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THE EFFECT OF TEMPERATURE AND ADRENALINE ON THE RELATIVE IMPORTANCE OF THE SARCOPLASMIC RETICULUM IN CONTRIBUTING Ca^{2+} TO FORCE DEVELOPMENT IN ISOLATED VENTRICULAR TRABECULAE FROM RAINBOW TROUT

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Summary

The sarcoplasmic reticulum (SR) is central to intracellular Ca^{2+} regulation during excitation–contraction (E-C) coupling in mammalian cardiac tissue. The importance of the SR to E-C coupling in lower vertebrates is less certain. This uncertainty can be attributed, in part, to the temperature-dependency of the SR Ca^{2+}-release channel and to interspecific differences in the ryanodine-sensitivity of ectotherm cardiac muscle. Furthermore, the relative importance of the SR in contributing intracellular Ca^{2+} to force development may be influenced by adrenergic stimulation, which increases trans-sarcolemmal (extracellular) Ca^{2+} influx. The objective of this study was to assess the relative importance of SR (intracellular) and sarcolemmal (SL; extracellular) Ca^{2+} fluxes during the isometric contraction of isolated ventricular trabeculae from rainbow trout *Oncorhynchus mykiss*. To approximate *in vivo* Ca^{2+} availability to the muscle better, a tonic level (10 nmol l^{-1}) of adrenaline was used in all control experiments, and SL Ca^{2+} influx was stimulated with high levels (10 μmol l^{-1}) of adrenaline. Ryanodine, a noted blocker of SR Ca^{2+} release in mammals, was used to assess SR involvement. To examine the role of temperature on the relative Ca^{2+} contribution from each source, experiments were performed at two temperatures (12 and 22 °C), using ventricular trabeculae from fish acclimated to both 12 and 22 °C.

Under all test conditions studied, SL Ca^{2+} influx was the primary source of activator Ca^{2+}, as assessed by the change in isometric force after ryanodine application. Even so, the SR contribution of activator Ca^{2+} was significantly greater at a test temperature of 22 °C than at 12 °C. We attribute this observation to the temperature-dependent nature of the SR Ca^{2+}-release channel. At 22 °C and under control conditions, ryanodine reduced peak tension at all pacing frequencies (by approximately 50 % at 0.2 Hz, approximately 25 % at 1.2 Hz and approximately 20 % at 2.0 Hz), regardless of acclimation temperature. Therefore, the SR is a significant, but secondary, contributor of activator Ca^{2+} for tension development at warm temperatures. The magnitude of SR Ca^{2+} contribution was inversely related to pacing frequency, but remained significant at physiological pacing frequencies. This was a novel finding. The degree of ryanodine-sensitivity in the present study was greater than that reported previously for the rainbow trout. We attribute this difference to the use of tonic adrenergic stimulation in the present study. In contrast to the experiments at the warmer test temperature, at 12 °C and under control conditions, ryanodine significantly reduced peak tension only at low frequencies (by approximately 25 % at 0.2 Hz), regardless of acclimation temperature. These findings suggest that at cold temperatures, and at physiologically relevant pacing frequencies, the SR may not be important in supplying Ca^{2+} to the contractile elements of the trout heart. At both test temperatures and regardless of acclimation temperature, stimulation with 10 μmol l^{-1} adrenaline caused positive inotropy of sufficient magnitude to ameliorate the negative inotropic effect of ryanodine completely, with the exception of high pacing frequencies (>1.2 Hz) at 22 °C, where adrenergic stimulation did not fully compensate for the effects of ryanodine. This exception is discussed in relation to the reduced adrenergic sensitivity of the trout myocardium at warm temperatures. The adrenergically mediated compensation for the loss of the SR Ca^{2+} supply is a novel finding for fish hearts. Therefore, while our study clearly demonstrates that the relative importance of SR Ca^{2+} release is subject to temperature and frequency, adrenaline-mediated increases in SL Ca^{2+} influx decrease the importance of the SR in contributing Ca^{2+} to E-C coupling in trout ventricular myofilaments.

Key words: trout, *Oncorhynchus mykiss*, heart, sarcoplasmic reticulum, Ca^{2+}, adrenaline, ryanodine, frequency, force, temperature.

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Introduction

Examination of excitation–contraction (E-C) coupling in the teleost heart suggests that, unlike mammals, release of intracellular Ca^{2+} from the SR is not necessary to activate the tropomyosin complex, rather contraction is initiated by a large trans-sarcolemmal Ca^{2+} influx (Tibbits *et al.* 1990). This assertion is supported by ultrastructural studies of fish myocytes which indicate the absence of a well-developed SR (Santer, 1985). However, the most compelling evidence for the lack of SR involvement in teleost E-C coupling is the absence of a ryanodine response. Ryanodine is a neutral plant alkaloid which, when applied in high concentrations (10 μmol l^{-1}), binds specifically and irreversibly to the SR Ca^{2+}-release channel, locking it closed (Rousseau *et al.* 1987). This renders the SR ineffective in contributing Ca^{2+} to E-C coupling. As a result, contractile force is typically reduced in adult mammalian cardiac muscle after ryanodine application. In contrast, no substantial reduction in force occurs at physiological pacing frequencies when ryanodine is applied to teleost ventricular muscle strips (Driedzic and Gesser, 1988; Hove-Madsen and Gesser, 1989; Vornanen, 1996) or to perfused hearts *in situ* (Keen *et al.* 1994) at temperatures between 5 and 20 °C. Mammalian cardiac studies demonstrate that the SR Ca^{2+}-release channel is highly temperature-sensitive (Bers, 1987, 1989; Sitsapesan *et al.* 1991), spending an increasingly greater proportion of time in the 'open-state' as temperature decreases. If the SR Ca^{2+}-release channel is also temperature-dependent in lower vertebrates, as has been suggested (Keen *et al.* 1992, 1994; Tibbits *et al.* 1992; Møller-Nielsen and Gesser, 1992), then the lack of a ryanodine response may be merely a result of the temperatures at which they were tested.

Catecholamines present another confounding factor in assessing the Ca^{2+} dynamics of teleost E-C coupling. In most fish, adrenaline (AD) is the predominant catecholamine affecting contractility (Farrell and Jones, 1992), acting through stimulation of β-adrenergic receptors. Studies demonstrate the presence of a tonic adrenaline concentration (5 nmol l^{-1}) in the circulation of resting fish (Milligan *et al.* 1989), with concentrations increasing to 1000 nmol l^{-1} under conditions of extreme stress (McDonald and Milligan, 1992). The importance of tonic adrenergic stimulation in maintaining regular contractions in both teleost and elasmobranch hearts has been demonstrated with *in situ* perfused heart preparations (Graham and Farrell, 1989; Davie and Farrell, 1991). In the myocyte, AD stimulates β-adrenergic receptors, causing the phosphorylation of the SL L-type Ca^{2+} channel *via* cyclic AMP and protein kinase A pathways (Tibbits *et al.* 1992). This phosphorylation increases the open probability of the channel (Bers, 1991), allowing for greater trans-sarcolemmal Ca^{2+} influx with each depolarization and producing, in part, the positive inotropic effect of AD. Since SL Ca^{2+} influx is so central to E-C coupling in teleost hearts, any assessment of the physiological importance of the SR Ca^{2+}-release channel must consider physiologically relevant states (i.e. tonic and maximal) of adrenergic stimulation. This has not been done in previous studies. In addition, the adrenergic sensitivity of the myocardium decreases with increased temperature (Graham and Farrell, 1989) owing to a reduction in the number of β-adrenoreceptors on the SL membrane (Keen *et al.* 1993; A. K. Gamperl, M. M. Vijayan, C. Pereira and A. P. Farrell, in preparation). Thus, while adrenergic stimulation may affect the relative importance of SR Ca^{2+} release in E-C coupling, temperature-induced modulation of β-adrenoreceptors may alter the efficacy of adrenergic effects.

Our experiments reassess the significance of SR Ca^{2+} release in contributing to ventricular force development in rainbow trout under tonic and maximal adrenergic stimulation at 'cold' (12 °C) and 'warm' (22 °C) temperatures. Resting heart rates for rainbow trout are approximately 55 beats min^{-1} (approximately 0.9 Hz) (Wood *et al.* 1979) at 12 °C and 83 beats min^{-1} (approximately 1.4 Hz) (Farrell *et al.* 1996) at 22 °C. Adrenergic stimulation can increase resting heart rate to 102 beats min^{-1} (approximately 1.7 Hz) at 22 °C (Farrell *et al.* 1996). Moreover, heart rate in trout can increase by as much as 40 % after prolonged swimming at critical speeds (U_{crit}) (H. Thorarensen, unpublished observation). Therefore, experiments were conducted over a range of pacing frequencies (0.2–2.0 Hz) to ensure that physiologically relevant contraction rates were studied.

Materials and methods

Fish origin and maintenance

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] (mean mass 1224±102 g, both sexes) were obtained from West Creek Trout Farms, British Columbia, Canada, and held outdoors in a 2000 l fibreglass tank receiving aerated, dechlorinated municipal water. The water was maintained at 12 °C (±2 °C) throughout the acclimation and experimental periods. On completion of the 12 °C acclimation experiments, the remaining fish were moved indoors to a similar tank to begin the acclimation to 22 °C. A water temperature of 22 °C was achieved *via* a countercurrent heat exchanger of local construction. The minimum acclimation time was 2 weeks. Fish were offered food three times a week and, while indoors, were exposed to a neutral photoperiod (12 h:12 h L:D).

