented (Farrell 1991) and is an important factor in the determination of SV. However, the tuna heart may also exhibit important differences in myocyte contractile function that are consistent with both the higher HR and SV. These would include the rates of Ca2+ delivery to and removal from the contractile element that determine the strength of cardiac contraction and the minimum duration of the cardiac cycle. Virtually nothing is known about these functions in the tuna heart.

It is well documented that myocardial sarcolemma (SL) is an important regulator of the intracellular calcium concentration ([Ca2+]), which is crucial in the regulation of contractility (Langer 1980). At least three SL proteins are involved in the...
regulation of [Ca\(^{2+}\)], in the mammalian heart: Ca\(^{2+}\) ATPase, Na\(^+\)-Ca\(^{2+}\) exchange, and voltage-dependent Ca\(^{2+}\) channels (Carafoli 1987). Both Na\(^+\)-Ca\(^{2+}\) exchange and L-type voltage-dependent Ca\(^{2+}\) channels (LCC) were recently demonstrated in the trout heart (Tibbits et al. 1990). LCC are known to bind dihydropyridines (DHP) with high affinity (Mikami et al. 1989) and thus can be quantified by DHP ligand binding. Furthermore, in the mammalian heart, the DHP receptor of the SL comes in proximity to and is implicated in the opening of the Ca\(^{2+}\) release channel of the sarcoplasmic reticulum (SR). Thus, in the mammalian heart, the opening of the LCC and the subsequent Ca\(^{2+}\) influx are prerequisites for the release of SR Ca\(^{2+}\), the major source of contractile Ca\(^{2+}\) (Fabio 1983). In lower vertebrates, however, the importance of SR Ca\(^{2+}\) release in contractility is not well established (Klitzner and Morad 1983; Tibbits et al. 1991). Electron micrographs indicate that T-tubules are absent and that the SR is both sparse and poorly organized relative to that of higher vertebrates (Santer 1985). Furthermore, tension generation in cardiac tissue from lower vertebrates is insensitive to Ryanodine in concentrations known to block the release of SR Ca\(^{2+}\) (Bers 1985; Driedzic and Gesser 1988).

The tuna heart, with a high HR and SV, may have a necessarily more complex system of Ca\(^{2+}\) regulation than other teleosts in order to meet these stringent demands. Thus this investigation was undertaken to obtain a better understanding of the molecular basis of Ca\(^{2+}\) regulation in the high-performance tuna heart. This article describes a suitable means of isolating large quantities of highly purified sarcolemma and some preliminary determinations of its properties.

Methods

Animal care and handling

Skipjack tuna, Katsuwonus pelamis, weighing 1–2 kg, were obtained from a local commercial fishing vessel and maintained in outdoor tanks at 24–26°C typically for 2–3 days at the Kewalo Research Facility in Honolulu.

Sarcolemmal isolation and characterization

Myocardial sarcolemma was isolated using modifications of techniques described for teleosts by Tibbits et al. (1990) and for mammals by Philipson and Ward (1987). The principal solution used in the isolation procedure was homogenizing medium (HM), which contained, in mM: sucrose, 350; N-tris(hydroxymethyl)-2,2-aminomethylpropane-1-sulfonic acid (TES), 20 (pH 7.6 at 25°C); and dithiothreitol (DTT), 1. A second solution (HMI) was produced by adding the following protease inhibitors (with final concentrations indicated in μM) to the HM: phenylmethylsulfonylfluoride (PMSF), 100; pepstatin A, 0.1; iodoacetamide, 1; benzamidine, 0.75; and the following (in mg/mL): trypsin soybean inhibitor, 0.1; leupeptin, 0.05; phenanthroline, 0.1; and aprotinin, 0.05.

