The Journal of Experimental Biology 205, 3641–3650 (2002)
Printed in Great Britain © The Company of Biologists Limited
JEB4426

Effects of temperature on intracellular [Ca^{2+}] in trout atrial myocytes

Holly A. Shiels[1,*], Matti Vornanen[2], Anthony P. Farrell[1]

[1]*Simon Fraser University, Biological Sciences, Burnaby, British Columbia, V5A 1S6, Canada* and
[2]*University of Joensuu, Department of Biology, PO Box 111, 80101 Joensuu, Finland*

*Author for correspondence at present address: School of Biomedical Sciences, University of Leeds, Leeds, LS2 9JT, UK (e-mail: hollys@sfu.ca)

Accepted 21 August 2002

Summary

Acute temperature change can be cardioplegic to mammals, yet certain ectotherms maintain their cardiac scope over a wide temperature range. To better understand the acute effects of temperature on the ectothermic heart, we investigated the stimulus-induced change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; cytosolic Ca^{2+} transient) in isolated rainbow trout myocytes at 7°C, 14°C and 21°C. Myocytes were voltage-clamped and loaded with Fura-2 to measure the L-type Ca^{2+} channel current (I_{Ca}) and $[Ca^{2+}]_i$ during physiological action potential (AP) pulses at frequencies that correspond to trout heart rates *in vivo* at 7°C, 14°C and 21°C. Additionally, $[Ca^{2+}]_i$ and I_{Ca} were examined with square (SQ) pulses at slow (0.2 Hz) and physiologically relevant contraction frequencies. The amplitude of $[Ca^{2+}]_i$ decreased with increasing temperature for both SQ and AP pulses, which may contribute to the well-known negative inotropic effect of warm temperature on contractile strength in trout hearts. With SQ pulses, $[Ca^{2+}]_i$ decreased from 474±53 nmol l^{-1} at 7°C to 198±21 nmol l^{-1} at 21°C, while the decrease in $[Ca^{2+}]_i$ with AP pulses was from 234±49 nmol l^{-1} to 79±12 nmol l^{-1}, respectively. Sarcolemmal Ca^{2+} influx was increased slightly at cold temperatures with AP pulses (charge transfer was 0.27±0.04 pC pF^{-1}, 0.19±0.03 pC pF^{-1} and 0.13±0.03 pC pF^{-1} at 7°C, 14°C and 21°C, respectively). At all temperatures, cells were better able to maintain diastolic Ca^{2+} levels at physiological frequencies with AP pulses compared with 500 ms SQ pulses. We suggest that temperature-dependent modulation of the AP is important for cellular Ca^{2+} regulation during temperature and frequency change in rainbow trout heart.

Key words: L-type Ca^{2+} current (I_{Ca}), calcium transient, sarcoplasmic reticulum, action potential, frequency, fish hearts, temperature, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Cardiac contractility is profoundly influenced by temperature in all vertebrates. This is not surprising as the transient rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which underlies excitation–contraction coupling, represents the culmination of many temperature-sensitive cellular processes; i.e. action potential configuration (Møller-Nielsen and Gesser, 1992; Puglisi et al., 1999; Coyne et al., 2000), myofilament Ca^{2+} sensitivity (Harrison and Bers, 1990; Churcott et al., 1994; Gillis et al., 2000) and Q_{10} effects on protein pumps and ion channels (Sitsapesan et al., 1991; Herve et al., 1992; Xue et al., 1999; Kim et al., 2000; Shiels et al., 2000). In most vertebrates, the transient change in $[Ca^{2+}]_i$ in response to stimulation is the product of both extracellular Ca^{2+} influx across the sarcolemmal membrane (SL) and intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR), although the relative Ca^{2+} contribution from each source may vary (Fabiato and Fabiato, 1978; Driedzic and Gesser, 1985; Bers, 1985, 1989; Tanaka and Shigenobu, 1989; Hove-Madsen, 1992; Bassani et al., 1994).

In mammals, the temperature sensitivity of extracellular and intracellular Ca^{2+} fluxes are well characterized. Transsarcolemmal Ca^{2+} influx *via* the L-type Ca^{2+} channel current (I_{Ca}) is strongly influenced by temperature, with a Q_{10} ranging from 2 to 3.5 (Cavalie et al., 1985; Herve et al., 1992). Cold temperatures reduce SR function in many mammalian hearts, impairing both release (Bers, 1987; Sitsapesan et al., 1991) and uptake (Liu et al., 1991, 1997; Wang et al., 2000) of SR Ca^{2+} stores. Under most conditions, excitation–contraction coupling in rainbow trout heart is thought to be driven primarily by extracellular Ca^{2+} influx *via* I_{Ca}, with SR Ca^{2+} release playing a secondary role (Tibbits et al., 1992; Hove-Madsen, 1992; Keen et al., 1994; Shiels and Farrell, 1997). Recent studies of I_{Ca} in trout myocytes indicate that the temperature sensitivity of peak current amplitude is not very different from that of mammals (Q_{10} of approximately 1.7–2.1; Kim et al., 2000; Shiels et al., 2000) but that the total transsarcolemmal Ca^{2+} influx is fairly well maintained during temperature change (Shiels et al., 2000). Furthermore, the fish

cardiac SR has been shown to uptake and release Ca^{2+} at temperatures well below those known to inhibit SR function in most mammals (Aho and Vornanen, 1999; Hove-Madsen et al., 2001; Shiels et al., 2002a; Tiitu and Vornanen, 2002).

Despite the fact that the individual Ca^{2+} flux pathways in trout hearts seem to demonstrate some tolerance to temperature change, *in situ* heart and *in vitro* cardiac muscle strip studies show both inotropic and chronotropic responses to temperature change (Keen et al., 1994; Shiels and Farrell, 1997; Aho and Vornanen, 1999). We wanted to understand to what extent these responses might be mediated by changes in cellular Ca^{2+}. The transient change in $[Ca^{2+}]_i$ during excitation–contraction coupling represents the sum of both extracellular and intracellular Ca^{2+} flux, and its amplitude is directly related to the strength of cardiac contraction (Yue, 1987). Thus, the focus of the present study was to examine $[Ca^{2+}]_i$ at different temperatures in rainbow trout atrial myocytes. Previous studies have shown that trout experience acute temperature fluctuations of as much as ±10°C either while transversing thermoclines or as a result of diurnal changes in shallow streams (Reid et al., 1997; Matthews and Berg, 1997). Thus, we conducted our studies on 14°C-acclimated rainbow trout at 7°C, 14°C and 21°C. By simultaneously quantifying $[Ca^{2+}]_i$ and I_{Ca} at each of these temperatures, we were able to assess the effects of temperature on $[Ca^{2+}]_i$ and indirectly separate the effect of temperature on extracellular and intracellular Ca^{2+} fluxes.

In fish hearts, ambient temperature also directly modulates heart rate (Farrell and Jones, 1992). In turn, changes in contraction frequency affect many of the cellular processes that underlie the transient change in $[Ca^{2+}]_i$ (Møller-Nielsen and Gesser, 1992; Hove-Madsen and Tort, 1998). For example, increasing the contraction frequency from 0.2 Hz to 1.4 Hz causes significant reductions in the amplitude of I_{Ca}, as well as reductions in both the amplitude and duration of the AP in trout ventricular myocytes (Harwood et al., 2000). Because the frequency of stimulation will modulate cellular Ca^{2+} cycling, it is critical to consider the physiologically relevant contraction frequency associated with a particular temperature when trying to understand the implications of temperature change on $[Ca^{2+}]_i$ *in vivo*. Thus, we examined $[Ca^{2+}]_i$ and I_{Ca} in trout atrial myocytes that were stimulated with physiological APs at physiologically relevant contraction frequencies for the applied temperature. We also examined the effect of temperature on $[Ca^{2+}]_i$ and I_{Ca} in response to conventional square (SQ) voltage clamp pulses at slow (0.2 Hz) and physiologically relevant frequencies to gain mechanistic insight and to facilitate comparison of our results with earlier studies.