Tissue preparation

Trout were killed by a sharp blow to the head and the heart was excised and placed in ice-cooled physiological saline (pH 7.78 at 12 °C and pH 7.82 at 22 °C) of the following composition (in mmol l^{-1}): NaCl, 124.1; KCl, 3.1; $CaCl_2$, 2.5; $MgSO_4$, 0.9; D-glucose, 5.0. Solutions were buffered with 20 mmol l^{-1} Tes (Na^+ salt and free acid combinations). The ventricle was isolated and cut lengthwise to expose the lumen. Three trabeculae (mean length 0.17 cm, and ⩽1 mm in diameter; mean mass 2 mg) were dissected out and tied at either end with single strands of 5-0 gauge surgical silk. One end of the trabeculae was attached to a fixed post, and the other was tied to a fine gold chain which hung from an isometric force transducer (Kulite Semiconductor Products,

Leonia, NJ, USA). The muscle was then lowered into a water-jacketed organ bath containing 20 ml of oxygenated physiological saline. The organ bath was maintained at either 12 or 22 °C. Trabeculae were stimulated (paced) using a Harvard Student Stimulator (Harvard Apparatus Ltd, Edenbridge, Kent) delivering charge *via* two flattened platinum electrodes positioned on either side of the muscle (0.2–2.0 Hz, 10 V, 10 ms duration). Signals from the force transducer were displayed on a Gould RS3400 chart recorder (Gould, Cleveland, OH, USA). Prior to experiments L_{max} (the muscle length at which active tension is maximized) was established. The muscle was allowed to equilibrate at this length for 30 min under basal stimulation (0.2 Hz) before being subjected to one of the protocols described below.

Experimental protocols

The protocols were intended to test the effects of ryanodine in the presence of high (maximal) (10 μmol l^{-1}) and low (tonic) (10 nmol l^{-1}) adrenaline levels on cardiac muscle properties at the two acclimation temperatures (12 and 22 °C) or with an acute temperature change (to 12 from 22 °C or to 22 from 12 °C). Because ryanodine binds irreversibly, two protocols were necessary to separate the effects of ryanodine under both tonic and maximal adrenaline stimulation. Each trabecular muscle was subjected to either protocol 1 or protocol 2 (defined below). Performance measured with the low AD concentration was intended to represent performance at the tonic level of AD found *in vivo*. This measurement therefore acted as the control for the subsequent tests on (1) the effect of ryanodine and (2) the effect of maximal adrenergic stimulation (10 μmol l^{-1} AD). The adrenaline doses used in our study were the minimum (10 nmol l^{-1}) and maximum (10 μmol l^{-1}) values of the dose–response curve generated by Keen *et al.* (1993). The sample sizes for each group were as follows: for 12 °C-acclimated fish, N=7 for protocol 1, N=4 for protocol 2; for 22 °C-acclimated fish, N=8 for protocol 1, N=7 for protocol 2. Some preparations became irregular at the highest pacing frequency under each protocol, so the sample size at the high frequencies is sometimes lower than the value given above; however, no mean values represent N<4.

Protocol 1

Low AD concentration

Graham and Farrell (1989) established that a tonic level of AD is needed to maintain tone in perfused hearts *in situ*; therefore, after the 30 min equilibration period (see above), fresh saline containing 10 nmol l^{-1} AD was added to the bath. Following a 5 min equilibration period, the muscle was subjected to a force–frequency trial in which pacing frequency was increased in 0.2 Hz increments from 0.2 Hz either to 2.0 Hz or until the muscle failed to show regular contractions. The muscle was stimulated at each frequency until peak force stabilized (usually within 30–60 s). At the end of the force–frequency trial, pacing frequency was returned to the basal level (0.2 Hz).

Ryanodine and low AD concentration

To examine the relative contribution of the SR in force development, the bath saline was replaced with saline containing 10 μmol l^{-1} ryanodine. The preparation was then left to equilibrate at 0.2 Hz (for 30 min at a bath temperature of 22 °C and for 45 min at 12 °C). It was assumed that, after these equilibration periods, ryanodine was irreversibly bound to the SR Ca^{2+}-release channel, rendering the SR ineffective in contributing Ca^{2+} to force development. After incubation with ryanodine, fresh saline containing 10 nmol l^{-1} AD was added to the bath and the force–frequency trial was repeated (as described above).

Ryanodine and high AD concentration

The final test in protocol 1 determined the effects of a high AD concentration on ryanodine-treated muscle to ascertain how increased trans-sarcolemmal Ca^{2+} influx affects force development during inhibition of SR Ca^{2+} release. Fresh saline containing 10 μmol l^{-1} AD was added and the force–frequency trial was repeated.

Protocol 2

Protocol 2 had a similar format to protocol 1; however, the purpose of protocol 2 was to observe the relative importance of SR Ca^{2+} release in the presence of maximal trans-sarcolemmal Ca^{2+} influx. Therfore, ryanodine effects were assessed under maximal adrenergic stimulation. The first force–frequency trial with low AD concentration established the control value against which two further trials, one with high AD concentration and the other with high AD concentration after ryanodine treatment, were evaluated.

Standardization

Force is expressed as mN mm^{-2}. Mean cross-sectional area was calculated using muscle mass, trabecular length and an assumed muscle density of 1.06 g cm^{-3} (Layland et al., 1995). The length of the trabeculae was measured (to 0.1 mm) using a pair of vernier calipers prior to removing the specimen from the apparatus. After removal, the tissue wet mass was determined (to the nearest 0.01 mg).

Drugs

All chemicals and drugs were purchased from either Sigma (St Louis, MO, USA) or BDH (Toronto, Ontario, Canada), with the exception of ryanodine which was purchased from Calbiochem (San Diego, CA, USA).

Data analysis and statistics

Peak tension, time to peak tension (TPT), time to half-relaxation (THR) and changes in resting tension were measured from an expanded (10 mm s^{-1}) chart recorder trace. Approximations of rates of contraction and relaxation were made by dividing peak tension by TPT or THR, respectively, and are expressed as mN s^{-1}. The effects of pacing frequency, test temperature and acclimation temperature on measured variables under control conditions (low [AD]) are displayed in

Table 1. *The effect of acclimation temperature, test temperature and pacing frequency on measured variables under control conditions (10 nmol l^{-1} adrenaline) in ventricular trabeculae from rainbow trout*

	12 °C acclimation				22 °C acclimation					
	12 °C test		22 °C test		12 °C test			22 °C test		
Variables	0.2 Hz	1.2 Hz	0.2 Hz	1.2 Hz	0.2 Hz	1.2 Hz	2.0 Hz	0.2 Hz	1.2 Hz	2.0 Hz
Peak tension (mN mm^{-2})	1.6±0.33^{a,e} (11)	0.57±0.16^{a,e} (5)	1.04±0.2^{a,e} (11)	0.33±0.08^{a,e} (8)	2.5±0.31^{a,e,d,b} (15)	1.28±0.15^{a,c,e} (14)	0.74±0.23^{b,c} (4)	1.79±0.17^{a,e,d,b} (15)	1.04±0.11^{a,c} (14)	0.77±0.08^{b,c} (12)
Normalized peak tension (%)	100±0^{a} (11)	39.2±4.0^{a,e} (5)	100±0^{a} (11)	35.5±2.06^{a,e} (8)	100±0^{a,b,d} (15)	52.3±3.0^{a,c,e} (14)	31.3±10.7^{b,c} (4)	100±0^{a,b,d} (15)	59±1.82^{a,c,e} (14)	42±2.51^{b,c} (12)
Time to peak tension, TPT (s)	0.54±0.01^{a,d} (11)	0.37±0.01^{a,e,d} (5)	0.27±0.01^{a,d} (11)	0.22±0^{a,e,d} (8)	0.51±0.02^{a,b,d} (15)	0.33±0.01^{a,c,d,e} (14)	0.23±0.01^{c,b,d} (4)	0.28±0.006^{a,b,d} (15)	0.21±0.004^{a,e,d} (14)	0.19±0.002^{b,d} (12)
Time to half-relaxation, THR (s)	0.36±0.03^{a,e,d} (11)	0.2±0^{a,e,d} (5)	0.24±0.01^{a,e,d} (11)	0.14±0.02^{a,d} (8)	0.26±0.01^{a,b,d,e} (15)	0.16±0.01^{a,e,d} (14)	0.13±0.01^{b,d} (4)	0.17±0.01^{a,b,d,e} (15)	0.11±0.004^{a,d} (14)	0.1±0.002^{b,d} (12)
Peak tension/TPT (mN s^{-1})	2.93±0.57^{e} (11)	1.58±0.45^{e} (5)	3.8±0.70^{a,e} (11)	1.51±0.40^{a,e} (8)	4.87±0.59^{e} (15)	3.88±0.49^{e} (14)	3.46±1.12 (4)	6.3±0.54^{e,b} (15)	5.03±0.54^{e} (14)	4.01±0.41^{b} (12)
Peak tension/THR (mN s^{-1})	4.02±0.92^{e} (11)	2.82±0.72^{e} (5)	3.72±0.61^{e} (11)	2.24±0.49^{e} (8)	9.42±1.05^{e} (15)	8.05±0.99^{e} (14)	6.67±2.18 (4)	10.9±1.24^{e} (15)	9.65±1.09^{e} (14)	7.88±0.88 (12)

Values are group means ± S.E.M. (*N*).

