Voltage-dependent calcium channels in ventricular cells of rainbow trout: effect of temperature changes in vitro

Catherine S. Kim, Mary D. Coyne, and Judith K. Gwathmey. Voltage-dependent calcium channels in ventricular cells of rainbow trout: effect of temperature changes in vitro. Am J Physiol Regulatory Integrative Comp Physiol 278: R1524–R1534, 2000.—Voltage-dependent calcium channels (VDCC) in ventricular myocytes from rainbow trout (Oncorhynchus mykiss) were investigated in vitro using the perforated patch-clamp technique, which maintains the integrity of the intracellular milieu. First, we characterized the current using barium as the charge carrier and established the doses of various pharmacological agents to use these agents in additional studies. Second, we examined the current at several physiological temperatures to determine temperature dependency. The calcium currents at 10°C (acclimation temperature) were identified as L-type calcium currents based on their kinetic behavior and response to various calcium channel agonists and antagonists. Myocytes were chilled (4°C) and warmed (18 and 22°C), and the response of VDCC to varying temperatures was observed. There was no significant dependency of the current amplitude and kinetics on temperature. Amplitude decreased 25–36% at 4°C (Q10 1.23–1.89) and increased 18% at 18°C (Q10 1.23–1.23) in control, Bay K8644 (Bay K), and forskolin-enhanced currents. The inactivation rates (τi) did not demonstrate a temperature sensitivity for the VDCC (Q10 1.23–1.92); Bay K treatment, however, increased temperature sensitivity of τi, between 10 and 18°C (Q10 3.98). The low Q10 values for VDCC are consistent with a minimal temperature sensitivity of trout myocytes between 4 and 22°C. This low-temperature dependency may provide an important role for sarcolemmal calcium channels in adaptation to varying environmental temperatures in trout.Heart failure and diabetes mellitus 278: R1524–R1534, 2000.—Voltage-dependent calcium channels (VDCC) in ventricular myocytes from rainbow trout (Oncorhynchus mykiss) were investigated in vitro using the perforated patch-clamp technique, which maintains the integrity of the intracellular milieu. First, we characterized the current using barium as the charge carrier and established the doses of various pharmacological agents to use these agents in additional studies. Second, we examined the current at several physiological temperatures to determine temperature dependency. The calcium currents at 10°C (acclimation temperature) were identified as L-type calcium currents based on their kinetic behavior and response to various calcium channel agonists and antagonists. Myocytes were chilled (4°C) and warmed (18 and 22°C), and the response of VDCC to varying temperatures was observed. There was no significant dependency of the current amplitude and kinetics on temperature. Amplitude decreased 25–36% at 4°C (Q10 1.23–1.89) and increased 18% at 18°C (Q10 1.23–1.23) in control, Bay K8644 (Bay K), and forskolin-enhanced currents. The inactivation rates (τi) did not demonstrate a temperature sensitivity for the VDCC (Q10 1.23–1.92); Bay K treatment, however, increased temperature sensitivity of τi, between 10 and 18°C (Q10 3.98). The low Q10 values for VDCC are consistent with a minimal temperature sensitivity of trout myocytes between 4 and 22°C. This low-temperature dependency may provide an important role for sarcolemmal calcium channels in adaptation to varying environmental temperatures in trout.

EXCITATION-CONTRACTION COUPLING (E-C coupling) in cardiac muscle is activated by an increase in intracellular calcium concentration ([Ca2+]i), which in turn is dependent on two sources: 1) internal calcium mobilization from the sarcoplasmic reticulum (SR) and 2) external calcium influx across the sarcolemma through voltage-dependent calcium channels (VDCC). Intracellular calcium is lowered by reuptake into the SR and extrusion through the sarcolemma via the Na+/Ca2+ exchanger. The magnitude of the increase in [Ca2+]i depends on the contribution from each compartment, which varies with species and temperature (11, 16). For example, fast transient increases in intracellular calcium are seen in mammalian and avian species, suggesting that immediate release of calcium from the SR is the primary source of [Ca2+]i (16). In contrast, in amphibians such as the salamander, the calcium transient has a more prolonged profile, suggesting the additional contribution of [Ca2+]i from another source, most likely diffusion across the sarcolemma from the extracellular compartment (16). At the other end of the spectrum, myocytes from frog ventricles have been shown to rely solely on a transsarcolemmal influx of calcium (11).

To date studies in teleost cardiac myocytes have shown that E-C coupling in trout is a ryanodine-insensitive mechanism at physiological heart rates and temperatures (10°C), implying an important role for transsarcolemmal calcium movement. However, at higher temperatures, the reverse is true, i.e., blockage of the SR by ryanodine has a significant effect on cardiac muscle function (19). This suggests that, depending on the temperature, the E-C coupling mechanism in trout hearts can vary its dependency on calcium from the extracellular compartment or from the intracellular SR. Therefore, one might predict that the VDCC in teleost hearts would be particularly sensitive to temperature changes.

