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RESEARCH ARTICLE

Rainbow trout myocardium does not exhibit a slow inotropic response to stretch

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SUMMARY

Mammalian myocardial studies reveal a biphasic increase in the force of contraction due to stretch. The first rapid response, known as the Frank–Starling response, occurs within one heartbeat of stretch. A second positive inotropic response occurs over the minutes following the initial stretch and is known as the slow force response (SFR). The SFR has been observed in mammalian isolated whole hearts, muscle preparations and individual myocytes. We present the first direct study into the SFR in the heart of a non-mammalian vertebrate, the rainbow trout (*Oncorhynchus mykiss*). We stretched ventricular trabecular muscle preparations from 88% to 98% of their optimal length and individual ventricular myocytes by 7% of their slack sarcomere length (SL). Stretch caused an immediate increase in force in both preparations, indicative of the Frank–Starling response. However, we found no significant effect of prolonged stretch on the force of contraction in either the ventricular trabecular preparations or the single myocytes. This indicates that rainbow trout ventricular myocardium does not exhibit a SFR and that, in contrast to mammals, the piscine Frank–Starling response may not be associated with the SFR. We speculate that this is due to the fish myocardium modulating cardiac output *via* changes in stroke volume to a larger extent than heart rate.

Key words: slow force response, Frank–Starling response, ventricle, myocyte, carbon fibres, trabeculae.

INTRODUCTION

During the cardiac cycle the heart is routinely stretched. In mammals this stretch has been shown to cause a biphasic increase in the force of contraction generated by the cardiac muscle (Calaghan et al., 2003). Initially, the force of contraction increases within one heartbeat of being stretched; this rapid response to stretch is known as the Frank–Starling response (Frank, 1895; Patterson and Starling, 1914). The Frank–Starling response occurs because of an increase in the Ca^{2+} sensitivity of the myofilaments rather than an increase in the amplitude of the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transient in both fish (Shiels et al., 2006) and mammals (Allen and Kurihara, 1982; Kentish and Wrzosek, 1998). The Frank–Starling response links cardiac filling to cardiac ejection and plays a major physiological role in adjusting output between the left and right sides of the heart in mammals (Kockskamper et al., 2008).

A second, slower, stretch-induced increase in contractility was identified in cat papillary muscle (Parmley and Chuck, 1973) and has since been observed in preparations ranging from single cardiac cells to whole hearts in a number of mammalian species (Allen and Kurihara, 1982; Alvarez et al., 1999; von Lewinski et al., 2004; Calaghan and White, 2004; Caldiz et al., 2007). This slow positive inotropic response occurs over the minutes following the initial stretch and is known as the slow force response (SFR). The SFR is associated with an increase in the magnitude of the $[Ca^{2+}]_i$ transient (Allen and Kurihara, 1982). Because the SFR occurs with a prolonged increase in end diastolic volume in mammals, it has been equated with the 'Anrep effect' (Sarnoff et al., 1960). The Anrep effect is the positive inotropy invoked in response to a prolonged increase in end diastolic volume due an increase in aortic pressure (von Anrep, 1912). It has also been suggested that the SFR is a protective mechanism that supplements cardiac force in a diseased myocardium in circumstances of increased preload and/or afterload (Kockskamper et al., 2008). The physiological relevance of the SFR is still under debate as is the precise balance of mechanisms by which it occurs.