Significance (P<0.05) is denoted by paired letters, which represent the following: for differences between frequencies within a test temperature, a indicates that 1.2 Hz is different from 0.2 Hz, b indicates that 2.0 Hz is different from 0.2 Hz, c indicates that 1.2 Hz is different from 2.0 Hz; d denotes test temperature differences at the same frequency and same acclimation temperature, and e denotes acclimation temperature differences at the same frequency and same test temperature.

Table 1. Student's *t*-tests and factorial analyses of variance (ANOVAs) were used to establish significant differences (P<0.05). Differences between percentage (normalized) data were tested non-parametrically using either Kruskal–Wallis tests or Mann–Whitney *U*-tests (P<0.05). An estimation of the muscle power output was made by multiplying peak tension by frequency as described by Matikainen and Vornanen (1992). This calculation was termed 'pumping capacity' (Matikainen and Vornanen, 1992).

Results

Acclimation to 12 °C: tests at 12 °C

Frequency effects

Under control conditions (low [AD]), increasing the stimulation frequency from 0.2 to 1.2 Hz decreased peak tension by 70 %, from 1.6 mN mm^{-2} to 0.57 mN mm^{-2} (Table 1; Fig. 1A). TPT decreased from 0.54 to 0.37 s as pacing frequency was increased from 0.2 to 1.2 Hz. Similarly, THR decreased from 0.36 to 0.2 s as frequency was increased from 0.2 to 1.2 Hz. Peak tension/TPT and peak tension/THR increased significantly with decreased pacing frequency (Table 1). Only two of 11 preparations maintained regular contractions at pacing frequencies higher than 1.2 Hz.

Protocol 1

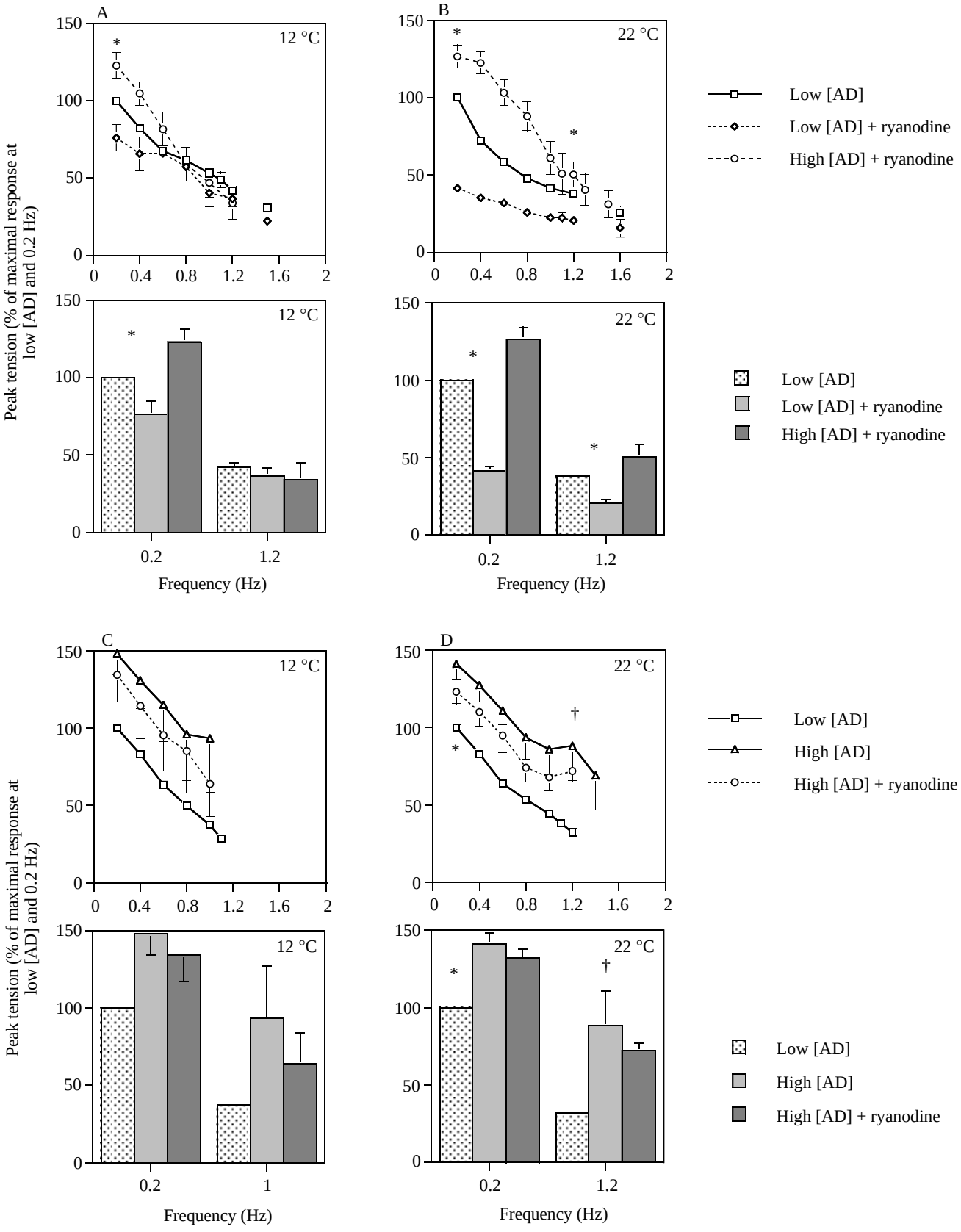
Addition of ryanodine, in the presence of low [AD] significantly decreased peak tension at low (0.2 Hz) frequencies but not at high (1.2 Hz) frequencies (Fig. 1A). Other measured variables were unchanged. Addition of high [AD] induced a significant positive inotropic response at 0.2 Hz, but not at 1.2 Hz. As a result, the negative inotropic effect of ryanodine was reversed at low frequencies and contractility improved beyond that observed under control conditions (low [AD] stimulation) (Fig. 1A).

Protocol 2

Protocol 2 specifically examined whether the effects of ryanodine could be detected in the presence of high [AD]. As with protocol 1, the force generated with high [AD] stimulation was greater than that observed under control conditions (low

Fig. 1. Force–frequency relationships for ventricular trabeculae from rainbow trout normalized to the low [AD] (values obtained at 10 nmol l^{-1} adrenaline) and 0.2 Hz treatment for fish acclimated to 12 °C at each test temperature (upper right corner) and each protocol (see Materials and methods) (protocol 1, A,B; protocol 2, C,D). The results are displayed as line graphs to show progressive changes in peak tension with increased frequency and as bar graphs to highlight the effects of drugs at high and low frequencies. Values are group means; vertical bars represent ±1 S.E.M. Values of *N* are given in Materials and methods. * denotes a significant difference (P<0.05) between all drug treatments within each frequency. † denotes a significant difference between low [AD] and both high [AD] (10 mmol l^{-1} adrenaline) and high [AD] + ryanodine groups, with no significant differences between the latter two. Within each drug treatment category, there is a significant change in peak tension when pacing frequency is increased from 0.2 to 1.2 Hz (or 1.0 Hz), with the exception of the high [AD] treatment in C.

[AD]) even after ryanodine treatment in all preparations, but these results could not be resolved statistically (Fig. 1C). Addition of ryanodine tended to reduce force relative to the high [AD] treatment in all four preparations, but this was not



statistically resolvable. Preparations could not be consistently paced at 1.2 Hz; therefore, the high pacing frequency comparisons were made at 1.0 Hz in this case.

Acclimation to 12 °C: tests at 22 °C

Acute temperature change effects

Increasing the test temperature by 10 °C significantly altered ventricular contractility. In seven of 11 preparations, regular contractions could be maintained at frequencies above 1.2 Hz. Under control conditions at 0.2 Hz, peak tension was 35 % lower at 22 °C than at 12 °C (1.04 *versus* 1.6 mN mm^{-2}, respectively). However, there was no significant difference in peak tension between test temperatures at 1.2 Hz (Table 1). TPT and THR at a test temperature of 22 °C were significantly shorter than at 12 °C, being 50 % and 35 % shorter, respectively, at 0.2 Hz, and 40 % and 30 % shorter, respectively, at 1.2 Hz. Peak tension/TPT and peak tension/THR were not significantly different from those in the 12 °C tests (Table 1).

Frequency effects

The effects of increasing pacing frequency from 0.2 to 1.2 Hz at a test temperature of 22 °C were similar to those observed at 12 °C. Peak tension decreased by 60 % (Table 1; Fig. 1B). Likewise, TPT and THR decreased from 0.27 to 0.22 s and from 0.24 to 0.14 s, respectively. Peak tension/TPT decreased significantly with increased pacing frequency (Table 1).