In these studies we identified the L-type calcium currents as the predominant calcium current in trout myocytes and characterized their responsivity to various agents. This information will be used in further physiological studies on trout myocardial contractility. In addition, in contrast to hearts of other species, trout myocardium shows little temperature dependence when subjected to warmer or cooler experimental temperatures in vitro.

METHODS

Cell isolation. Rainbow trout (Oncorhynchus mykiss) 8–10 inches in length were obtained from the Mohawk Trout Hatchery (Sunderland, MA). The fish were raised outdoors in open tanks maintained at 10°C by a continuous flow of fresh well water and were maintained indoors at 10°C in an aquarium containing aerated, dechlorinated water (pH 7.0, Q2 7.5 parts/million) for 3–14 days until euthanasia.

The trout were anesthetized with 3-aminobenzoic acid ethyl ester placed in an aliquot of aquarium water (0.2 g/l, pH 7.4). After excision of the heart and removal of excess tissue and blood clots, the hearts were placed in oxygenated 0.2 mM calcium buffer (CaB) at 5°C. The heart was quickly mounted in the primary source of [Ca2+]i (16). In contrast, in amphibians such as the salamander, the calcium transient has a more prolonged profile, suggesting the additional contribution of [Ca2+]i from another source, most likely diffusion across the sarcolemma from the extracellular compartment (16). At the other end of the spectrum, myocytes from frog ventricles have been shown to rely solely on a transsarcolemmal influx of calcium (11).

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to a perfusion apparatus by inserting a cannula with an olive-shaped tip (27 gauge) through the bulbus arteriosus into the ventricle and tying a ligature between the atria and the ventricle.

The cell isolation procedure was modified from that of Mitra and Morad (29) and Kattunen and Tirri (20). Solutions for isolation were as follows: standard buffer (SB) contained (in mM) 137 NaCl, 4.6 KCl, 1.2 MgCl₂, 3.5 NaH₂PO₄·H₂O, 11 glucose, and 10 HEPES, pH to 7.4 with 1 M NaOH. CaB contained SB with 0.2 mM CaCl₂. Enzyme buffer was composed of 50 mM CaB, 0.033 g collagenase (type 1), and 0.025 g BSA (fraction V). The heart was perfused for 20 min with calcium-free SB and then with enzyme buffer containing collagenase (type 1, 300 U/ml) and 0.2 mM Ca²⁺ for 20 min. After digestion, the heart was perfused with 0.2 mM CaB for 10 min to wash out the enzyme solution. All solutions were oxygenated at room temperature. The heart was then placed into CaB, atria and bulbus arteriosus were excised, and the remaining tissue was teased apart into small pieces and triturated with fresh CaB. The cells in CaB were oxygenated and stored at 2–8°C until use.

Chemicals. The following stock solutions were kept frozen in aliquots and thawed and diluted just before use: Bay K8644 1, 10, and 100 µM; Bay K; 1,4-dihydropyridine-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]pyridine-3-carboxylic acid methyl ester in DMSO, a gift of Dr. A. Scriabine (Miles Laboratories; New Haven, CT); nifedipine 100 µM in DMSO; 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP) 0.058 M. The following stock solutions were refrigerated (2–4°C) until used: FPL-64176 100 µM [FPL; 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxlic acid methyl ester] in DMSO; a gift of Dr. C. M. Strickland (Research Biochemicals International; Natick, MA) and 1 mM nicardipine. A stock solution of isoproterenol (HCl) (1 mM) was kept in a dark bottle at room temperature and was diluted before use. Unless indicated otherwise, chemicals were obtained from Sigma (St. Louis, MO) and were of the highest analytic grade.

Electrophysiology. Currents were recorded using the perforated-patch clamp version of the original patch-clamp technique (32). Myocytes were plated on 35-mm tissue culture dishes pretreated with 10% poly-L-lysine solution. The composition of the extracellular (bath) solution and the pipette solution was designed to minimize currents through Na⁺ and K⁺ channels. Tetrodotoxin (TTX, 1–2 µM) was applied to the bath to block Na⁺ channels. The predominant use of barium as the charge carrier and Cs⁺ in the pipette solution blocked K⁺ currents. Barium or calcium was used as the charge carrier in the bath solution, which contained (in mM) 138 NaCl, 5 BaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES with 1 M NaOH or 115.5 NaCl, 20 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES. The pH was set to 7.3 with 1 M NaOH at room temperature and varied from 7.2 to 7.4 over the experimental temperature range (4–22°C). These pH ranges reflect those for catfish (7.2) (29) myocardium and myocytes. The pipette solution contained (in mM) 123 Cs-glutamate, 20 tetraethylammonium chloride, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, pH to 7.3 with 1 M CsOH. All solutions were passed through a 0.2-µm filter before use. Amphotericin B for the perforated patch was made fresh daily (30 mg/ml DMSO). The final concentration was 240 µg/ml in the pipette solution.