After the tuna was killed by a sharp blow to the head and an incision made ventrally, the heart (approximately 5 g) was excised quickly and then rinsed in HMI maintained at 4°C. The heart was trimmed of connective tissue, atria, and fat and then weighed. Twelve tuna ventricles were pooled to give a mean starting wet weight of about 6 g for each of the three separate isolations. The pooled ventricles were minced in about three volumes of HMI with scissors to about 2 mm\(^3\) and homogenized twice for 10 s each time in about four volumes of HMI in a chilled Waring blender set at high speed. The homogenate was brought to a volume of 290 mL with HMI, and 2 mL was removed for subsequent biochemical analyses of the crude homogenate (CH). The contractile proteins were solubilized by adding 32 mL of a KCl and NaPPO\(_4\) solution to the 288 mL of CH, bringing the final concentrations of KCl and NaPPO\(_4\) to 100 and 25 mM, respectively. The CH was spun at 20,000 × g for 35 min at 4°C. After the supernatants were discarded, the pellets were homogenized in HM with a Polytron homogenizer at setting 5–6 until the pellets were resuspended. This suspension was spun at 20,000 × g for 35 min, and once again the supernatant was discarded and the pellet resuspended in HM with a Polytron homogenizer at a setting of 5–6. The resultant suspension was spun at 38,000 × g for 45 min at 4°C.

These pellets were resuspended in six 5-mL aliquots of 45% sucrose. Six discontinuous sucrose gradients comprising 32, 30, 28, and 8% sucrose steps were layered on top. The gradients were spun in a swing bucket rotor (SW 28) at 122,000 × g for 16 h and each was fractionated into four tubes labeled F1 to F4 in order of increasing density. All fractions were diluted with roughly equal volumes of an ice-cold solution of 560 mM NaCl and 40 mM TES in order to avoid osmotic shock. The tubes were allowed to equilibrate for approximately 90 min on ice, after which they were slowly diluted with an ice-cold loading medium (LM) that contained 140 mM NaCl and 10 mM TES (pH 7.6 at 25°C). The suspensions were centrifuged at 180,000 × g for 1 h, and the pellets from F2 and F3 were resuspended in 3 mL LM (to give a final protein concentration of ~6 mg·mL\(^{-1}\)), characterized as described below, and then frozen in liquid N\(_2\). Protein determinations on all fractions were done by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The SL marker K\(^{+}\)-stimulated p-nitrophenolphosphatase (K\(^{+}\)pNPPase) was assayed, as described by Tibbits et al. (1991), in the crude homogenate and all fractions derived from the sucrose gradient. Purification index (PI) was defined as the ratio of the specific activity of K\(^{+}\)pNPPase in the sarcolemmal fraction to that in the crude homogenate. Recovery was defined as the percentage of the total K\(^{+}\)pNPPase activity (μmol·h\(^{-1}\)) in the SL fraction compared with the crude homogenate.

Dihydropyridine binding

The binding of the dihydropyridine [\(^{3}H\) NPN200-110] was measured in both the crude homogenate and the F2 (SL-enriched) fraction derived from the sucrose gradients. The tubes contained (in a final volume of 5 mL) 20 mM TES (pH 7.6 at 25°C), 0.05–0.8 nM [\(^{3}H\) NPN200-110], and 2.5 mM CaCl\(_2\). The amount of protein in each tube was approximately 25 μg for F2 and 250 μg for CH, and the binding reaction was allowed to proceed for 90 min in the dark at room temperature. Assays were performed in both the presence and the absence of 1 μM unlabeled nifedipine to determine specific binding. All binding assays were conducted in dim (10 W) red light to prevent photoinactivation of the dihydropyridines. Binding was terminated by rapid vacuum filtration through GF/C (Whatman) filters. The filters were washed with three 4.5 mL aliquots of ice-cold buffer, placed in a vial, dried, and counted by standard liquid scintillation procedures.

Ryanodine binding

The binding of [\(^{3}H\) Ryanodine to crude homogenates was carried out with minor modifications of the procedure described by Chu et al. (1988). The binding reaction was carried out with 160 μg homogenate protein (at 28°C for 1 h) in a tube with a 1-mL final volume that contained the following: 11 nM [\(^{3}H\) Ryanodine, nonradioactive ryanodine (Calbiochem) in concentrations of 0–500 nM, 150 mM KCl, 20 mM TES (pH 7.6 at 25°C), and 25 mM CaCl\(_2\). In pilot studies, we determined that these conditions resulted in equilibrium binding. No buffers were added in an attempt to regulate free Ca\(^{2+}\). The ryanodine was dissolved in ETOH and the final [ETOH] was less than 0.5%, which we determined previously to have no effect on the binding. After 1 h, the binding reaction was stopped by filtration through 0.45-μm cellulose acetate filters. The filters were rinsed first with 2 mL of a solution containing 150 mM KCl and 10 mM TES (pH 7.6 at 25°C), and finally with 1 mL of a 10% ETOH solution. The dried filters were counted by conventional liquid scintillation procedures.