Protocol 1

Under control conditions (low [AD]), application of ryanodine significantly reduced peak tension at 0.2 and 1.2 Hz (Fig. 1B). This response differs from the 12 °C tests where the effects of ryanodine were significant only at low (0.2 Hz) pacing frequencies. Also in contrast to the 12 °C tests, ryanodine application at 22 °C caused significant reductions in THR at 0.2 Hz (but not at 1.2 Hz) (Fig. 2A). As in the 12 °C tests, high [AD] reversed the negative inotropic effect of ryanodine by increasing peak tension and improving contractility beyond that observed under control conditions (Fig. 1B). However, in contrast to the 12 °C tests, at a test temperature of 22 °C, this AD-mediated restoration of tension was evident at both high and low pacing frequencies (Fig. 1B). Thus, cold-acclimated muscles tested at warm (22 °C) temperatures demonstrate significant effects of adrenaline and ryanodine at physiologically realistic (1.2 Hz) contraction frequencies.

Protocol 2

Increasing AD concentration from 10 nmol l^{-1} to 10 µmol l^{-1} increased peak tension at both 0.2 and 1.2 Hz (Fig. 1D). Other variables remained unchanged. Addition of ryanodine under high [AD] stimulation caused a significant decrease in peak tension at 0.2 Hz but not at higher pacing frequencies. Other variables were not significantly affected by ryanodine treatment.

Acclimation to 22 °C: tests at 12 °C

Frequency effects

Acclimation to 22 °C resulted in significantly higher attainable pacing frequencies (2.0 Hz at 22 °C *versus* 1.2 Hz at 12 °C) and thus statistical comparisons are made at three pacing frequencies (see Table 1). As in 12 °C-acclimated trabeculae, increasing pacing frequency in 22 °C-acclimated trabeculae significantly decreased peak tension, TPT and THR. Under control conditions, peak tension fell by 50 % from 2.5 mN mm^{-2} at 0.2 Hz to 1.27 mN mm^{-2} at 1.2 Hz, and by another 20 % to 0.74 mN mm^{-2} at 2.0 Hz (Table 1; Fig. 3A). TPT decreased significantly as pacing frequency was increased (from 0.51 at 0.2 Hz, to 0.33 s at 1.2 Hz and to 0.23 s at 2.0 Hz). THR also decreased significantly (from 0.26 s at 0.2 Hz, to 0.16 s at 1.2 Hz and to 0.13 s at 2.0 Hz) (Table 1; Fig. 2B). Additionally, peak tension/TPT and peak tension/THR decreased significantly with increased pacing frequency (Table 1).

Protocol 1

As in the 12 °C tests following 12 °C acclimation, ryanodine decreased peak tension at 0.2 Hz but not at 1.2 Hz under control conditions (Fig. 3A). Ryanodine reduced THR at 0.2 Hz (Fig. 2B) and decreased peak tension/THR (data not shown). Ryanodine treatment did not exert significant effects on the other variables measured. Increasing the AD concentration to 10 µmol l^{-1} increased peak tension at 0.2 Hz (Fig. 3A), as in the 12 °C tests after 12 °C acclimation. Thus, irrespective of acclimation temperature, at a test temperature of 12 °C and at 0.2 Hz, increased trans-sarcolemmal Ca^{2+} influx after high [AD] stimulation overwhelms the ryanodine-induced loss in SR Ca^{2+} release, resulting in greater peak tension than that observed under control conditions.

Protocol 2

Increasing the AD concentration from 10 nmol l^{-1} to 10 µmol l^{-1} increased peak tension significantly at 0.2 Hz (Fig. 3C). This positive inotropy was associated with a significantly longer THR at 0.2 Hz (from 0.26 to 0.38 s). Other variables did not change significantly in response to high [AD]. Addition of ryanodine under high [AD] stimulation significantly decreased peak tension at 0.2 Hz, but not at either 1.2 or 2.0 Hz (Fig. 3C), again demonstrating that, at low (12 °C) test temperatures, ryanodine effects are discernible only at low (0.2 Hz) pacing frequencies irrespective of previous acclimation temperature.

Acclimation to 22 °C: tests at 22 °C

Acute temperature change effects

Under control conditions, peak tension was 1.79 mN mm^{-2} at 0.2 Hz, a value significantly lower than the 2.5 mN mm^{-2} observed at the 12 °C test temperature (Table 1). TPT was significantly shortened by the 22 °C test temperature, being 55 % shorter than the 12 °C test group at 0.2 Hz, 35 % shorter at 1.2 Hz and 20 % shorter at 2.0 Hz. THR was also significantly shorter at all frequencies compared with the 12 °C test temperature (Table 1).

Frequency effects

In accordance with other test conditions, a negative

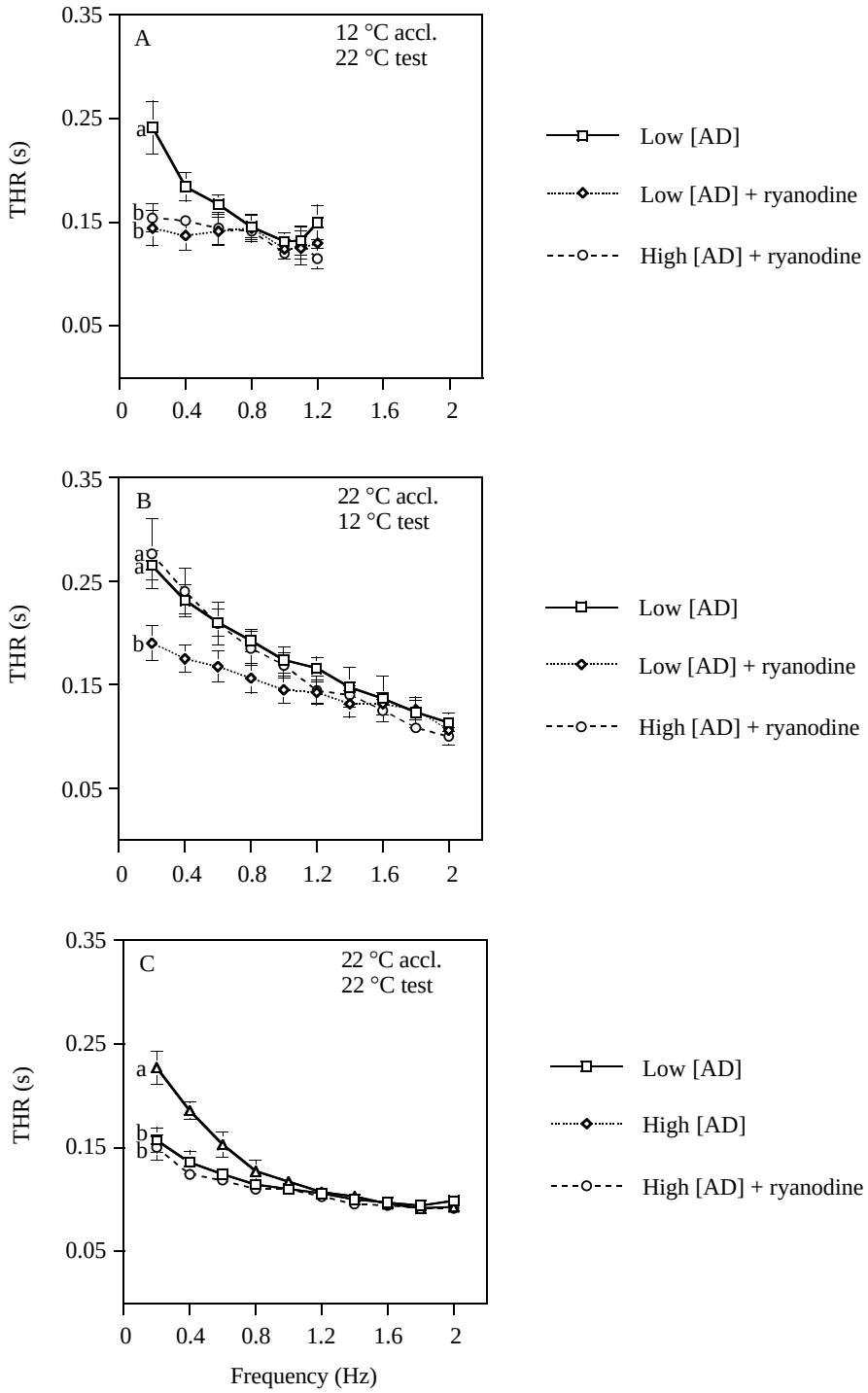


Fig. 2. Effects of increased pacing frequency and drug application on the time to half-relaxation (THR) in ventricular trabeculae from rainbow trout. Test temperature and acclimation (Accl.) temperature are indicated in the upper right corner of each graph. (A,B) Protocol 1; (C) protocol 2. Values are group means. Vertical bars represent ±1 S.E.M. Values of *N* are given in Materials and methods. Within each frequency, significant differences ($P<0.05$) between drug treatments are denoted by dissimilar letters. Within each drug treatment, significant differences exist between 0.2 and 1.2 Hz, but not between 1.2 and 2.0 Hz.

force–frequency relationship was observed in response to increased pacing frequency. Under control conditions, peak tension decreased by 55 % as pacing frequency increased from 0.2 to 2.0 Hz. (Table 1; Fig. 3B,D). Increased pacing frequency also significantly decreased TPT and THR. TPT fell from 0.28 s at 0.2 Hz to 0.21 s at 1.2 Hz. THR fell from 0.17 s at 0.2 Hz to 0.11 s at 1.2 Hz. Neither TPT nor THR changed significantly as pacing frequency increased above 1.2 Hz.