For perforated patch recording, the tip of the pipette was filled with amphotericin B-free pipette solution and was backfilled with the amphotericin B solution. Electrode resistance varied from 2–5 MΩ. The liquid junction potential was adjusted before seal formation, and capacitive transients were compensated after seal formation but not after perforation. Once an electrical continuity was established, the currents were stable for up to 2 h. Data were filtered at 5 kHz using an Axopatch 200A patch-clamp amplifier (Axon Instruments; Foster City, CA), collected on a Gateway 2000 4DX2–50 computer, and analyzed using pCLAMP software (version 5.5, Axon Instruments). Patch electrodes were made from soft glass hematocrit tubes (Drummond Scientific; Broomall, PA; 100 µl) with a double pull (David Kopf Instruments; Tujunga, CA) coated with Sylgard (Dow Corning; Midland, MI) and fire-polished on a homemade microlathe (36).

Under voltage clamp conditions, the cells (n = 57) were held at −100 or −50 mV and stepped in 10-mV increments to different potentials for 300 ms. Voltage-dependent activation was taken as the average response of cells held at −100 mV and stepped to voltages between −40 to +20 mV. For steady-state inactivation, cells were held at a given voltage for 1.5 s before stepping to 20 mV for 300 ms. Linear leakage current was determined at the different potentials and subtracted from the individual records. The temperature was set during the experiments with a heating and cooling temperature controller (TC-10, Dagan; Minneapolis, MN) with a stability of ±0.2°C. The cooling apparatus surrounding the culture dish containing the cells, which were brought to temperature over a 10-min period and then held at each temperature for at least 5 min before testing. The cells were perfused by gravity flow except during the equilibration and test periods. All drugs, including TTX, were added directly to the bath during the test period. The temperature probe was resin-coated so metal was not in contact with the bath solution.

Data analysis. Curve fitting to data was accomplished using Sigma Plot Scientific Graphing Software (Jandel Scientific; Corte Madera, CA). The half-maximum activation and inactivation of the barium currents were obtained by fitting the data to the Boltzmann equation for activation or for inactivation (15). The time constants (τ) for the inactivation of the barium currents were determined using pCLAMP software. Inactivation was fit with a single or a double exponential. An R value of 0.97 or greater determined the best fit. The value for Qₑ for the temperature analyses was determined with the equation

\[ \log Qₑ = (10/(t₂ - t₁)) \log X₁₁ - \log X₁₂ \]

where \( t₂ \) and \( t₁ \) represent the temperatures at which the parameters (X) were measured (e.g., peak current amplitude, rate of inactivation) (26).

RESULTS

All experiments were conducted at 10°C, the acclimated body temperature of the fish, unless otherwise noted. Freshly isolated rainbow trout ventricular myocytes were narrow, elongated, and striated. Two inward currents were expressed in myocytes, a low-threshold, voltage-dependent Na⁺ current as well as a high-threshold, voltage-dependent calcium current. The Na⁺ current activated between −30 and −40 mV and was blocked with 1–2 µM TTX, which did not affect the remaining inward current (data not shown). The inward high-voltage activating calcium current was present in all cells tested (n = 57) and was investigated in the presence of TTX at 10°C.

Calcium and barium were compared as charge carriers by holding the potential of the cell at −50 mV and stepping to voltages from −60 to +70 mV in 10-mV increments for 300 ms. Figure 1 shows current traces with the two different carriers recorded from two
different cells. At 10°C, the calcium current (Fig. 1A) was small even with 20 mM Ca\(^{2+}\) and inactivated rapidly (\(\tau_i = 52.10\) ms at voltage step \(V_s = 50\) mV). The peak current with 20 mM Ca\(^{2+}\) was at 50 mV. In contrast, with barium as the charge carrier (Fig. 1B), the amplitude of the inward current was larger and did not completely inactivate during the depolarizing step (\(\tau_i = 80.52\) ms at \(V_s = 20\) mV). The faster inactivation with calcium as the charge carrier could be due to calcium-dependent inactivation of L-type calcium channels. The peak current was shifted to a more hyperpolarized voltage (+20 mV) with barium. The larger current, slower inactivation, and shift of peak voltage is characteristic of an L-type calcium current seen in mammalian cells with barium as a charge carrier vs. calcium (12, 18). Because barium provided a larger current in these cells, it was used as the charge carrier in subsequent experiments.

Voltage-dependent kinetics. Figure 2A shows a family of barium currents obtained under perforated patch-clamp conditions. Cells were held at −100 mV with 10-mV steps between −10 and 40 mV. As the cell was depolarized by increasing voltage steps, the voltage-dependent current demonstrated an increased amplitude and increased rate of inactivation up to the peak voltages. As seen in Fig. 2B, activation occurred at voltages more positive than −20 mV. The mean voltage eliciting a peak current was 10 mV, although with barium it varied between 10 and 20 mV for any given cell. This may be due to the normal variability from cell to cell because the perforated patch maintains cellular integrity.

