TEMPERATURE-DEPENDENCE OF L-TYPE Ca\textsuperscript{2+} CHANNEL CURRENT IN ATRIAL MYOCYTES FROM RAINBOW TROUT

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Summary

Rainbow trout, \textit{Oncorhynchus mykiss}, inhabit eurythermal environments and must therefore be able to cope with changes in environmental temperature. As ectotherms, their heart is required to maintain cardiac function over a range of ambient water temperatures. This raises important questions concerning the temperature-dependence of cardiac ion channel function in fish hearts, in particular, the channels involved in Ca\textsuperscript{2+} transport. Thus, we studied the effects of acute, physiologically relevant temperature changes on the density and kinetics of the L-type Ca\textsuperscript{2+} channel current (I\textsubscript{Ca}) in rainbow trout atrial myocytes using the whole-cell patch-clamp technique. Myocytes from fish acclimated to 14 °C were first tested at 14 °C, then at 21 °C and finally at 7 °C. Using a square-pulse voltage-clamp in the first series of experiments, the peak density of I\textsubscript{Ca} increased (Q\textsubscript{10}=1.9) as temperature was increased from 14 to 21 °C and decreased (Q\textsubscript{10}=2.1) as temperature was decreased from 14 to 7 °C. In contrast to current density, the charge carried by I\textsubscript{Ca} was inversely related to temperature as a result of changes in the kinetic properties of the channel; both the fast (\tau\textsubscript{f}) and slow (\tau\textsubscript{s}) components of inactivation were slower at 7 °C than at 14 and 21 °C. Action potentials were recorded at the three test temperatures and then used as voltage-clamp stimulus waveforms to reassess I\textsubscript{Ca} in a second series of experiments. While the temperature-dependency of I\textsubscript{Ca} was similar to that found with the square-pulse voltage-clamp, the charge carried by I\textsubscript{Ca} was temperature-independent. These results show that the temperature-dependency of I\textsubscript{Ca} in rainbow trout is in the lower range of that reported in mammals and, although this could have profound effects on Ca\textsuperscript{2+} delivery to the myofilaments, the temperature-induced modifications in the action potential may help to maintain a fairly constant Ca\textsuperscript{2+} delivery during an acute temperature change in rainbow trout.

Key words: rainbow trout, \textit{Oncorhynchus mykiss}, electrophysiology, I\textsubscript{Ca}, temperature, L-type Ca\textsuperscript{2+} channel, Ca\textsuperscript{2+} cycling, sarcoplasmic reticulum.

Introduction

Many fish inhabit eurythermal environments and must therefore be able to cope with changes in environmental temperature. With seasonal temperature changes, fish can acclimatize, often adjusting their physiological and biochemical processes in a compensatory manner. However, temperature changes can be more rapid, either as a result of diurnal cycles (Matthews and Berg, 1997) or as the fish moves vertically through the water column, often in association with feeding or escaping predation (Reid et al., 1997). Acute temperature changes are of considerable interest for cardiac physiologists because the heart is required to maintain cardiac function while it reaches a new temperature equilibrium with the surrounding environment. The temperature tolerance of eurythermal fish, therefore, raises important questions concerning the temperature-dependency of cardiac ion channel function, particularly because acute temperature changes can be cardioplegic to mammals. We were specifically interested in the ion channels involved in Ca\textsuperscript{2+} transport during excitation–contraction coupling of the heart.

In most fish, including rainbow trout, transsarcolemmal Ca\textsuperscript{2+} influx is considered to be the primary source of Ca\textsuperscript{2+} for activation of the myofilaments during excitation–contraction coupling (Vornanen, 1989; Tibbits et al., 1990; Keen et al., 1994; Shiel and Farrell, 1997). The majority of this Ca\textsuperscript{2+} enters the myocyte through the L-type Ca\textsuperscript{2+} channel (Vornanen, 1997, 1998; Hove-Madsen and Tort, 1998), although the contribution of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger can also be quite significant (contributing up to 50 % of the activator Ca\textsuperscript{2+}; Vornanen, 1999). Mammalian studies indicate that the L-type Ca\textsuperscript{2+} channel is temperature-dependent, with Q\textsubscript{10} values ranging from 1.8 (Herve et al., 1992; guinea pig and ground squirrel) to 2.9 (Cavalié et al., 1985; guinea pig). If the temperature-dependency of the L-type Ca\textsuperscript{2+} channel is similar in fish, then there must be other mechanisms in place that allow...
fish to maintain cardiac viability during acute temperature change. To date, however, the temperature-dependence of the L-type Ca\(^{2+}\) channel has not been investigated in fish cardiac myocytes. Thus, the aim of this study was to characterize the temperature-dependence of the L-type Ca\(^{2+}\) channel in trout atrial myocytes during physiologically relevant acute temperature changes. The temperature-dependence of the L-type Ca\(^{2+}\) channel was studied at 14 °C, the thermal preference for rainbow trout (Coutant, 1977), and after a temperature change of ±7 °C, encompassing the range of temperatures trout experience in nature (Kaya, 1978; DeVera and Priede, 1991; Farrell et al., 1996). The results indicate that the temperature-sensitivity of ICa for trout atrial myocytes is within the range found in most mammals, exhibiting a Q10 of approximately 2. However, when cells are stimulated with an action potential waveform appropriate for the test temperature, the total charge carried by ICa is temperature-independent, suggesting a possible compensatory mechanism for coping with acute temperature change.