Materials

[\(^{3}H\) NPN200-110 and [\(^{3}H\) Ryanodine were purchased from New England Nuclear and were used without further purification. With the
CAN. J. ZOOL. VOL. 70, 1992

**FIG. 1.** Amount of [³H]PN200-110 bound (bnd) to purified sarcotelia as a function of free [³H]PN200-110 concentration. Both the total (○) and the specific (●) binding are indicated.

**FIG. 2.** Scatchard plot of the specific binding data shown in Fig. 1. A least-squares linear regression line is drawn through the data with a correlation coefficient of >0.95. B/F, bound/free; bnd, bound.

**Table 1.** Protein yield (normalized per unit of ventricular wet weight) and specific K⁺pNPPase activity at 37°C (normalized per unit of protein) in the crude homogenate and various fractions from the sucrose gradient.

<table>
<thead>
<tr>
<th>Protein yield</th>
<th>Specific K⁺pNPPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>103.59 ± 4.97</td>
</tr>
<tr>
<td>F2</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>F3</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>F4</td>
<td>10.45 ± 2.56</td>
</tr>
</tbody>
</table>

**Table 2.** Recovery and purification index based on the activities of K⁺pNPPase in the crude homogenate and various fractions from the sucrose gradient.

<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>Purification index</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2</td>
<td>14.7</td>
</tr>
<tr>
<td>F3</td>
<td>3.3</td>
</tr>
<tr>
<td>F4</td>
<td>8.8</td>
</tr>
</tbody>
</table>

**Results**

The large-scale SL isolation procedure developed for the tuna heart resulted in the isolation of large quantities of highly purified SL with a yield of 0.31 mg SL protein·g wet wt.⁻¹ (Table 1). The activities of the SL marker K⁺pNPPase in the crude homogenate and SL fraction were 0.20 and 8.66 µmol·mg protein⁻¹·h⁻¹ (Table 1). From these data, the calculated recovery of SL in F2 from the total SL pool was 14.7% and the purification index was 43.3-fold (Table 2).

Figure 1 illustrates the binding of the DHP [³H](+)PN200-110 to highly purified SL as a function of the free concentration of [³H](+)PN200-110 in a typical experiment. The specific binding between 0.05 and 0.20 nM [³H](+)PN200-110 was greater than 81% of the total binding. Total [³H](+)PN200-110 binding involved no more than 3% of the total ligand concentration. The specific binding data shown in Fig. 1 are presented as a Scatchard plot in Fig. 2. The correlation coefficient exceeded 0.95. Using three different SL preparations, the derived binding density (B₅₀) and dissociation constant (Kᵦ) values for [³H](+)PN200-110 equilibrium binding to SL were 0.48 ± 0.07 pmol·mg protein⁻¹ and 0.09 ± 0.01 nM, respectively. The binding of [³H](+)PN200-110 to crude homogenates from tuna ventricles was carried out in an identical manner, except more protein was included per tube. From a Scatchard plot (not shown) of these data, it was calculated that the B₅₀ and Kᵦ values in the crude homogenate were 0.24 ± 0.04 pmol·mg⁻¹ and 0.15 ± 0.02 nM, respectively.