The time course of the current decay during depolarization at 10°C was best fit with a single exponential function at all voltages tested. The time constants for inactivation of the currents shown in Fig. 2A are plotted in Fig. 2C where the value decreased from 589 ms at \(V_s = 10\) mV to about 70–75 ms between \(V_s = 20\) and 40 mV. The fast inactivation at the peak voltage was not due to calcium-induced inactivation because barium was used as the charge carrier. Although the rate of decay for L-type channels is much slower in many tissues, gener-
ally moderately fast inactivation is characteristic of \(L\)-type current in ventricular myocytes (18).

Data from multiple cells for voltage-dependent activation and steady-state inactivation were normalized to the maximum current and the means ± SE were plotted as current amplitude/maximum current (\(I/I_{\text{max}}\)) vs. the holding potential (Fig. 3). Both sets of data were fit with the appropriate form of the Boltzmann equation, which is shown as a smooth line. The inward barium current activated at voltages greater than \(-20\) mV with a peak at \(10\) mV and a midpoint potential (\(V_{1/2}\)) for activation at \(-8.05\) mV, slope parameter (\(k\)) 4.37. Steady-state inactivation began around \(-30\) mV with complete inactivation at 5–10 mV; \(V_{1/2}\) for inactivation was \(-14.11\) mV, \(k\) 5.16. Our values, however, for \(V_{1/2}\) for steady-state inactivation are about 12 mV more depolarized than those values for rainbow trout under whole cell patch clamp was used compared with whole cell patch clamp conditions (9).

The steady-state inactivation curve shows that the inward current was not inactivated at \(-50\) mV. This is also illustrated in Fig. 4 where no difference was observed in the amplitude or characteristic shape of the inward current when myocytes were held at \(-100\) or at \(-50\) mV (\(V_h\) 20 mV). The consistency of this response suggests that only one type of inward calcium current is present in teleost ventricular myocytes.

Dihydropyridinesensitivity. Myocytes (–70%) tended to have very small inward currents under control conditions at 10°C compared with those seen by Vornanen (40) in trout that were studied at 22°C using the whole cell patch clamp. This response raised the question of whether calcium channels in these trout ventricular myocytes were inactive because of either the low temperature (10°C compared with 22°C) (40) or the patch-clamp conditions vs. whole cell. Bay K, a dihydropyridine (DHP) calcium channel agonist, which enhances open time of \(L\)-type channels (18), was applied to the myocytes to expose latent \(L\)-type calcium channels. As seen in a typical cell in Fig. 5A, a much larger current was expressed after application of 20 nM Bay K. The calcium channels in trout myocytes were very sensitive to Bay K, showing a response to doses as small as 5 nM. Figure 5A shows that at holding potential (\(V_h\)) \(-100\) mV and \(V_s\) 0 mV, the barium currents were enhanced with increasing doses of Bay K from 5 nM up to a concentration of 20 nM Bay K; no significant change in the current amplitude was observed beyond this concentration. The inward barium current was also sensitive to nifedipine, a DHP calcium channel antagonist, as illustrated in Fig. 5B.

To see inhibition with DHP antagonists, we first generated a larger current with 80 nM Bay K. Increasing doses of the antagonist nifedipine were then used to block the Bay K effects, but the concentrations necessary were substantially higher than expected. At \(V_h\) –100 mV, 1 µM nifedipine (12.5 times greater concentration than Bay K) suppressed only 62% of the Bay K-enhanced current (Fig. 5B); at \(V_h\) –50 mV, however, 86% of the current was suppressed (data not shown). The greater effectiveness of nifedipine at \(V_h\) –50 mV is most likely due to the fact that DHP binding is greatest at more depolarized holding potentials (4). We approached the problem another way by increasing the current using forskolin, which increases phosphorylation of \(L\)-type calcium channels by directly activating adenylate cyclase. Forskolin activation of the current eliminated binding competition between two DHPs, and in addition, we used a more potent DHP antagonist, nicardipine. At 4°C and \(V_h\) –100 mV, 6 µM nicardipine, but not 3 µM, blocked the inward current by 87.9% (Fig 6D). At \(V_h\) –50 mV, however, 92.9% of the current was suppressed by nicardipine. In summary, the inward current in trout myocytes was as sensitive to DHP agonists as cardiac cells in other species (3, 30, 31) but demonstrated less sensitivity to antagonists as reported earlier in carp and trout myocytes (39, 40).

Response to other agonists and inhibitors. A non-DHP agonist for the \(L\)-type calcium channel FPL was also tested in trout cardiac myocytes with 20 mM barium in the bath. A dose of 30 nM FPL was ineffective, but 60 nM produced a large current similar to the response seen with Bay K but with considerably slower rates of activation, inactivation, and deactivation (Fig. 6A; \(V_h\) –100 mV, \(V_s\) 30 mV). FPL also shifted the voltage peak of the inward barium current to a relatively more depolarized voltage (30 mV). Similar differences in the three rates in response to Bay K and FPL have been demonstrated in rat and guinea pig ventricular myocytes (33, 34).
Calcium channels are differentially blocked by specific inorganic ions. Cadmium has a potent inhibitory effect on VDCC. As seen in Fig. 6B, 250 µM Cd²⁺ completely suppressed the Bay K-enhanced barium current in trout myocytes (Vₕ = −100 mV, Vₛ = 10 mV). This is similar to the effect of cadmium observed in frog (3, 13), carp (39), and rat (22) myocardium. In trout myocytes, with an FPL-enhanced barium current, 250 µM Cd²⁺ also completely blocked the inward current (Fig. 6C).

