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Electrical excitability of roach (*Rutilus rutilus*) ventricular myocytes: effects of extracellular K^+, temperature, and pacing frequency

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Badr A, Abu-Amra ES, El-Sayed MF, Vornanen M. Electrical excitability of roach (*Rutilus rutilus*) ventricular myocytes: effects of extracellular K^+, temperature, and pacing frequency. *Am J Physiol Regul Integr Comp Physiol* 315: R303–R311, 2018. First published May 2, 2018; doi:10.1152/ajpregu.00436.2017.—Exercise, capture, and handling stress in fish can elevate extracellular K^+ concentration ($[K^+]_o$) with potential impact on heart function in a temperature- and frequency-dependent manner. To this end, the effects of $[K^+]_o$ on the excitability of ventricular myocytes of winter-acclimatized roach (*Rutilus rutilus*) (4 ± 0.5°C) were examined at different test temperatures and varying pacing rates. Frequencies corresponding to in vivo heart rates at 4°C (0.37 Hz), 14°C (1.16 Hz), and 24°C (1.96 Hz) had no significant effect on the excitability of ventricular myocytes. Acute increase of temperature from 4 to 14°C did not affect excitability, but a further rise to 24 markedly decreased excitability: stimulus current and critical depolarization needed to elicit an action potential (AP) were ~25 and 14% higher, respectively, at 24°C than at 4°C and 14°C ($P < 0.05$). This depression could be due to temperature-related mismatch between inward Na^+ and outward K^+ currents. In contrast, an increase of $[K^+]_o$ from 3 to 5.4 or 8 mM at 24°C reduced the stimulus current needed to trigger AP. However, other aspects of excitability were strongly depressed by high $[K^+]_o$: maximum rate of AP upstroke and AP duration were drastically (89 and 50%, respectively) reduced at 8 mM $[K^+]_o$ in comparison with 3 mM ($P < 0.05$). As an extreme case, some myocytes completely failed to elicit all-or-none AP at 8 mM $[K^+]_o$ at 24°C. Also, amplitude and overshoot of AP were reduced by elevation of $[K^+]_o$ ($P < 0.05$). Although high $[K^+]_o$ antagonizes the negative effects of high temperature on excitation threshold, the precipitous depression of the rate of AP upstroke and complete loss of excitability in some myocytes suggest that the combination of high temperature and high $[K^+]_o$ will severely impair ventricular excitability in roach.

cardiac action potential; catch-and-release fishing; excitation threshold; exercise; fish heart

INTRODUCTION

Electrical excitation of the sarcolemma triggers contraction of cardiac myocytes. Excitation originates from a small group of pacemaker cells, which in teleost fish heart comprise a ring-liked structure at the border between the sinus venosus and atrium (29, 49). From there the excitation spreads via interconnected cardiac myocytes throughout the heart, first into the atrium and then along a specialized nodal tissues into the ventricle (34, 36). The orderly sequence of electrical excitation is based on a well-balanced interaction among several Na^+, K^+, and Ca^{2+} specific ion channels of the myocyte sarcolemma, which generate a fast-propagating cardiac action potential (AP). In each functionally specialized cardiac tissue, the AP has a characteristic shape generated by chamber-specific ion currents and ion channel compositions (43). However, the shape of the chamber-specific AP is far from constant; neuronal inputs, hormones, local tissue factors, temperature changes, and stretch-modifying AP waveform so that the pump function of the heart is optimally adjusted to the circulatory demands (20, 28, 32, 33, 43). The delicate and complex balance between interacting cardiac ion channels is affected, and sometimes severely disturbed, by acute temperature changes and stresses that alter ion concentrations of the external fluid around cardiac myocytes (25, 46).