The [³H]ryanodine binding was performed only with crude homogenate protein. The binding to crude homogenates proved to be difficult and not all experiments were successful, as judged by the consistency of the duplicates. The data presented represent those experiments in which the duplicates and goodness of fit were satisfactory. In Fig. 3, the specific binding of [³H]ryanodine to crude homogenates is shown as a function of free ryanodine concentration from 22 to 511 nM. The specific binding at 22 nM represents more than 60% of the total binding of [³H]ryanodine to crude homogenates. The Scatchard representation of these data is shown in Fig. 4, which has a correlation coefficient of >0.99. In three different preparations, the derived B₅₀ of ryanodine binding to crude homogenates was 0.12 ± 0.04 pmol·mg protein⁻¹.
that observed in cold-adapted trout hearts (Tibbits et al. 1990) and mammalian hearts (Tibbits et al. 1989) ventricles. The specific activity of $K^+pNPP$ase in the crude homogenate, $0.20 \mu mol \cdot mg^{-1} \cdot h^{-1}$, is only 31% of that observed in cold-adapted trout hearts (Tibbits et al. 1990). Both of these assays were conducted at 37°C for comparison with mammalian values; however, the temperature dependence of this enzyme was evaluated in both teleost species, to determine whether $K^+pNPP$ase is denatured at 37°C and to confirm this statement. Since the $K^+pNPP$ase activity reflects the Na+-K+ pump (Schwartz et al. 1990), we have evidence that the high $K^+pNPP$ase activity in trout ventricles is about one-half that in cold-adapted heart (G. F. Tibbits and H. Kashihara, unpublished observations) and thus may not require the high pump densities that are apparent in the cold-adapted trout. The SL isolation procedure described in this paper was successful in producing a reasonable yield of highly purified membranes from the tuna heart. The fraction enriched in SL (F2), had a yield of 0.31 mg protein · g wet wt. $^{-1}$, which is about two-thirds that achieved with a much smaller scale procedure using trout ventricles. However, the advantages of obtaining $\sim 20$ mg SL per isolation with this procedure versus $\sim 3$ mg with the small-scale approach are obvious. Furthermore, the recovery and purification of SL from the tuna ventricles are about 2 and 3 times greater, respectively, than those obtained with trout by means of the procedure described previously (Tibbits et al. 1990). The high specific activity of $K^+pNPP$ase of the SL preparation indicates a high degree of purity. However, the extent of cross contamination from other organelles cannot be precisely stated without determinations of markers of SR and other organelles, which could not be performed because of prevailing conditions.

DHP binding was examined in both crude homogenates as well as in purified SL fractions from the tuna ventricles, using SPHIN200-110. The $K_a$ values derived from Scatchard analyses of these binding curves were similar to those reported in trout (Tibbits et al. 1990) and mammalian hearts (Lee et al. 1984), suggesting a similarity in receptor structure across these phyla. The mean $B_{max}$ values from homogenates and SL fractions were 0.24 and 0.48 pmol · mg protein $^{-1}$, respectively. The homogenate $B_{max}$ is about 40% lower than that observed in the cold-adapted trout heart. Again, there is reason to believe that the very high DHP $B_{max}$ observed in the cold-adapted trout heart homogenate is, at least in part, a consequence of the cold adaptation (G. F. Tibbits and H. Kashihara, unpublished observations). The $B_{max}$ values in the warm-adapted (15°C) trout are similar to those observed in tuna in the present study. The DHP $B_{max}$ observed in the SL-enriched fractions from tuna in the present study (0.48 pmol · mg protein $^{-1}$) is approximately 15% of that found in purified SL in the cold-adapted trout heart. There are probably two explanations for this disparity. The first is based on the differences in the homogenates. From these data, we would therefore expect the tuna SL DHP $B_{max}$ to be about 40% that of trout. We propose that this is a species difference, an explanation of which is offered below. The second reason may be related to the
higher SL purification realized in the present study and the observation that the DHP receptor does not copurify strictly with conventional SL markers. This is supported by what we and others have observed in mammalian species. Thus, the high purification observed in this study may be at the expense of recovered DHP receptor and components of the junctional complex. However, despite the fact that the DHP $B_{max}$ in SL in the tuna heart is lower than that of trout, it should be noted that it is comparable to that observed in mammals (Glossmann et al. 1984).