**Materials and methods**

_Fish origin and care_

Rainbow trout *Oncorhynchus mykiss* (Walbaum) (175.5±13.6 g, mean ± s.e.m., N=6) of both sexes were obtained from a local fish farm (Kontiolahti, Finland) and held in the laboratory in a 5001 tank receiving aerated tap water. The tank temperature was 14±1 °C. Fish were held for a minimum of 4 weeks prior to experimentation and were fed daily on commercial trout pellets (Ewos, Turku, Finland). The photoperiod was 12 h:12 h light:dark.

_Isolated myocyte preparation_

A detailed description of the cell preparation has been published previously (Vornanen, 1998, 1999). Briefly, fish were stunned with a blow to the head, the spine was then cut and the heart was carefully excised. All procedures were in accordance with local animal handling protocols. A cannula was inserted through the bulbus arteriosus into the lumen of the ventricle, and the heart was perfused from a height of 50 cm with the physiological saline used as the perfusate to block fast Na\(^{+}\) channels and 10 \(\mu\)mol l\(^{-1}\) tetrodotoxin (TTX) was added to the solution to record ICa. In addition, 1 \(\mu\)mol l\(^{-1}\) ryanodine (Hove-Madsen et al., 1999) was added to inhibit the sarcoplasmic reticulum Ca\(^{2+}\) release channel. These concentrations of TTX (Vornanen, 1998) and ryanodine (Hove-Madsen et al., 1999) have been shown to inhibit completely INa and sarcoplasmic reticulum function in rainbow trout myocytes, respectively. To record action potentials from the cells, the solution was modified by replacing the CsCl with equimolar KCl, pH balanced with KOH, and by omitting the TTX and ryanodine. The pipette solution for ICa recordings contained (mmol l\(^{-1}\)): CsCl, 130; MgATP, 5; tetrabutylammonium chloride (TEA\(^{+}\)), 15; MgCl\(_2\), 1; oxaloacetate, 5; Heps, 10; EGTA, 5; disodium GTP, 0.03, pH adjusted to 7.2 with CsOH. For action potential recordings, the pipette solution contained (mmol l\(^{-1}\)): potassium aspartate, 125; KCl, 15; MgCl\(_2\), 1; MgATP, 5; EGTA, 0.05; disodium phosphocreatine, 5; Heps, 10; and was adjusted to pH 7.2 with KOH. All drugs were purchased from Sigma (MO, USA) with the exception of ryanodine (Calbiochem, CA, USA) and TTX (Tocris, Bristol, UK).

**Experimental procedure**

The whole-cell patch-clamp technique was used to study the effects of temperature on the electrophysiological properties of the L-type Ca\(^{2+}\) channel in rainbow trout atrial myocytes. In the first series of experiments, square pulses were used to determine the effects of acute temperature change on the current–voltage relationship and the kinetics of activation and inactivation of ICa.

A sample of atrial cells (cell capacitance 38.9±1.88 pF, mean ± s.e.m., N=28) was added to the recording chamber (volume 0.5 ml) and allowed to settle on the bottom. The cells were then superfused at a rate of 2 ml min\(^{-1}\) with the physiological extracellular solution. 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 were pulled from borosilicate glass (Vitrex microhaematocrit tubing, Modulohm A/S, Denmark) using a vertical patch-pipette puller (List-Medical, Darmstadt, Germany) and had a resistance of 1.5–2.5 MΩ when filled with pipette solution. Junction potentials were zeroed prior to seal formation. Pipette capacitance (6–8 pF) was compensated after formation of a gigaohm seal. The membrane under the pipette was ruptured with a brief voltage pulse (zap), and capacitative transients were eliminated by iteratively adjusting the series resistance and cell capacitance compensation circuits. Mean series resistance was 7.2±0.7 MΩ. Currents were leakage-corrected using the P/N procedure of the software (Clampex, Axon Instruments) and were low-pass-filtered using the four-pole low-pass Bessel filter on the Axopatch-1D amplifier at a frequency of 2 kHz for ICa and 10 kHz for action potentials, and then analyzed off-line on a computer using pClamp 6.0 software (Axon Instruments). Membrane capacitance was corrected using the P/N procedure of the software (Clampex, Axon Instruments).
measured using the calibrated capacity compensation circuit of the Axopatch amplifier.