Capture-related exercise, air exposure, and handling stress cause significant changes in metabolite and ion composition of the extracellular fluid and may result in significant poststress mortality of fish as in pike (*Esox Lucius*) and Atlantic salmon (*Salmo salar*) (14, 24, 38). In particular, increases in external K^+ concentration ($[K^+]_o$) (7–20 mM) are often marked and detrimental for postexercise recovery and survival of the fish (11, 41, 47). For example, in capture-stressed marine gamefish, including both teleost and elasmobranch species like yellowfin tuna (*Thunnus albacares*), striped marlin (*Tetrapturus audax*), and blue shark (*Prionace glauca*), $[K^+]_o$ ranged from 7 to 20 mM, which markedly exceeds the $[K^+]_o$ values of nonstressed fish (usually 3–4 mM) (47). Notably, the $[K^+]_o$ remains elevated for several hours after exercise and handling stress (38, 48). Furthermore, the exercise-related increase in $[K^+]_o$ and mortality of the fish are dependent on temperature and thermal history of the animal (11, 14, 23): e.g., the exercise-induced increase in $[K^+]_o$ is much higher in warm-acclimated rainbow trout (*Oncorhynchus mykiss*) (18.9°C; ~5 mM) than in cold-acclimated trout (4.9°C; ~3 mM) (23). Indeed, the postexercise mortality in Atlantic salmon and coral grouper (*Cephalopholis miniata*) is more frequent at high than low temperatures (1, 7, 24).

The exercise-induced increase in $[K^+]_o$ is mainly due to K^+ leakage from the intensely working skeletal muscle fibers (27). In addition, $[K^+]_o$ in the immediate microenvironment of the cardiac myocyte is altered by heart's own activity. In the intact heart, cardiac myocytes are tightly packed leaving only a small and diffusion-restricted "paracellular" space around cells, where $[K^+]_o$ tends to accumulate at high heart rates. In the frog (*Rana pipiens*, *R. catesbeina*, and *R. ridibunda*) hearts, the

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magnitude of K^+ accumulation depends not only on the frequency of stimulation but also on temperature (25, 31). In the *R. pipiens* ventricle at 22°C, the paracellular $[K^+]_o$ can rise from ~3 to 9–12 mM, when contraction frequency increases from 30 to 60 beats/min (25). Since the heart rate in fish is strongly dependent on temperature, it is possible that temperature-dependent increases in heart rate are associated with increases in paracellular $[K^+]_o$, similar to the frog hearts. This could have a significant impact on electrical excitability, since changes in $[K^+]_o$ directly affect membrane potential of excitable cells. Hyperkalemia is potentially cardiotoxic by depolarizing the resting membrane potential (RMP), depressing cardiac contractility, and inducing arrhythmias (15, 16, 22).

It is obvious that $[K^+]_o$, temperature, and heart rate are closely interconnected factors in stress responses, which may exert synergistic or antagonistic effects on cardiac excitability in ectotherms. Although the importance of $[K^+]_o$ on electrical excitability of the vertebrate heart is well realized, only a few studies have been conducted on fish hearts (rainbow trout) (22, 30) and nothing is known about the effects of $[K^+]_o$ on cellular excitability. Therefore, the aim of the current study was to examine how these factors affect cardiac excitability of the roach (*Rutilus rutilus*), an eurythermal fish species (9). Roach was selected as the target species, since there is sufficient background information about the temperature dependence of electrical excitability of the roach heart and its ionic and molecular basis (2–4). Based on the existing knowledge from mammalian literature, it was hypothesized that high $[K^+]_o$ will depress excitability of fish cardiac myocytes (15), possibly in a frequency- and temperature-dependent manner. To this end, patch-clamp experiments were conducted on enzymatically isolated ventricular myocytes of the roach heart at three different $[K^+]_o$, at three different acute test temperatures and at four different pacing rates.

MATERIALS AND METHODS

Animals. Roach (*Rutilus rutilus*) (56.48 ± 3.51 g, $n = 18$) were caught in February and March from the ice-covered Lake Pyhäselkä (water temperature 0–4°C) in Central Finland (62°35 ′N, 21°34 ′E). In the animal facilities of the University of Eastern Finland, the fish were maintained in 500 liters of metal aquaria for a minimum of 3 wk before being used in the experiments. Water temperature was regulated at 4 ± 0.5°C (Computec Technologies, Joensuu, Finland), and oxygen saturation was maintained by aeration with compressed air. Ground water was constantly flowing through the aquaria at the rate of ~200 liters per day. Roach were fed commercial trout fodder (EWOS, Turku, Finland) five times a week. Experiments were authorized by the National Animal Experimental Board in Finland (Permission No. ESAVI/2832/04.10.07/2015).