Protocol 1

Under control conditions, ryanodine significantly reduced peak tension at 0.2, 1.2 and 2.0 Hz (Fig. 3B). This contrasts with results from the 12 °C tests, in which ryanodine only had an effect at low pacing frequencies. These data suggest that warm (22 °C) ambient temperatures are necessary for significant effects of ryanodine to be observed at physiological pacing frequencies (1.2–2.0 Hz). Ryanodine incubation also significantly shortened THR at 0.2 Hz but not

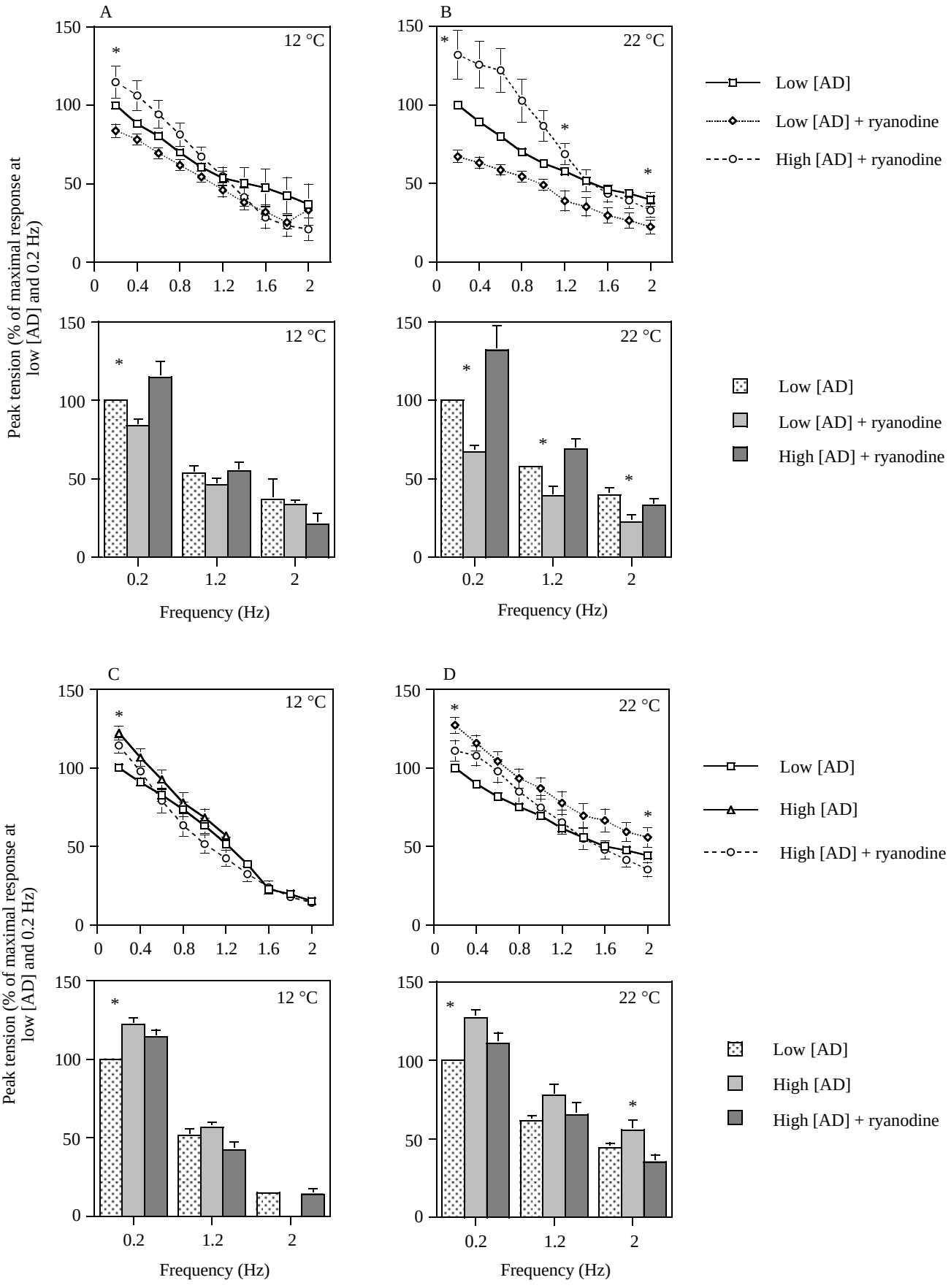

A
B
C
D
12 °C
22 °C
Low [AD]
Low [AD] + ryanodine
High [AD] + ryanodine
High [AD]
Peak tension (% of maximal response at low [AD] and 0.2 Hz)
Frequency (Hz)
0
50
100
150
0.4
0.8
1.2
1.6
2
0.2
*

Fig. 3. Force–frequency relationships for ventricular trabeculae from rainbow trout normalized to the low [AD] and 0.2 Hz treatment for fish acclimated to 22 °C at each test temperature (upper right corner) and each protocol (protocol 1, A,B; protocol 2, C,D). Results are displayed as line graphs to show progressive changes with increased frequency and as bar graphs to highlight the effects of the drug at high and low frequencies. Values are group means; vertical bars represent ±1 S.E.M. Values of *N* are given in Materials and methods. * denotes a significant difference ($P<0.05$) between all drug treatments within each frequency. Within each drug treatment, significant differences were found between pacing frequencies (0.2, 1.2 and 2.0 Hz).

at either 1.2 or 2.0 Hz (data not shown). Ryanodine incubation was without significant effects on the other variables measured. As observed in the previous treatments, the loss of force due to ryanodine application could be compensated for by increasing adrenergic stimulation. High [AD] increased peak tension at 0.2, 1.2 and 2.0 Hz at 22 °C (Fig. 3B). At 0.2 and 1.2 Hz, high [AD] increased peak tension beyond control levels. However, at 2.0 Hz, although force was increased by high [AD], peak tension remained below that under control (low [AD]) conditions. This suggests that adrenaline is less effective in compensating for SR inhibition at high pacing frequencies. Peak tension/THR was significantly faster at 0.2 Hz (but not 1.2 or 2.0 Hz) after high [AD] stimulation (data not shown).

Protocol 2

Increasing the AD concentration from 10 nmol l^{-1} to 10 μmol l^{-1} significantly increased peak tension at both 0.2 and 2.0 Hz (Fig. 3D). This is in contrast to the 12 °C tests, where positive inotropic effects were found only at low stimulation frequencies (Fig. 3C). THR was significantly increased at 0.2 Hz by high [AD] (Fig. 2D), but other measured variables were not changed significantly. Application of ryanodine, in the presence of high [AD], significantly decreased peak tension at both 0.2 and 2.0 Hz (Fig. 3D) which, again, is in contrast to the 12 °C tests where ryanodine effects occurred only at 0.2 Hz (Fig. 3C). Thus, at physiological pacing frequencies, acute exposure to warm temperature (22 °C) increased the muscle's sensitivity to ryanodine. Accompanying these ryanodine-mediated decreases in peak tension were significant decreases in THR relative to control conditions (Fig. 2C).

Effects of temperature acclimation

Temperature acclimation significantly affected the force development capabilities of the muscle. In all cases, 22 °C acclimation resulted in increased peak tension under control conditions compared with 12 °C acclimation (Table 1). For instance, with 22 °C acclimation, peak tension increased by approximately 65 % at 0.2 Hz (from 1.6 to 2.5 mN mm^{-2}) and by approximately 125 % at 1.2 Hz (from 0.57 to 1.28 mN mm^{-2}). Additionally, 22 °C acclimation allowed consistent pacing at higher frequencies (2.0 Hz). However, despite the increase in attainable pacing frequency at 22 °C acclimation, contractions became irregular when peak tension had decreased by 65–70 % of the 0.2 Hz value in both acclimation groups and at both test temperatures (Table 1).

Temperature acclimation also influenced the responsiveness of the muscles to ryanodine. Peak tension was reduced by ryanodine treatment at high and low frequencies (Fig. 3) after 22 °C acclimation but only at low frequencies after 12 °C acclimation (Fig. 1). These data suggest that, as in mammals, SR activity in rainbow trout is temperature-sensitive. Nonetheless, test temperature, rather than acclimation temperature, seems to be the predominant factor influencing the high-frequency pervasiveness of the ryanodine response. This point is shown by the significant reductions in peak tension after ryanodine treatment at both high (1.2 or 2.0 Hz) and low (0.2 Hz) frequencies when tested at 22 °C, irrespective of acclimation temperature (Figs 1, 3).

