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Temperature acclimation modifies Na^+ current in fish cardiac myocytes

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Summary

The present study was designed to test the hypothesis that temperature acclimation modifies sarcolemmal Na^+ current (I_{Na}) of the fish cardiac myocytes differently depending on the animal's lifestyle in the cold. Two eurythermal fish species with different physiological strategies for surviving in the cold, a cold-dormant crucian carp (*Carassius carassius* L.) and a cold-active rainbow trout (*Oncorhynchus mykiss*), were used in acclimation experiments. The I_{Na} of carp and trout were also compared with I_{Na} of a cold stenothermal burbot (*Lota lota*). In accordance with the hypothesis, cold-acclimation decreased the density of I_{Na} in crucian carp and increased it in rainbow trout, suggesting depression of impulse conduction in cold-acclimated carp and positive compensation of impulse propagation in cold-acclimated trout. The steady-state activation curve of trout I_{Na} was shifted by 6 mV to more negative voltages by cold acclimation, which probably lowers the stimulus threshold for action potentials and further improves cardiac excitability in the cold. In burbot myocytes, the I_{Na} density was high and the position of the steady-state activation curve on the voltage axis was even more negative than in trout or carp myocytes, suggesting that the burbot I_{Na} is adapted to maintain high excitability and conductivity in the cold. The I_{Na} of the burbot heart differed from those of carp and trout in causing four times larger charge influx per excitation, which suggests that I_{Na} may also have a significant role in cardiac excitation–contraction coupling of the burbot heart. In summary, I_{Na} of fish cardiac myocytes shows thermal plasticity that is different in several respects in cold-dormant and cold-active species and thus has a physiologically meaningful role in supporting the variable life styles and habitat conditions of each species.

Key words: fish heart, thermal acclimation, sodium current, *Carassius carassius*, *Oncorhynchus mykiss*, *Lota lota*.

Introduction

While endothermic animals maintain constant body temperature and thus constant body functions by using massive energy expenditure, ectothermic animals regulate selected and vital body functions in a temperature-dependent manner and are able to cope with variable thermal environments using minimal energy consumption. Temperature acclimation requires physiological plasticity, which may be achieved by expressing different protein isoforms in a temperature-dependent manner or possessing proteins that are relatively temperature insensitive (Johnston, 1982; Driedzic and Gesser, 1994; Johnson and Bennett, 1995; Wakeling et al., 2000; Johnston and Temple, 2002; Rosenthal and Bezanilla, 2002; Vornanen et al., 2002; Watabe, 2002). Moreover, the physiological response of an animal to temperature acclimation may vary depending on habitat conditions. Some ectotherms become inactive during harsh winter conditions, which may be the only successful strategy in severely hypoxic environments (Crawshaw, 1984; Vornanen, 1994; Lutz and Nilsson, 1997; Jackson, 2000; Boutilier, 2001). Others try to keep active and compensate for the decelerating effects of low temperature by physiological mechanisms (Lagerspetz, 1974; Driedzic et al., 1996; Aho and Vornanen, 1999, 2001).

In previous studies we characterised cardiac function in three teleost fish that have different temperature preferences and seem to use partially different overwintering strategies in cold-temperate climates (Matikainen and Vornanen, 1992; Aho and Vornanen, 1999, 2001; Tiitu and Vornanen, 2001, 2002a,b). Crucian carp *Carassius carassius* L. inhabit small and anoxic water bodies where other fish are unable to survive. The crucian carp is a very anoxia resistant and eurythermic species tolerating temperatures between 0°C and +36°C (Blažka, 1958; Horoszewich, 1973). This anoxia resistance is based on huge glycogen stores (Hyvärinen et al., 1985; Vornanen, 1994), alternative metabolic pathways (Johnston and Bernard, 1983; Holopainen and Hyvärinen, 1985) and especially on small energy consumption that is reflected as inverse compensation in the heart function (Matikainen and Vornanen, 1992, 1994; Tiitu and Vornanen, 2001). In contrast, rainbow trout *Oncorhynchus mykiss* is active in cold waters and expresses several compensatory changes that are reflected in heart rate, heart size, myofibrillar function as well as in the

activity of sarcolemmal ion channels (Graham and Farrell, 1992; Driedzic et al., 1996; Aho and Vornanen, 1999, 2001). Rainbow trout is not as eurythermic as crucian carp, but is able to tolerate temperatures between 0°C and +25°C (Taylor et al., 1996). Burbot *Lota lota* are cold-stenothermal fish that are most active in the middle of winter and try to avoid warm waters (above +13°C) in summer (Bernard et al., 1993). Structurally and functionally the burbot heart is more similar to the heart of rainbow trout than crucian carp in that it is relatively large and remains highly active in the cold (Tiitu and Vornanen, 2002a,b).

Voltage-gated Na^+ channels are crucial for the excitability of the heart by allowing fast Na^+ influx. Sodium current (I_{Na}) determines the amplitude and slope of the action potential upstroke, which affect the threshold of excitability and are especially important in the control of impulse conduction velocity (Fozzard and Hanck, 1996). I_{Na} is also involved in excitation–contraction coupling of cardiac myocytes (Leblanc and Hume, 1990; Maier et al., 2001). Considering the wide differences in cardiac activity between cold-active (trout, burbot) and cold-dormant (crucian carp) fish species, it would be expected that these differences might be reflected in the properties of cardiac I_{Na}. Therefore, we examined I_{Na} in ventricular myocytes of these fish to test the effects of thermal acclimation on I_{Na}. More specifically, we hypothesised that the density of I_{Na} would be larger and kinetics faster in cold-active than cold-dormant species, and that thermal acclimation might induce opposite changes in the function of I_{Na} in eurythermal crucian carp and rainbow trout.

Materials and methods

Fish

Crucian carp *Carassius carassius* L. were caught in June and November from a small pond near the University of Joensuu. Burbot *Lota lota* L. were caught during spawning time in February 2003 from the lake Orivesi (62°30′N, 29°46′E) in Finland. Trout *Oncorhynchus mykiss* Walbaum were obtained from a local fish farm and were brought to the laboratory in September. In the laboratory, the fish were reared in temperature controlled 500 or 1000 l stainless tanks with a continuous supply of aerated groundwater circulating at a rate of about 0.5 l min^{-1}. Crucian carp and rainbow trout were acclimated at two different temperatures, +20°C and +4°C for warm- (w.a.) and cold- (c.a.) acclimated crucian carp, and +17°C and +4°C for w.a. and c.a. trout, respectively. Seasonally acclimatised crucian carp were put directly into their acclimation temperature, which corresponded to the water temperature of the pond at the time of catching. Cold-stenothermal burbot were acclimated only at +4°C, and because of this are called c.a. burbot. All fish were held for at least 3 weeks at the acclimation temperatures under a 15 h:9 h light:dark photoperiod before experimentation. During that time trout were fed five times a week *ad libitum* with commercial fodder (Biomar, Brande, Denmark) and w.a. carp three times week with aquarium fish food (Tetra, Melle, Germany). Cold-acclimated carp do not feed. Burbot were fed two or three times a week with dead vendace (*Coregonus albula*).