Myocyte isolation. All experiments were conducted in vitro on enzymatically isolated ventricular myocytes. The fish were killed by a cranial concussion and pithing, and the heart was rapidly excised. Ventricular myocytes were isolated using the methods developed in our laboratory for fish hearts as recently reported also for the roach (3, 45). Freshly isolated myocytes were used in the experiments within 8 h from isolation.

Whole cell patch clamp. The whole cell current-clamp recordings of ventricular APs and voltage-clamp measurements of sarcolemmal K^+ currents were conducted with the use of an Axopatch 1D amplifier (Axon Instruments, Saratoga, CA) equipped with a CV-4 1/100 head-stage. During experiments, myocytes were continuously superfused with external saline solutions at the rate of 1.5–2 ml/min. The temperature of the external solution was regulated at 4, 14, or 24°C with the use of a Peltier device (HCC-100A; Dagan, Minneapolis, MN) and continuously recorded on the same file with electrophysiological data. Clampex 9.2 and Clampfit 10.4 software (Axon) were used for data acquisition and offline analysis of the recordings, respectively.

Patch pipettes were pulled (PP-83; Narishige, Tokyo, Japan) from borosilicate glass (King Precision, Claremont, CA) and had a mean (±SE) resistance of 2.53 ± 0.06 MΩ ($n = 93$) when filled with the electrode solutions. After a giga ohm seal was achieved, the membrane was ruptured by a short voltage pulse (zap) to get access to the cell, transients due to series resistance (8.86 ± 0.06 MΩ) and pipette capacitance (6.14 ± 0.28 pF) were canceled, and capacitive size of ventricular myocytes (39.13 ± 0.92 pF, $n = 93$) was determined.

The same external saline solution was used for current-clamp recordings of APs and voltage-clamp recordings of K^+ currents. The external solution contained (in mmol/l): 150 NaCl, 3 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose at pH adjusted to 7.6 at 20°C with NaOH. When the effects of $[K^+]_o$ on APs or K^+ currents were examined, KCl concentration of this saline was raised from 3 to 5.4 or 8 mM. When K^+ currents were measured, the external saline was supplied with 0.5 μmol/l tetrodotoxin (Tocris Cookson, Bristol, UK) and 10 μmol/l nifedipine (Sigma, St. Louis, MO) to block sodium (I_{Na}) and calcium (I_{Ca}) currents, respectively. E-4031 (2 μmol/l; Tocris Cookson) or Ba^{2+} (0.2 mmol/l) was also included depending on whether inward rectifier K^+ current (I_{K1}) or delayed rectifier K^+ current (I_{Kr}) was recorded (see *Voltage-clamp recordings*). In current-clamp experiments, an EGTA-free pipette solution was used (in mmol/l): 140 KCl, 5 Na_2ATP, 1 $MgCl_2$, 0.03 Tris-GTP, and 10 HEPES, pH 7.2 at 20°C with KOH. In K^+ current recordings, intracellular calcium was buffered with 5 mM EGTA in the pipette solution (mmol/l): 140 KCl, 4 MgATP, 1 $MgCl_2$, 5 EGTA, and 10 HEPES, pH 7.2 at 20°C with KOH.

Current-clamp recordings. The effects of temperature, pacing frequency, and $[K^+]_o$ on the excitability of ventricular myocytes were studied in current-clamp experiments. To this end, ventricular myocytes were stimulated with current pulses of constant duration (4 ms) and varying amplitude. The initial stimulus strength was 300 pA, and it was raised with 20-pA increments until an all-or-none AP was elicited (Fig. 1).

In the first series of experiments, the effects of temperature and pacing frequency on the excitability of roach ventricular myocytes were examined. These experiments were conducted in the external saline solution containing 3 mM K^+. Three different temperatures (4, 14, and 24°C) and four pacing frequencies were tested. Temperature-specific physiological pacing rates of 0.37, 1.16, and 1.96 Hz were used at 4, 14, and 24°C, respectively. These frequencies correspond to physiological heart rates of the winter-acclimatized roach at the respective temperatures (2). Experiments were started from the acclimatization temperature of the fish (4°C) followed by an acute increase of temperature (14 and 24°C). In addition to temperature-specific pacing rates, the effects of a constant frequency of 0.25 Hz were examined at all three temperatures.