Each cell was tested at three temperatures (7 °C, 14 °C and 21 °C). Experimental temperature was maintained (±0.5 °C) by adjusting the temperature of the perfusion solution. Three water baths were set to the three experimental temperatures. The inflow tube carrying the perfusion solution was heated or chilled by the water bath circuit before emptying into the recording chamber. A thermocouple was placed inside the recording chamber and positioned no less than 5 mm from the cell to determine when the desired temperature had been achieved. Each experiment began at 14 °C, the acclimation temperature of the fish. The current–voltage relationship and the steady-state activation and inactivation curves for I_{Ca} were obtained using a double-pulse protocol. Every 6 s, a 1000 ms prepulse depolarized the membrane from a holding potential of –70 mV to +70 mV in 10 mV steps. Each voltage step was followed by a test pulse to +10 mV for 500 ms. The prepulse and test pulse were separated by a 5 ms return to the holding potential. After the protocol had been completed at 14 °C, the perfusate temperature was changed to 21 °C. It took approximately 1 min for the chamber to stabilize at the new temperature. At this point, the double-pulse protocol was repeated. To assess for possible run-down and any side effects of the temperature change, the temperature was returned to 14 °C, and the protocol was repeated. The temperature was then changed to 7 °C, and the protocol was repeated. A fifth and final protocol was performed on the same cell after the temperature had been returned to 14 °C. The duration of each experiment was approximately 11 min. The successive 14 °C trials are referred to as 14 °C’ for the second trial and 14 °C” for the third trial at this temperature. The order of the temperature change was reversed in some experiments (i.e. 14 °C to 7 °C to 14 °C’ to 21 °C to 14 °C”), but this did not influence the effect of temperature on I_{Ca}.

The effects of temperature on the kinetics of inactivation of I_{Ca} were assessed by fitting the decay of I_{Ca} with a second-order exponential function using the Chebyshev transformation procedure from the Clampfit software (Axon Instruments). From this, the time constants for the fast (τ_{f}) and slow (τ_{s}) components of inactivation were derived.

The effects of temperature on steady-state activation and inactivation were studied by fitting the curves with Boltzmann functions. The methods for calculating the voltage-dependence of steady-state activation and inactivation have been reported elsewhere (Vornanen, 1998). Briefly, steady-state inactivation was calculated by dividing the amplitude of the test current by the maximal current elicited. The voltage-dependence of activation was determined as the normalized Ca^{2+} conductance (g_{Ca}/g_{max}), where g_{max} is the maximum value for I_{Ca} conductance. The voltage-dependence of the peak conductance of Ca^{2+} channels was calculated from the equation: g_{Ca}=I_{Ca}/(V–V_{rev}), where g_{Ca} is the membrane Ca^{2+} conductance, I_{Ca} is the peak Ca^{2+} current at the given potential (V) and V_{rev} is the apparent reversal potential obtained from the current–voltage relationship, or by extrapolating the ascending portion of the current–voltage relationship to zero current. Steady-state kinetic parameters were obtained by fitting the data to Boltzmann equations to determine the half-activating or half-inactivating potential (V_{0}) and the slope of activation and inactivation (k) (for details, see Vornanen, 1998).

The contribution of I_{Ca} to total cellular [Ca^{2+}] was calculated from the transferred charges and cell volume. Charge transfer through L-type Ca^{2+} channels was determined by integrating the inactivating portion of the Ca^{2+} current obtained from the

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**Fig. 1.** Current–voltage relationship for trout atrial myocytes at 21, 14 and 7 °C. Three individual trials at 14 °C (shown in the inset) were averaged to form the 14 °C line on the main figure. * indicates a significant difference between 14 and 21 °C. ‡ indicates a significant difference between 14 and 7 °C. All values are means ± s.e.m. The values for I_{Ca} (pA) are normalized from the measured cell capacitance to give the value in pA pF\(^{-1}\), N=13 for 21 and 7 °C, and N=39 for 14 °C; N=13 for each 14 °C trial shown in the inset. Significance (P<0.05) was determined before averaging the 14 °C trials using a repeated-measures ANOVA.
prepulse depolarization to +10 mV. Cell volume was calculated from the measured cell capacitance and the surface-to-volume ratio of the cell (see Vornanen, 1998). The myocytes were considered to be flat elliptical cylinders with an axis ratio of 1:2 for the elliptical cross section (Vornanen, 1997, 1998). The change in total cellular [Ca\textsuperscript{2+}] due to Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels was expressed as a function of myofibrillar volume (40%; Vornanen, 1998).

**Action potential clamp**

To provide a more physiologically relevant indication of how temperature affects I\textsubscript{Ca}, a second series of experiments was conducted. Action potentials from rainbow trout atrial cells were elicited by a short (2 ms) 1 V pulse at 7, 14 or 21 °C. Representative action potential recordings for each temperature were then used as stimulus waveforms. The shape of the action potential varied significantly with temperature, so each myocyte was tested with the three action potential waveforms and with a square pulse waveform as in the first series of experiments. The protocol was as follows. Starting at 14 °C, the cell was voltage-clamped first with the 14 °C action potential waveform, second with the 21 °C action potential waveform, third with the 7 °C action potential waveform, and finally with a 500 ms square pulse waveform from a holding potential of −70 to +10 mV. Each of these waveforms was applied three times before the next waveform was applied. The average of the three recordings for each individual waveform was used to give N=1 for each waveform for that cell. The bath temperature was then changed to 21 °C, and the four stimulation waveforms were repeated. As with the first series of experiments, the stimulation protocol was then repeated at 14 °C, 7 °C and finally at 14 °C*.

**Statistical analyses**

In general, comparisons among temperatures were made using one-way repeated-measures analysis of variance (ANOVA) with SigmaStat 1.0 software (Jandel Scientific). In some cases, the data did not fit a normal distribution, and a Friedman repeated-measures ANOVA on ranks was then conducted. Differences were considered significant when P<0.05 and were assessed using a Student–Newman–Keuls *post-hoc* analysis. Details of statistical procedures for each data set are found in the corresponding figure legend. The three tests at 14 °C (14 °C, 14 °C* and 14 °C†) were tested as individual groups to ensure that there were no statistical differences between them before the values were pooled to make an average 14 °C data point. For this reason, the N value for the 14 °C data is three times that for the 7 °C and 21 °C groups.