It is surprising that the SFR has been largely ignored in non-mammalian myocardium, as non-mammalian vertebrates in general, and fish in particular, are known to be exquisitely sensitive to cardiac stretch (Farrell, 1991; Shiels et al., 2006; Shiels and White, 2008). Rainbow trout (*Oncorhynchus mykiss*) modulate their cardiac output primarily *via* changes in stroke volume, and can increase stroke volume by up to 300% during strenuous activity with little change in heart rate (Farrell and Olson, 2000). This differs from mammals, which, for the most part, modulate cardiac output *via* larger changes in heart rate than stroke volume. These comparisons suggest a shift in the role of volume (and so myocardial stretch) in the regulation of cardiac output during vertebrate evolution (Burggren et al., 1997; Shiels and White, 2008). Indeed, we have recently shown that the fish heart is specialized for large extensions during diastolic filling and for active tension development during systolic emptying from a wide range of lengths (Patrick et al., 2010b). Therefore the SFR may not be of significant benefit to fish; it may have evolved after fish diverged from the vertebrate lineage and reflect the evolutionary shift in cardiac output modulation from stroke volume to heart rate. Conversely, if the SFR is associated with a prolonged increase in venous return, an enhanced SFR may be expected as fish routinely experience large stroke volumes. In order to address these questions we have investigated whether the SFR is present in the rainbow trout heart by measuring the temporal change in force upon stretching trout ventricular trabeculae and trout ventricular myocytes. We found no time-dependent change in force after stretch in either preparation and therefore conclude that the SFR is absent in the trout ventricle.

MATERIALS AND METHODS

Animals

Female rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were purchased from Chirk Trout Farm (Wrexham, UK). The trout were kept in re-circulated freshwater tanks at 11–13°C with a 12 h:12 h light:dark photo-cycle and fed with commercial trout pellets to satiation three times a week. The trout were held for a minimum of 3 weeks prior to the experiments. All procedures were in accordance with local animal handling protocols and adhere to UK Home Office legislation. All chemicals were from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

Stretch of ventricular trabeculae

Rainbow trout (334.5±15.5 g, N=3) were killed by concussion of the brain and severance of the cervical spinal cord. The heart was excised and the ventricle was carefully removed. Four roughly cylindrical trabecular bundles, no more than 1.5 mm in width, were cut from each ventricle. Bundles (1.7±0.1 mg, N=11) were hung from 25 g force transducers in a Myobath II multi-channel tissue bath system (World Precision Instruments, Sarasota, FL, USA) and lowered into separate tissue baths containing oxygenated physiological saline with the following composition (in mmol l^{-1}): NaCl, 150; KCl, 5.4; $MgSO_4$, 1.5; NaH_2PO_4, 0.4; $CaCl_2$, 2.0; glucose, 10; and Hepes, 10; pH adjusted with KOH to 7.8, the pH of interstitial fluid in fresh water teleosts. The trabecular preparations were left for 10 min before being stimulated to contract at 0.2 Hz, with 5–10 ms square voltage pulses at 50% above the threshold voltage for activation (Grass SD9B stimulator, Grass Medical Instruments, Quincy, MA, USA). All experiments were carried out at room temperature (19–21°C). Analogue signals were amplified (Transbridge 4M; World Precision Instruments), A/D converted (LT4/16-S; World Precision Instruments) and then stored on a computer using the DataTrax data acquisition/analysis program (World Precision Instruments).

Trabecular preparations were stretched to quantify the SFR in fish myocardium by adaptation of protocols previously used in mammalian myocardium (Calaghan and White, 2004). Briefly, the length at which maximum tension was generated (L_{max}) was established. The trabecular preparations were then maintained at 98% L_{max} ($L_{98\%}$) until the force of contraction was stable for 10 min and then reduced to 88% L_{max} ($L_{88\%}$) and again allowed to stabilize for 10 min. Muscle preparations were then stretched from $L_{88\%}$ to $L_{98\%}$ and the rapid and slow increases in force were recorded at 10 s and 600 s after stretch, respectively. Time to peak force (T_{peak}), time to 50% relaxation ($T_{50\%}$), the mean rate of contraction, the mean rate of 50% relaxation and the passive (resting) tension were also recorded. Means of six force traces were used to determine force and contractile characteristics for each trabecular muscle preparation at each length.

Stretch of ventricular myocytes

Rainbow trout (125.3±12.3 g, N=6) were killed as described above after which the heart was carefully excised. The heart was cannulated through the bulbus arteriosus and perfused from a height of 50 cm for 10 min with a nominally Ca^{2+}-free isolation solution to clear the heart of blood and to stop the heart contracting. The isolation solution contained (in mmol l^{-1}): NaCl, 100; KCl, 10; KH_2PO_4, 1.2; $MgSO_4$, 4; taurine, 50; glucose, 20; and Hepes, 10 (adjusted to pH 6.9 using KOH). Next, BSA and proteolytic enzymes (collagenase and trypsin) were added to the solution and the heart was perfused for an additional 15 min. The bulbus arteriosus and atria were then removed and the ventricle was cut open and splayed out in a Petri dish containing fresh isolation solution. Individual myocytes were obtained by rinsing the partially digested ventricle with isolation solution. The myocyte suspension was filtered through a nylon mesh and the liberated myocytes were used within 8 h.