In the second series of experiments, the effects of $[K^+]_o$ on the excitability of ventricular myocytes were studied at the constant temperature of 24°C. Temperature-specific pacing frequency of 1.96 Hz was used. The same stimulus protocol was used as above. AP recordings were started at 3 mM $[K^+]_o$ and then accomplished at 5.4 and 8 mM $[K^+]_o$.

In both series of experiments, the strength of stimulus (pA) required to trigger AP and several AP parameters were measured. RMP (mV); threshold potential, i.e., the take-off potential of AP (TP, mV); critical depolarization (CD = TP-RMP, mV); AP overshoot (mV); AP amplitude (mV); AP duration at 50% repolarization level (APD_{50}, ms); maximum rate of AP upstroke ($+V_{max}$, mV/ms); and the maximum rate of AP repolarization ($-V_{max}$, mV/ms) were analyzed offline.

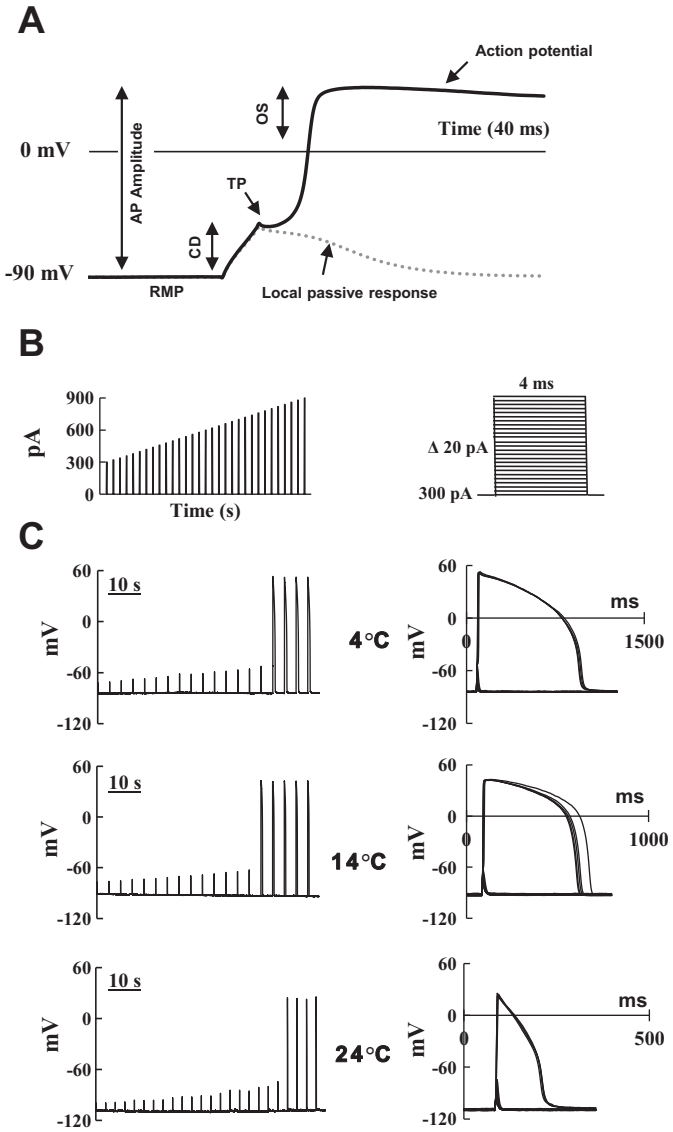

Fig. 1. Effects of experimental temperature and pacing rate on excitability of roach ventricular myocytes. *A*: the first 40 ms of a roach ventricular action potential (AP) at 4°C indicating the parameters that were measured from the current-clamp recordings. CD, critical depolarization; RMP, resting membrane potential; TP, threshold potential; OS, overshoot of AP and AP amplitude. Dotted line indicates the passive voltage change of membrane in response to subminimal stimulus. *B*: stimulus protocol of increasing current strengths (duration 4 ms) used to trigger APs. *C*: examples of voltage responses to increasing stimulus strength at 4, 14 and 24°C. *Left* and *right*: slow and fast time-base recordings of AP, respectively. Note the different time scales at different temperatures (*right*).