Isoproterenol, a potent stimulator of cardiac contraction, acts by phosphorylating L-type calcium channels via a cAMP-dependent kinase. The inward current in trout ventricular myocytes exhibited a positive response to isoproterenol at 30 nM with a maximum 10-fold increase in current at 300 nM (Fig. 7A; Vₕ = −100 mV, Vₛ = 0 mV) with an EC₅₀ of approximately 60 nM (Fig. 7B). Although the magnitude of the response to isoproterenol was greater than that previously reported for trout and carp (40), the sensitivity appeared to be somewhat less than that reported, although the full range of doses was not tested in earlier reports (40).

8-BrcAMP, which easily penetrates the cell membrane and is not degraded, was added to the bath solution to bypass the receptor-adenylate cyclase complex and to directly stimulate protein kinase A for channel phosphorylation. At 10°C 8-BrcAMP (100 µM) elicited a positive response from trout ventricular myocytes (Fig. 8A; Vₕ = −100 mV, Vₛ = 10 mV). The amplitude of the inward current increased progressively with additional 8-BrcAMP, reaching an eightfold increase at 1 mM. With these data EC₅₀ was ~250 µM (Fig. 8B).

We noticed that there were differences in the time course of the inward barium current inactivation with isoproterenol and 8-BrcAMP stimulation compared with Bay K. Isoproterenol and 8-BrcAMP promoted increases in channel phosphorylation and hence current amplitude, with nominal inactivation during the 300-ms depolarization, whereas the current in the presence of Bay K, which also increased in amplitude, had a comparatively rapid rate of inactivation.

Temperature effects on current amplitude. The effect of temperature on barium currents in trout ventricular myocytes was tested in vitro in ventricular myocytes from trout kept at their standard environmental temperature of 10°C. The myocytes were held at the following temperatures within their physiologically relevant temperature range: 10°C (control, acclimation temperature) and 4, 18, and 22°C, with 22°C being considered a stress temperature. Each cell was tested at two to four different temperatures: 10°C and then 4, 10, 18, and 22°C, except for the control cell in Fig. 9A, which was tested in the following sequence: 22°C and then 18, 10, and 4°C. No difference in the results was noted with the order of temperatures studied. Figure 9A demonstrates traces obtained from a cell stepped to 20 mV from a holding potential of −100 mV. From the current-voltage plot shown in Fig. 9B, there was a significant decrease (32%) in current amplitude at 4°C compared with the current at 10°C (Table 1). This difference also held true in subsequent experiments when the cell was first held at 10°C and then changed to 4°C. The amplitude of the currents at 18 and 22°C was not significantly different from the control at 10°C. The average Q₁₀ for change in amplitude of the peak current from 10 to 4°C was 1.73 ± 0.30 (n = 4). This is less than that seen in mammalian cardiac ventricular...
myocytes, which have Q₁₀ values between 2.4–3.0 at temperatures between 22 and 37°C (26), but comparable to the Q₁₀ for peak current between 10 and 20°C in frog skeletal muscle (1.6) (7).

Bay K (100 nM) was added to prolong the open state of L-type calcium channels to see whether the changes in current at the different temperatures remained. As expected the current was significantly enhanced with Bay K. The temperature effects, nevertheless, were still present. At 4°C, the current was reduced by 36% compared with the control at 10°C (Table 1); this was similar to the depression in amplitude expressed in the control cell (Fig. 9A). The magnitude of the current at 22°C was similar to that at 10°C, and a small increase in the barium current occurred at 18°C (Fig. 9D).

β-Agonists act by a cAMP-dependent phosphorylation of L-type channels, which is a result of the sequential activation of adenylate cyclase and protein kinase A. Forskolin was added to bypass the β-adrenergic receptor and to stimulate cAMP production via direct activation of adenylate cyclase. This treatment should provide a large population of phosphorylated L-type calcium channels. At 10°C 10 µM forskolin significantly enhanced the inward current and displayed a noninactivating current characteristic of phosphorylated channels (Vₘ -80 mV, Vₛ 10 mV) (Fig. 10A). At 4°C, the current was again suppressed by 25% compared with that seen at 10°C (Table 1) but its profile was not changed (Fig 10A). At 18°C, the forskolin-stimulated current was increased by 23% (Vₛ 10 mV) as seen in the current-voltage plot (Fig. 10B). The Q₁₀ values remained similar to that seen with primarily unphosphorylated control cells.

In summary, the colder temperature resulted in a suppression of the peak current by about 28%, whereas the warmer temperature yielded an average increment of 23% and the Q₁₀ values indicate minimal temperature sensitivity beyond that of diffusion alone.