Materials and methods

Fish origin and care

Rainbow trout *Oncorhynchus mykiss* Walbaum (mean mass 416.7±17.2 g, *N*=12) were obtained from a local fish farm (Kontiolahti, Finland) and held in the laboratory at 14±1°C for a minimum of four weeks prior to experimentation. They were fed daily on commercial trout pellets (Biomar, Brande, Denmark) and exposed to a 15 h:9 h L:D cycle. All procedures were in accordance with local animal handling protocols.

Isolated myocyte preparation

A detailed description of the rainbow trout myocyte preparation has been published previously (Vornanen, 1998; Shiels et al., 2000). Briefly, myocytes were obtained by retrograde perfusion of the heart, first with an isolating solution for 8–10 min and then with a proteolytic enzyme solution for 20 min. After enzymatic treatment, the atrium was removed from the ventricle, cut into small pieces and then triturated through the opening of a Pasteur pipette. The isolated cells were stored for up to 8 h in fresh isolating solution at 14°C.

Solutions

The isolating solution contained: NaCl, 100 mmol l^{-1}; KCl, 10 mmol l^{-1}; KH_2PO_4, 1.2 mmol l^{-1}; $MgSO_4$, 4 mmol l^{-1}; taurine, 50 mmol l^{-1}; glucose, 20 mmol l^{-1}; and Hepes, 10 mmol l^{-1}; adjusted to pH 6.9 with KOH. For enzymatic digestion, collagenase (Type IA), trypsin (Type III) and fatty-acid-free bovine serum albumin (BSA) were added to the isolating solution. Standard internal and external solutions were used to eliminate contaminating Na^+ and K^+ currents. Tetrodotoxin (TTX, 1 μmol l^{-1}) was included in all external solutions to effectively eliminate fast Na^+ currents (Vornanen, 1998). Cs^+-based internal and external solutions with 15 mmol l^{-1} tetraethylammonium chloride (TEA) in the pipette were used to block K^+ currents (Hove-Madsen and Tort, 1998). The extracellular solution for recording I_{Ca} contained: NaCl, 150 mmol l^{-1}; CsCl, 5.4 mmol l^{-1}; $MgSO_4$, 1.2 mmol l^{-1}; NaH_2PO_4, 0.4 mmol l^{-1}; $CaCl_2$, 1.8 mmol l^{-1}; glucose, 10 mmol l^{-1}; and Hepes, 10 mmol l^{-1}; adjusted to pH 7.7 with CsOH. Additionally, 5 nmol l^{-1} adrenaline was added to emulate the tonic level of adrenaline circulating in the blood of resting rainbow trout *in vivo* (Milligan et al., 1989). The pipette solution contained: CsCl, 130 mmol l^{-1}; MgATP, 5 mmol l^{-1}; TEA, 15 mmol l^{-1}; $MgCl_2$, 1 mmol l^{-1}; oxaloacetate, 5 mmol l^{-1}; Hepes, 10 mmol l^{-1}; EGTA, 0.025 mmol l^{-1}; Na_2GTP, 0.03 mmol l^{-1}; and K_5-Fura-2, 0.1 mmol l^{-1}; pH adjusted to 7.2 with CsOH. All drugs, with the exception of TTX (Tocris, Bristol, UK), were purchased from Sigma (St Louis, MO, USA).

Patch-clamp recordings

Whole-cell voltage-clamp experiments were performed using an Axopatch 1D amplifier with a CV-4 1/100 headstage (Axon Instruments, Foster City, CA, USA). Pipettes had a resistance of 2.2±0.30 MΩ when filled with pipette solution. Junction potentials were zeroed prior to seal formation. Pipette capacitance (9.3±0.2 pF) was compensated after formation of a GΩ seal. Mean series resistance was 6.5±0.3 MΩ. Membrane capacitance (31.2±0.8 pF, *N*=76) was measured using the calibrated capacity compensation circuit of the Axopatch amplifier. Signals were low-pass filtered using the 4-pole

lowpass Bessel filter on the Axopatch-1D amplifier at a frequency of 2 kHz and were then analyzed off-line using pClamp 6.0 software (Axon Instruments).

In a separate series of experiments, we recorded APs from rainbow trout atrial myocytes (Fig. 1). Cells were stimulated to elicit APs at 7°C, 14°C or 21°C and at a frequency that corresponds to the resting heart rate of rainbow trout *in vivo* at each of these temperatures. The temperature/frequency pairs were as follows: 0.6 Hz at 7°C, 1.0 Hz at 14°C and 1.4 Hz at 21°C (Tuurala et al., 1982; Farrell et al., 1996; Aho and Vornanen, 2001). APs were elicited by the minimum voltage pulse able to trigger a self-sustained AP (approximately 1 ms, 0.8 nA). The resultant AP waveforms were subsequently used to provide a more physiologically relevant stimulus under which to evaluate I_{Ca} and $[Ca^{2+}]_i$ during temperature change. In all experiments, the capacitive transients resulting from the AP waveform were compensated for by the amplifier.

Peak current amplitude of I_{Ca} was calculated as the difference between the peak inward current and the current recorded at the end of the depolarizing pulse. The charge (pC) carried by I_{Ca} was calculated from the time integral of the current and then normalized to cell capacitance (pC pF^{-1}).

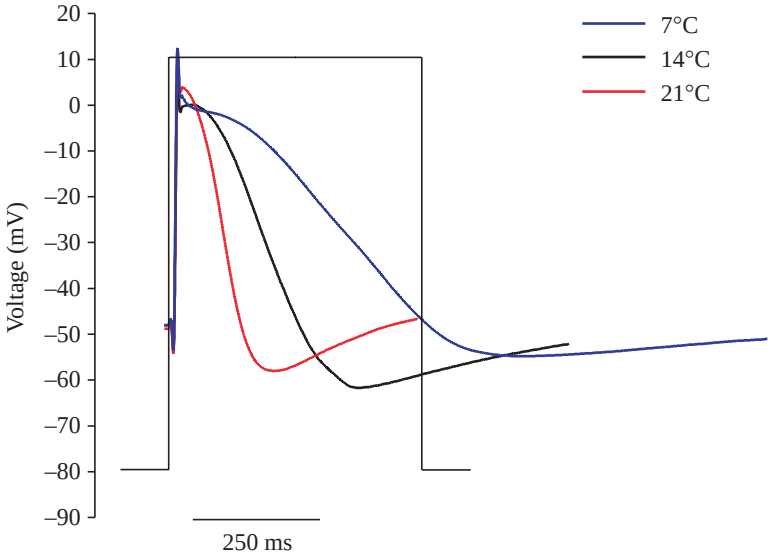

Fig. 1. Action potential (AP) waveforms used to elicit L-type Ca^{2+} channel current (I_{Ca}) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The APs were recorded by current-clamp in a separate series of experiments (Shiels et al., 2002a). AP waveforms were obtained by averaging recordings from 6–10 cells at each temperature. The relatively depolarized resting potential is typical for isolated atrial myocytes from rainbow trout (Shiels et al., 2000) and reflects both a low density of the inward rectifier K^+ current (I_{K1}) and the lack of cholinergic tone (Vornanen et al., 2002). The inter-pulse holding level during subsequent AP stimulation was –80 mV. The solid line shows the dimensions of the 500 ms square (SQ) pulse.

Fluorescent recordings

For each cell, after formation of the GΩ seal, and before rupture of the cell membrane, background fluorescence (approximately 40% and 25% of the total signal at 340 nm and 380 nm, respectively) was recorded and then subtracted from all subsequent measurements. Temperature change had no effect on background fluorescence. Myocytes were alternately illuminated (optical chopper, 0.2 kHz, 75 W Xenon arc lamp) at 340 nm and 380 nm with a high-speed dual-wavelength filter-based illuminator (PowerFilter, Photon Technology International, Brunswick, NJ, USA). Emitted light was filtered at 510 nm and detected by a photon-counting photomultiplier tube, the output of which was connected to a computer *via* an I/O board and saved to disk for later analysis using the FeliX acquisition and analysis software (7-point smoothing and trace averaging, Photon Technology International). The emission aperture window was kept as small as possible and was focused close to, but out of view of, the pipette tip to minimize any effect of cell movement during contraction (cells typically contracted approximately 10–15% of the resting length).