**Results**

**Effects of temperature on current–voltage relationships for a square wave pulse**

The average peak I\textsubscript{Ca} at 21 °C ranged from $-5.43\pm0.78\ \text{pA}\text{pF}^{-1}$ in the first series of experiments (see Table 1) to $-1.77\pm0.21\ \text{pA}\text{pF}^{-1}$ in the second series of experiments (see Table 3). This range is within that reported previously for peak current amplitude in rainbow trout atrial (Hove-Madsen and Tort, 1998) and ventricular (Vornanen, 1998) myocytes at room temperature (19–21 °C) using slightly different experimental conditions. Similarly, the charge carried by I\textsubscript{Ca} at 21 °C in our experiments ($-0.129\pm0.02$ to $-0.075\pm0.03\ \text{pC}\text{pF}^{-1}$) was within the range found previously for trout myocytes (Hove-Madsen and Tort, 1998; Vornanen, 1998).

The effects of temperature on the current–voltage relationship for I\textsubscript{Ca} in trout atrial myocytes are shown in Fig. 1. I\textsubscript{Ca} amplitude was positively correlated with temperature (Fig. 1; Table 1). When temperature was increased from 14 to 21 °C, peak current density increased from $-3.45\pm0.34$ to $-5.43\pm0.78\ \text{pA}\text{pF}^{-1}$, which corresponds to a $Q_{10}$ of 1.9. Similarly, peak current density decreased from $-3.45\pm0.34$ to $-2.05\pm0.43\ \text{pA}\text{pF}^{-1}$ on cooling from 14 to 7 °C, yielding a $Q_{10}$ of 2.1. The shape of the current–voltage relationship was unchanged by temperature. There was no significant rundown of I\textsubscript{Ca} over the course of the experiment, as shown by the inset of the three 14 °C trials in Fig. 1.

In contrast to peak current amplitude, the charge carried by I\textsubscript{Ca} (at peak current; +10 mV) was negatively correlated with temperature (Table 1). As temperature was decreased from 21 °C through 14 °C to 7 °C, charge density increased from $-0.129\pm0.02$ to $-0.194\pm0.017$ and $-0.213\pm0.04\ \text{pC}\text{pF}^{-1}$. Similarly, the change in total cellular [Ca\textsuperscript{2+}] increased from 30.5±4.8 μmol l\textsuperscript{-1} at 21 °C to 46.1±3.9 μmol l\textsuperscript{-1} at 14 °C and to

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>N</th>
<th>I\textsubscript{Ca} (pA)</th>
<th>I\textsubscript{Ca} density (pA pF\textsuperscript{-1})</th>
<th>Q\textsubscript{Ca} (pC)</th>
<th>Charge density (pC pF\textsuperscript{-1})</th>
<th>Δ[Ca\textsuperscript{2+}]\textsubscript{tot} (μmol l\textsuperscript{-1})</th>
</tr>
</thead>
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<tr>
<td>21</td>
<td>13</td>
<td>$-205.6\pm24.7$</td>
<td>$-5.43\pm0.78$</td>
<td>$-4.88\pm0.67$</td>
<td>$-0.129\pm0.02$</td>
<td>30.5±4.8</td>
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<tr>
<td>14</td>
<td>39</td>
<td>$-127.7\pm11.8$</td>
<td>$-3.45\pm0.34$</td>
<td>$-7.46\pm0.61$</td>
<td>$-0.194\pm0.02$</td>
<td>46.1±3.9</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>$-72.1\pm14.9$</td>
<td>$-2.05\pm0.43$</td>
<td>$-8.09\pm1.42$</td>
<td>$-0.213\pm0.04$</td>
<td>50.6±9.7</td>
</tr>
</tbody>
</table>

Δ[Ca\textsuperscript{2+}]\textsubscript{tot}, the change in total cellular [Ca\textsuperscript{2+}] expressed as a function of myofilament space (see Vornanen, 1998). Dissimilar letters indicate significant differences between test temperatures.

All values are means ± S.E.M.
50.6±9.7 μmol l⁻¹ at 7°C. Thus, although the peak current amplitude decreased with temperature, the amount of Ca²⁺ entering the atrial myocyte increased. These results are explained by a temperature-induced change in the kinetics of ICa.

Effects of temperature on the kinetics of ICa

Fig. 2 shows the effect of temperature on the kinetics of ICa. The time constants for the fast (τf) and slow (τs) components of inactivation were obtained by fitting a double exponential function to the decay of ICa. Over a range of membrane potentials (−10, 0, +10 mV), both τf and τs increased as temperature decreased (Fig. 2). These longer time constants explain why the charge carried by ICa increased while the amplitude decreased. At peak current (+10 mV), τf increased from 22.9±2.1 ms at 21°C to 39.6±3.1 ms at 14°C and further to 60.9±7.3 at 7°C, a 2.7-fold increase in the time required for fast inactivation. The effect of temperature on the time constant for the slow inactivation was a 3.4-fold increase. At peak current, τs increased from 161.9±18.6 at 21°C and 213.9±17.2 at 14°C to 543.9±98.3 at 7°C. The effect of temperature on τf and τs was greater between 14 and 7°C than between 14 and 21°C.