Myocyte isolation

Single ventricular myocytes were enzymatically isolated using previously published methods (Vornanen and Tuomennoro, 1999). Briefly, fish were stunned with a quick blow to the head, the spine was cut and the heart was excised. A metallic cannula was slipped through the bulbus arteriosus into the ventricle and the heart was retrogradely perfused with a nominally Ca^{2+}-free, low-Na^+ solution for 10 min and then with proteolytic enzyme solution for 10–15 min from a height of 50 cm. Solutions were continuously gassed with 100% O_2 and the enzyme solution was recycled using a peristaltic pump. After enzymatic digestion the ventricle was excised and placed in fresh isolating solution in a Petri dish. The ventricle was chopped in small pieces with scissors and single cells were released by agitating tissue pieces through the opening of a Pasteur pipette. Isolated myocytes were stored in isolating solution at +6°C and used within 8 h from the isolation. All procedures were in accordance with the local committee for animal experimentation.

Solutions

A nominally Ca^{2+}-free saline used for cell isolation contained ($mmol^{-1}$): 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose and 10 Hepes at pH 6.9 at 20°C. For enzymatic digestion, 0.75 mg ml^{-1} collagenase (Type IA; Sigma, St Louis, MO, USA), 0.5 mg ml^{-1} trypsin (Type IX; Sigma) and 0.5 mg ml^{-1} fatty-acid-free bovine serum albumin (BSA) were added to this saline. The physiological K^+-based external saline solution contained ($mmol^{-1}$): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 Hepes (pH adjusted to 7.7 with NaOH). The actual extracellular Cs-based solution used for recording I_{Na} contained ($mmol^{-1}$): 20 NaCl, 120 CsCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose and 10 Hepes (pH adjusted to 7.7 with CsOH). In addition, 10 μmol l^{-1} nifedipine (Sigma) was added to both solutions to block L-type Ca^{2+}-currents. The pipette solution contained ($mmol^{-1}$): 5 NaCl, 130 CsCl, 1 $MgCl_2$, 5 EGTA, 5 Mg_2ATP and 5 Hepes (pH adjusted to 7.2 with CsOH).

Patch-clamp experiments

A small portion of myocyte suspension was transferred to a recording chamber (RC-26, Warner Instrument Corporation, Brunswick USA; volume 150 μl) and cells were allowed to settle on the chamber bottom before superfusing with the external saline solution at the rate of 1.5–2.0 ml min^{-1}. First, the myocytes were perfused with normal K^+-based saline so that gigaseal and whole-cell patch clamp recording of the myocytes were established. Internal perfusion of the myocytes with pipette solution was continued in this solution for at least for 3 min in order to allow buffering of intracellular Ca^{2+} with 5 mmol l^{-1} EGTA. Then, solution flow could be switched to a low-Na^+ external solution without inducing contracture in the

patched myocyte. The experiments with myocytes of c.a. animals were conducted at +4°C and +11°C and those with w.a. animals at +11°C and +18°C. Accordingly, results were obtained not only at the physiological body temperatures of the animals, but also at the common experimental temperature of +11°C, which enabled direct comparison of results between the two acclimation groups. The temperature of the saline was adjusted to the desired temperatures by using two circulating water baths and was continuously monitored by thermocouple positioned closed to the myocyte.

The whole-cell voltage clamp measurements of I_{Na} were performed using an Axopatch 1-D amplifier with a CV-4 1/100 headstage (Axon Instruments, CA, USA). The digitised data were stored on the hard drive of the computer using the Clampex 8.2 software (Axon). The recordings were analysed off-line with Clampfit 8.2 and SigmaPlot 6.0 (SPSS) software. Patch pipettes were pulled from borosilicate glass (Garner, Claremont, CA, USA) using a vertical two-stage puller (List-Medical, L/M-3P-A). Off-set potentials were zeroed just before the formation of gigaohm seal and the pipette capacitance (8.22±0.10 pF, N=101) was compensated for after the seal formation. The membrane was ruptured by a short voltage pulse (zap) and capacitive transients were eliminated by adjusting series resistance and cell capacitance compensation circuits. Mean resistance of the electrodes and total access resistance before compensation were 3.02±0.07 and 10.20±0.33 MΩ (N=101), respectively. To ensure adequate voltage control a minimum of 80% series resistance compensation was applied. I_{Na} was elicited from the holding potential of –120 mV with different pulse protocols and recorded at sampling rate of 10 kHz. The recordings were low-pass filtered at 5 kHz. The calculated liquid–junction potential of the electrodes was about 1.5 mV and was not corrected in the results.

Steady-state activation and inactivation of I_{Na}

Steady-state inactivation was determined using a two-step protocol where a 500 ms conditioning pulse to potentials between –110 mV and –20 mV was followed by a 15 ms test pulse to –20 mV. For the voltage dependence of steady-state inactivation the normalized test pulse currents (I/I_{max}) were plotted as a function of membrane potential and fitted to the Boltzmann function:

$$y = 1 / (1+\exp(V–V_{0.5}) / –S) , \quad (1)$$

where V is membrane potential, $V_{0.5}$ is the midpoint and $–S$ is the slope of the curve. The steady-state voltage dependence of activation was obtained by plotting the normalized conductance (G/G_{max}) as a function of membrane potential and fitting it to the Boltzmann distribution (above) with a positive slope (S). The voltage dependence of Na^+ channel conductance was obtained from the current–voltage relationships (Fig. 1) according to the equation:

$$G_{Na} = I_{Na} / (V–V_{rev}) , \quad (2)$$

where G_{Na} is the Na^+ conductance of the membrane, I_{Na} is the peak Na^+ current at a given membrane potential (V) and V_{rev} is the reversal potential of I_{Na}.

Recovery and development of I_{Na} inactivation

Time-dependent recovery of I_{Na} from inactivation was examined using a paired-pulse protocol where two successive 100 ms pulses from –120 to –20 mV were separated by a variable (40–400 ms) delay at holding potential. The peak I_{Na} during the latter pulse was plotted as a function of time and fit to a single exponential function ($y=y_0+a^{–bt}$) to obtain the time constant (τ=1/–b) of recovery from inactivation.

The development of rested-state inactivation was examined using a protocol consisting of a conditioning prepulse from –120 mV to –60 mV with variable (30–360 ms) duration, followed by a short return (3 ms) to the holding potential and a test pulse to –20 mV for 30 ms. The peak I_{Na} elicited by test pulses was plotted as a function of the prepulse duration and fitted to a single exponential function to obtain the time constant for the development of rested-state inactivation.

Kinetics of I_{Na} inactivation

The time constants of I_{Na} inactivation kinetics were derived by fitting the decay phase of the I_{Na} at different membrane potentials (–40 mV to +10 mV) with a single exponential equation using the Chebyshev transformation procedure of the Clampfit software package.