The ratio of emitted fluorescence at 340 nm/380 nm (R) was calculated to give an index of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was calculated using the acquired fluorescent ratios and calibration parameters derived both experimentally and through calculation. To measure R_{max} and R_{min}, cells were patched-clamped and perfused with an extracellular solution containing metabolic inhibitors (rotenone, 2 µmol l^{-1}; carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), 5 µmol l^{-1}; jodo acetic acid, 5 mmol l^{-1}). Pipette and extracellular solutions were the same as those described above except that nucleosides and sulphates were omitted, respectively. To estimate R_{max}, 10 µmol l^{-1} Ca^{2+} ionophore (4-bromo A23187) and 1.8 mmol l^{-1} $CaCl_2$ were added to the perfusate. To estimate R_{min}, cells were then perfused with a solution containing 10 mmol l^{-1} EGTA. The values obtained are as follows: R_{max}=9.80±0.67, R_{min}=0.99±0.01, β (signal fluorescence at 380 nm/background fluorescence at 380 nm)=4.62±0.44; N=17 (A. Ryökkynen and M. Vornanen, unpublished data). The affinity constant K_d of Fura-2 has been measured *in vivo* over a range of temperatures (5–37°C) in enterocytes from the Atlantic cod (*Gadus morhua*; Larsson et al., 1999). We adjusted the K_d values obtained in that study to match our experimental conditions of pH, buffer and ionic strength by calculating the effects of pH, buffer and ionic strength on 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (Harrison and Bers, 1987) using the MaxChelator program (Bers et al., 1994). The calculated K_d values were as follows: 395 nmol l^{-1} at 7°C, 366 nmol l^{-1} at 14°C and 336 nmol l^{-1} at 21°C. The fluorescence ratios obtained during the experiments were converted into $[Ca^{2+}]_i$ using these K_d values and the above measurements of R_{max}, R_{min} and β, as described by Grynkiewicz et al. (1985).

Experimental procedures

Atrial myocytes were superfused at a rate of 2 ml min^{-1} with extracellular solution at 7°C, 14°C or 21°C. Each cell was tested at one experimental temperature only. The extracellular

solution was heated or chilled by a water bath circuit before emptying into the recording chamber (RC-26, Warner Instrument Corp., Hamden, CT, USA; volume 150 µl). A thermocouple was placed inside the recording chamber and positioned no less than 5 mm from the cell to ensure it was experiencing the desired temperature. At the onset of each experiment, cells were stimulated with 25 physiological APs at a frequency that corresponded to the *in vivo* heart rate of trout at each temperature (see Fig. 1) to ensure that SR Ca^{2+} content was similar in all cells (Shiels et al., 2002a). However, the subsequent stimulation protocols may alter SR Ca^{2+} content as the experiment proceeds, which is a limitation of our approach.

Cells were first stimulated to contract with five SQ depolarizing pulses (from –80 mV to +10 mV, 500 ms duration) at a frequency of 0.2 Hz ($SQ_{0.2Hz}$), and I_{Ca} and $[Ca^{2+}]_i$ were simultaneously recorded. The cells were then stimulated to contract with five SQ depolarizing pulses at a frequency that corresponded to the *in vivo* heart rate for rainbow trout at the given test temperature ($SQ_{physiol}$). SQ voltage-clamp protocols are vital for characterizing channel kinetics and simplify interpretation of mechanistic relationships, but they may have only limited relevance to the condition *in vivo* (Linz and Meyer, 1998; Puglisi et al., 1999). Therefore, after stimulation under $SQ_{physiol}$ conditions, the cells were stimulated with an AP waveform (see Fig. 1) appropriate for the test temperature and at a frequency appropriate for the test temperature ($AP_{physiol}$). I_{Ca} and $[Ca^{2+}]_i$ were simultaneously recorded throughout (see Fig. 4). Although the $SQ_{physiol}$ part of the protocol is of little physiological significance, we believe that comparing the results obtained at physiological stimulation frequencies from both a 500 ms SQ ($SQ_{physiol}$) and a physiological AP ($AP_{physiol}$) allows us to evaluate the importance of the AP and the possible