Effects of temperature on steady-state activation and inactivation

The temperature-induced changes in the kinetics of ICa revealed by the fast and slow components of inactivation are reinforced by the Boltzmann fits for steady-state activation and inactivation (Fig. 3; Table 2). It was possible to superimpose the steady-state activation curves at 21 and 14°C (Fig. 3). However, at 7°C, the activation curve was significantly shifted such that half-maximal activation (V1/2,act) occurred at more depolarized voltages (see Fig. 3; Table 2), probably indicating slower channel activation at the low temperature. This is supported by the more gradual slope of the Boltzmann fit for activation (kact) at 7°C. There was no difference in Vh for inactivation between temperatures; however, the slope of inactivation was also greater at 7°C (Table 2), suggesting that the L-type Ca²⁺ channel makes the transition from the activated to the inactivated state more gradually at the colder temperature. In addition, there was a clear difference in the steady-state inactivation curves at positive membrane potentials. Between +30 and +70 mV, the attenuation of inactivation of ICa was significantly greater at 7°C than at 21°C (Fig. 3), which can probably be explained by a reduction in Ca²⁺-dependent inactivation at cold temperatures, where the density of ICa is significantly reduced. Furthermore, because the activation and inactivation curves overlap, there is a small non-inactivating component of ICa, known as a window current, which peaks at approximately −10 mV (see Fig. 3 inset). The size of the window current is greatest at 7°C, which again is probably due to the lower current density found at cold temperatures in trout atrial myocytes.

Effects of temperature on the action potential

The shape of the action potential recorded from trout atrial myocytes was significantly altered by temperature (see

<table>
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<tr>
<th>Temperature (°C)</th>
<th>Vh (mV)</th>
<th>k (mV)</th>
<th>Vh (mV)</th>
<th>k (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
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<td>6.63±0.5</td>
</tr>
<tr>
<td>7</td>
<td>−2.09±2.2*</td>
<td>9.89±1.0*</td>
<td>−23.28±1.7</td>
<td>10.14±1.5*</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.05) from the value at 14 and 21°C (one-way repeated-measures ANOVA). All values are means ± s.e.m. (N=13).
Methods for fitting data with Boltzmann functions are described in the text. The inset shows the relative L-type Ca\(^{2+}\) channel inactivation and window current calculated as the product of the activation and inactivation curves at each temperature. * indicates significant differences (\(P<0.05\)) between the 7 and 21 °C treatments. At membrane potentials of +30 and +40 mV, the value at 7 °C is also significantly different from the 14 °C values. At membrane potentials of +50, +60 and +70 mV, the 21 °C values are significantly different from both the 7 and 14 °C values.

Discussion for an explanation). Representative action potential recordings at each temperature from one cell were selected and converted into a series of stimulus waveforms for subsequent activation of the L-type Ca\(^{2+}\) channel (Fig. 4, inset). The action potential plateau duration at 50 % repolarization from peak increased from 70 ms at 21 °C to 134 ms at 14 °C and to 288 ms at 7 °C. The size of the hyperpolarizing phase decreased (from resting potential) with decreasing temperature from ~24 mV at 21 °C, to ~19 mV at 14 °C and to ~11 mV at 7 °C. In addition, the resting potential became progressively depolarized as temperatures were increased from ~55 mV at 7 °C, to ~41 mV at 14 °C and to ~38 mV at 21 °C. Given such profound changes in the action potential, it was necessary to examine \(I_{\text{Ca}}\) with action potential waveforms for different temperatures and test whether these temperature-induced modifications of the action potential influenced \(I_{\text{Ca}}\).

**Effects of temperature on action potential-clamp**

This series of experiments demonstrated that \(I_{\text{Ca}}\) is sensitive to the shape of the stimulus waveform. At 7 and 14 °C, but not at 21 °C, peak current density elicited from the square-pulse stimulus waveform was greater than that elicited by the action potential waveform (Table 3; compare values vertically). This suggests that the typical square pulse (usually to 0 or +10 mV) used to study \(I_{\text{Ca}}\) may overestimate current amplitude because the driving force for Ca\(^{2+}\) entry is greater at these voltages than with the more depolarized action potential overshoot (+20 to +40 mV). Even so, and in accordance with the first series of experiments, the peak amplitude of \(I_{\text{Ca}}\) elicited from the action potential waveforms was significantly temperature-dependent, with a Q\(_{10}\) of approximately 2 (Fig. 4; Table 3). Furthermore, there was a trend towards increasing charge transfer with decreasing temperature using the square-pulse waveforms (compare charge densities at the three test temperatures with the square pulse in Table 3) but, unlike in the first series of experiments, statistical significance could not be established. In contrast, a physiologically relevant action potential clamp did not result in an increase in charge transfer with decreasing temperature (Table 3).