A suspension of trout ventricular myocytes was placed on the stage of an inverted microscope and superfused with physiological saline (see above). Carbon fibres were then attached to a single myocyte in order to stretch the myocyte along its longitudinal axis and record tension (Le Guennec et al., 1990; Calaghan and White, 2004; Shiels et al., 2006). One end of the myocyte was attached to a flexible carbon fibre (diameter 12 μm, length 2.0 mm, compliance 80 m N^{-1}) and the other end was attached to a stiff carbon fibre (diameter 12 μm, length 0.5 mm, compliance 1.2 m N^{-1}). Fibres were mounted in the ends of microelectrodes, which were attached to micromanipulators (Sutter Instruments, Novato, CA, USA). All experiments were carried out at room temperature (19–21°C) and myocytes were field stimulated to contract, using platinum electrodes delivering suprathreshold pulses of 5–10 ms duration, at 0.2 Hz (Grass SD9B stimulator). Cells were stimulated to contract at slack sarcomere length (SL) for 5 min and then stretched to the greatest extent possible within a period of 5 s. Stretches were typically 7% of resting SL. The stiff fibre was used to stretch the cell. The Frank–Starling response to stretch was calculated from the increase in force 10 s after the myocyte was stretched and the SFR was calculated as the increase in force 300 s after stretch. Force was measured from the mean of six traces for each cell (N=9) at each length. The displacement of the flexible fibre during auxotonic contractions was used to calculate the force developed. T_{peak}, $T_{50\%}$, the mean rate of contraction and the mean rate of 50% relaxation were recorded for comparison with the trabecular measurements. The position of the carbon fibres and the SL of the myocytes was acquired at a sampling frequency of 60 Hz and analysed in real time using IonOptix equipment and software (IonOptix, Milton, MA, USA). Passive tension was measured as end diastolic force assuming zero passive tension at slack SL; active tension was measured as the additional force developed during systole.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test or, in the case of the passive tension of the trabecular preparation, by a paired *t*-test (Sigmastat 3.5, SysStat software, Waldbronn, Germany). $P<0.05$ indicated a significant difference. We present both representative raw data and mean data ± s.e.m.

RESULTS

Effect of prolonged stretch on ventricular trabeculae

The effect of stretching a ventricular trabecular preparation from $L_{88\%}$ to $L_{98\%}$ is shown in Fig. 1A. From this raw trace the Frank–Starling response is evident as the immediate increase in contractile force upon stretching. We did not observe a secondary increase in the 600 s after the muscle was stretched, indicating the SFR was absent. There was an elastic element of passive tension immediately visible upon stretching as a slow decline in passive tension. Expanded time scale traces of the active force produced before stretch and 10 and 600 s after stretch during a single twitch are shown in Fig. 1B. Again, the immediate Frank–Starling response was apparent but there was no evidence of the SFR. The 10 s and 600 s traces are nearly identical. Mean peak active contractile forces are shown in Fig. 1C. The Frank–Starling response was seen as a significant increase in maximum active force produced by the contracting trabecular preparations 10 s after the muscle was stretched to $L_{98\%}$ ($P<0.05$). We observed no significant difference in the active force produced by the trabecular preparations after they were held at $L_{98\%}$ for 600 s and so no evidence for the SFR. There were significant increases in the mean

