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

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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+}]; 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+}] 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+}] and I_{Ca} were examined with square (SQ) pulses at slow (0.2 Hz) and physiologically relevant contraction frequencies. The amplitude of [Ca^{2+}] 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+}] 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+}] 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+}]; 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+}] 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+}] and I_{Ca} were examined with square (SQ) pulses at slow (0.2 Hz) and physiologically relevant contraction frequencies. The amplitude of [Ca^{2+}] 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.

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 (Cavalié 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 et al., 2000). 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$\text{Ca}$ 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$\text{Ca}$, 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$^{2+}$], *in vivo*. Thus, we examined [Ca$^{2+}$]$_i$ and I$\text{Ca}$ 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$\text{Ca}$ 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 tritutated 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$_2$PO$_4$, 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$\text{Ca}$ contained: NaCl, 150 mmol l$^{-1}$; CsCl, 5.4 mmol l$^{-1}$; MgSO$_4$, 1.2 mmol l$^{-1}$; NaH$_2$PO$_4$, 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 mmol 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$_2$GTP, 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±0.3 MΩ when filled with pipette solution. Junction potentials were zeroed prior to seal formation. Pipette capacitance (9.3±2.0 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
calculated to give an index of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was calculated using these $d$ values and the above measurements of $R_{\text{max}}$, $R_{\text{min}}$, cells were then

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$^{-1}$).

**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 $R_{\text{max}}$ and $R_{\text{min}}$, 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 $R_{\text{max}}$, 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 $R_{\text{min}}$, cells were then perfused with a solution containing 10 mmol l$^{-1}$ EGTA. The values obtained are as follows: $R_{\text{max}}$=9.80±0.67, $R_{\text{min}}$=0.99±0.01, $\beta$ (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 $R_{\text{max}}$, $R_{\text{min}}$ and $\beta$, 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 Ca²⁺ content was similar in all cells (Shiels et al., 2002a). However, the subsequent stimulation protocols may alter SR Ca²⁺ 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 (SQ₀.2Hz), and I_Ca and [Ca²⁺]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 (SQphysiol). 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 SQphysiol 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 (APphysiol). I_Ca and [Ca²⁺]ᵢ were simultaneously recorded throughout (see Fig. 4). Although the SQphysiol 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 (SQphysiol) and a physiological AP (APphysiol) allows us to evaluate the importance of the AP and the possible

Fig. 2. The effect of temperature on intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) and L-type Ca²⁺ channel current (I_Ca) in trout atrial myocytes. (A) The effect of temperature on [Ca²⁺]ᵢ during SQ₀.2Hz, SQphysiol and APphysiol (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 SQphysiol reflects the increase in diastolic Ca²⁺ 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 SQ₀.2Hz were leakage corrected using the P/N procedure of the software (Clampex, Axon Instruments). Leakage correction was not employed at SQphysiol or APphysiol. Mean data and statistical analysis is given in Tables 1–3 and in Fig. 3. SQ₀.2Hz, square pulses at a frequency of 0.2 Hz; SQphysiol, square pulses at physiological frequency; APphysiol, 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, SQphysiol and APphysiol; 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).

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.](image-url)
profoundly altered cellular Ca\(^{2+}\) dynamics. As a detailed example, Fig. 4 illustrates the frequency dependence and the influence of pulse shape on \(I_{\text{Ca}}\) and \([\text{Ca}^{2+}]_i\) 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±0.11 μmol l\(^{-1}\) s\(^{-1}\) to 1.16±0.84 μmol 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±0.17 μmol 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 (SQphysiol) led to a significant increase in [Ca\(^{2+}\)] \(_i\) and diastolic Ca\(^{2+}\) levels.

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

| Table 2. Time to peak of [Ca\(^{2+}\)] (ms) in trout atrial myocytes |
|---|---|---|
|          | SQ\(_{0.2}\)Hz | SQphysiol | APphysiol |
| 7°C       | 601±15\(^{a,e}\) | 483±44\(^{a,f}\) | 453±30\(^{a,f}\) |
| 14°C      | 540±36\(^{a,b,e}\) | 425±57\(^{a,b,f}\) | 394±29\(^{a,f}\) |
| 21°C      | 502±68\(^{a,b,e}\) | 291±46\(^{a,b,f}\) | 252±24\(^{a,b,f}\) |

The effect of temperature and the frequency/stimulus waveform on diastolic [Ca\(^{2+}\)]. 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 \([\text{Ca}^{2+}]_i\) and in diastolic \(\text{Ca}^{2+}\) levels with 500 ms SQ stimulation pulses. By contrast, the AP stimulus, which is much shorter in duration, reduced \([\text{Ca}^{2+}]_i\) and diastolic \(\text{Ca}^{2+}\) levels compared with SQ pulses at the same frequency.