Table 3 summarizes the effects of the three action potential waveforms at the three test temperature on the peak density of \(I_{\text{Ca}}\) and the charge carried by \(I_{\text{Ca}}\). While there were significant differences among these values, the most physiologically important comparison is for the three action potential waveforms corresponding to the comparable test temperature. The three \(I_{\text{Ca}}\) curves are compared in Fig. 4. While peak current density was temperature-dependent, the charges carried by these currents were not significantly different (Fig. 4; Table 3). As a result, provided that the cell is stimulated with an
action potential waveform appropriate for the test temperature, the cell maintains a relatively constant Ca$^{2+}$ influx via the L-type Ca$^{2+}$ channel over the temperature range 7–21 °C. This finding has important physiological implications when trying to understand how trout maintain cardiac function during acute temperature change.

**Discussion**

The hearts of fish continue to function at temperatures that are cold enough to be cardioplegic to most mammals. Possible explanations for this temperature tolerance include (i) changes in myocyte metabolic rate, although this form of adaptation usually occurs with seasonal acclimatization; (ii) changes in protein–protein interactions during excitation–contraction coupling; (iii) changes in Ca$^{2+}$ uptake and release from the sarcoplasmic reticulum and (iv) changes in sarcolemmal ion channel function. Because contractility is dependent on both the rate and magnitude of Ca$^{2+}$ delivery to the myofilaments, and because Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channel is vital both for activating the myofilaments directly and for triggering the possible release of Ca$^{2+}$ from the sarcoplasmic reticulum, we studied the effects of temperature on the electrophysiological properties of the L-type Ca$^{2+}$ channel in rainbow trout atrial myocytes.

Given their ability to maintain cardiac function at low temperatures and during acute temperature changes above and below their acclimatization temperature, we had anticipated that the temperature-sensitivity of the trout L-type Ca$^{2+}$ channel would be less than that reported for mammals. Indeed, the Q$10$ of approximately 2 is similar to the lowest of Q$10$ values reported for mammals (1.8, Herve et al., 1992; guinea pig and ground squirrel ventricle; 2.9, Cavalié et al., 1985; guinea pig ventricle; 2.3, Kiyosue et al., 1993, guinea pig ventricle; 2.7, Shimon and Banno, 1993, rabbit ventricle; 5.8, Allen and Mikala, 1998, human L-type Ca$^{2+}$ channel expressed in *Xenopus laevis* oocytes conducting Ba$^{2+}$; 1.9, Rosen, 1996, GH3 cells).

Although the temperature-dependence of peak current through the L-type Ca$^{2+}$ channel is not as great as that found in most mammals, this alone can hardly account for the temperature tolerance observed in fish in nature. Thus, other mechanisms regulating contractility must help to confer the relative temperature tolerance observed in rainbow trout. Here, we have established that the shape of the action potential is critical in determining Ca$^{2+}$ delivery across the sarcolemma. In fact, provided that we used an appropriate action potential waveform for a given test temperature, Ca$^{2+}$ delivery was apparently independent of temperature. Therefore, this observation helps explain the normal temperature tolerance observed in rainbow trout hearts.

In addition to the sarcolemma, the other primary organelle involved in handling Ca$^{2+}$ during excitation–contraction coupling is the sarcoplasmic reticulum. On the basis of studies with multicellular preparations, it has been proposed that the Ca$^{2+}$ contribution made by the sarcoplasmic reticulum is temperature-dependent in rainbow trout, contributing more significantly at warmer temperatures (Hove-Madsen, 1992; Möller-Nielsen and Gesser, 1992; Shiels and Farrell, 1997). Furthermore, using cardiac homogenates or suspensions of ventricular myocytes from rainbow trout, it has been demonstrated that Ca$^{2+}$ uptake by the sarcoplasmic reticulum is also temperature-sensitive, with faster uptake rates at higher temperatures (Aho and Vorman, 1998; Hove-Madsen and Tort, 1998). It is expected, therefore, that the Ca$^{2+}$ contribution of the sarcoplasmic reticulum may be significantly altered by
acute temperature change. Indeed, if sarcoplasmic reticulum Ca\(^{2+}\) release changes with temperature, it may also significantly affect the Ca\(^{2+}\)-dependent inactivation of the L-type Ca\(^{2+}\) channel (Hove-Madsen et al., 1999; Harwood et al., 2000). However, to prevent this confounding effect of possible feedback from the sarcoplasmic reticulum, we included 10\(\mu\)mol\(^{-1}\) ryanodine in all experiments when examining the properties of the trout L-type Ca\(^{2+}\) channel. Thus, for the purpose of this paper, we must examine other possible mechanisms that may affect Ca\(^{2+}\) delivery to the myofilaments under conditions of changing ambient temperature.

The results of this study suggest that the kinetics of I\(_{\text{Ca}}\) plays an important role in maintaining adequate Ca\(^{2+}\) influx during temperature change in rainbow trout. The slow component of inactivation (time constant \(t_s\)) was especially sensitive, demonstrating a 3.4-fold increase in the duration of inactivation with a 14°C decrease in temperature. This powerful temperature-dependence of the inactivation time constants is a novel finding. Our slow time constants for an acute temperature change to 21°C (161.9±18.9 ms) are longer than those reported in a previous study performed only at room temperature using ventricular myocytes from rainbow trout (101 ms for fish acclimated to 18°C and 73 ms for fish acclimated to 4°C; Vornanen, 1998). However, our values for fast inactivation (22.9±2.1 ms) are within the range reported for trout ventricular myocytes (27 ms for fish acclimated to 18°C and 18 ms for fish acclimated to 4°C; Vornanen, 1998) and for atrial myocytes (approximately 13 ms; Hove-Madsen et al., 1999) at room temperature.