Voltage-dependent kinetics at different temperatures. To determine whether the calcium channel kinetics as well as current amplitude were altered by the change in temperature, we looked at τₑ at the experimental temperatures. This parameter has been examined in other species, that is, in rat (26, 28) and guinea pig hearts (2, 6, 17, 23), as well as frog skeletal muscle (7) and is particularly sensitive to temperature changes. We looked at this same parameter to compare the responses of the trout with other species.

β-Adrenergic agonist. A: traces of inward barium current (5 mM) at 10°C in response to increasing doses of isoproterenol from 30 to 300 nM. B: dose-response curve to isoproterenol with current amplitudes normalized to maximum current. Current amplitude reached maximum at 300 nM with an EC₅₀ of ~60 nM.
Inactivation of the barium current was easily quantitated and was best fit to a single exponential in the control and forskolin-treated groups. At the acclimation temperature of 10°C, the inactivation rate in both the control and Bay K-enhanced currents were similar. As temperature warmed, \( t \) became longer in untreated control cells and was the reverse direction of change expected with an increase in temperature. Conversely, Bay K had a shorter \( t \), indicating an increased rate of decay with increasing temperature. Unlike the other curve fits for \( t \), inactivation in the presence of Bay K at the higher temperatures was best fit with a double exponential curve, suggesting two components of the current under these conditions, a slower and faster phase. When the current was enhanced by phosphorylation, i.e., forskolin treatment (Fig. 10), there was very little decay during the depolarizing step. However, by estimating the slope of a line, a slight increase in the \( t \) was apparent at the higher temperatures (Q\(_{10}\) 1.3, Table 2). All temperature-related changes in the untreated control and forskolin-treated cells had Q\(_{10}\) between 1.2 and 1.4 (Table 2), implying that both unphosphorylated and phosphorylated L-type channels did not show any temperature dependence beyond that resulting from thermal effect on ion diffusion. In the presence of Bay K between 10 and 22°C, the values for Q\(_{10}\) were higher (>3) than between 4 and 10°C. It is important to note that the rate of inactivation in phosphorylated or unphosphorylated L-type calcium currents, which are very sensitive to temperature change in other species (Q\(_{10}\) >3), do not display this temperature dependence in trout cardiac myocytes.

**DISCUSSION**

Characterization of L-type current in fish. A high-voltage activating current was found in trout ventricular myocytes, which is an L-type calcium current based on its kinetic behavior and response to various calcium channel agonists and antagonists. Similar currents have been reported in both carp and trout (39, 40), but there are some differences in the characteristics of the current amplitude, sensitivity to DHP, and current decay after phosphorylation. The current amplitude in control cells using either calcium or barium as the

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**Fig. 8.** Channel phosphorylation. 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) was used to promote calcium channel phosphorylation. A: traces of inward barium current (5 mM) at 10°C in response to increasing doses of 8-Br-cAMP from 0.1 to 1 mM. B: dose-response to 8-Br-cAMP with current amplitudes normalized to maximum current. Current amplitude reached maximum at 1 mM with EC\(_{50}\) of ~250 µM.

**Fig. 9.** Response to in vitro changes in temperature. Recordings were taken in following temperature sequence: A and B: 22, 18, 10, 4°C. C and D: 10, 4, 18, 22°C. A and B: untreated cell. A: traces at 4 temperatures. B: current-voltage plot at 4 temperatures. C and D: cell exposed to 100 nM Bay K8644. C: traces at 4 temperatures. D: current-voltage plot at 4 temperatures. □, 4°C; ●, 10°C; ○, 18°C; △, 22°C.
charge carrier is substantially smaller in this report compared with that reported earlier for trout and carp under whole cell patch-clamp conditions (39, 40). The small current under perforated patch-clamp conditions might suggest a lesser density of channels, but when the population of L-type channels was fully activated by either Bay K or forskolin, the currents increased substantially. The low activity of VDCC at rest in the trout myocytes in the present study, in comparison to the stimulatory response seen with isoproterenol, cAMP, and forskolin, could be due to multiple controls on the phosphorylation of VDCC. For example, in rat ventricular myocytes, β2-receptors couple to a G protein. The adenylate cyclase system has both a stimulatory (Gs) and inhibitory (Gi) control, as shown by pertussis toxin inhibition of Gi (41). If the Gi system predominates in resting conditions, which are more likely to be seen with use of the perforated patch technique, then a smaller proportion of the population of L-type channels would be active. However, with β-adrenergic stimulation using isoproterenol and subsequently activation of the Gs pathway, VDCC would be significantly increased as more channels were phosphorylated. This hypothesis needs to be tested further.

There also appears to be a large discrepancy in trout cells in their comparable sensitivity to DHP agonists and antagonists. The sensitivity to Bay K was in the nanomolar range, similar to that found in both mammalian cardiac and skeletal muscle cells (14). The antagonists, however, appeared to have a reduced effectiveness in trout cardiac cells. We found that concentrations greater than 1–6 µM were required for suppression of the current, whereas the usual dissociation constants are in the nanomolar range (14). Even though it has been reported that DHP antagonists are less effective on barium currents (24), similar µM doses of nifedipine were needed to inhibit calcium currents in carp as well as in trout myocytes (39, 40).