Voltage-clamp recordings. Two major sarcolemmal K^+ currents of the fish heart, the inward rectifier K^+ current (I_{K1}) and the rapid component of the delayed rectifier K^+ current (I_{Kr}), were examined at 4°C and 24°C in the presence of 3, 5.4, and 8 mM $[K^+]_o$ (for solutions see *Whole cell patch clamp*). The external solution was supplemented with 0.5 μmol/l tetrodotoxin and 10 μmol/l nifedipine to block Na^+ and Ca^{2+} currents, respectively. When I_{K1} was recorded, 2 μmol/l E-4031 were always included in the external solution to prevent I_{Kr}, while 0.2 mmol/l Ba^{2+} was included when recording I_{Kr} to prevent I_{K1} (3).

Statistics. Data are presented as mean values ± SE from *n* cells. After normality of distribution and equality of variances was checked, paired samples *t*-test and one-way ANOVA were used to assess the statistically significant differences between AP variables and K^+ currents, as indicated in figure legends. Differences between mean values were deemed statistically significant if $P < 0.05$.

RESULTS

Effects of temperature and pacing rate on the excitability of ventricular myocytes. Ventricular myocytes were stimulated at constant and temperature-specific (physiological) pacing frequencies at 4°C (0.25 and 0.37 Hz), 14°C (0.25 and 1.16 Hz), and 24°C (0.25 and 1.96 Hz) to find out the minimal stimulus current (with a constant duration of 4 ms) needed to trigger APs. $[K^+]_o$ in these experiments was 3 mM. Small current pulses elicited only passive local depolarization of membrane potential, which decayed back to the resting level when the stimulus pulse was turned off (Fig. 1). When the stimulus was sufficiently strong to depolarize the membrane to the TP, an all-or-none AP with a fast upstroke was elicited (Fig. 1*C*). Notably, a significantly stronger stimulus was required for activation of APs at 24°C than at 4°C or 14°C ($P < 0.05$), both at the constant stimulation frequency (608.0 ± 44.7 vs. 467.7 ± 27.2 and 445.3 ± 19.9 pA, respectively) and at the temperature-specific stimulation rates (628 ± 50.4 vs. 475.4 ± 28.4 and 469.3 ± 20.8 pA, respectively; Fig. 2*B*). In brief, excitability of ventricular myocytes was reduced at the highest test temperature.

RMP, TP, and critical depolarization are important determinants of electrical excitability. Increasing temperatures made RMP and TP progressively more negative ($P < 0.05$; Fig. 2*C*). In contrast, critical depolarization was initially reduced by a temperature increase from 4 to 14°C but significantly increased with a further rise in temperature from 14 to 24°C ($P < 0.05$) (Fig. 2*C*). These responses were practically independent of stimulus frequency. Thus the reduced excitability of ventricular myocytes at 24°C was correlated with the increased critical depolarization.

Other AP characteristics were also modified by temperature. AP duration (APD_{50}) was drastically reduced and the maximum rate of AP depolarization ($+V_{max}$) was strongly increased by rising temperatures (Fig. 2, *D* and *E*). The maximum rate of AP repolarization ($-V_{max}$) was not significantly changed by temperature changes (Fig. 2*E*). Overshoot and amplitude of AP were not changed by rising temperature when the stimulation frequency was 0.25 Hz (Fig. 2*F*, *left*). In myocytes paced at temperature-specific frequencies, the overshoot of AP was slightly depressed at 24°C and the amplitude slightly increased at 14°C (Fig. 2*F*, *right*).

Effects of $[K^+]_o$ on the excitability of ventricular myocytes. Ventricular myocytes were stimulated by short (4 ms) current pulses at the temperature-specific pacing frequency of 1.96 Hz at 24°C while $[K^+]_o$ was varied. Three different $[K^+]_o$ were tested, 3, 5.4, and 8 mM. Stimulus current was increased with 20-pA increments until the minimal current strength for AP initiation was found (Fig. 3). The current strength needed to trigger an AP reduced with increasing $[K^+]_o$ (Fig. 4*B*; $P < 0.05$). The currents needed to elicit APs at 3, 5.4, and 8 mM $[K^+]_o$ were 628.0 ± 50.4, 427.9 ± 30.1, and 470.6 ± 26.0 pA, respectively (Fig. 4*B*).