The amplitude of \(I_{\text{Ca}}\) 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 \(I_{\text{Ca}}\) 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 \(\text{Ca}^{2+}\) influx during the AP plateau and because of \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]_i\) increases with decreasing temperature, (2) the transient change in \([\text{Ca}^{2+}]_i\) is smaller in response to physiological AP stimulation than to 500 ms SQ stimulus pulses, and (3) diastolic \(\text{Ca}^{2+}\) 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 \(I_{\text{Ca}}\) alone cannot account for the temperature-dependent changes in \([\text{Ca}^{2+}]_i\). Thus, although we made no direct measurements of SR \(\text{Ca}^{2+}\) flux, our results suggest temperature may modulate SR \(\text{Ca}^{2+}\) cycling in trout atrial myocytes.

**Effects of temperature on \([\text{Ca}^{2+}]_i\) and \(I_{\text{Ca}}\)**

With both SQ and AP pulses, \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) with decreases in temperature (Wang et al., 2000). The small amplitude of \([\text{Ca}^{2+}]_i\) and the elevated diastolic \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]_i\) 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 \(I_{\text{Ca}}\) with decreasing temperature, and our present study supports these earlier findings. The similar \(\text{Ca}^{2+}\) influx via \(I_{\text{Ca}}\) at 7°C, 14°C and 21°C probably resulted from a combination of higher and faster peak \(I_{\text{Ca}}\) offset by more rapid \(I_{\text{Ca}}\) inactivation as temperatures increased (see Fig. 2 and Shiels et al., 2000). A similar lack of effect of temperature on the charge density of \(I_{\text{Ca}}\) 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 \(\text{Ca}^{2+}\) influx via reverse-mode sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchange (NCX; Hove-Madsen et al., 2000). During AP stimulation, the charge density of \(I_{\text{Ca}}\) 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 \([\text{Ca}^{2+}]_i\) 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 \(\text{Ca}^{2+}\) influx and \([\text{Ca}^{2+}]_i\) suggests that temperature must modulate another \(\text{Ca}^{2+}\) flux pathway in trout atrial myocytes.

**Effects of increasing SQ pulse stimulation frequency on \([\text{Ca}^{2+}]_i\)**

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

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

The myocytes were better able to manage diastolic \(\text{Ca}^{2+}\) levels at physiological frequencies with AP pulses (Table 3) but not as effectively as at 0.2 Hz. This suggests that the \(\text{Ca}^{2+}\) efflux mechanisms are not able to remove all of the \(\text{Ca}^{2+}\) effectively during diastole at physiological pacing frequencies. However, it is possible that resting diastolic \(\text{Ca}^{2+}\) concentration in the absence of stimulation and at 0.2 Hz is subphysiological. The true ‘diastolic \(\text{Ca}^{2+}\) 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 \(V_{\text{max}}\)), the trout SR \(\text{Ca}^{2+}\)-ATPase should be able to remove the total intracellular \(\text{Ca}^{2+}\) transient between depolarizations at physiological heart rates (Hove-Madsen et al., 1998). However, our study reveals that, at room temperature, the trout intracellular \(\text{Ca}^{2+}\) transient is small compared with cooler temperatures and suggests that the SR \(\text{Ca}^{2+}\)-ATPase is unlikely to be operating near \(V_{\text{max}}\). Furthermore, estimates of the maximal \(\text{Ca}^{2+}\) efflux rate of the rainbow trout NCX at room temperature (Hove-Madsen and Tort, 2001) suggest that the NCX can remove a physiological \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) during the 700 ms between depolarizations at 1.4 Hz in the present study at 21°C. The observed increase in the diastolic \(\text{Ca}^{2+}\) levels in the present study are in contrast to these estimates of \(\text{Ca}^{2+}\) 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 \(I_{\text{Ca}}\) 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 \([\text{Ca}^{2+}]_i\) with AP pulses in our study at 14°C (approximately 250 ms) with the ratiometric results from the only other published study of \([\text{Ca}^{2+}]_i\) 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 \(\text{Ca}^{2+}\) efflux pathways and the effect of exogenous buffering and temperature change therein.

Effects of stimulus change on \(I_{\text{Ca}}\)

The frequency-dependent decrease in \(I_{\text{Ca}}\) 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 \(\text{Ca}^{2+}\) flux via the \(I_{\text{Ca}}\) 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 voltage range for \(I_{\text{Ca}}\) 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 \(I_{\text{Ca}}\) 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 (\(I_{\text{K1}}\)) 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 \([\text{Ca}^{2+}]_i\) and \(I_{\text{Ca}}\) that exist in rainbow trout atrial myocytes. By calibrating the Fura-2 signal, we were able to make meaningful comparisons of \([\text{Ca}^{2+}]_i\) at physiologically relevant temperatures for rainbow trout. We have shown that increased temperature causes a decrease in \([\text{Ca}^{2+}]_i\). This is the first cellular observation of a temperature-dependent change in \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) are not solely dependent on temperature-dependent changes in \(I_{\text{Ca}}\). 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 Kerrys 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


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