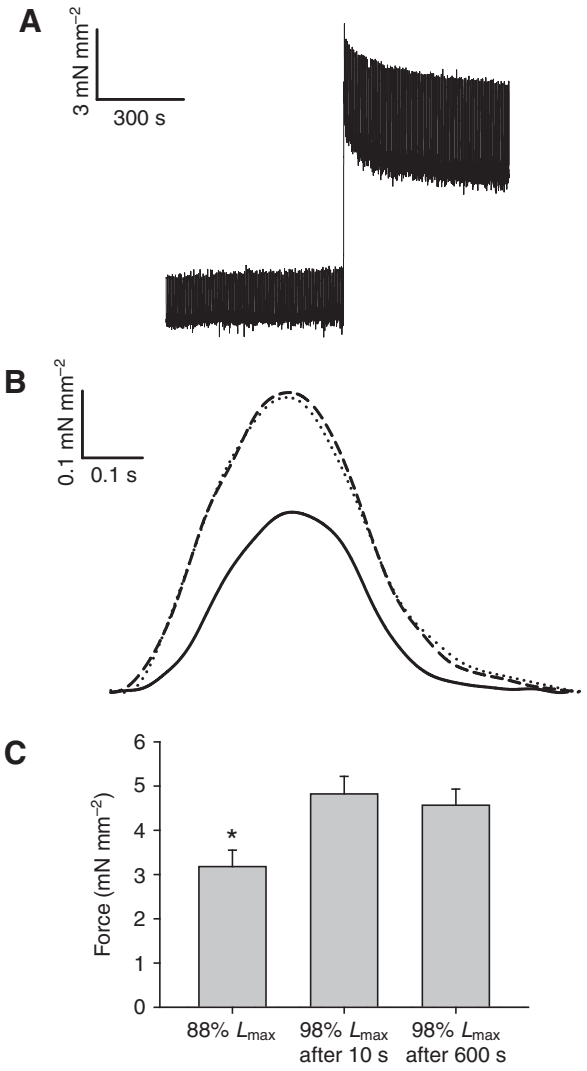

Fig. 1. The effect of stretch on ventricle trabecular muscle preparations from the rainbow trout. (A) A representative trace of the response of a trabecular muscle preparation that has been stretched from 88% ($L_{88\%}$) to 98% ($L_{98\%}$) of the length at which maximum tension was generated. (B) Expanded time scales of representative traces of the active force produced in a single contraction at $L_{88\%}$ (solid line), 10 s after stretch to $L_{98\%}$ (dashed line) and 600 s after stretch to $L_{98\%}$ (dotted line). (C) Mean force generated by the trabecular preparations. *Significant difference from other values (N=11, P<0.05; one-way ANOVA, Student–Newman–Keuls).

rate of contraction and the mean rate of 50% relaxation 10 s after the trabecular preparation was stretched from $L_{88\%}$ to $L_{98\%}$ (P<0.05) but T_{peak} and $T_{50\%}$ were unchanged (see Table 1). Passive tension significantly increased from 8.24±0.96 to 11.75±1.38 mN mm^{-2} as the trabecular preparation was stretched from $L_{88\%}$ to $L_{98\%}$ (P<0.05, not shown). These passive tension measurements are similar to those recorded previously (Harwood et al., 1998).

Effect of prolonged stretch on isolated myocytes

The trabecular tissue data suggested that the trout ventricle does not exhibit the SFR. To be sure of this result we moved to a cellular preparation where SL could be precisely measured and controlled. In addition, moving from the multicellular to the single cell approach allowed us to rule out extracellular and/or paracrine effects in the response to stretch. The slack SL of the cardiac myocytes used in our study was 1.97±0.02 μm (N=9), which is similar to that observed in a recent study in rainbow trout (Patrick et al., 2010b). Typically, we were able to stretch the myocytes by 7% from slack SL. This stretch was held for 300 s. This equated to a mean stretch of 0.14±0.02 μm from a SL of 1.97±0.02 μm to a SL of 2.11±0.03 μm (N=9). Stretching of myocytes beyond this length caused the carbon fibres to detach from the myocytes. In mammals a SL of ~2.1 μm is within the peak (plateau) response of the sarcomere length–tension relationship (Allen and Kentish, 1985). We have previously shown that the trout myocyte has an extended sarcomere length–tension relationship (Shiels et al., 2006), suggesting that the stretches imposed in the current study were on the ascending limb but probably not the peak. Regardless, the ~7% stretches from slack SL resulted in significant length-dependent activation as illustrated by the 185% increase in active force 10 s after myocyte stretch (see Table 1). Moreover, as the SFR in mammals is proportional to the size of the stretch, we would expect a 7% stretch to evoke some SFR if the mechanism was present.