Phosphorylation of the L-type calcium channels in trout with either isoproterenol, cAMP, or forskolin not only increased current amplitude but also reduced current decay during the depolarizing step. These responses are characteristic of phosphorylated channels, whether from mammals or amphibians (25, 38). Although these changes in VDCC were seen in trout ventricular myocytes using the perforated patch, the slower current inactivation was not visually observed in current traces of carp or trout myocytes under whole cell conditions using either isoproterenol or forskolin, as reported by Vornanen (40). This may reflect differences in experimental techniques, i.e., whole cell vs. perforated patch.

Temperature responses. Cardiac tissue in mammals is very sensitive to a fall in body temperature, which results in an increased risk of cardiac failure. However, poikilotherms, such as trout, may experience a range of body temperatures otherwise found only in hibernating animals (17) and yet are able to maintain adequate

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**Table 1. Effects of temperature on amplitude of barium currents and related Q10 values from representative cells**

<table>
<thead>
<tr>
<th>Temp, °C</th>
<th>Q10 (4–10°C)</th>
<th>Q10 (10–18°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.20</td>
<td>1.21</td>
</tr>
<tr>
<td>Bay K</td>
<td>1.21</td>
<td>1.21</td>
</tr>
<tr>
<td>Forskolin</td>
<td>1.21</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Data were from peak currents at voltage step (Vd) 10 mV. Q10 values were calculated with formula described in METHODS: Q10 = (current at 18°C/current at 10°C)^(1/10°C). Calculations between 4 and 10°C; Q10–10, calculations between 10 and 18°C. Responses at different temperatures were recorded from the same cell for each experimental condition. Control, untreated cell; Bay K, 100 nM; forskolin, 10 µM.

**Table 2. Current inactivation rate and Q10 values for unphosphorylated (untreated) and phosphorylated (treated with 10 µM forskolin) channels**

<table>
<thead>
<tr>
<th>Temp, °C</th>
<th>Untreated, ms</th>
<th>Forskolin, pA/100 ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>68.55</td>
<td>0.75</td>
</tr>
<tr>
<td>10</td>
<td>76.64</td>
<td>2.25</td>
</tr>
<tr>
<td>18</td>
<td>84.16</td>
<td>1.25</td>
</tr>
<tr>
<td>22</td>
<td>112.57</td>
<td>ND</td>
</tr>
</tbody>
</table>

Currents were analyzed for inactivation. Q10 was calculated from these representative data. Data were from peak currents at Vd 10 mV. For forskolin, estimation of slope of line since decay rate was slow. Consequently, values will be larger with faster inactivation rather than smaller (pA/100 ms). ND, not done.

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**Fig. 10. Activation of adenylate cyclase. Forskolin was used to directly activate adenylate cyclase in these cells. A: 10 µM forskolin significantly stimulated inward barium current at 10°C. At 4°C current was reduced by 25% compared with that at 10°C. Shifting to 18°C increased amplitude of current by 21%. B: current-voltage relationship of forskolin-stimulated current at 3 temperatures. □, 4°C; ○, 10°C; ●, 18°C.**
cardiac function. In this study, we attempted to explore the adaptability of VDCC in trout ventricular cells to changes in temperature. There are two ways to approach this problem: 1) acute studies in vitro and 2) long-term adaptive studies in vivo. We began by taking the former approach and determined that in the temperature range for trout from 4 to 22°C, their ventricular cells did not demonstrate temperature sensitivity beyond that which could be attributed to thermal effects on ion diffusion.

The first parameter measured in trout ventricular myocytes was amplitude of the inward barium current. The amplitude decreased as conditions shifted from the control acclimation temperature of 10°C to cooler conditions. Because of the detrimental effects of higher temperatures on the trout myocardium we were not able to do a complete range of temperatures for an Arrhenius plot. However, we estimated Q10 using a calculation that has been utilized to assess temperature effects on calcium currents in rat ventricular tissue (26). With use of this method, the estimated Q10 for current amplitude with cooling was only 1.73, whereas the Q10 found in mammalian ventricular cells is usually between 2 and 3 (17, 26), indicating a more temperature-sensitive response. Conversely, as the temperature was raised to 18 or 22°C, the amplitude increased, again with a low value for the Q10. It appears that using the perforated patch clamp, the change in amplitude in response to temperature shifts is not much greater than the Q10 for the diffusion of ions (−1.3–1.6) (18) and implies a low sensitivity of trout myocardial cells to changes in temperature within the environmental range.

In addition to amplitude, we measured rates of inactivation in trout myocytes because this parameter is particularly sensitive to temperature variation with reported Q10 of 3 or greater (17, 26). Inactivation rates were quantitated in all three groups (untreated, forskolin, and Bay K) and demonstrated low values for Q10. This indicates that although there were modifications in inactivation of VDCC in trout with temperature, the changes did not have a Q10 large enough to indicate an enhanced sensitivity to temperature. These data in trout are contrary to those seen in other species. In addition, inactivation of calcium uptake by cardiac SR in crucian carp and rainbow trout has been shown to express an insensitivity to temperature as well (1). In the hearts of other species, even in hibernators (7, 17, 26, 28), however, the rate of current decay of L-type current is the one response that is consistently temperature sensitive. Therefore, trout appear to be a special case.