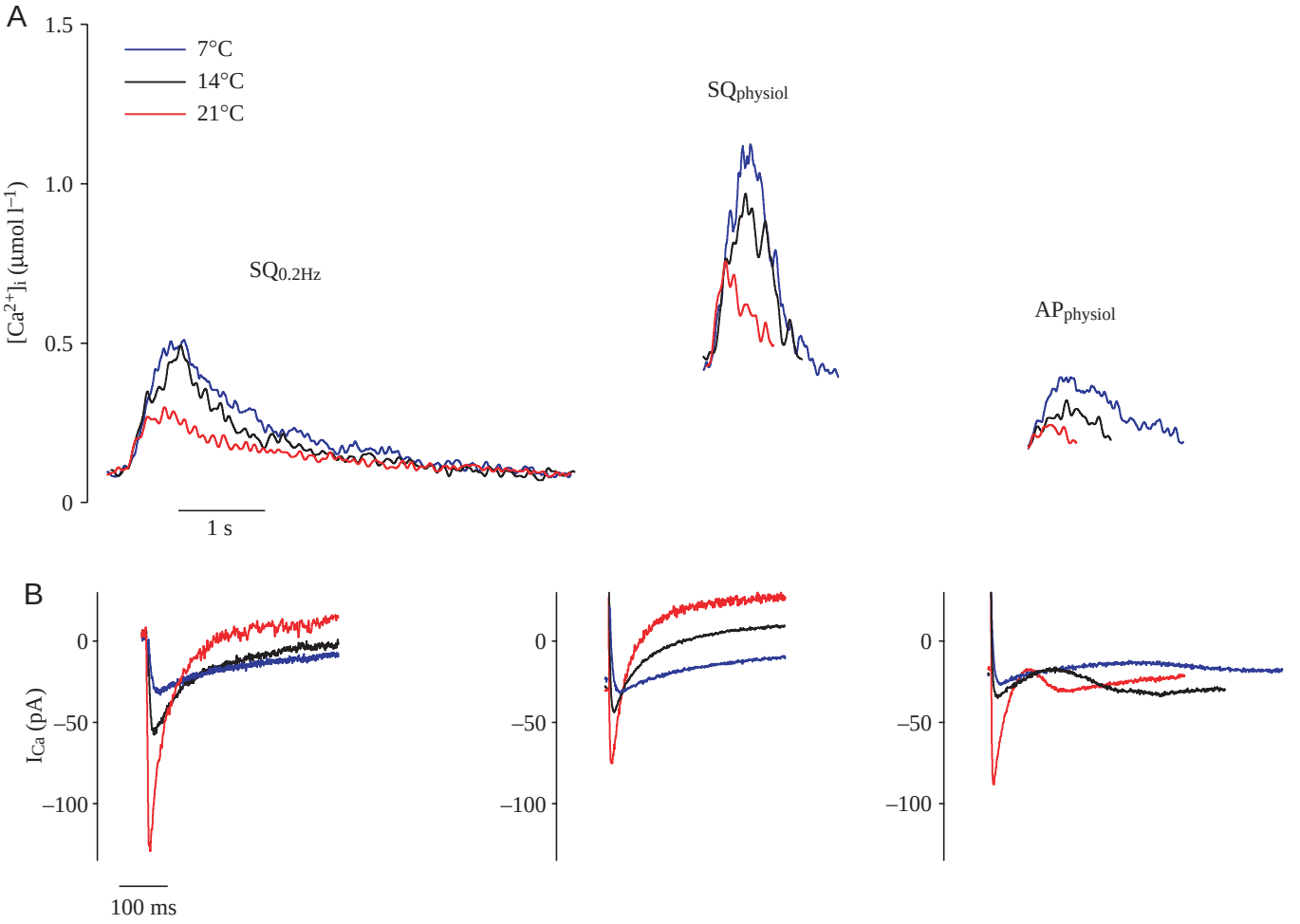

Fig. 2. The effect of temperature on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and L-type Ca^{2+} channel current (I_{Ca}) in trout atrial myocytes. (A) The effect of temperature on $[Ca^{2+}]_i$ during $SQ_{0.2Hz}$, $SQ_{physiol}$ and $AP_{physiol}$ (the physiological frequencies were: 0.6 Hz at 7°C, 1.0 Hz at 14°C and 1.4 Hz at 21°C). The increase in the resting level of the transient at $SQ_{physiol}$ reflects the increase in diastolic Ca^{2+} concentration (see Fig. 4, Table 3). (B) Current recordings of I_{Ca} under the same conditions. Peak current amplitude was calculated as the difference between the peak inward current and the current recorded at the end of the depolarizing pulse. The currents elicited at $SQ_{0.2Hz}$ were leakage corrected using the P/N procedure of the software (Clampex, Axon Instruments). Leakage correction was not employed at $SQ_{physiol}$ or $AP_{physiol}$. Mean data and statistical analysis is given in Tables 1–3 and in Fig. 3. $SQ_{0.2Hz}$, square pulses at a frequency of 0.2 Hz; $SQ_{physiol}$, square pulses at physiological frequency; $AP_{physiol}$, action potential at physiological frequency.

limitations of the SQ pulse in assessing physiological processes. The duration of each protocol was approximately 1 min and we did not observe any changes in intracellular buffering by Fura-2 or run-down of I_{Ca} over this time period.

Statistical analysis

One-way repeated measures analysis of variance (RM ANOVA) were used to compare the effects of changing the frequency and the stimulus waveform on I_{Ca} and $[Ca^{2+}]_i$. One-way ANOVAs were used to test the effects of temperature on I_{Ca} and $[Ca^{2+}]_i$. Differences were considered significant at $P<0.05$, as assessed by Student–Newman–Keuls (SNK) *post-hoc* analysis. The mean values ± S.E.M. and the statistical analysis for $[Ca^{2+}]_i$ appear in the tables. Five transients were recorded under each experimental pulse condition (i.e. $SQ_{0.2Hz}$, $SQ_{physiol}$ and $AP_{physiol}$; see Fig. 4). The diastolic $[Ca^{2+}]_i$ at the end of the 4th transient and the peak $[Ca^{2+}]_i$ of the 5th transient were used to represent a steady-state value for each condition (Tables 1–3). For future experiments, it would, however, be prudent to provide a longer train of stimulation pulses, especially at warm temperatures, to ensure a steady-state level had been attained. On the other hand, excursions by fish across thermoclines can be very rapid and transitory in nature and therefore I_{Ca} may not reach a steady-state *in vivo* before temperature (and thus heart rate) change again.

Results

Effects of temperature

For both SQ and AP pulses, the amplitude of the stimulus-induced change in $[Ca^{2+}]_i$ decreased with increasing temperature (Fig. 2, Table 1). The time required to reach peak $[Ca^{2+}]_i$ also decreased with increasing temperature (Table 2), probably due to the reduced amplitude because the rate of rise did not change significantly (0.625±0.33 μmol l^{-1} s^{-1} at 7°C and 0.460±0.07 μmol l^{-1} s^{-1} at 21°C with SQ pulses at 0.2 Hz).

Table 1. *Amplitude of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (nmol l^{-1}) in trout atrial myocytes*

	$SQ_{0.2Hz}$	$SQ_{physiol}$	$AP_{physiol}$
7°C	474±53^{a,e}	662±65^{a,f}	234±49^{a,g}
14°C	398±40^{a,e}	506±48^{a,f}	122±41^{b,g}
21°C	198±27^{b,e}	278±41^{b,f}	79±12^{b,g}

The effect of temperature and the frequency/stimulus waveform on the amplitude of the stimulus-induced change in $[Ca^{2+}]_i$, calculated as the difference between the diastolic and systolic Ca^{2+} concentration with each depolarization. Values are means of 4–8 cells at each temperature.

The frequency of stimulation during $SQ_{physiol}$ (square pulses) and $AP_{physiol}$ (action potential) was 0.6 Hz at 7°C, 1.0 Hz at 14°C and 1.4 Hz at 21°C.

The effect of temperature is indicated by dissimilar letters a, b and c (compare vertically within each frequency/stimulus treatment) [analysis of variance (ANOVA), Student–Newman–Keuls (SNK) test]. Dissimilar letters e, f and g indicate significant differences between frequency/stimulus protocols (compare horizontally within each temperature) [repeated measures ANOVA, SNK].

The peak amplitude of I_{Ca} increased with temperature (Figs 2B, 3A). The charge carried across the SL *via* I_{Ca} was unchanged by temperature during SQ pulse stimulation and decreased with temperature during AP stimulation (Fig. 3B).

During SQ pulse stimulation, we observed a temperature-dependent change in the amplitude of $[Ca^{2+}]_i$ but no change in the charge density calculated by integrating I_{Ca}. This suggests that, in addition to Ca^{2+} flux across the SL, other Ca^{2+} flux pathways, most probably Ca^{2+} release from the SR, must be changing with respect to temperature in these cells. During AP stimulation, the reduced charge density of I_{Ca} at warm temperatures (Fig. 3B) may underlie the large percentage reduction in $[Ca^{2+}]_i$ observed under these conditions (Fig. 2, Table 1).

Effects of stimulus change

The frequency and shape of the depolarizing stimulus

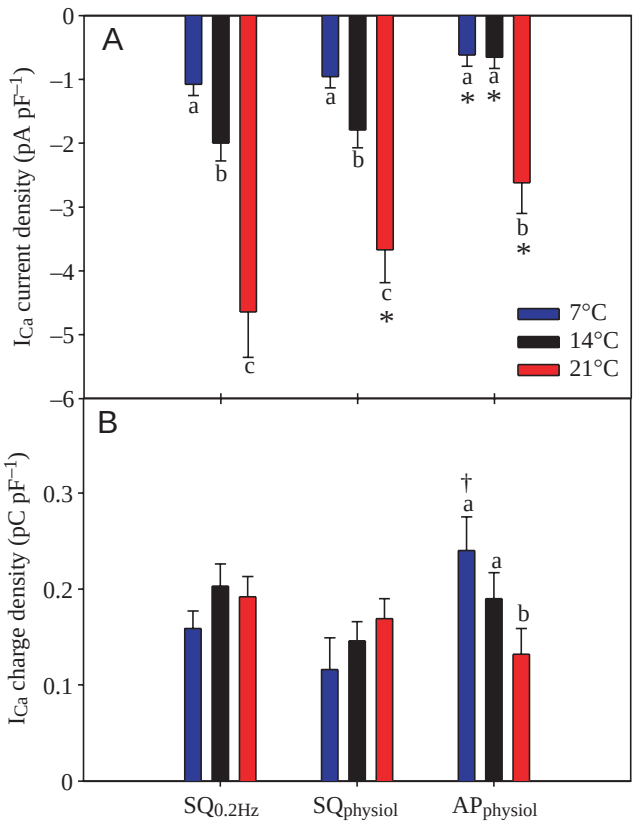

Fig. 3. The effect of temperature and stimulus change on (A) the peak current density of L-type Ca^{2+} channel current (I_{Ca}) and (B) the charge density of I_{Ca} in trout atrial myocytes. Dissimilar letters indicate significant effects of temperature within each stimulus treatment (analysis of variance, Student–Newman–Keuls test). * indicates a significant decrease in I_{Ca} during AP stimulation at all temperatures and with each successive stimulus protocol at 21°C. † indicates greater I_{Ca} charge density at 7°C during AP stimulation compared with SQ stimulation. Values are means ± S.E.M.; N=7 for 7°C and 21°C, and N=8 for 14°C.