A ventricular myocyte attached to the carbon fibres is shown in Fig. 2A. An example of a representative trace of the force generated by a myocyte at slack SL and after stretch is shown in Fig. 2B. The Frank–Starling response was apparent as the increase in force immediately upon stretch. However, in keeping with the multicellular results, there was no secondary increase in force over the following 300 s, indicating the lack of the SFR at the cellular level. The elastic element of passive tension was also present at the cellular level (Fig. 2B). Fig. 2C shows representative traces of the active force generated by a myocyte during a contraction at slack SL, and 10 s and 300 s after stretch was applied. Mean maximum forces are shown in Fig. 2D. The Frank–Starling response can be clearly seen 10 s after the cell was stretched as the significant increase in active force (P<0.05). The lack of a significant increase in force 300 s after the myocytes were stretched indicates that the SFR was not present. The mean increase in passive tension with stretch was 0.62±0.07 nN μm^{-2}, which is similar to that recorded previously in trout ventricular myocytes using the same technique (Shiels et al., 2006). There was a significant increase in the mean rate of contraction and the mean rate of 50% relaxation 10 s after stretch of the myocytes but no significant difference between the slack and stretched cells for T_{peak} or $T_{50\%}$ (see Table 1).

DISCUSSION

Here we have presented the first direct study of the SFR in a non-mammalian vertebrate, the rainbow trout. We found no significant effect of prolonged stretch on the force of contraction in either the ventricular trabecular preparations or the single ventricular myocytes. The rationale for this dual approach was that an absence of the SFR in myocytes but the presence of the SFR in trabeculae would have pointed to an extracellular and or paracrine mechanism of action. Both of these approaches have been employed in previous mammalian studies to investigate the SFR (Calaghan and White, 2004). We stretched the trout myocytes to an extent known to induce the SFR in mammals and stretched the trabecular preparations to 98% of L_{max}. Thus, we are confident that we would have detected the SFR in the trout ventricular tissue and/or myocytes if such a response was present. The lack of the SFR at both levels of cardiac organization in the rainbow trout heart suggests that in fish, the Frank–Starling response is not associated with the SFR, as it is in mammals, and that it may have evolved after fish diverged from the vertebrate lineage.

The SFR has been found in all mammalian tissues in which it has been investigated. However, to our knowledge, it has not been investigated in any non-mammalian vertebrate or invertebrate heart tissue. In mammals, the SFR may be a protective mechanism that

Table 1. Contractile characteristics of trout ventricular myocytes and trabecular muscle preparations in response to stretch

		N	Active force (mN mm^{-2})	T_{peak} (s)	Mean rate of contraction (mN mm^{-2} s^{-1})	$T_{50\%}$ (s)	Mean rate of 50% relaxation (mN mm^{-2} s^{-1})
Trabeculae							
Resting	$L_{88\%}$	11	3.18±0.37*	0.26±0.01	12.01±1.33*	0.12±0.01	13.07±1.46*
FSR	10 s after $L_{98\%}$	11	4.82±0.40	0.26±0.01	18.57±1.59	0.13±0.01	19.37±2.06
SFR	600 s after $L_{98\%}$	11	4.57±0.36	0.27±0.01	16.81±1.33	0.13±0.01	17.80±1.50
Single cell							
Slack	Resting	9	0.27±0.05*	0.21±0.02	1.37±0.28*	0.08±0.01	2.12±0.45*
FSR	10 s after stretch	9	0.77±0.12	0.23±0.02	3.45±0.52	0.08±0.01	5.06±0.79
SFR	300 s after stretch	9	0.69± 0.10	0.21±0.01	3.37±0.61	0.07±0.01	4.63±0.69