The determinants of AP excitation RMP, TP, and critical depolarization were markedly modified by $[K^+]_o$. Increasing $[K^+]_o$ made RMP progressively more positive due to the

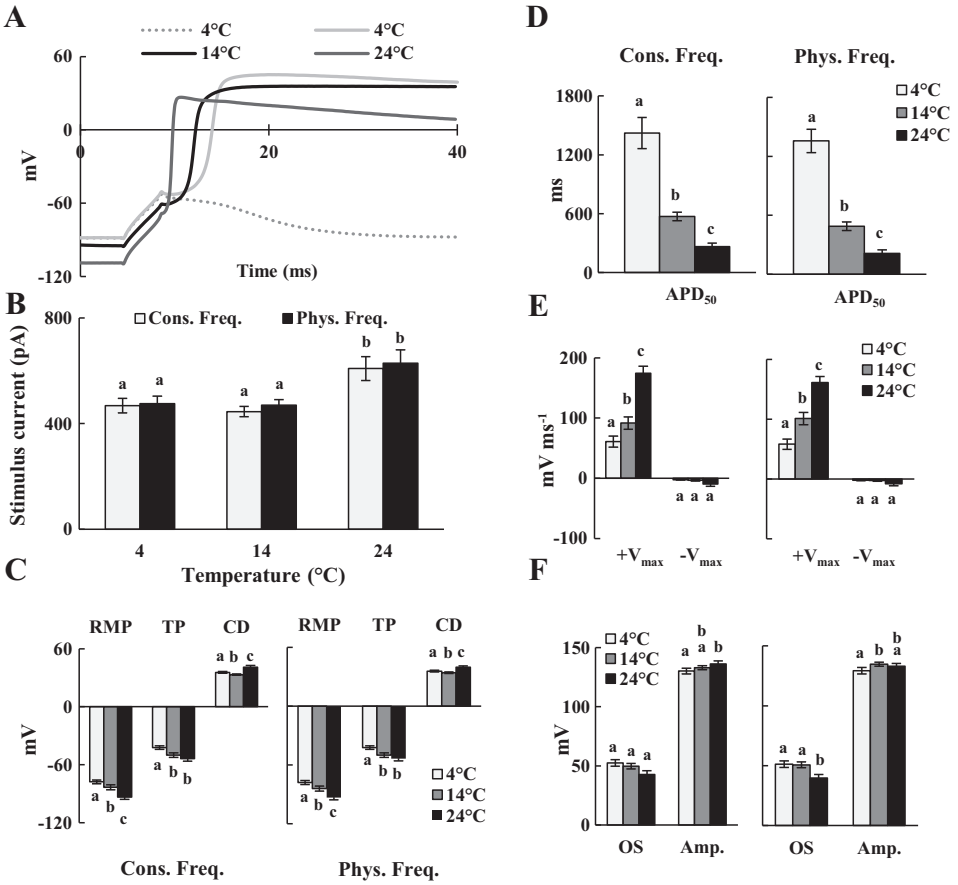

Fig. 2. Effects of experimental temperature and pacing rate on action potential parameters of roach ventricular myocytes at an extracellular K^+ concentration ($[K^+]_o$) of 3 mM. *A*: representative recordings of ventricular action potential at 4, 14, and 24°C at the frequency of 0.25 Hz. Only the first 40 ms are shown. Dotted line represents the passive membrane response to the subminimal stimulus current. *B*: strength of stimulus current needed to trigger action potentials at constant (0.25 Hz; Cons. Freq.) and temperature-specific (Phys. Freq.) pacing rates at 3 different temperatures. *C*: resting membrane potential (RMP), threshold potential (TP), and critical depolarization (CD). *D*: action potential duration (APD_{50}). *E*: maximum rate of action potential upstroke ($+V_{max}$) and repolarization ($-V_{max}$). *F*: overshoot (OS) and AP amplitude (Amp.) of action potential. All parameters in *C*–*F* were measured at the constant frequency of 0.25 Hz (*left*) and at the temperature-specific stimulation rate (*right*) at 3 different temperatures (4, 14, and 24°C). The results are means ± SE of 10–15 cells from 4 fish. Dissimilar letters indicate statistically significant differences ($P < 0.05$) between experimental temperatures (4, 14, and 24°C; *t*-test for paired samples).