Statistical analyses

Data are expressed as means ± S.E.M. Analysis of variance (one-way ANOVA) with Tukey's HSD *post hoc* test was used for multiple comparisons between species and acclimations groups. Effects of acute temperature changes on I_{Na} were assessed by paired or unpaired t-test. P<0.05 was considered statistically significant.

Results

The myocytes of crucian carp and burbot heart were similar in size and clearly smaller than trout ventricular myocytes (Table 1). Interestingly, the myocyte capacitance was 42% larger in c.a. trout in comparison to w.a. trout, suggesting a significant cold-induced hypertrophy of trout cardiac myocytes. The small cell size (15–30% of the size of mammalian cardiac myocytes) and the absence of t-tubuli (Santer, 1985) make fish cardiac myocytes a suitable preparation for clamping large and fast membrane currents such as I_{Na}. Despite these experimental advantages of fish myocytes, the concentration of external Na^+ was lowered to 20 mmol $l^{–1}$ in order to reduce I_{Na} and thereby improve the voltage control. Low experimental temperatures further reduced the current amplitude. Under these experimental conditions the largest currents were less than 3 nA and therefore the voltage error with 10 MΩ series resistance would be maximally 3 mV.

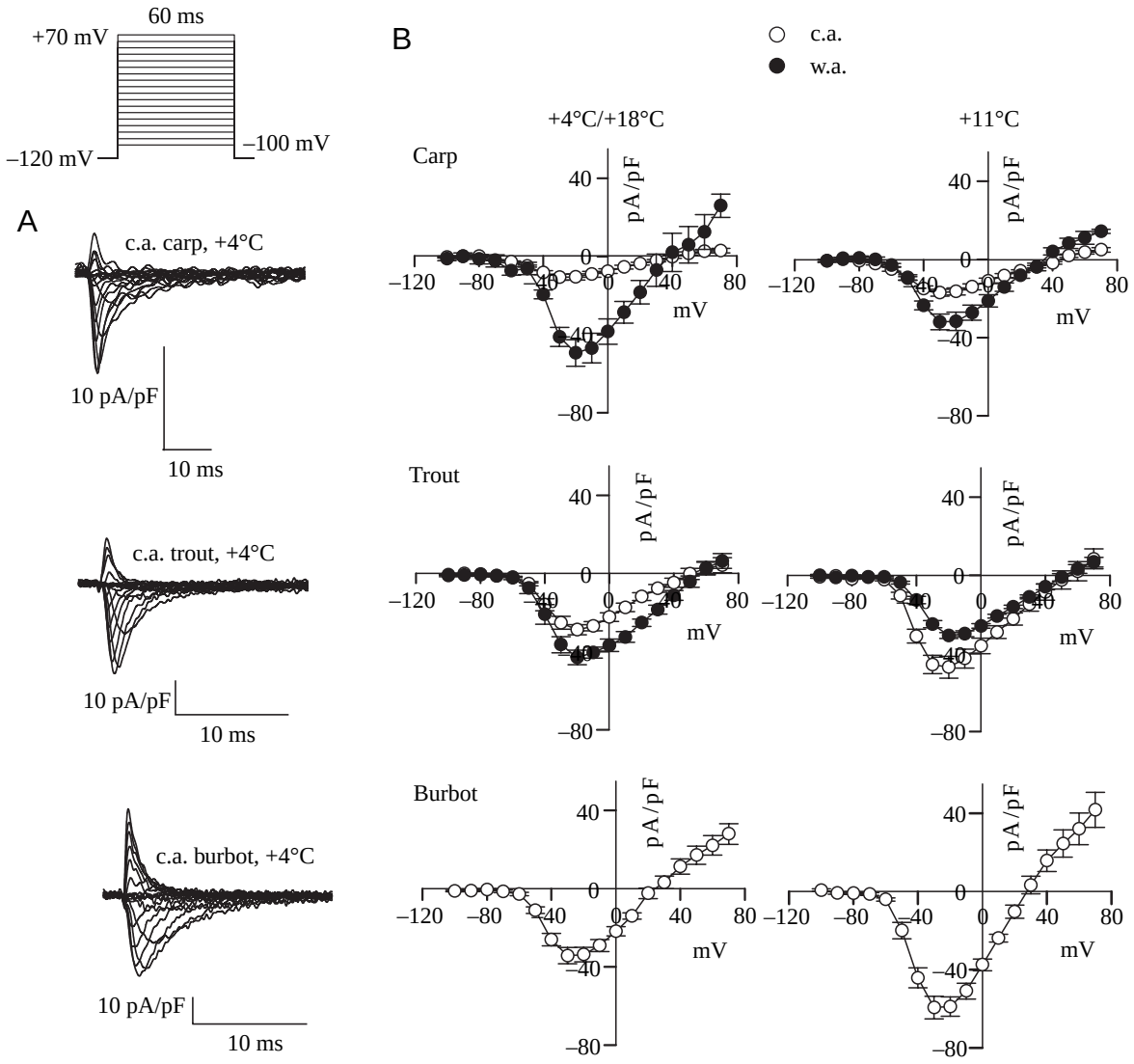

Fig. 1. Voltage-dependence of I_{Na} in ventricular myocytes of crucian carp, rainbow trout and burbot hearts. Currents were elicited from a holding potential of –120 mV with 60 ms depolarising pulse between –100 and +70 mV (top). (A) Representative recordings of I_{Na} from cold-acclimated (c.a.) fish at +4°C. (B) Results shown as means ± S.E.M. (N=9–16). *I–V* curves from warm-acclimated (w.a.; closed symbols) and c.a. (open symbols) fish are superimposed to allow direct comparisons between acclimation groups at physiological body temperatures (+4°C/+18°C) and at the common experimental temperature (11°C). See Materials and methods for details.

Size of I_{Na}

The current–voltage relationships and maximal conductance (G_{max}) of I_{Na} are shown in Fig. 1 and Table 2, respectively. In all three species, I_{Na} activated between –70 and –60 mV, reached the peak value at –20 mV and reversed close to the theoretical reversal potential (30.5–32 mV, depending on temperature) of I_{Na}. In rainbow trout, cold-acclimation slightly shifted the activation threshold of I_{Na} to more negative values. There were marked species-specific differences in I_{Na} density and G_{max} of c.a. fish at the physiological body temperature (+4°C). I_{Na} was clearly smallest in crucian carp and largest in burbot, whereas the I_{Na} of trout heart was intermediate between those of carp and burbot. Interestingly, thermal acclimation modified I_{Na} in opposite manner in carp and trout; in the carp cold-acclimation reduced the density of I_{Na}, while in the trout it increased I_{Na} (Fig. 1B).

Acute temperature effects on the density of I_{Na} were bigger in c.a. than w.a. fish both for crucian carp (Q_{10}=1.97 and 1.74, respectively) and rainbow trout (Q_{10}=2.33 and 1.61, respectively). The Q_{10} for burbot I_{Na} was 2.20 between +4° and +11°C.