The 12 °C acclimation group were more responsive to high [AD] stimulation, both in the presence and in the absence of ryanodine. Greater adrenaline-mediated increases in peak tension after ryanodine treatment were observed after 12 °C acclimation than after 22 °C acclimation. Additionally, high [AD] (without ryanodine in protocol 2) increased peak tension by 50 % (albeit not statistically significant because of the low *N* value) at both 0.2 and 1.2 Hz (Fig. 1C,D) after 12 °C acclimation, compared with an approximately 25 % increase at the same frequencies after 22 °C acclimation (Fig. 3C,D). This observation is consistent with the cold-acclimation-induced increase in adrenaline sensitivity reported by Keen *et al.* (1993) and is related to an increased number of β-adrenoreceptors in cold-acclimated animals.

Of the other variables measured, only THR was significantly affected by temperature acclimation and then only at low (0.2 Hz) pacing frequencies (Fig. 2). Acclimation to 22 °C significantly shortened THR (Table 1). Additionally, THR was shortened by ryanodine application after 22 °C acclimation, irrespective of test temperature (Fig. 2B,C), but only at the 22 °C test temperature under 12 °C acclimation (Fig. 2A). High [AD] increased THR after it had been decreased by ryanodine application, but only after 22 °C acclimation and at a 12 °C test temperature (Fig. 2B). Ryanodine-induced reductions in THR under high [AD] stimulation were only evident in the 22 °C tests (Fig. 2C). In the absence of ryanodine, high [AD] increased THR significantly at both test temperatures under 22 °C acclimation. These results suggest that, at 0.2 Hz, THR is more responsive to both AD and ryanodine treatment when tested at 22 °C after acclimation at 22 °C. The physiological relevance of the changes in THR at such low frequencies are unclear.

Resting tension

An increase in resting tension suggests a compromise in the Ca^{2+} extrusion capabilities of the cell, with Ca^{2+} remaining available to the contractile apparatus after the development of peak tension (Bailey and Driedzic, 1990). Changes in resting tension, from the onset of the force–frequency test (0.2 Hz) to the point at which contractions became irregular (approximately 1.2 Hz at 12 °C acclimation temperature or 2.0 Hz at 22 °C acclimation temperature), were quantified

Table 2. *Changes in resting tension, expressed as a percentage of active tension, observed when pacing frequency was increased from 0.2 Hz to either 1.2 Hz (12 °C acclimation temperature) or 2.0 Hz (22 °C acclimation temperature)*

	12 °C acclimation		22 °C acclimation	
Treatment	12 °C test	22 °C test	12 °C test	22 °C test
Low [AD]	7.1 (6/11) 0.09±0.03	4.1 (4/11) 0.03±0.02	10.0 (4/15) 0.1±0.06	4.7 (3/15) 0.06±0.04
Low [AD]+Ry	17.0 (6/7) 0.17±0.07	0 (0/7) 0	31.8 (5/8) 0.25±0.14	12.0 (3/8) 0.07±0.05
High [AD]+Ry	23.5 (9/11) 0.24±0.07	13.2 (7/11) 0.11±0.03	35.3 (9/15) 0.25±0.07	19.5 (10/15) 0.19±0.07
High [AD]	6.7 (2/4) 0.12±0.09	1.9 (1/4) 0.02±0	0 (0/7) 0	2.6 (2/7) 0.05±0.04

The percentage change includes the results from all the experiments under each test condition (i.e. not just the responders).

Beside each percentage value, in parentheses, the number of responders over the total number of preparations tested is presented as a fraction. Below each percentage value is the mean increase in resting tension ± S.E.M. from all preparations (in mN).

Low [AD], 10 nmol l^{-1} adrenaline; Low [AD]+Ry, 10 nmol l^{-1} adrenaline plus ryanodine, High [AD], 10 μmol l^{-1} adrenaline.

(Table 2). Under control conditions (low [AD]) and for all four temperature treatments, resting tension did not change with increased frequency in the majority of preparations [see ratio of responders (i.e. muscles that demonstrated changes in resting tension) to total number of preparations in Table 2]. The mean (including both responders and non-responders) increase in resting tension under control conditions was ⩽10 % of active tension. With high [AD], increases in resting tension prior to arrhythmia were fewer in number and lower in percentage (Table 2). This suggests that, under both control conditions and conditions of increased SL Ca^{2+} influx, Ca^{2+} was removed successfully from the cytosol during relaxation, even at high pacing frequencies. With the exception of the 12 °C-acclimated, 22 °C test group, ryanodine application, in the presence of low [AD], increased both the number of responders and the magnitude of the response. This observation may reflect limited Ca^{2+} sequestering by the SR after ryanodine treatment. High [AD] exacerbated the effect of ryanodine on resting tension in all four temperature groups, increasing both the mean change in resting tension and the number of preparations that responded. These results suggest increased difficulty in removing Ca^{2+} during relaxation under conditions of increased SL Ca^{2+} influx and reduced SR Ca^{2+}-sequestering abilities, and they suggest that the SR plays an important role in relaxation at pacing frequencies approaching the maximum possible for a given set of test conditions.

Discussion

Force–frequency response

This study has demonstrated a consistent decline in peak tension with increased pacing frequency irrespective of adrenaline concentration, ryanodine treatment or temperature change (Table 1). This negative force–frequency response is consistent with the ‘typical’ teleost force–frequency response reported by Driedzic and Gesser (1988), Hove-Madsen (1992) and Matikainen and Vornanen (1992). However, although the negative force–frequency response is considered typical for fish, other studies report both temperature- and ryanodine-dependent changes in the shape of the response curve. For example, Matikainen and Vornanen (1992) found the typical negative force–frequency relationship in cold-acclimated (5 °C) carp, but a positive force–frequency relationship in warm-acclimated (15 °C) carp. Hove-Madsen (1992) reported that the addition of ryanodine reversed the negative force–frequency response to give a positive force–frequency response in trout tested at 25 °C. In agreement with our results, Bailey and Driedzic (1990) observed a negative force–frequency response in yellow perch (*Perca flavescens*) and smallmouth bass (*Micropterus dolomieui*) after cold (5 °C) and warm (20 °C) acclimation. Interestingly, they found that, in fish acclimated to low temperature and tested at high temperature, increasing the Ca^{2+} concentration in the bathing medium ameliorated the negative force–frequency response. They hypothesized that acute temperature change may trigger the release of catecholamines which increase Ca^{2+} delivery to the myofilaments, facilitating increased cardiac performance. In our study, adrenaline application increased peak tension at all frequencies but, as for ryanodine treatment and temperature acclimation, did not affect the shape of the negative force–frequency response. The differences between these studies and ours may relate to the fact that none of the earlier work utilized a tonic level of adrenergic stimulation.

Peak tension

Peak tension reliably indicates the inotropic capabilities of the heart. The present study illustrates that test temperature, acclimation temperature, adrenaline concentration and ryanodine application all affect peak tension, with the degree of the effect varying with pacing frequency. Since ryanodine decreased peak tension, we conclude that the SR is involved in supplying the contractile elements with Ca^{2+}. Under all test

conditions (12 or 22 °C acclimation temperature, 12 or 22 °C test temperature, and high or low [AD]), the role of the SR is secondary to that of the SL Ca^{2+} influx in supplying Ca^{2+} for tension development in trout myocardium. This finding is in agreement with previous results (Keen *et al.* 1994; Tibbits *et al.* 1991, 1992; Møller-Nielsen and Gesser, 1992). However, the contraction frequency at which the SR became involved, and the magnitude of that involvement, varied with test conditions. Our study showed that, at cold (12 °C) temperatures, SR involvement is limited to low (0.2 Hz) pacing frequencies, with peak tension falling by approximately 25 % after ryanodine treatment. This is in contrast to previous studies which were unable to demonstrate any ryanodine response, even at low pacing frequencies at temperatures below 15 °C [Driedzic and Gesser, 1988 (sea raven (*Hemitripterus americanus*) and cod (*Gadus mornua*) at 10 °C); El-Sayed and Gesser, 1989; Hove-Madsen and Gesser, 1989 (rainbow trout and plaice (*Pleuronectes flesus*) at 15 °C); Møller-Nielsen and Gesser, 1992 (rainbow trout at 10 °C); Keen *et al.* 1994 (rainbow trout at 8 °C)]. We attribute this difference to the use of a tonic [AD] in our studies. The physiological significance of this response to ryanodine in our study is unclear, since 0.2 Hz is well below resting heart rate unless there is severe bradycardia. The corollary to this conclusion is a significant involvement of the SR at warm temperatures. At a test temperature of 22 °C, ryanodine application decreased peak tension by approximately 50 % at 0.2 Hz, demonstrating that the SR Ca^{2+} contribution can approach that of the SL. These results agree with previous studies which showed decreases in tension associated with ryanodine treatment at warm temperatures and 0.2 Hz (Møller-Nielsen and Gesser, 1992, 32 % reduction in tension at 20 °C; Keen *et al.* 1994, 60 % reduction in tension at 18 °C).