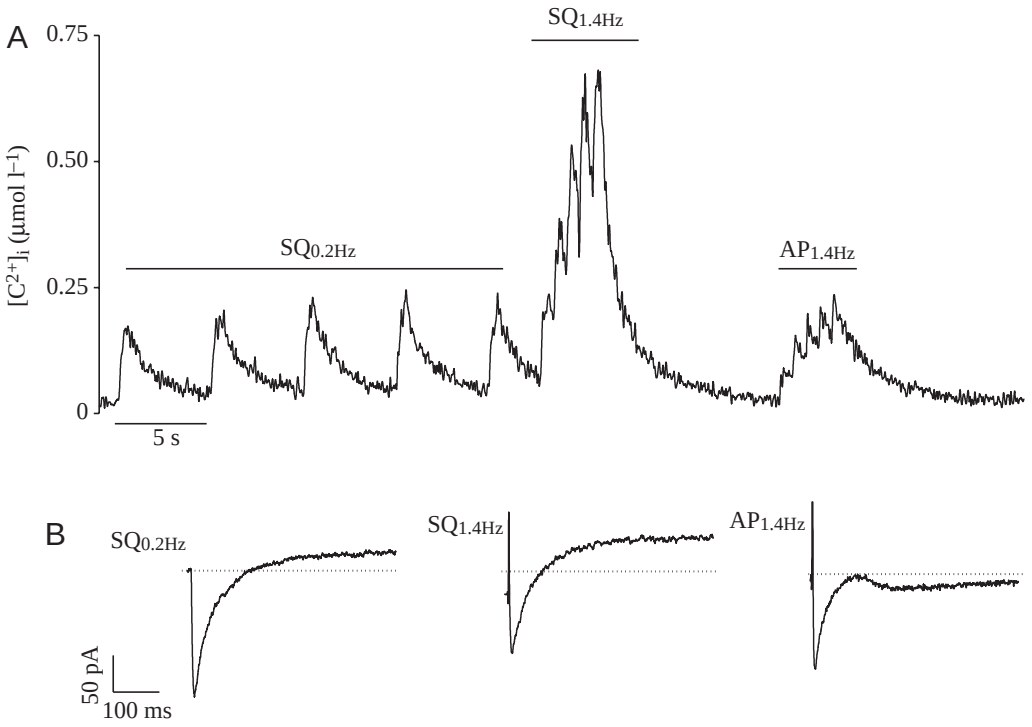

Fig. 4. This figure is an example of a recording for a single myocyte at 21°C. Part (A) shows intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and part (B) shows the corresponding L-type Ca^{2+} channel current (I_{Ca}) at each stimulus change. Each panel is marked according to the frequency and waveform used to elicit currents and transients. The first five transients at each temperature were elicited by square pulses at 0.2 Hz ($SQ_{0.2Hz}$). The middle five transients were elicited by SQ pulses applied at a physiologically relevant frequency for the test temperature ($SQ_{1.4Hz}$), but with a long pulse duration (500 ms). Together these factors contribute to the dramatic rise in diastolic Ca^{2+} levels. The last five transients were elicited by temperature- and frequency-dependent action potentials ($AP_{1.4Hz}$). The dotted line in the lower panel indicates 0 mV. Leakage correction was employed at $SQ_{0.2Hz}$, but not at $SQ_{1.4Hz}$ or $AP_{1.4Hz}$.

profoundly altered cellular Ca^{2+} dynamics. As a detailed example, Fig. 4 illustrates the frequency dependence and the influence of pulse shape on I_{Ca} and $[Ca^{2+}]_i$ for an experiment conducted at 21°C. During $SQ_{0.2Hz}$, diastolic Ca^{2+} concentrations returned to rest between depolarizing pulses (Fig. 4A). When stimulation frequency was increased from $SQ_{0.2Hz}$ to $SQ_{1.4Hz}$, diastolic Ca^{2+} concentration increased significantly due to the long (500 ms) and high-frequency (1.4 Hz) depolarization of the membrane (also see Table 3). In addition, $[Ca^{2+}]_i$ increased by approximately 30% (Fig. 4, Table 1). The rate of rise also increased significantly (from $0.73\pm0.11\,\mu mol\,l^{-1}\,s^{-1}$ to $1.16\pm0.84\,\mu mol\,l^{-1}\,s^{-1}$), and the time required to reach the peak of the transient decreased during $SQ_{1.4Hz}$ compared with that during $SQ_{0.2Hz}$ (Table 2). When the stimulus waveform was changed from $SQ_{1.4Hz}$ to $AP_{1.4Hz}$, $[Ca^{2+}]_i$ and diastolic Ca^{2+} levels both decreased significantly (Tables 1, 2), and the rate of rise of the transient decreased significantly to a value ($0.59\pm0.17\,\mu mol\,l^{-1}\,s^{-1}$) that was not significantly different from that for the $SQ_{0.2Hz}$ pulse. Qualitatively similar effects of frequency and stimulus shape were observed at 14°C and 7°C (Tables 1–3). Thus, independent of temperature, an increase in stimulation frequency from 0.2 Hz to a physiologically relevant frequency for the experimental temperature ($SQ_{physiol}$)

Table 2. *Time to peak of $[Ca^{2+}]_i$ (ms) in trout atrial myocytes*

	$SQ_{0.2Hz}$	$SQ_{physiol}$	$AP_{physiol}$
7°C	$601\pm15^{a,e}$	$483\pm44^{a,f}$	$453\pm30^{a,f}$
14°C	$540\pm36^{a,b,e}$	$425\pm57^{a,b,f}$	$394\pm29^{a,f}$
21°C	$502\pm682^{b,e}$	$291\pm46^{b,f}$	$252\pm24^{b,f}$

The effect of temperature and the frequency/stimulus waveform on the time required to reach the peak amplitude of $[Ca^{2+}]_i$. All other features are as in Table 1.

Table 3. *Diastolic Ca^{2+} levels (nmol l^{-1}) in trout atrial myocytes*

	$SQ_{0.2Hz}$	$SQ_{physiol}$	$AP_{physiol}$
7°C	92 ± 20^{e}	401 ± 63^{f}	158 ± 37^{e}
14°C	76 ± 8^{e}	450 ± 15^{f}	176 ± 32^{g}
21°C	80 ± 5^{e}	444 ± 42^{f}	186 ± 22^{g}

The effect of temperature and the frequency/stimulus waveform on diastolic $[Ca^{2+}]_i$. Diastolic Ca^{2+} levels were significantly affected by frequency/stimulus changes but not by temperature changes. All other features are as in Table 1.

produced a large increase in both $[Ca^{2+}]_i$ and in diastolic Ca^{2+} levels with 500 ms SQ stimulation pulses. By contrast, the AP stimulus, which is much shorter in duration, reduced $[Ca^{2+}]_i$ and diastolic Ca^{2+} levels compared with SQ pulses at the same frequency.

The amplitude of I_{Ca} was significantly smaller with an AP stimulus compared with a SQ stimulus at the same frequency (Figs 2–4; Table 1). The charge carried by I_{Ca} did not differ significantly at 14°C and 21°C but was greater at 7°C (Fig. 3B). This situation probably arose as a result of sustained Ca^{2+} influx during the AP plateau and because of Ca^{2+} influx occurring *via* the window current (see Fig. 2B and below).

Discussion

This is the first study we are aware of to measure cytosolic free Ca^{2+} in trout atrial myocytes and examine how it changes with temperature, stimulus shape and frequency. Furthermore, an effort was made to simulate conditions that were more physiologically relevant with respect to temperature, as well as stimulation waveform and frequency. There were three principle findings: (1) the transient change in $[Ca^{2+}]_i$ increases with decreasing temperature, (2) the transient change in $[Ca^{2+}]_i$ is smaller in response to physiological AP stimulation than to 500 ms SQ stimulus pulses, and (3) diastolic Ca^{2+} levels are lower at physiological frequencies when contraction is stimulated with APs compared with 500 ms SQ pulses. Our results indicate that temperature-dependent changes in I_{Ca} alone cannot account for the temperature-dependent changes in $[Ca^{2+}]_i$. Thus, although we made no direct measurements of SR Ca^{2+} flux, our results suggest temperature may modulate SR Ca^{2+} cycling in trout atrial myocytes.