All data are means ± s.e.m. of *N* cells/trabecular preparations.
FSR, Frank–Starling response; SFR, slow force response; T_{peak}, time to peak force; $T_{50\%}$, time to 50% relaxation; 1 nN μm^{-2}=1 mN mm^{-2}.
Trabeculae were stretched to 88% L_{max} ($L_{88\%}$) and 98% L_{max} ($L_{98\%}$), where L_{max} is the length at which maximum tension was generated.
Cells were stretched by 7% of their sarcomere length at slack length.
*Significant difference (P<0.05; one-way ANOVA, Student–Newman–Keuls).

comes into play to protect a diseased heart and allow it to continue to generate force in circumstances of increased venous pressure (Kockskamper et al., 2008). If this is the origin of the SFR, then it may not be surprising that an organism which relies on stretch of the ventricle to regulate cardiac output and exhibits such an exquisitely sensitive Frank–Starling response does not exhibit the SFR. The maximum possible increase in systolic force as a consequence of stretch may be required to enable a stroke volume-regulated heart to function and eject close to 100% of its blood during systole (Franklin and Davie, 1992). The enhanced Ca^{2+} sensitivity and length-dependent activation of fish myocytes (Patrick et al., 2010b) allows the heart to maximize the force produced *via* the Frank–Starling response during large stretches (Shiels et al., 2006) and may preclude the need for the SFR. Further support for the lack of a SFR in the fish heart can be gleaned by scrutinizing whole-heart studies where pre-load has been increased and held for a period of time. Imbrogno and colleagues found no change in stroke volume in the *in vitro* heart of *Anguilla anguilla* 30 min after an increase in pre-load (Imbrogno et al., 2003).

The active forces and kinetic time courses produced by both the trabecular preparations and the cellular preparations are similar to those found in previous studies in this species (Gesser, 1996; Harwood et al., 1998; Shiels et al., 2006). It is thought that as the muscle is stretched, more cross-bridges are formed per unit time because of an increase in myofilament sensitivity to Ca^{2+}; thus, both the amplitude and rate of force development are increased by stretch. These effects are also seen in both multicellular (Allen and Kurihara, 1982) and single myocyte preparations of mammalian myocardium (White et