Steady-state activation and inactivation of I_{Na}

Steady-state activation and inactivation curves are shown in Fig. 2 and statistical evaluation of the data is presented in Table 2. There were no species-specific differences in half-voltage ($V_{0.5}$) of steady-state activation for c.a. fish at +4°C. In contrast, the $V_{0.5}$ of steady-state inactivation of c.a. crucian

Table 1. *Body mass and membrane capacitance of ventricular myocyte in crucian carp, rainbow trout and burbot*

	Acclimation			
	Cold	*N*	Warm	*N*
Crucian carp				
Body mass (g)	66.85±4.86	7	134.00±17.25	7
Cell capacitance (pA)	21.46±1.77[a]	16	24.41±1.85[a]	18
Rainbow trout				
Body mass (g)	230.31±24.40	7	216.09±18.54	8
Cell capacitance (pA)	41.94±1.91[b,*]	16	29.59±1.55[a]	29
Burbot				
Body mass (g)	229.34±10.73	7		
Cell capacitance (pA)	23.31±1.34[a]	22		

A statistically significant difference (P<0.05) between species is indicated by a dissimilar letter and between warm-acclimated and cold-acclimated animals of the same species by an asterisk.

carp and c.a. burbot were –16 and –10 mV more negative, respectively, than that of the c.a. trout.

In rainbow trout myocytes, acclimation to cold caused a 6 mV shift of steady-state activation curve to left (Fig. 2B). This effect was specific for steady-state activation, since there was no difference in $V_{0.5}$ of the steady-state inactivation between the acclimation groups.

Acute temperature change from the acclimation temperature to the common temperature of 11°C did not have any significant effect on $V_{0.5}$ of steady-state activation or steady-state inactivation in any of the fish species. However, due to the tendency of rising temperature to shift the inactivation curve to right, the $V_{0.5}$ of the steady-state inactivation curve of the c.a. carp was almost 10 mV more negative than in w.a. carp, when measured at the physiological body temperatures of the fish (Fig. 3B). Furthermore, acute increases in experimental temperature slightly increased the slope factor (S) of steady-state activation in w.a. and c.a trout and the slope factor of steady-state inactivation in w.a. trout.

Development of rested-state inactivation of I_{Na}

The amplitude of I_{Na} decreased as a function of prepulse duration, possibly due to direct transfer of Na^+ channels from resting closed state to inactivated closed state without intervening opening (Fig. 3, Table 3). The large residual currents after long prepulses indicate that a significant proportion of the Na^+ channels failed to enter the inactivated state within 360 ms. The proportion of non-inactivated channels was especially large in w.a. and c.a. trout and in w.a. carp. Neither thermal acclimation nor acute temperature changes affected the time constant of rested-state inactivation. There were no species-specific differences in the time constant of rested-state inactivation.

Recovery of I_{Na} from inactivation

The recovery of I_{Na} from inactivation at +4°C was

Table 2. *Conductance of maximum current, half-voltage and slope factor of steady-state activation and steady-state inactivation for I_{Na} in fish ventricular myocytes*

	Acclimation temperature (°C)							
	Cold-acclimated (c.a.)				Warm-acclimated (w.a.)			
	+4	*N*	+11	*N*	+11	*N*	+18	*N*
Crucian carp								
Conductance (pS/pF)	0.18±0.03[a,†,‡]	11	0.26±0.03[a,*]	15	0.51±0.08[a,†]	9	0.79±0.10[a]	11
$V_{0.5}$ steady-state activation (mV)	–41.45±2.45[a]	11	–42.32±1.47[a]	15	–38.92±1.68[a]	9	–39.27±2.45[a]	11
Slope	9.59±0.62[a,‡]	11	8.50±0.44[b,*]	15	6.70±0.60[a]	9	7.73±0.57[a]	11
$V_{0.5}$ steady-state inactivation (mV)	–98.09±1.42[a,‡]	10	–94.53±1.79[a]	11	–91.12±1.32[a]	7	–89.58±1.02[a]	10
Slope	3.67±0.81[a,‡]	10	5.53±0.64[a]	11	6.10±0.16[a]	7	5.85±0.09[a]	10
Rainbow trout								
Conductance (pS/pF)	0.49±0.04[b,†,‡]	12	0.75±0.10[b,*]	10	0.54±0.03[a,†]	16	0.78±0.06[a]	10
$V_{0.5}$ steady-state activation (mV)	–36.51±1.80[a,‡]	14	–37.78±1.16[a,*]	13	–31.45±1.25[a]	17	–31.72±1.02[a]	18
Slope	7.32±0.30[b,‡]	14	8.50±0.39[b]	13	7.32±0.20[a,†]	17	9.05±0.39[a]	18
$V_{0.5}$ steady-state inactivation (mV)	–81.91±2.21[b]	11	–82.81±1.79[b]	14	–79.58±1.28[a]	21	–79.39±1.59[a]	19
Slope	6.42±0.23[b]	11	6.60±0.29[a]	14	6.05±0.12[a,†]	21	6.64±0.25[a]	19
Burbot								
Conductance (pS/pF)	0.54±0.06[b,†]	16	0.95±0.07[b]	11				
$V_{0.5}$ steady-state activation (mV)	–40.45±1.65[a]	16	–40.09±2.16[a]	11				
Slope	6.61±0.34[b]	16	5.77±0.37[a]	11				
$V_{0.5}$ steady-state inactivation (mV)	–92.06±2.21[a]	12	–90.35±2.67[a]	10				
Slope	5.21±0.28[a]	12	5.34±0.24[a]	10				

Dissimilar letters indicate a statistically significant difference between species; * between w.a. and c.a. animals of the same species; † between experimental temperatures; ‡ between c.a. and w.a. fish in their physiological body temperatures.