The SR Ca$^{2+}$ release channel, or ryanodine receptor, controls the efflux of Ca$^{2+}$ from the SR to the cytosol (Meissner 1986), which in the mammalian heart is the largest source of Ca$^{2+}$ delivered to the myofilaments (Wier 1990). Ryanodine inhibits force production in the mammalian heart (Satko and Kenyon 1983) by binding with very high affinity to the release channel and affecting the opening of the channel in a dose-dependent manner (Meissner 1986). The binding of [3H]ryanodine to crude homogenates allowed us to determine the relative density of the ryanodine receptor in this tissue. The $B_{max}$ observed in this study, 0.12 pmol mg protein$^{-1}$, is similar to that observed by others (e.g., Fessah et al. 1985) in mammalian hearts. Because of the difficulty with the ryanodine binding and a variety of other complications, we would prefer, at this stage, not to speculate about the stoichiometry of these receptors. We are not aware of ryanodine binding having been reported in cardiac tissue from other teleosts. These data suggest that there is a reasonable amount of SR Ca$^{2+}$-release channel protein in the tuna ventricle despite the apparent sparsity of SR observed in electron micrographs (Breisch et al. 1983). The function of the SR as a contributing source of Ca$^{2+}$ is indicated further by the inhibition of atrial contractions by ryanodine in the study by Keen et al. (1992). As suggested previously (Tibbets et al. 1991), a reasonable hypothesis may be that the magnitude of SR releasable Ca$^{2+}$ in the teleost heart is temperature dependent. It may be postulated that at lower (<20°C) temperatures the channel remains open and the SR is unable to sequester Ca$^{2+}$, thus eliminating the SR as a functional Ca$^{2+}$-release site. Preliminary evidence to support this hypothesis comes from studies (L. Hove-Madsen, unpublished observations) using ventricular strips from the trout heart studied at different temperatures.

We propose, therefore, that the tuna heart may have a higher reliance on SR Ca$^{2+}$ for contraction than other lower vertebrates. This may be the consequence of a more fully developed SR or simply the fact that the tuna physiological temperature range (~25°C) allows the SR Ca$^{2+}$-release channel to function appropriately. This hypothesis offers an explanation for the lower DHP $B_{max}$ observed in this study; namely, there is less dependence on SL Ca$^{2+}$ influx for contraction in the tuna heart. However, considering the observation that the L-type Ca$^{2+}$ channel has a high $Q_{10} (~3)$ in mammals (Cavalli et al. 1985), it is also possible that elevated operational temperatures in tuna do not necessitate high channel densities for adequate Ca$^{2+}$ current. The notion of greater reliance on SR Ca$^{2+}$ release for contraction offers some advantages to the tuna heart, most notably, higher rates of delivery and removal of Ca$^{2+}$ from the contractile element. The latter would be consistent with the shorter cardiac cycle required for the high heart rates observed in these species (Brill and Bushnell 1991). It should be stated that because of the limited data from this species, these inferences must be considered as no more than working hypotheses at this time. Much more extensive investigation (involving both biochemical and physiological approaches) is required before these hypotheses can gain acceptance.

In summary, we have been able to isolate highly purified SL in large quantities from the tuna ventricle. This procedure should permit experiments in the future that will offer a much greater understanding of the role of SL in myocardial function in teleosts than has heretofore been possible.

**Acknowledgements**

The SL isolation was conducted for the most part in the laboratory of Dr. Howard Mower in the Department of Biochemistry at the University of Hawaii, and the radioligand binding experiments were carried out in the laboratory of Dr. David Karl in the Department of Oceanography at the University of Hawaii. Without the generous hospitality of Drs. Mower and Karl and Ms. Georgia Tien, these experiments would not have been possible. The captain and crew of the *Corsair*, the staff at Kewalo Research Facility, and especially Mr. Shigeru Yano, played a critical role in the provision of skipjack tuna. The coordinating efforts of Drs. A. P. Farrell and P. Hochachka are also appreciated. The investigators gratefully acknowledge grant support from the Natural Sciences and Engineering Research Council of Canada to G. F. T. (No. 2231).


Fabian, A. 1985. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am. J. Physiol. 248: C1–C14.


Keen, J. E., Farrell, A. P., Tibbets, G. F., and Brill, R. W. 1992. Cardiac physiology in tunas. II. Effect of ryanodine, calcium, and