Effects of temperature on $[Ca^{2+}]_i$ and I_{Ca}

With both SQ and AP pulses, $[Ca^{2+}]_i$ was slowest and largest at 7°C compared with at 14°C and 21°C. This observation agrees with results from multicellular cardiac muscle studies from rainbow trout, where isometric contraction is slower and stronger as temperature is decreased (Driedzic and Gesser, 1994; Shiels and Farrell, 1997; Aho and Vornanen, 2001). These findings also agree with mammalian studies that demonstrate an increase in the amplitude of the transient change in $[Ca^{2+}]_i$ with decreases in temperature (Wang et al., 2000). The small amplitude of $[Ca^{2+}]_i$ and the elevated diastolic Ca^{2+} levels during physiological AP pulses at 21°C is striking and is consistent with the finding that the maximum power output of trout heart preparations *in situ* falls off at temperatures above 18°C (Farrell et al., 1996). Indeed, the poor maximum cardiac performance at temperatures approaching the upper incipient lethal temperature for salmonids (23–25°C; Black, 1953), coupled with the observed reduction in maximum swimming capabilities of salmonids as temperatures approach 21°C in both lab (Brett, 1971) and field (Tierney, 2000) studies, has led to the suggestion (Farrell, 1997) that cardiac failure may be a critical factor when salmon are exposed to temperatures that exceed their preferred temperature (approximately 15°C; Brett, 1971). The implication of the present study is that the small amplitude of $[Ca^{2+}]_i$ in the cardiac myocyte may be critical to setting the upper thermal regime in fishes. Thus, it follows that data obtained from trout cardiac myocytes at room temperature should be regarded with some caution.

Previous studies with trout atrial (Shiels et al., 2000) and ventricular (Kim et al., 2000) myocytes have shown a reduction in the peak current amplitude of I_{Ca} with decreasing temperature, and our present study supports these earlier findings. The similar Ca^{2+} influx *via* I_{Ca} at 7°C, 14°C and 21°C probably resulted from a combination of higher and faster peak I_{Ca}, offset by more rapid I_{Ca} inactivation as temperatures increased (see Fig. 2 and Shiels et al., 2000). A similar lack of effect of temperature on the charge density of I_{Ca} has been reported for rabbit myocytes at 25°C and 35°C (Puglisi et al., 1999). We observed an increase in the outward current at the end of the stimulus pulse at 21°C compared with at 14°C and 7°C (see Fig. 2B), which is probably attributable to increased Ca^{2+} influx *via* reverse-mode sarcolemmal Na^+/Ca^{2+} exchange (NCX; Hove-Madsen et al., 2000). During AP stimulation, the charge density of I_{Ca} was reduced at warm temperatures (Fig. 3B), probably due to the shorter AP duration. This will have contributed to the reduction in the transient change in $[Ca^{2+}]_i$ observed during stimulation at 21°C. However, certainly during the SQ pulse stimulus protocol, and also during the AP stimulus protocol, the 'mismatch' between temperature-induced changes in SL Ca^{2+} influx and $[Ca^{2+}]_i$ suggests that temperature must modulate another Ca^{2+} flux pathway in trout atrial myocytes.

Effects of increasing SQ pulse stimulation frequency on $[Ca^{2+}]_i$

Increasing SQ pulse stimulation frequency (from $SQ_{0.2Hz}$ to $SQ_{physiol}$) caused an approximately 30% increase in $[Ca^{2+}]_i$ and an approximately 75% increase in the diastolic Ca^{2+} level (Figs 2, 4; Tables 1, 3), indicating that more Ca^{2+} enters the cytosol than exits *via* SR uptake or SL efflux. The high-frequency application of long SQ pulses (500 ms) increased Ca^{2+} influx *via* reverse-mode NCX, as evidenced by the increased outward current at the end of the stimulation pulse (see Fig. 4B, $SQ_{1.4Hz}$). In avian (Lee and Clusin, 1987) and mammalian cardiomyocytes (Frampton et al., 1991), the frequency-dependent increase in $[Ca^{2+}]_i$ has been attributed, in part, to increased SR Ca^{2+} load and thus an increased SR Ca^{2+} release. Increased Ca^{2+} influx *via* NCX may have resulted in greater SR Ca^{2+} loading and thus greater Ca^{2+}-induced Ca^{2+} release, contributing further to the increase in the amplitude of the transient change in $[Ca^{2+}]_i$ in the present study. An increase in the frequency of field stimulation from 0.6 Hz to 1.0 Hz at 14°C increased diastolic Ca^{2+} (approximately 25–50%) without changing the amplitude of $[Ca^{2+}]_i$ in trout ventricular myocytes (Harwood et al., 2000). In the same study, the authors found that application of caffeine revealed a greater SR Ca^{2+} content at 1.0 Hz compared with at 0.6 Hz, indicating that SR Ca^{2+} content increased with pacing frequency but was not releasable

during a field-stimulated twitch (Harwood et al., 2000). The differences between studies may indicate that a SQ pulse is a better trigger for SR Ca^{2+} release than is field stimulation, where the membrane is depolarized with an AP (see below). Indeed, Hove-Madsen et al. (2001) have demonstrated a similar degree of CICR at 7°C and 21°C with SQ pulses in trout atrial myocytes.

Effects of action potential stimulation on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ and diastolic Ca^{2+} levels decreased dramatically when the SQ pulse was changed to an AP pulse at the same frequency. This reflects a reduction in SL Ca^{2+} influx and also possibly a reduction in CICR with AP pulses, as the smaller amplitude of I_{Ca} during AP pulses (see Figs 2, 3) may be a less effective trigger for CICR.

The myocytes were better able to manage diastolic Ca^{2+} levels at physiological frequencies with AP pulses (Table 3) but not as effectively as at 0.2 Hz. This suggests that the Ca^{2+} efflux mechanisms are not able to remove all of the Ca^{2+} effectively during diastole at physiological pacing frequencies. However, it is possible that resting diastolic Ca^{2+} concentration in the absence of stimulation and at 0.2 Hz is sub-physiological. The true 'diastolic Ca^{2+} concentration' for fish hearts remains unknown and is worthy of future investigation. In any case, visual observations indicate that the cells were relaxing between stimulation pulses at physiological frequencies with AP pulses at all temperatures.

It has been estimated from trout ventricular myocytes at room temperature that, when operating maximally (i.e. at or near V_{max}), the trout SR Ca^{2+}-ATPase should be able to remove the total intracellular Ca^{2+} transient between depolarizations at physiological heart rates (Hove-Madsen et al., 1998). However, our study reveals that, at room temperature, the trout intracellular Ca^{2+} transient is small compared with cooler temperatures and suggests that the SR Ca^{2+}-ATPase is unlikely to be operating near V_{max}. Furthermore, estimates of the maximal Ca^{2+} efflux rate of the rainbow trout NCX at room temperature (Hove-Madsen and Tort, 2001) suggest that the NCX can remove a physiological Ca^{2+} transient from the cytosol in a few hundred milliseconds. Based on those estimations, the NCX should have been able to remove all of the cytosolic Ca^{2+} during the 700 ms between depolarizations at 1.4 Hz in the present study at 21°C. The observed increase in the diastolic Ca^{2+} levels in the present study are in contrast to these estimates of Ca^{2+} efflux and may reflect exogenous buffering by Fura-2. The *in vivo* buffering capacity of the fish myoplasm and its modulation by temperature is unknown. However, because both contractility and the time course of I_{Ca} were maintained in the presence of Fura-2, it is unlikely that exogenous buffering was large relative to the measurements being made. A comparison of the time to 50% relaxation of $[Ca^{2+}]_i$ with AP pulses in our study at 14°C (approximately 250 ms) with the ratiometric results from the only other published study of $[Ca^{2+}]_i$ in fish hearts, which examined field-stimulated trout ventricular myocytes loaded with Fura-2-AM at 15°C (approximately 230 ms; Harwood et al., 2000), suggests comparable time courses of the transients. Additional studies are needed to resolve the physiological efficacy of trout Ca^{2+} efflux pathways and the effect of exogenous buffering and temperature change therein.