Recent studies with caffeine blockade of sarcoplasmic reticulum Ca\(^{2+}\) release in rainbow trout (Hove-Madsen et al., 1999, atrial cells at room temperature; Harwood et al., 2000, ventricular cells at 15°C) indicate that \(t_\text{r}\) may be related to the sarcoplasmic reticulum Ca\(^{2+}\)-dependent component of inactivation of the L-type Ca\(^{2+}\) channel in rainbow trout. We observed slower inactivation of I\(_{\text{Ca}}\) at low temperatures despite the absence of sarcoplasmic reticulum Ca\(^{2+}\)-dependent inactivation (due to ryanodine). This observation could be partly related to Ca\(^{2+}\)-dependent inactivation from Ca\(^{2+}\) entering through the L-type Ca\(^{2+}\) channel itself, a mechanism already established for mammalian myocytes (McDonald et al., 1994). This idea is supported by the observation that the recovery from steady-state inactivation was greatly facilitated between +30 and +70 mV at cold temperature (see Fig. 3) when the density of I\(_{\text{Ca}}\) was small and inactivation of I\(_{\text{Ca}}\) was slow. Because the density of I\(_{\text{Ca}}\) at 7°C is greatly reduced, Ca\(^{2+}\)-dependent inactivation can also be expected to be reduced.

Another mechanism might involve greater intracellular Ca\(^{2+}\) buffering at cold temperatures, which could further decrease the degree of Ca\(^{2+}\)-dependent inactivation of I\(_{\text{Ca}}\). The myofilaments are one of the largest Ca\(^{2+}\) sinks, and the Ca\(^{2+}\) affinity of trout cardiac troponin C changes significantly with a change in temperature from 21 to 7°C (Gillis et al., 2000). Furthermore, we observed a more prominent window current at cold temperatures (see inset in Fig. 3), a result often described under conditions where Ca\(^{2+}\)-dependent inactivation is reduced (i.e. rabbit versus rat, neonate versus adult, Bers, 1991). Indeed, the potential physiological significance of a larger window current at colder temperatures can be appreciated when one considers the role of the window current during an action potential. At the onset of the action potential, depolarization initiates opening of L-type Ca\(^{2+}\) channels. The subsequent repolarization will facilitate channel inactivation. However, during the action potential plateau (which usually corresponds to voltages in the window current range; McDonald et al., 1994), some of the inactivated channels recover and re-open (Hirano et al., 1992), providing the non-inactivating current.

The recovery from inactivation is kinetically slow, so lengthening the time that the action plateau remains in the window current voltage range increases the probability of Ca\(^{2+}\) channels reopening within the window (Hirano et al., 1992). Indeed, when the duration of the action potential ‘plateau’ was held constant (i.e. with the square pulse protocols in the first series of experiments), we observed both an increase in recovery from inactivation at positive potentials and an increase in the window current at 7°C compared with 14 and 21°C. This may have a profound effect on transsarcolemmal Ca\(^{2+}\) influx at cold temperatures (see Perspectives) and may help to account for the larger charge transfer observed at low temperature in our study. Furthermore, when we recorded action potentials from myocytes at different temperatures in the second series of experiments, we observed a significant increase in the action potential plateau as temperature decreased. Thus, it is possible that, in addition to temperature-induced changes in K\(^{+}\) currents (see below), there are temperature-induced changes in the I\(_{\text{Ca}}\) window current which, along with the slower decay of I\(_{\text{Ca}}\), may also contribute to the lengthening of the action potential plateau at lower temperatures.

Recent studies on the temperature-dependency of the two primary K\(^{+}\) currents involved in shaping the action potential (I\(_{\text{Kr}}\)) and maintaining the resting potential (I\(_{\text{K1}}\)) shed more light on the temperature-induced changes in the action potential we observed in trout atrial myocytes. First, the density of the inwardly rectifying K\(^{+}\) current (I\(_{\text{Kr}}\)) was very low in trout atrial myocytes, resulting in a depolarized and relatively unstable resting membrane potential (A. Nurmi and M. Vornanen, personal communication). Indeed, the resting membrane potential in our study was significantly more depolarized than that found in most mammalian cells and in trout ventricular cells (Harwood et al., 2000). In contrast to the inward rectifier, the density of the rapid component of the delayed rectifier (I\(_{\text{K1}}\)) was fairly high in atrial cells (A. Nurmi and M. Vornanen, personal communication) and contributed not only to the plateau and hyperpolarizing phases of the action potential but also to the maintenance of the diastolic resting membrane potential because specific blockade of this channel resulted in almost complete loss of resting membrane potential. In addition, A. Nurmi and M. Vornanen (personal communication) observed a higher density and faster kinetics
of $I_{Kr}$ at warmer temperatures, which may explain the faster repolarization and transient hyperpolarization of the trout atrial action potentials recorded at 21 °C in our study. In short, when the temperature-induced changes in the $K^+$ currents are combined with our results concerning the temperature sensitivity of the L-type Ca$^{2+}$ channel, the changes in the shape of the action potential appear to culminate in the maintenance of electrical excitability in trout cardiac muscle over a range of temperatures. Indeed, when the cells were stimulated with an appropriate action potential waveform for the applied test temperature, the charge carried by $I_{Ca}$ did not differ significantly among temperatures. Thus, factors that change the shape of the action potential appear to have a large impact on $I_{Ca}$ because they may help to compensate for the temperature-dependence of peak $I_{Ca}$.