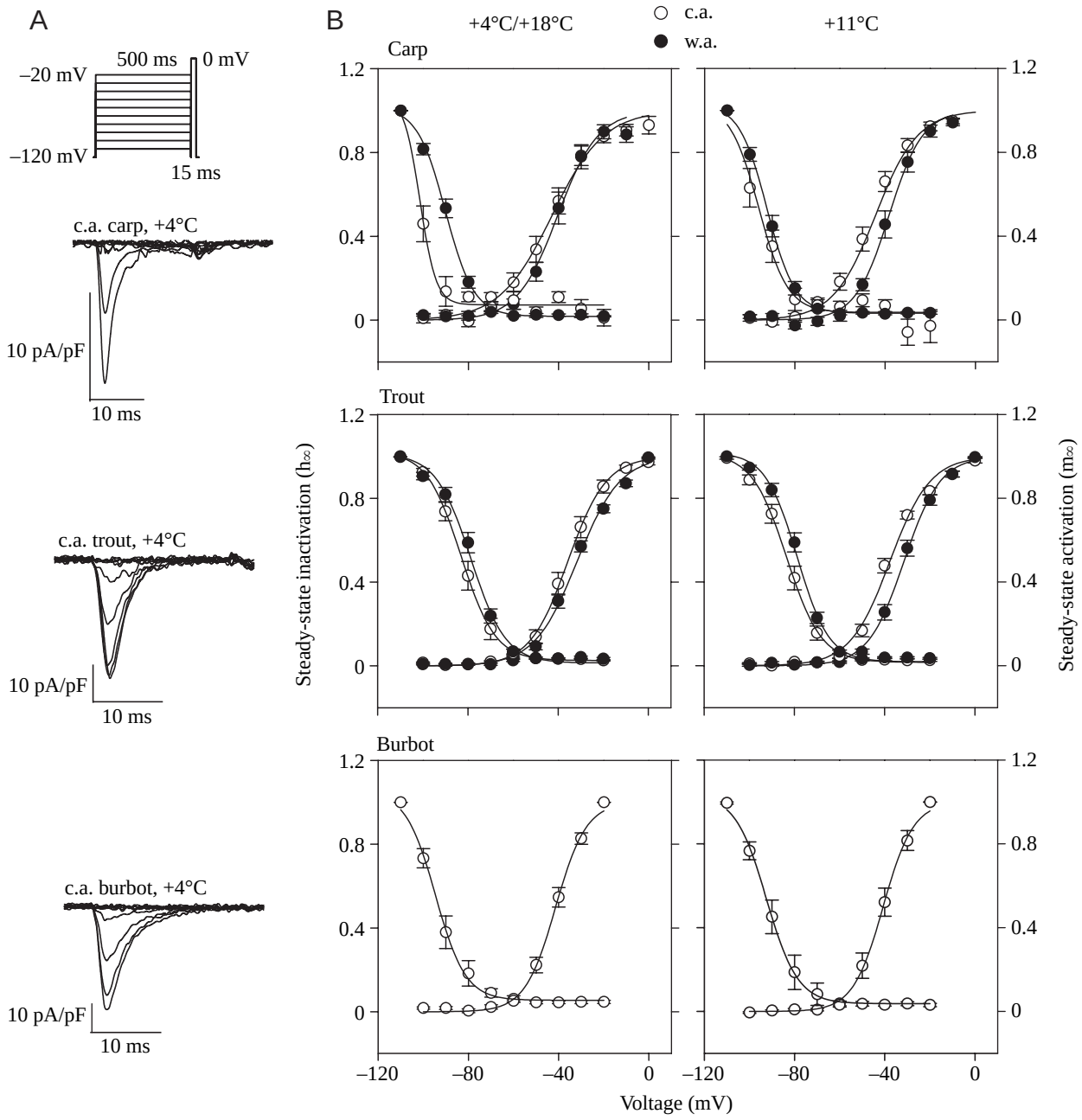

Fig. 2. Steady-state activation (m_∞) and inactivation (h_∞) of I_{Na} in ventricular myocytes of crucian carp, rainbow trout and burbot heart. (A) Voltage protocol for steady-state inactivation and representative recordings of I_{Na} from c.a. fish at +4°C. Steady-state activation curves were constructed from the *I–V* curves of Fig. 1 as explained in Materials and methods. (B) Voltage-dependence of steady-state activation and inactivation for w.a. (closed symbols) and c.a. (open symbols) fish (means ± S.E.M.; *N*=7–21).

significantly faster in c.a. trout in comparison to c.a. carp and c.a. burbot (Table 3, Fig. 4). Acclimation to cold made the recovery of Na^+ channels from inactivation slower in carp ventricular myocytes, but did not affect the I_{Na} of the trout myocytes. Acute increases in temperature slightly accelerated the recovery of the channels from inactivation in w.a. carp and c.a. burbot.

Inactivation kinetics of I_{Na}

There were some prominent species-specific differences in the rate of inactivation (Fig. 5, see also Table 3). In c.a. fish at +4°C, inactivation kinetics was slower in burbot and carp in comparison to trout. Furthermore, cold-acclimation increased the inactivation rate of Na^+ channels in rainbow trout ventricular myocytes, but did not have any effect on Na^+ channels of the carp cardiac myocytes.

Acute temperature increases accelerated the inactivation kinetics of Na^+ channels in all species. The Q_{10}-values at –20 mV were 0.48 and 0.40 for c.a. and w.a. carp, and 0.53