Effects of stimulus change on I_{Ca}

The frequency-dependent decrease in I_{Ca} at 21°C reported here agrees well with previous studies that report a 15–20% decrease in peak current amplitude over a similar frequency range at 21°C in trout myocytes (Hove-Madsen and Tort, 1998; Harwood et al., 2000). These responses are akin to the negative force-frequency response observed independent of temperature in the myocardium of a number of fish species (Driedzic and Gesser, 1985; Keen et al., 1994; Shiels and Farrell, 1997; see Shiels et al., 2002b for a recent review). The smaller amplitude of the peak current during an AP pulse compared with during a SQ pulse is qualitatively similar to what we have found previously in trout atrial myocytes (Shiels et al., 2000) and similar to what has been described in mammals (Linz and Meyer, 1998; Puglisi et al., 1999).

The shape of the trout AP, in combination with the relatively depolarized resting membrane potential (–50 mV), may have led to an overestimation of SL Ca^{2+} flux *via* the I_{Ca} window current in the present study (Fig. 2B). This is because the late phase depolarization after the initial hyperpolarization (Fig. 1) brings the membrane potential close to the window current voltage range for I_{Ca} in trout atrial myocytes (–40 mV to +30 mV, with a peak at –10 mV; see Shiels et al., 2000). Recent measurements *in vivo* suggest that the resting membrane potential in rainbow trout atrial cells is approximately –65 mV (Vornanen et al., 2002). At this membrane potential, the late phase depolarization would not enter the window voltage range and thus I_{Ca} inactivation would be maintained. The relatively depolarized membrane potential in isolated myocytes from our study results from the loss of cholinergic tonus and also reflects the lower density of inward rectifier K^+ channel current (I_{K1}) in atrial compared with ventricular cells (Vornanen et al., 2002). Future studies with isolated trout myocytes could consider including tonic levels of acetylcholine when measuring APs to account for this.

Summary

Collectively, we have characterized some of the temperature-, frequency- and waveform-dependent changes in $[Ca^{2+}]_i$ and I_{Ca} that exist in rainbow trout atrial myocytes. By calibrating the Fura-2 signal, we were able to make meaningful comparisons of $[Ca^{2+}]_i$ at physiologically relevant temperatures for rainbow trout. We have shown that increased temperature causes a decrease in $[Ca^{2+}]_i$. This is the first cellular observation of a temperature-dependent change in $[Ca^{2+}]_i$ in fish myocytes and may help to explain the well-known negative inotropic effect of warm temperatures on trout heart function. Our results suggest that temperature-dependent changes in $[Ca^{2+}]_i$ are not solely dependent on temperature-dependent changes in I_{Ca}. Therefore, temperature and

frequency modulation of SR Ca^{2+} accumulation and release may be involved, and future studies should be directed at understanding these mechanisms. Finally, we have shown that the amplitude of $[Ca^{2+}]_i$ and the level of the diastolic Ca^{2+} concentration is dependent on the shape and the rate of stimulation. At all temperatures, cells were better able to maintain diastolic Ca^{2+} levels at physiological frequencies with AP pulses compared with 500 ms SQ pulses. This suggests that temperature-dependent modulation of the shape of the AP is important for cellular Ca^{2+} regulation during temperature and frequency changes in rainbow trout heart.

We acknowledge Kontiolahti fish farm for donating the trout and Ari Ryökkynen for his efforts with the Fura-2 calibrations. We also thank Dr Kerry Delaney for his technical advice and Dr Leif Hove-Madsen for his constructive comments on earlier versions of this manuscript. The study was supported by NSERC to A.P.F., Simon Fraser University Steele Memorial Fellowship to H.A.S., and the Academy of Finland (project #63090) to M.V.

References

Aho, E. and Vornanen, M. (1999). Contractile properties of atrial and ventricular myocardium of the heart of rainbow trout (*Oncorhynchus mykiss*): Effects of thermal acclimation. *J. Exp. Biol.* **202**, 2663-2677.

Aho, E. and Vornanen, M. (2001). Cold acclimation increases basal heart rate but decreases its thermal tolerance in rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B* **171**, 173-179.

Bassani, J. W., Bassani, R. A. and Bers, D. M. (1994). Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *J. Physiol.* **476**, 279-293.

Bers, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during post-rest recovery. *Am. J. Physiol.* **248**, H366-H381.

Bers, D. M. (1987). Ryanodine and the calcium content of cardiac SR assessed by caffeine and rapid cooling contractures. *Am. J. Physiol.* **253**, C408-C415.

Bers, D. M. (1989). SR Ca loading in cardiac muscle preparations based on rapid-cooling contractures. *Am. J. Physiol.* **256**, C109-C120.

Bers, D. M., Patton, C. W. and Nuccitelli, R. (1994). A practical guide to the preparation of Ca^{2+} buffers. *Methods Cell Biol.* **40**, 3-29.

Black, E. C. (1953). Upper lethal temperatures of some British Columbian fresh water fish species. *J. Fish. Res. Bd. Canada* **10**, 196-210.

Brett, J. R. (1971). Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and fresh water ecology of sockeye salmon. *Am. Zool.* **11**, 99-113.

Cavalie, A., McDonald, T. F., Pelzer, D. and Trautwein, W. (1985). Temperature-induced transitory and steady-state changes in the calcium current of guinea pig ventricular myocytes. *Pflugers Arch.* **405**, 294-296.

Churcott, C. S., Moyes, C. D., Bressler, B. H., Baldwin, K. M. and Tibbits, G. F. (1994). Temperature and pH effects on Ca^{2+} sensitivity of cardiac myofibrils: a comparison of trout with mammals. *Am. J. Physiol.* **267**, R62-R70.

Coyne, M. D., Kim, C. S., Cameron, J. S. and Gwathmey, J. K. (2000). Effects of temperature and calcium availability on ventricular myocardium from rainbow trout. *Am. J. Physiol.* **278**, R1535-R1544.

Driedzic, W. R. and Gesser, H. (1985). Ca^{2+} protection from the negative inotropic effect of contraction frequency on teleost hearts. *J. Comp. Physiol. B* **156**, 135-142.

Driedzic, W. R. and Gesser, H. (1994). Energy-metabolism and contractility in ectothermic vertebrate hearts: hypoxia; acidosis; and low-temperature. *Physiol. Rev.* **74**, 221-258.

Fabiato, A. and Fabiato, F. (1978). Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit, rat, and frog hearts and from fetal and new-born rat ventricles. *Ann. N. Y. Acad. Sci.* **307**, 491-522.

Farrell, A. P. (1997). Effects of temperature on cardiovascular performance. In *Global Warming Implications for Freshwater and Marine Fish*. (ed. C. M. Wood and D. G. McDonald), pp. 135-158. Cambridge, UK: Cambridge University Press.

Farrell, A. P., Gamperl, A. K., Hicks, J. M. T., Shiels, H. A. and Jain, K. E. (1996). Maximum cardiac performance of rainbow trout (*Oncorhynchus mykiss*) at temperatures approaching their upper lethal limit. *J. Exp. Biol.* **199**, 663-672.

Farrell, A. P. and Jones, D. R. (1992). The Heart. In *Fish Physiology, The Cardiovascular System* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 1-88. London: Academic Press.

Frampton, J. E., Orchard, C. H. and Boyett, M. R. (1991). Diastolic, systolic and sarcoplasmic reticulum $[Ca^{2+}]$ during inotropic interventions in isolated rat myocytes. *J. Physiol.* **437**, 351-375.

Gillis, T. E., Marshall, C. R., Xue, X. H., Borgford, T. J. and Tibbits, G. F. (2000). Ca^{2+} binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. *Am. J. Physiol.* **279**, R1707-R1715.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.