Our study is the first to demonstrate a ryanodine-induced reduction in tension at physiologically realistic contraction frequencies in a temperate fish species. We demonstrate that, at a test temperature of 22 °C (irrespective of acclimation temperature), the SR releases a significant, albeit small, proportion of activator Ca^{2+} for routine (>0.6 Hz) cardiac contractions. This is a novel finding. Previous studies (with the skipjack tuna (*Katsuwonus pelamis*) being a notable exception; Keen *et al.* 1992) have demonstrated ryanodine-sensitivity at warm test temperatures but not at physiological pacing frequencies (Keen *et al.* 1994; Hove-Madsen, 1992). We attribute the differences between the results from our study and those from previous studies to the use of tonic adrenergic stimulation. The use of a tonic adrenaline concentration (10 nmol l^{-1}) increases attainable peak tension at control levels, which may facilitate observations of changes in force that are undetectable in the absence of adrenaline.

The finding that acute (test) warm-temperature exposure amplified the ryanodine response to a greater extent than did chronic (acclimation) warm-temperature exposure agrees with results from Keen *et al.* (1994). At a test temperature of 8 °C, Keen *et al.* (1994) were unable to demonstrate a significant effect of ryanodine regardless of previous acclimation temperature (8 or 18 °C); however, when tested at 18 °C, ryanodine reduced tension at 0.2 Hz. This thermal dependence of the ryanodine response is also consistent with the gating and conductance properties of the SR Ca^{2+}-release channel in mammals, where the open-state probability of the channel increases as temperatures decrease (Sitsapesan *et al.* 1991). Our results are also in accordance with the effects of rapid cooling on SR Ca^{2+} release in mammals, where rapid tissue cooling to 1 °C results in a prolonged contracture, equated with the release of Ca^{2+} from SR stores (Bers, 1987, 1989). If this is a general property of all vertebrate cardiac tissue, then the low ambient temperatures experienced by ectotherms would promote the open-state probability of the SR Ca^{2+}-release channel, reducing the effectiveness of the SR in sequestering and releasing Ca^{2+} during contraction (Tibbits *et al.* 1991; Keen *et al.* 1992).

This rationale can be used to explain the absence of an effect of ryanodine at high pacing frequencies in our 12 °C groups. The acquisition of a response to ryanodine at 0.2 Hz in the 12 °C group may be attributed to the fact that temperatures lower than 12 °C are necessary for complete inactivation of the SR Ca^{2+}-release channels in fish. Therefore, enough channels remain functional at 12 °C such that, at slow contraction rates (0.2 Hz), the SR may be able to accumulate sufficient Ca^{2+} between depolarizations to contribute significantly to force development. However, *in vivo* and at cold temperatures, this SR contribution is probably insignificant at physiological heart rates and is easily overwhelmed by adrenergic stimulation (discussed below). In contrast, when trout explore water temperatures near their upper thermal tolerance level, SR Ca^{2+} plays a secondary role (25 %) in cardiac contractility at physiological heart rates. Because this involvement is independent of temperature acclimation, and since salmonids exploit thermoclines behaviourally (Brett, 1971), SR Ca^{2+} release may become an important factor when trout move from the colder depths of a lake to feed in the warmer upper waters as well as for fish routinely living in warm water.

The interplay between SR and SL Ca^{2+} flux for force development was seen clearly when high [AD] concentrations were used to stimulate SL Ca^{2+} influx maximally. The positive inotropy resulting from high [AD] stimulation increased peak tension to beyond control levels, overwhelming the negative inotropy associated with ryanodine treatment at 0.2 Hz at both acclimation temperatures and at 1.2 Hz in the tests at 22 °C. These results suggest that trout ventricle can dramatically increase its force-generating ability by increasing Ca^{2+} influx *via* SL channels, negating the necessity of an intracellular (SR) contribution. The striking (50–95 %) adrenaline-mediated increase in force in 12 °C-acclimated fish (Fig. 1A,B) may reflect an increased adrenergic sensitivity of the myocardium and an increased SL β-receptor density, which is known to occur with cold acclimation in trout (Keen *et al.* 1993). However, although increasing Ca^{2+} influx *via* SL channels compensates for the loss of SR Ca^{2+} at most pacing frequencies, this compensation may be inadequate at high (2.0 Hz) frequencies. For example, high [AD] ameliorates the negative inotropic effect of ryanodine to a lesser extent at

1.2 Hz than at 0.2 Hz at both acclimation temperatures and both test temperatures. Furthermore, at an acclimation temperature of 22 °C, at which the trabeculae were able to contract at 2.0 Hz, the ryanodine-induced reduction in peak tension was not fully compensated for by application of high [AD]. This finding, consistent with the reduced inotropic effects of adrenaline observed in perfused rainbow trout hearts after 18 °C acclimation compared with 8 °C acclimation, is probably due to the presence of fewer β-adrenoreceptors (Keen *et al.* 1993). Thus, it is conceivable that, in order to maintain tension at the faster maximal contraction frequencies associated with warm temperatures, the SR must be involved in sequestering and releasing Ca^{2+} to compensate for the reduced adrenergic sensitivity of the myocardium.

The positive inotropy observed with adrenergic stimulation is normally linked to increased trans-sarcolemmal Ca^{2+} influx mediated by the β-adrenergic signal-transduction pathway. Certainly, we interpret our data in this way. However, we cannot exclude a role for positive inotropy *via* an α-adrenergic pathway. Stimulation of the α-adrenoreceptors in mammals increases the Ca^{2+}-sensitivity of the myofilaments and can increase intracellular Ca^{2+} concentrations through the actions of inositol 1,4,5-trisphosphate (InsP_3) (Benfey, 1990). InsP_3 induces the release of Ca^{2+} from the SR in smooth muscle cells, but has been ruled out as the primary catalyst for SR Ca^{2+} release in mammalian cardiac cells because the rate and degree of InsP_3-induced Ca^{2+} release are significantly lower than that of Ca^{2+}-induced Ca^{2+}-release (Kentish *et al.* 1990). The role of InsP_3 in E-C coupling in fish is still unknown. Indeed, although α-receptors may be present on the SL membrane, they do not mediate positive inotropy or chronotropy in rainbow trout (Ask, 1983; Farrell *et al.* 1986), flounder (*Pleuronectes platessa*) (Ask, 1983) and carp (*Carassius carassius*) (Vornanen, 1989), but may do so in eel *Anguilla anguilla* (Peyraud-Waitzenegger *et al.* 1980) and perch (*Perca fluviatilis*) (Tirri and Lehto, 1984). Additionally, it is possible that, as in rainbow trout hepatocytes, myocyte cytosolic InsP_3 level is not modulated by adrenergic stimulation of the α-adrenoreceptor (Fabbri *et al.* 1995).

Other experimental approaches, such as the use of specific β-agonists, could have been used in this study to avoid the possibility of activating both α- and β-adrenoreceptors. Additionally, increasing extracellular Ca^{2+} levels could increase trans-sarcolemmal Ca^{2+} influx without activating either signal-transduction pathway. Both of these approaches, in conjunction with ryanodine application, could be used in future studies to provide additional information on the relative importance of intracellular and extracellular Ca^{2+} flux during E-C coupling. In this study, we have attempted to use realistic levels of adrenergic stimulation to provide a more relevant physiological framework within which to assess SL and SR Ca^{2+} flux.

Contraction kinetics

TPT and THR provide information on contraction kinetics. This study demonstrates changes in the duration of both variables with changes in test temperature, acclimation temperature and pacing frequency (Table 1). However, only THR was significantly affected by adrenaline and ryanodine treatment and then only at low (0.2 Hz) pacing frequencies. In all cases, ryanodine shortened THR at 0.2 Hz, and the magnitude of this response paralleled the ryanodine-induced reductions in force (i.e. the decrease in THR was greatest for a 22 °C test temperature after 12 °C acclimation and at both test temperatures after 22 °C acclimation). The most probable explanation for the faster relaxation in the presence of ryanodine is a more rapid complete Ca^{2+} extrusion as a result of the lower intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) reflected in the lower peak tension. Additionally, the relatively large time interval between depolarizations at 0.2 Hz may allow Ca^{2+} to leak out of the SR and be extruded by the SL Ca^{2+} pump, the SR Ca^{2+} pump or the Na^+/Ca^{2+} exchanger. Locking the SR Ca^{2+}-release channel in a closed state with ryanodine may prevent or slow Ca^{2+} leakage, allowing extrusion mechanisms to be devoted entirely to the removal of activator Ca^{2+} and thus decreasing THR.

Mammalian research demonstrates faster rates of relaxation with adrenergic stimulation owing to increased activity of the SR Ca^{2+} pump and increased stimulation of the Na^+ pump. This ultimately increases Ca^{2+} efflux *via* Na^+/Ca^{2+} exchange (Bers, 1991). Such processes have yet to be studied in fish, and the effects of temperature acclimation may further complicate the situation. However, the slower rates of relaxation and the longer THR at 0.2 Hz after adrenergic stimulation in the present study suggest a different adrenergic effect on Ca^{2+} extrusion from that found in mammals. We feel that variable extrusion capabilities between acclimation temperatures would not account for the slower relaxation at 22 °C after adrenergic stimulation because temperature change causes little variation in the activity of the rainbow trout Na^+/Ca^{2+} exchanger (between 4 and 15 °C) (McKnight *et al.* 1989) and the SR Ca^{2+} pump (between 0 and 20 °C) (skeletal muscle, Toledo *et al.* 1995). We have no explanations for these findings and are unclear as to the physiological relevance of longer relaxation times at such low contraction frequencies. Other effects on relaxation were noted from the changes in resting tension and are discussed below.