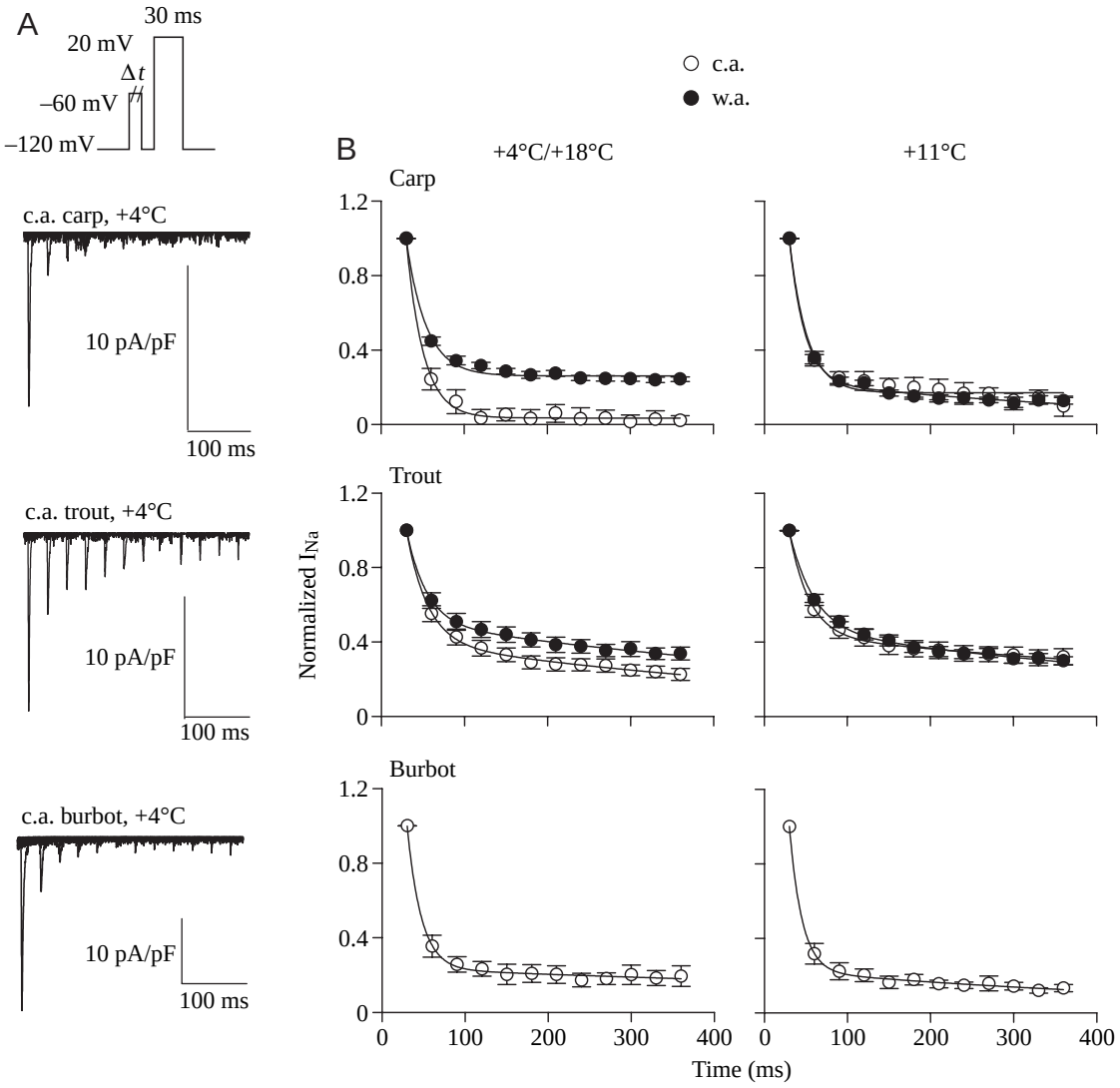

Fig. 3. Development of rested-state inactivation of I_{Na} in ventricular myocytes of crucian carp, rainbow trout and burbot hearts. (A) Voltage protocol and representative recordings of I_{Na}; (B) results shown as means ± S.E.M. (*N*=6–13). Increased duration of the prepulse at –60 mV reduces I_{Na} elicited by the test pulse to –20 mV for 30 ms.

and 0.50 for c.a. and w.a. trout, respectively. The Q_{10} for the burbot I_{Na} was 0.32 between +4°C and +11°C.

Charge transfer by I_{Na}

Na^+ channels are the most significant Na^+ entry pathway in the myocyte, and this may have an affect on excitation–contraction coupling and Na^+ load imposed on the Na^+/K^+-pump. Integration of I_{Na} at –20 mV indicated that the charge transfer per excitation through Na^+ channels is about four times larger in burbot ventricular myocytes than in trout or carp myocytes (Fig. 6).

Discussion

The present results show that temperature acclimation modifies the I_{Na} of fish cardiac myocytes and the effects are in several cases opposite in cold-dormant carp and in cold-active trout. Indeed, the I_{Na} of w.a. carp and w.a. trout were almost indistinguishable from another, while significant differences appeared in the I_{Na} of c.a. fish. The I_{Na} of cold stenothermal burbot is characterized by high current density and slow inactivation kinetics resulting in large charge influx. Possible physiological implications of these findings are discussed below.

Density of I_{Na}

In agreement with our hypothesis, cold acclimation reduced the density of I_{Na} in crucian carp ventricular myocytes and increased it in rainbow trout myocytes. The cold-induced increase in the density of trout I_{Na} is in line with the well-known compensatory changes in size and function of the trout heart that counteract the direct decelerating effects of low temperature on circulation (Farrell and Jones, 1992; Driedzic et al., 1996; Aho and Vornanen, 1999, 2001). However,

Table 3. *Kinetics of the Na^+ current in crucian carp, rainbow trout and burbot ventricular myocytes*

	Acclimation temperature (°C)							
	Cold-acclimated (c.a.)				Warm-acclimated (w.a.)			
Time constant τ (ms)	+4	*N*	+11	*N*	+11	*N*	+18	*N*
Crucian carp								
Recovery from inactivation	40.27±3.72[a,‡]	9	42.69±3.30[a,*]	12	29.61±3.16[a,†]	6	19.25±2.05[a]	10
Rested-state inactivation	22.85±3.38[a,‡]	11	24.39±4.13[a]	13	21.67±3.96[a]	6	32.48±3.78[a]	12
Kinetics of inactivation	1.69±0.02[a,†,‡]	10	1.32±0.11[a]	14	1.25±0.05[a,†]	6	0.66±0.02[a]	8
Rainbow trout								
Recovery from inactivation	28.17±2.39[b,‡]	10	22.29±1.72[b]	12	21.69±1.79[a]	19	17.26±1.63[a]	13
Rested-state inactivation	24.36±1.37[a]	10	25.25±1.89[a]	11	29.60±2.25[a]	12	29.85±4.02[a]	13
Kinetics of inactivation	1.12±0.05[b,†,‡]	11	0.64±0.02[b,*]	10	0.99±0.05[b,†]	16	0.62±0.03[a]	10
Burbot								
Recovery from inactivation	47.36±3.10[a,†]	10	24.22±2.20[b]	10				
Rested-state inactivation	19.39±4.01[a]	10	18.26±3.66[a]	8				
Kinetics of inactivation	1.86±0.08[a,†]	14	0.89±0.05[b]	11				

Dissimilar letters indicate a statistically significant difference between species; * between w.a. and c.a. animals of the same species; † between experimental temperatures; ‡ between c.a. and w.a. fish in their physiological body temperatures.