**Perspectives**

Previous studies (Hove-Madsen, 1992; Keen et al., 1994; Shiels and Farrell, 1997) have suggested a degree of interplay between Ca$^{2+}$ flux across the sarcolemma and Ca$^{2+}$ flux through the sarcoplasmic reticulum at different temperatures in rainbow trout. The results of the present study may help to explain some of the potential mechanisms underlying this interplay between the sarcoplasmic reticulum and sarcolemma. Studies with isolated ventricular muscle preparations from rainbow trout have revealed a greater ryanodine sensitivity at warm temperatures. The effect of ryanodine is often abolished at temperatures below 15 °C (El-Sayed and Gesser, 1989; Hove-Madsen, 1992; Möller-Nielsen and Gesser, 1992; Keen et al., 1994; Gesser, 1996; Shiels and Farrell, 1997). Studies with isolated atrial tissue have suggested that the ryanodine sensitivity may be less warm-temperature-dependent than in ventricular tissue (trout, Gesser, 1996; yellowfin tuna, Shiels et al., 1999). If the initial magnitude of $I_{Ca}$ is the trigger for sarcoplasmic reticulum Ca$^{2+}$ release, as suggested by Fabiato (1983, 1985), then at warm temperatures where the density of $I_{Ca}$ in trout cells is high, it is quite feasible that, under physiological conditions, a sizeable Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) could occur. Indeed, a recent study at room temperature with trout atrial myocytes has provided evidence for CICR and has even shown that it may contribute to Ca$^{2+}$-dependent inactivation of $I_{Ca}$ (Hove-Madsen et al., 1999). The pumping capabilities of the sarcoplasmic reticulum Ca$^{2+}$-ATPase are also significantly greater at warm temperatures (Aho and Vornanen, 1998; Hove-Madsen et al., 1998), so sarcoplasmic reticulum Ca$^{2+}$-loading could be faster. Taken together, the faster and larger $I_{Ca}$ and the increased sarcoplasmic reticulum Ca$^{2+}$-ATPase activity may enable the sarcoplasmic reticulum to contribute Ca$^{2+}$ on a beat-to-beat basis at warm temperatures.

At cold temperatures, when the initial magnitude of $I_{Ca}$ is small, it may be impossible to trigger sarcoplasmic reticulum Ca$^{2+}$ release under physiological conditions. However, because of (i) the slower decay of $I_{Ca}$ and (ii) the increased recovery from inactivation during window current voltages at low temperatures, there may be sufficient Ca$^{2+}$ influx across the sarcolemma to meet the demands of the myofilaments. Correspondingly, we report an increase in the total Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channel at low temperatures. This increased Ca$^{2+}$ influx at colder temperatures could result in increased sarcoplasmic reticulum loading. Moreover, if the slow component of inactivation ($\tau_s$) is the sarcoplasmic reticulum loading component of $I_{Ca}$, as suggested by Fabiato (1983, 1985), then the drastically slower $\tau_s$ observed at 7 °C in the present study would greatly facilitate sarcoplasmic reticulum loading. This contention is supported by the recent discovery that, unlike the sarcoplasmic reticulum of mammals, the sarcoplasmic reticulum of rainbow trout does not leak Ca$^{2+}$ at low temperature (Hove-Madsen et al., 1998). Taken together, these observations may help to explain why rainbow trout appear to have a large sarcoplasmic reticulum Ca$^{2+}$ load at low temperatures, but generally do not seem to release it under physiological conditions (for an exception in trout atrium acclimated to 4 °C, see Aho and Vornanen, 1999). Clearly, a complete study of the effects of temperature on sarcoplasmic reticulum loading and release in trout myocardium is needed to elucidate these relationships properly.

In summary, $I_{Ca}$ plays many roles in maintaining contractility in rainbow trout myocardium. First, it provides a transsarcolemmal Ca$^{2+}$ influx that can directly activate the myofilaments. Second, it can function as the trigger for the release of Ca$^{2+}$ from the intracellular stores of the sarcoplasmic reticulum, and lastly it can serve to increase sarcoplasmic reticulum Ca$^{2+}$ loading. All these potential roles are modulated by temperature. The results of this study indicate that the temperature-sensitivity ($Q_{10}$) of $I_{Ca}$ for trout atrial myocytes is within the lower range of those found for most mammals. This could compromise Ca$^{2+}$ cycling at the myofilaments and affect cardiac performance during an acute temperature change. However, we also discovered that, when the cells were stimulated with an appropriate action potential waveform for the applied test temperature, the charge carried by $I_{Ca}$ did not differ significantly among temperatures. This suggests that the temperature-induced modifications in the action potential, and the resultant changes in the density and kinetics of $I_{Ca}$, may help to ensure adequate Ca$^{2+}$ cycling for the maintenance of cardiac performance during acute temperature change in rainbow trout.

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**References**