Harrison, S. M. and Bers, D. M. (1987). The effect of temperature and ionic strength on the apparent Ca- affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochim. Biophys. Acta* **925**, 133-143.

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

Harwood, C. L., Howarth, F. C., Altringham, J. D. and White, E. (2000). Rate-dependent changes in cell shortening; intracellular Ca^{2+} levels and membrane potential in single isolated rainbow trout (*Oncorhynchus mykiss*) ventricular myocytes. *J. Exp. Biol.* **203**, 493-504.

Herve, J. C., Yamaoka, K., Twist, V. W., Powell, T., Ellory, J. C. and Wang, L. C. (1992). Temperature dependence of electrophysiological properties of guinea pig and ground squirrel myocytes. *Am. J. Physiol.* **263**, 177-184.

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

Hove-Madsen, L., Llach, A. and Tort, L. (1998). Quantification of Ca^{2+} uptake in the sarcoplasmic reticulum of trout ventricular myocytes. *Am. J. Physiol.* **44**, R2070-R2080.

Hove-Madsen, L., Llach, A. and Tort, L. (2000). Na/Ca-exchange activity regulates contraction and SR Ca(2+) content in rainbow trout atrial myocytes. *Am. J. Physiol.* **279**, 1856-1864.

Hove-Madsen, L., Llach, A. and Tort, L. (2001). The function of the sarcoplasmic reticulum is not inhibited by low temperatures in trout atrial myocytes. *Am. J. Physiol.* **281**, R1902-R1906.

Hove-Madsen, L. and Tort, L. (1998). L-type Ca^{2+} current and excitation–contraction coupling in single atrial myocytes from rainbow trout. *Am. J. Physiol.* **275**, 2061-2069.

Hove-Madsen, L. and Tort, L. (2001). Characterization of the relationship between Na–Ca exchange rate and cytosolic calcium in trout cardiac myocytes. *Pflugers Arch.* **441**, 701-708.

Keen, J. E., Vianzon, D. M., Farrell, A. P. and Tibbits, G. F. (1994). Effect of temperature and temperature-acclimation on the ryanodine sensitivity of the trout myocardium. *J. Comp. Physiol. B* **164**, 438-443.

Kim, C. S., Coyne, M. D. and Gwathmey, J. K. (2000). Voltage-dependent calcium channels in ventricular cells of rainbow trout: effect of temperature changes in vitro. *Am. J. Physiol.* **278**, R1524-R1534.

Larsson, D., Larsson, B., Lundgren, T. and Sundell, K. (1999). The effect of pH and temperature on the dissociation constant for fura- 2 and their effects on [Ca(2+)](i) in enterocytes from a poikilothermic animal, Atlantic cod (*Gadus morhua*). *Anal. Biochem.* **273**, 60-65.

Lee, H. C. and Clusin, W. T. (1987). Cytosolic calcium staircase in cultured myocardial cells. *Circ. Res.* **61**, 934-939.

Linz, K. W. and Meyer, R. (1998). Control of L-type calcium current during the action potential of guinea- pig ventricular myocytes. *J. Physiol.* **513**, 425-442.

Liu, B., Belke, D. D. and Wang, L. C. H. (1997). Ca^{2+} uptake by cardiac sarcoplasmic reticulum at low temperatures in rat and ground squirrel. *Am. J. Physiol.* **41**, R1121-R1127.

Liu, B., Wang, L. C. H. and Belke, D. D. (1991). Effect of low temperature on the cytosolic free Ca^{2+} in rat ventricular myocytes. *Cell Calcium* **12**, 11-18.

Matthews, K. R. and Berg, N. H. (1997). Rainbow trout responses to water temperature and dissolved oxygen stress in two southern California streampools. *J. Fish Biol.* **50**, 50-67.

Milligan, C. L., Graham, M. S. and Farrell, A. P. (1989). The response of trout red cells to adrenaline during seasonal acclimation and changes in temperature. *J. Fish Biol.* **35**, 229-236.

Møller-Nielsen, T. and Gesser, H. (1992). Sarcoplasmic reticulum and excitation–contraction coupling at 20°C and 10°C in rainbow trout myocardium. *J. Comp. Physiol. B* **162**, 526-534.

Puglisi, J. L., Yuan, W. L., Bassani, J. W. M. and Bers, D. M. (1999). Ca^{2+} influx through Ca^{2+} channels in rabbit ventricular myocytes during action potential clamp: influence of temperature. *Circ. Res.* **85**, E7-E16.

Reid, S. D., McDonald, D. G. and Wood, C. M. (1997). Interactive effects of temperature and pollutant stress. In *Global Warming: Implications for Freshwater and Marine Fish* (ed. C. M. Wood and D. G. McDonald), pp. 235-249. Cambridge, UK: Cambridge University Press.

Shiels, H. A. and Farrell, A. P. (1997). 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. *J. Exp. Biol.* **200**, 1607-1621.

Shiels, H. A., Vornanen, M. and Farrell, A. P. (2000). Temperature-dependence of L-type Ca^{2+} channel current in atrial myocytes from rainbow trout. *J. Exp. Biol.* **203**, 2771-2780.

Shiels, H. A., Vornanen, M. and Farrell, A. P. (2002a). Temperature dependence of cardiac sarcoplasmic reticulum function in rainbow trout myocytes. *J. Exp. Biol.* **205**, 3631-3639.

Shiels, H. A., Vornanen, M. and Farrell, A. (2002b). The force-frequency relationship in fish hearts: a review. *Comp. Biochem. Physiol. A* **132**, 811-826.

Sitsapesan, R., Montgomery, R. A. P., Macleod, K. T. and Williams, A. J. (1991). Sheep cardiac sarcoplasmic-reticulum calcium-release channels: modification of conductance and gating by temperature. *J. Physiol.* **434**, 469-488.

Tanaka, H. and Shigenobu, K. (1989). Effect of ryanodine on neonatal and adult rat heart: developmental increase in sarcoplasmic reticulum function. *J. Mol. Cell Cardiol.* **21**, 1305-1313.

Tibbits, G. F., Moyes, C. D. and Hove-Madsen, L. (1992). Excitation–contraction coupling in the teleost heart. In *Fish Physiology, The Cardiovascular System* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 267-304. London: Academic Press.

Tierney, K. (2000). The repeated swimming performance of sockeye, coho and rainbow trout in varying conditions. *MSc Thesis*, Simon Fraser University, Burnaby, Canada.

Tiitu, V. and Vornanen, M. (2002). Regulation of cardiac contractility in a stenothermal fish, the burbot (*Lota lota*). *J. Exp. Biol.* **205**, 1597-1606.

Tuurala, H., Soivio, A. and Nikinmaa, M. (1982). The effects of adrenaline on heart rate and blood pressure in *Salmo Gairdneri* at two temperatures. *Ann. Zool. Fennici* **19**, 47-51.

Vornanen, M. (1998). L-type Ca^{2+} current in fish cardiac myocytes: effects of thermal acclimation and beta-adrenergic stimulation. *J. Exp. Biol.* **201**, 533-547.

Vornanen, M., Ryökkynen, A. and Nurmi, A. (2002). Temperature-dependent expression of sarcolemmal K^+ currents in rainbow trout atrial and ventricular myocytes. *Am. J. Physiol.* **282**, R1191-R1199.

Wang, S., Zhou, Z. and Qian, H. (2000). Recording of calcium transient and analysis of calcium removal mechanisms in cardiac myocytes from rats and ground squirrels. *Science In China (series C)* **43**, 191-199.

Xue, X. H., Hryshko, L. V., Nicoll, D. A., Philipson, K. D. and Tibbits, G. F. (1999). Cloning, expression, and characterization of the trout cardiac Na^+/Ca^{2+} exchanger. *Am. J. Physiol.* **277**, C693-C700.

Yue, D. T. (1987). Intracellular $[Ca^{2+}]$ related to rate of force development in twitch contraction of heart. *Am. J. Physiol.* **252**, H760-H770.