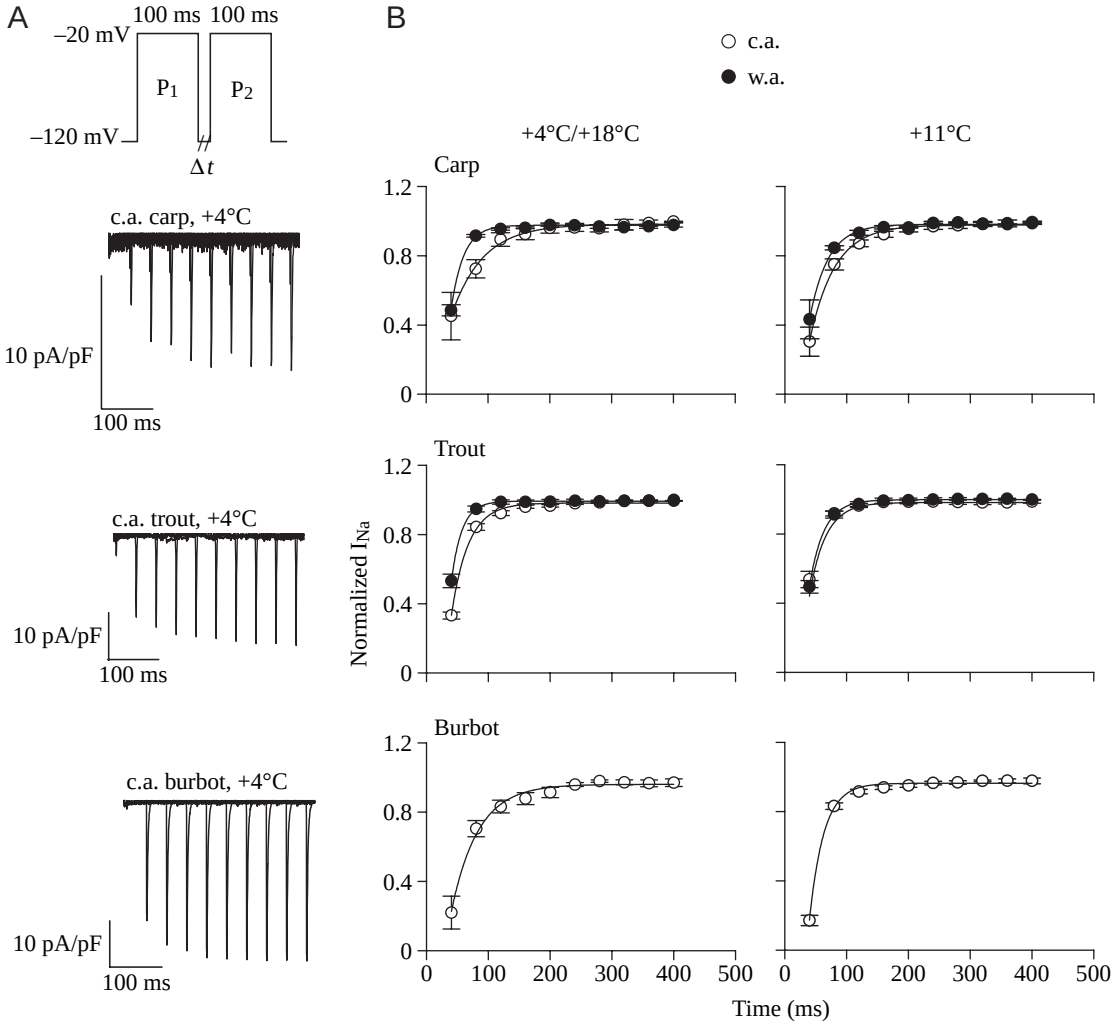

Fig. 4. Recovery of I_{Na} from inactivation (reactivation) in ventricular myocytes of crucian carp, rainbow trout and burbot heart. (A) Voltage protocol and representative recordings of I_{Na}; (B) values shown as means ± S.E.M. (*N*=6–19). The amplitude of I_{Na} elicited by test pulses to –20 mV for 100 ms increases as function of the time interval between the prepulse P_1 and the test pulse P_2; see Materials and methods for details.

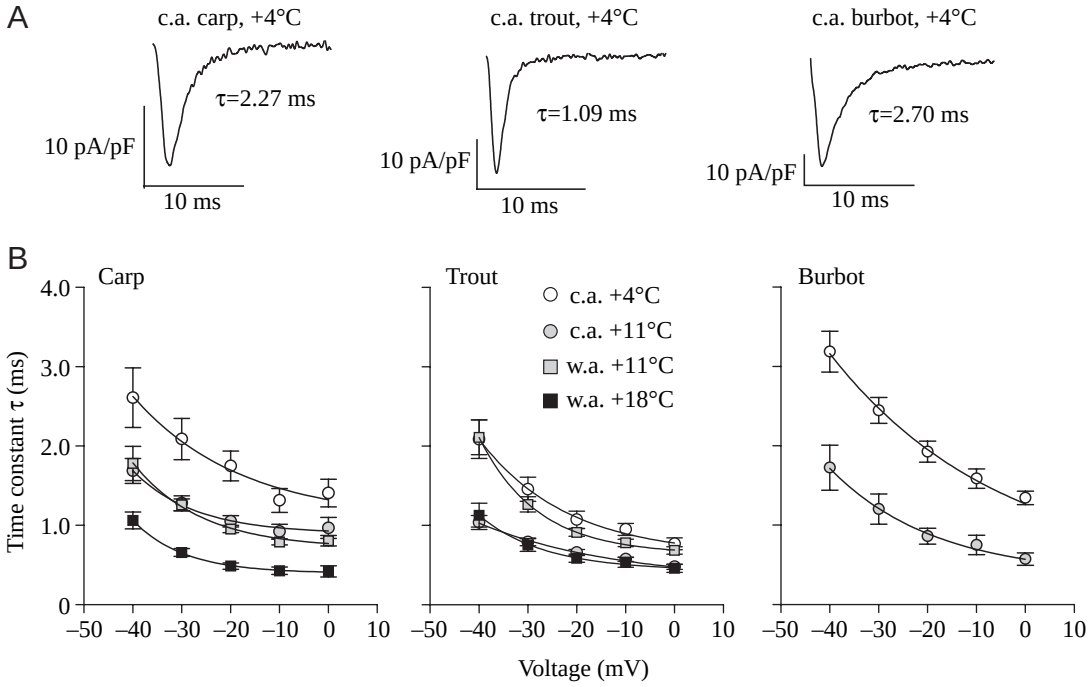

Fig. 5. Voltage-dependence of inactivation kinetics of I_{Na} in ventricular myocytes of crucian carp, rainbow trout and burbot heart. (A) Representative recordings of I_{Na} at –20 mV; (B) results from 5–16 myocytes (means ± S.E.M.).

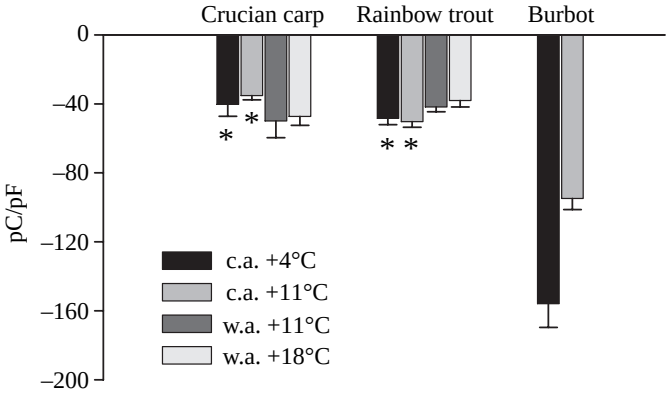

Fig. 6. Charge transfer by I_{Na} in ventricular myocytes of crucian carp, rainbow trout and burbot heart. The transferred charged was obtained by integrating the inward current at –20 mV. The values were normalized for cell size by dividing them by membrane capacitance. An asterisk denotes a statistically significant (P<0.01) difference from the values of the burbot heart.

thermal compensation was only partial as the density of I_{Na} at +4°C was 59% of the density at +18°C. Similarly, the decrease in the density of I_{Na} in carp myocytes is in agreement with the previously reported cold-induced depression in heart rate and velocity of cardiac contraction (Matikainen and Vornanen, 1992; Vornanen, 1994; Tiitu and Vornanen, 2001). In carp, the density of I_{Na} at +4°C was only 21% of the value at +18°C, suggesting that only one fifth of the current amplitude of the w.a. summer fish is needed to maintain cardiac excitability in c.a. winter fish. It should be noted that due to the very negative position of the steady-state inactivation curve in the voltage axis, all Na^+ channels might not be available for opening at –120 mV and therefore I_{Na} of the c.a. carp might have been slightly underestimated. This does not, however, affect the conclusions since a similar reduction in I_{Na} would be expected to occur in the physiological context. Indeed, a temperature-dependent shift in the availability may be the means of achieving downregulation of I_{Na}. In brief, acclimation-induced changes in the density of I_{Na} would mean that in winter the rate of upstroke and conduction velocity of action potentials are only moderately depressed in rainbow trout but severely depressed in the carp.