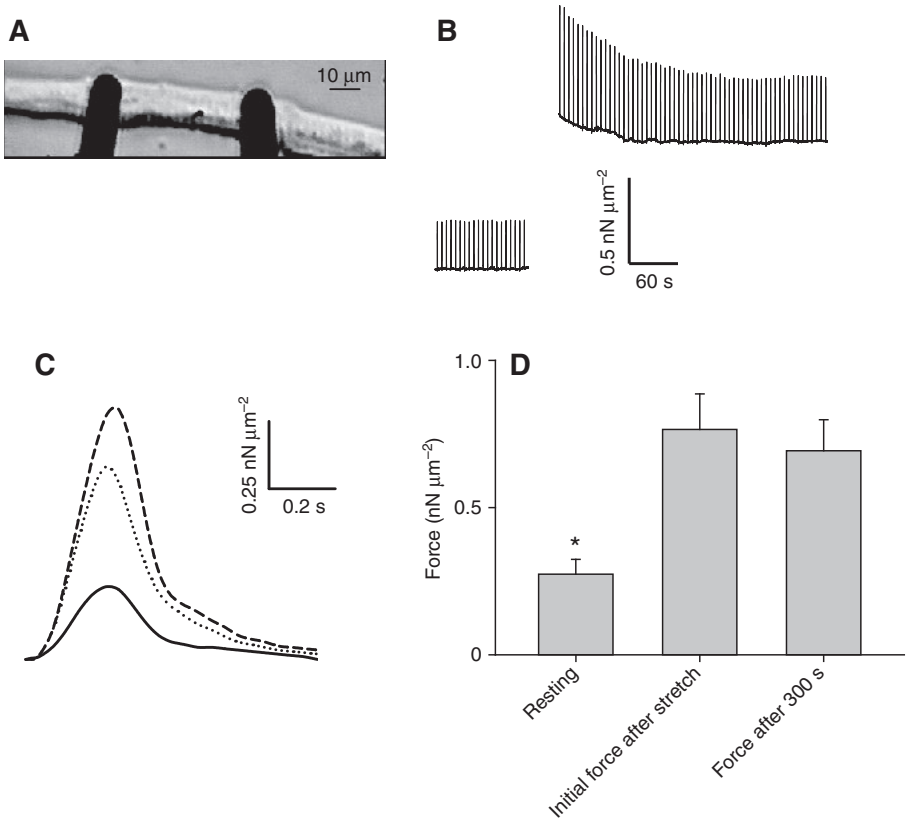

Fig. 2. The effect of stretch on individual ventricular myocytes from rainbow trout. (A) An individual ventricular myocyte attached to carbon fibres; the scale bar represents 10 μm. (B) A representative trace of the force generated by a myocyte at slack length and after stretch was applied. (C) Representative traces of the active force generated by a cell during one contraction at slack length (solid line), 10 s after stretch was applied (dashed line) and 300 s after stretch was applied (dotted line). (D) Mean force generated by the individual myocytes. Significant difference (N=9, P<0.05; one-way ANOVA, Student–Newman–Keuls).

al., 1995). Single myocytes develop lower peak active force than trabecular muscle per cross-sectional area, probably because the myocytes are contracting auxotonically whilst the trabecular preparations are isometric. In both cases, however, stretch caused marked inotropy *via* the Frank–Starling response.

Recently, increased effort has been made to identify the underlying mechanisms responsible for the SFR in the mammalian heart. The least controversial proposal is that stretch results in stimulation of the Na^+/H^+ exchanger (NHE), facilitating Na^+ entry into the cell and that this increase in intracellular $[Na^+]$ ($[Na^+]_i$) favours the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) (Alvarez et al., 1999; Perez et al., 2001; von Lewinski et al., 2003; Calaghan and White, 2004; Luers et al., 2005; Niederer and Smith, 2007). In this scenario, the rise in $[Na^+]_i$ due to NHE activation is followed by an increase in $[Ca^{2+}]_i$ *via* the NCX, resulting in the SFR (Perez et al., 2001; von Lewinski et al., 2003; Luers et al., 2005). Non-selective cationic mechanosensitive ion channels (MSC_{NS}) may also conduct Na^+ or Ca^{2+} into the myocyte and thus may be able to mediate stretch-dependent inotropy and the SFR (Calaghan and White, 2004; Niederer and Smith, 2007; Ward et al., 2008). Consensus over the precise balance of mechanisms leading to the SFR within each mammalian species is still lacking.

With regard to these mechanisms in fish, we have recently reported evidence to support the presence of the transient receptor potential (TRP) channel in trout myocardium (omTRPC1) (Patrick et al., 2010a). However, this work suggested this MSC_{NS} activates at extreme physiological levels of stretch. It is not known whether trout myocardium possess NHE, but our preliminary work (D. E. Warren and H.A.S., unpublished observations) suggests that, if present, it is not robust. Interestingly, a recent study has suggested a pivotal role for mitochondrial reactive oxygen species in the generation of the SFR by activation of NHE (Caldiz et al., 2007). If this is the case, the high antioxidant activity found within the trout heart, as opposed to a lack of NHE, may explain the lack of the SFR in trout ventricular tissue (Trenzado et al., 2006). Obviously, further studies in both mammalian and non-mammalian species are required to elucidate the mechanism underlying the differing time-dependent responses to stretch.

Our main finding is the lack of evidence for the SFR in isolated rainbow trout ventricular tissue and ventricular myocytes. This result suggests that the Frank–Starling response is not associated with the SFR in fish as it is in mammals and may relate to the relative importance of volume modulation of cardiac output between species. This suggestion must be tested by examining the time-dependent response to stretch in other fish species and other ectothermic vertebrates.

LIST OF ABBREVIATIONS

$L_{88\%}$	88% L_{max}
$L_{98\%}$	98% L_{max}
L_{max}	length at which maximum tension was generated
MSC_{NS}	non-selective cationic mechanosensitive ion channels
NCX	Na^+/Ca^{2+}-exchanger
NHE	Na^+/H^+-exchanger
SFR	slow force response
SL	sarcomere length
$T_{50\%}$	time to 50% relaxation
T_{peak}	time to peak force

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