We next asked whether the amplitude changes would still be present if the currents were enhanced with Bay K to prolong the open time of the L-type channels. We saw an increase in overall current amplitude as would be expected with longer open times in the presence of the agonist. Inactivation kinetics in the presence of Bay K was the one situation in which a temperature change (from 10 to 18 or 22°C) exaggerated the response. At 18 and 22°C, τi was fit with a double exponential curve rather than a single. The faster time constant for inactivation had a Q10 of −3, suggesting that Bay K increased the sensitivity of inactivation at the higher temperatures, but the mechanism for this change is unknown and contrary to what we found for trout myocytes in untreated and forskolin-treated groups.

Assessment of current amplitude in phosphorylated channels is simpler compared with the previous experiments because the current is uncontaminated by an inactivation process. The peak of the currents in forskolin-treated cells exhibited similar directional changes in current amplitude to that of previous groups, i.e., increasing with a rise in temperature with Q10 values between 1.27 and 1.85. This is in contrast to guinea pig ventricular cells in which the Q10 for changes in amplitude shifts from 2.57 to 1.85 after phosphorylation (2). The Q10 value for τi in forskolin-treated cells did not show a temperature dependency beyond that which might reflect changes in ionic flow.

The identification of calcium permeable stretch-sensitive cation channels (35) raises the question of whether such channels are present in the trout ventricle. Moreover, could such currents be activated by a potential change in cell volume at different temperatures? These mechanosensitive channels do not appear to be responsible for the inward currents in trout ventricular cells that we observed in response to temperature, because the change in cell volume (increasing at lower temperatures) varied inversely with the magnitude of the current rather than the reverse.

What does this apparent temperature independence of cardiac VDCC mean to the fish? At decreased temperatures the metabolic needs of the organism may be less, but blood pressure must be maintained to adequately perfuse the tissues. Heart rate usually decreases with temperature, which would decrease cardiac output (10). Therefore, to maintain blood flow, stroke volume would need to increase. This latter response would require an increase in [Ca2+], which in turn is dependent on release of calcium from the SR or entry via VDCC. We have demonstrated that VDCC show minimal temperature sensitivity so that the decrease in calcium current and channel kinetics would be small with falling temperatures. This would allow the fish to adapt to the oscillating environmental temperatures with only a small change in the activity of the channels and influx of calcium. In addition, if norepinephrine were released, phosphorylated channels would further increase calcium influx. Because the activity of Ca2+-ATPase in trout SR is not significantly altered at lower temperatures, reuptake of calcium would remain relatively stable (1). However, there may be less release of calcium from the SR since cardiac contraction in teleosts is ryanodine-insensitive at low temperatures (19, 21), implying that calcium release from the SR is not central to E-C coupling at low temperatures. In this case then, maintenance of calcium influx via VDCC becomes an important mechanism in trout cardiac function at low temperatures. Lowered temperatures might also decrease the activity of the Na+-K+-ATPase, leading to an increase in intra-
cellular Na⁺. The elevated Na⁺ could in turn promote further calcium influx via the reverse activity of the Na⁺/Ca²⁺ exchanger as has been shown in guinea pig myocytes (5, 37). Ultimately, the total influx of calcium per given unit of time and the chances of overloading the cell with calcium would depend on the heart rate and phosphorylation state of the channels. If the actin–myosin–troponin complex remained sensitive to [Ca²⁺], (27) then we could predict the maintenance or even enhancement of the stroke volume at lower temperatures sustaining adequate cardiac output.

Perspectives

Factors that need to be considered in evaluating the physiological response of ventricular muscle at different temperatures would include heart rate, the state of E–C coupling, potential release of norepinephrine under stress and hence channel phosphorylation, sodium reuptake by the SR, calcium extrusion via sarcoplasmic Na⁺/Ca²⁺ exchanger, Ca²⁺-ATPase, binding of calcium to calsequestrin in the SR, and the sensitivity of the actin–myosin–troponin complex to calcium signaling. Of all these parameters the availability of calcium plays a pivotal role in ventricular contraction. In contrast to mammalian hearts, which are severely impaired at lower temperatures, the ability of teleosts to maintain [Ca²⁺] may be an important mechanism in their ability to adapt to the cold. Part of the adaptive response appears to be an ability to maintain calcium entry via VDCC at lower temperatures, which may compensate for the declining availability of calcium influx from intracellular stores. Clearly, plasma membrane calcium channels may be an important consideration in adaptation of cardiac function to changes in temperature in teleosts. However, studies on the function of trout VDCC in the cold need to be coupled with additional experiments on cardiac function at different temperatures when calcium channel activity has been modified. These responses are demonstrated in the companion study (8), which investigates the contractile function of trout hearts in the cold in the presence of modulators of VDCC activity.

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