Resting tension

Although not the central focus of the present study, limited observations were made on changes in resting tension. An increase in resting tension suggests a reduced ability to extrude Ca^{2+} adequately from the cytosol after tension development (Bailey and Driedzic, 1990). The greatest increase in resting tension occurred under maximal adrenergic stimulation after ryanodine incubation. We attribute this to inadequate Ca^{2+} extrusion in the presence of the increased intracellular Ca^{2+} levels that occur after maximal adrenergic stimulation and decreased SR Ca^{2+} sequestering after ryanodine treatment. However, we have no explanation for why, under the various test conditions, only a proportion of the total muscles tested showed any changes in resting tension (see Table 2). Furthermore, there was no significant correlation between

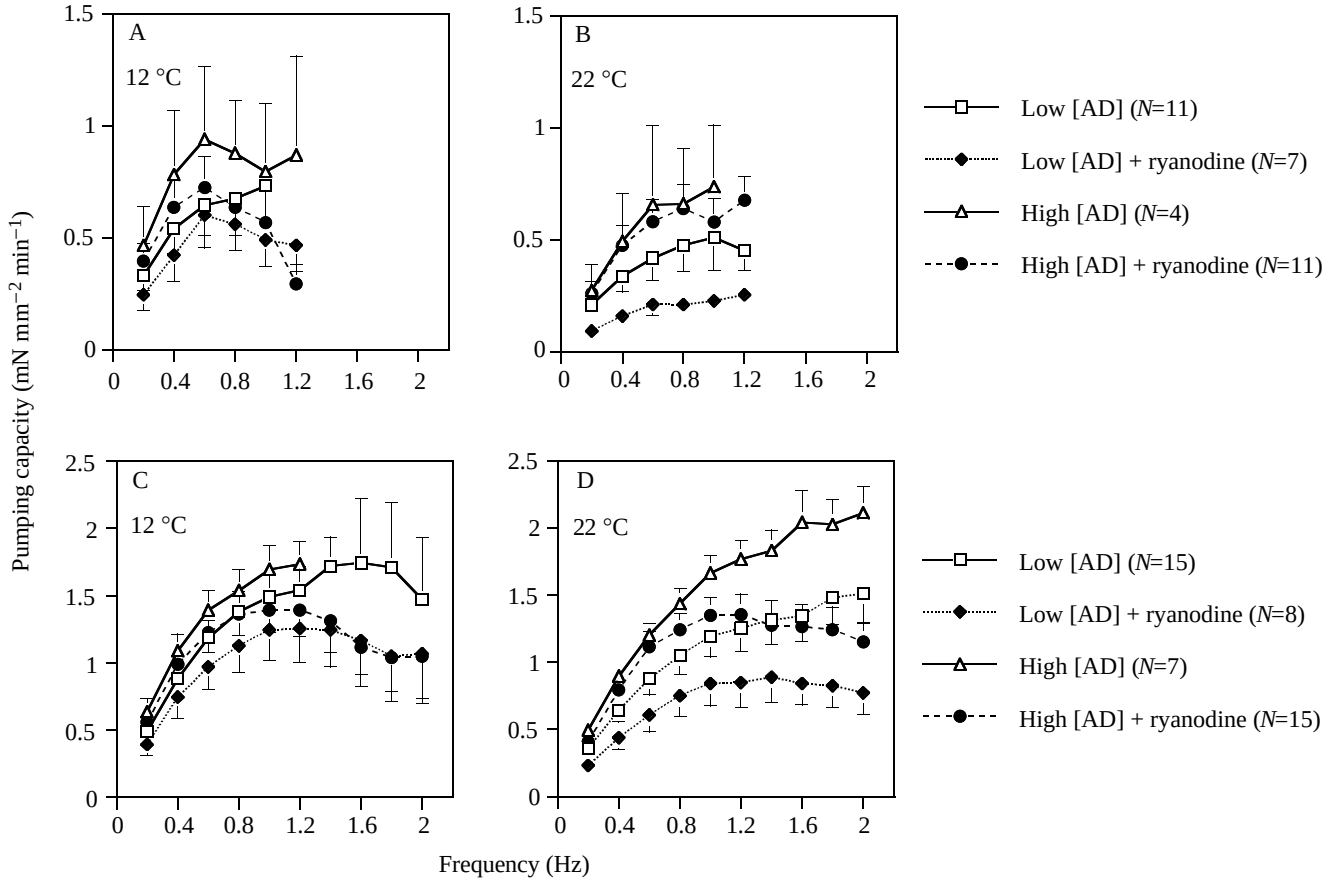


Fig. 4. Pumping capacity (the product of frequency and peak tension) for isolated ventricular trabeculae from rainbow trout. (A,B) 12 °C acclimation temperature; (C,D) 22 °C acclimation temperature. Note that the scales for each acclimation temperature are different. Test temperature is indicated in the upper left corner of each graph. Values are group means; vertical bars represent ±1 S.E.M. Values of *N* are given in Materials and methods. Significance ($P \leqslant 0.05$) was examined for D: there is a significant effect of ryanodine treatment for both low [AD] and high [AD] treatments at 1.6, 1.8 and 2.0 Hz. The effect of high [AD] treatment was significant at all frequencies.

changes in resting tension and changes in THR as might be expected. Therefore, although the changes in resting tension observed in this study can be roughly correlated with changes in SR function and extracellular Ca^{2+} influx, we are not confident that they reliably indicate intracellular Ca^{2+} handling.

Cardiac pumping capacity

Pumping capacity, the product of pacing rate and peak tension (Matikainen and Vornanen, 1992), may be calculated from the present data for isolated ventricular trabeculae (Fig. 4). Pumping capacity can be used as an index of power output for isolated muscle preparations since it integrates the effects of changes in tension and pacing frequency. It therefore provides an integrative way of comparing frequency, temperature and drug-treatment effects on ventricular performance. Moreover, it provides a means to evaluate the pacing frequency at which pumping capacity is highest. Consistent with the conclusions drawn from the inotropic responses, pumping capacity was reduced by ryanodine application and increased by adrenergic stimulation. After acclimation at 12 °C (Fig. 4A,B), the effect of ryanodine on pumping capacity appeared more pronounced under tonic adrenergic stimulation, but the differences were not statistically significant. Pumping capacity was very sensitive to acclimation temperature, with superior pumping ability after 22 °C acclimation (Fig. 4C,D) (note the different scales in Fig. 4), an observation in accordance with the results of Matikainen and Vornanen (1992), who reported increased heart rate, isometric force development and pumping capacity after warm acclimation (20 °C) compared with cold acclimation (4 °C) in crucian carp *Carassius carassius*. Additionally, pumping capacity is maintained at higher frequencies after 22 °C acclimation than after 12 °C acclimation, but is particularly sensitive to ryanodine. Ryanodine reduces the ability to pump at high frequencies and tends to move the apex of the pumping capacity *versus* frequency curves to the left (Fig. 4). Furthermore, calculations of pumping capacity indicate that maximal adrenergic stimulation cannot compensate for the ryanodine-induced loss of power at frequencies greater than 0.8–1.0 Hz. Above 1.0 Hz, pumping capacity falls, presumably as a result of a reduced

intracellular Ca^{2+} contribution, suggesting that inhibiting SR Ca^{2+} release reduces the pumping power of the ventricle at high frequencies.

Conclusions

SL Ca^{2+} influx dominates E-C coupling in trout heart at both warm (22 °C) and cold (12 °C) temperatures. However, the SR can contribute a significant proportion of Ca^{2+} to force development at 22 °C, the magnitude of which is inversely related to pacing frequency (approximately 50 % at 0.2 Hz, approximately 25 % at 1.2 Hz and approximately 20 % at 2.0 Hz). Adrenaline-mediated increases in SL Ca^{2+} influx decrease the importance of the SR in contributing Ca^{2+} to E-C coupling at all temperatures, with the exception of high pacing frequencies (>1.2 Hz) at 22 °C, where the positive inotropy resulting from adrenergic stimulation did not fully compensate for the negative inotropic effect of ryanodine. This suggests that the ability to maintain maximum force at high temperatures and high frequencies may involve recruitment of SR Ca^{2+} stores. The slight leftward shift in optimum frequency for pumping capacity and the increase in resting tension after ryanodine treatment at high temperatures lend support to this suggestion. From these results, it is tempting to suggest that warm temperatures allow fish to utilize SR Ca^{2+} stores that lie dormant at low temperatures owing to the temperature-dependency of the SR Ca^{2+}-release channel. This increased recruitment of SR Ca^{2+} stores at warm temperatures may correspond to the reduced ability of the muscle to increase Ca^{2+} delivery *via* adrenergic modulation of SL Ca^{2+} channels. Thus, the interplay between SR and SL Ca^{2+} flux is altered by adrenergic stimulation while, at the same time, being both frequency- and temperature-dependent. Interestingly, the almost complete dominance of SL Ca^{2+} flux occurs at temperatures at, and below, the trout's preferred temperature. In contrast, the relative importance of the SR increases as the temperature approaches the upper incipient lethal temperature, allowing the heart to maintain its pumping ability as the effectiveness of adrenaline wanes.

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