The differences in response to acclimation between trout and carp I_{Na} are not explained by the acute effects of temperature on I_{Na}, and therefore must involve acclimation-induced changes in the number of functional channels. Whether this is due to temperature-dependent changes in transcription, translation, rate of protein degradation or trafficking of channels to the sarcolemma remains to be clarified (Herfst et al., 2004). Furthermore, the significance of temperature related changes in membrane lipids on Na^+ channel function should be also examined.

Steady-state activation

In ventricular myocytes of rainbow trout, acclimation to cold caused a 6 mV shift of the steady-state activation curve to more negative voltages. The negative shift of steady-state activation will probably decrease stimulus threshold for eliciting an action potential. Thus, the temperature-induced change in voltage-dependence of steady-state activation will further improve on that obtained by the partial compensation of current density, and thereby maintain excitability in c.a. trout.

Such a change was not observed in crucian carp myocytes, whose Na^+ channels are inherently activated at slightly more negative voltages, as are those of the burbot myocytes. Furthermore, the shallow slope of the activation curve in c.a. carp actually also decreases the action potential threshold at negative voltages.

Inactivation of I_{Na}

Na^+ channels were completely inactivated by a 100 ms depolarising pulse to –20 mV and all channels recovered from inactivation within 100–200 ms. Accordingly, only relatively short diastolic intervals are needed for full recovery of cardiac excitability, and therefore it might be anticipated that little compensatory adjustments are required upon cold acclimation. Indeed, in rainbow trout there were no temperature-dependent changes in time constant of recovery from inactivation. In crucian carp, the recovery of I_{Na} from inactivation was about 44% slower in c.a. than w.a. fish, which is in line with the depressed cardiac function in cold-dormant crucian carp (Matikainen and Vornanen, 1992), although its physiological importance is not immediately clear. The slow recovery of channels from inactivation might, however, explain the negative shift of the steady-state inactivation of the c.a. carp at +4°C, and thus the depression of I_{Na} density.

It was hypothesised that, similar to the current density, the effect of temperature acclimation on I_{Na} kinetics might be opposite in trout and carp; however, this was found to be only partially correct. In rainbow trout, cold-acclimation induced a partial compensation in inactivation kinetics of I_{Na}, while no change was observed in carp. As a consequence of thermal acclimation the inactivation kinetics of Na^+ channels is decelerated 1.8-fold and 2.6-fold in c.a. trout and c.a. carp, respectively, in comparison to w.a. species mates.

Na^+ channels can directly enter the inactivated closed state from the resting closed state without opening. The rate of rested-state inactivation was measured at the prepulse voltage of –60 mV where Na^+ channels have a low probability of opening and where the decrease in I_{Na} is, therefore, likely to depend on a direct transition of Na^+ channels from the resting closed state to the inactivated state. If rested-state inactivation occurred at voltages close to the resting membrane potential (–80 mV; Paajanen and Vornanen, 2004), a small depolarisation of membrane would significantly compromise the excitability of the heart. Indeed, a significant proportion of the Na^+ channels in fish cardiac myocytes was inactivated by the –60 mV prepulse, and therefore transient subthreshold depolarisation would decrease excitability of the fish hearts. At physiological body temperatures, the rested-state inactivation was more extensive in c.a. carp than in w.a. carp, in agreement with more negative steady-state inactivation curve of the c.a. carp. In contrast, there were few differences in the rate of development of the rested-state inactivation either between acclimation groups or between different species.

Species-specific differences

All three species of fish were acclimated and examined at +4°C, which allowed direct comparison between their I_{Na} properties. I_{Na} of the burbot heart resembles that of the trout heart with respect to high current density. On the other hand, with respect to voltage dependence of steady-state activation and inactivation, it is closer to those of the crucian carp myocytes. The high current density and negative position of the steady-state activation curve make the Na^+ channels of the burbot heart especially suitable for maintaining membrane excitability in the cold.

Recovery from inactivation and the kinetics of inactivation of the burbot heart are similar to those of the crucian carp and slower than those of the rainbow trout. The slow kinetics of inactivation, together with high current density, results in an approximately fourfold larger charge transfer per excitation in burbot cardiac myocytes in comparison to those of carp and trout. This will impose a high Na^+ load on Na^+/K^+-pump of the burbot cardiac myocytes and will be energetically expensive. It might be expected that the evolutionary preservation of such an energetically expensive mechanism in cold-adapted species would serve some important physiological function. Intracellular Na^+ is needed for the reverse mode of Na^+/Ca^{2+} exchange, and therefore it could be speculated that large I_{Na} will be used to activate Ca^{2+} influx through Na^+/Ca^{2+} exchange. Sarcolemmal Ca^{2+} influx by Na^+/Ca^{2+} change might directly activate myofilaments or serve as a trigger for further Ca^{2+} release from the SR. It should be noted that in burbot ventricle significant portion of the Ca^{2+} comes from the SR (Tiitu and Vornanen, 2002a; Vornanen et al., 2002), while the sarcolemmal Ca^{2+} current is relatively small (M. Vornanen, unpublished results). Therefore, the reverse mode Na^+/Ca^{2+} exchange might serve as an additional trigger for Ca^{2+} release from the SR (Vornanen et al., 1994; Han et al., 2001). Increased Ca^{2+} handling capacity of the cardiac SR (for a recent review, see Vornanen et al., 2002) and enhanced aerobic ATP production of cardiac myocytes (Rodnick et al., 1997) in cold-acclimated fish has been documented, and could support such an excitation–contraction coupling mechanism. However, this possibility should be directly examined by appropriate methods.

Conclusions

In ventricular myocytes of rainbow trout heart, excitability of the heart is supported in the cold by an acclimation-induced increase in the density of I_{Na} and leftward shift of the steady-state activation curve. In the heart of c.a. crucian carp, the leftward shift of the steady-state activation was absent and I_{Na} density was depressed. This difference in response to cold acclimation may be associated with the different life styles and habitat conditions of the two eurythermal species in the cold. Depression of I_{Na} density and associated Na^+ pumping might be necessary manoeuvres for crucian carp to deal with the hypoxic energy shortage, but unnecessary for trout living in oxygen-rich waters.

Due to its high density and low voltage threshold, the I_{Na} of the cold stenothermal burbot is perhaps best able to maintain

cardiac excitability and conductivity in the cold. Unlike I_{Na} of trout and carp, the burbot I_{Na} allows a large Na^+ influx, which suggests an additional function of I_{Na} in the burbot heart.

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