decreasing K^+ gradient across the cell membrane and consistently with the Nernstian equilibrium potential. TP behaved qualitatively in a similar way to RMP and became increasingly more positive when $[K^+]_o$ was elevated (Fig. 4*C*). However, quantitatively TP changed more than RMP, and therefore critical depolarization (TP-RMP) was lower at 5.4 and 8 mM $[K^+]_o$ than at 3 mM $[K^+]_o$ (Fig. 4*C*; $P < 0.05$). Accordingly, the depolarization (stimulus current) needed to trigger AP at 5.4 and 8 mM $[K^+]_o$ was less than at 3 mM $[K^+]_o$.

$[K^+]_o$ had strong effects on other AP variables. APD_{50} was strongly reduced by high $[K^+]_o$ so that at 8 mM $[K^+]_o$, APD_{50} was only 49.7% of the value at 3 mM $[K^+]_o$ (Fig. 4*D*). The $+V_{max}$ was even more drastically depressed by $[K^+]_o$: at 8 mM $[K^+]_o$ $+V_{max}$ was only 11.2% of the value at 3 mM $[K^+]_o$ (Fig. 4*E*). The maximum rate of repolarization ($-V_{max}$) was not significantly changed at 5.4 or 8 mM $[K^+]_o$ (Fig. 4*E*). The overshoot and amplitude of AP were decreased by increasing $[K^+]_o$ (Fig. 4*F*). Notably, in 3 cells out of 17, all-or-none APs could not be triggered at all at 8 mM $[K^+]_o$ at 24°C. The response of these cells to increasing stimulus strength was a gradual increase in AP amplitude, and the APs were characterized by slow $+V_{max}$ (Fig. 5).

Potassium currents at different $[K^+]_o$. The effects of $[K^+]_o$ on the two major repolarizing K^+ currents I_{K1} and I_{Kr} of the fish cardiac myocytes were examined at 4 and 24°C (Fig. 6). At both experimental temperatures, increasing $[K^+]_o$ increased the density of both I_{K1} and I_{Kr}, even though the effects were clearly stronger at 24°C. At 24°C, the inward I_{K1} was increased from -8.47 ± 0.63 at 3 mM $[K^+]_o$ to -18.80 ± 1.33 and -30.09 ± 2.18 pA/pF at 5.4 and 8 mM $[K^+]_o$, respectively (Fig. 6*B*). Similarly, the outward I_{K1} was increased from 3.04 ± 0.17 at 3 mM $[K^+]_o$ to 4.03 ± 0.28 and 4.98 ± 0.35 pA/pF at 5.4 and 8 mM $[K^+]_o$, respectively (Fig. 6*C*). Similar changes in I_{K1} were noticed at 4°C, although the absolute densities of current were smaller than at 24°C ($P < 0.05$) (Fig. 6, *B* and *C*). Also, the density of I_{Kr} increased when $[K^+]_o$ was raised. At 24°C, the outward tail current of I_{Kr} was increased from 1.18 ± 0.24 pA/pF at 3 mM $[K^+]_o$ to 2.47 ± 4.7 and 3.46 ± 0.52 pA/pF at 5.4 and 8 mM $[K^+]_o$, respectively (Fig. 6*F*). Similar changes in I_{Kr} were noticed at 4°C, although the absolute densities of current were smaller than at 24°C ($P < 0.05$) (Fig. 6, *E* and *F*).

DISCUSSION

Electrical excitation of atrial and ventricular myocytes is mainly governed by two opposing and interdependent currents, the fast Na^+ current (I_{Na}) and the inward rectifier K^+ current (I_{K1}). I_{K1} determines the RMP and increase in I_{K1} raises the AP threshold, while I_{Na} determines the rate of AP propagation and increase in I_{Na} lowers the AP threshold (42). Furthermore, I_{K1} indirectly affects the density of I_{Na} via its effect on RMP: the more negative the RMP the larger number of Na^+ channels available for opening. Our previous studies on fish hearts suggest that electrical excitability, in particular, the function of Na^+ channels is compromised at critically high temperatures as in brown trout (*Salmo trutta fario*) and roach, as formulated in the hypothesis of temperature-dependent deterioration of

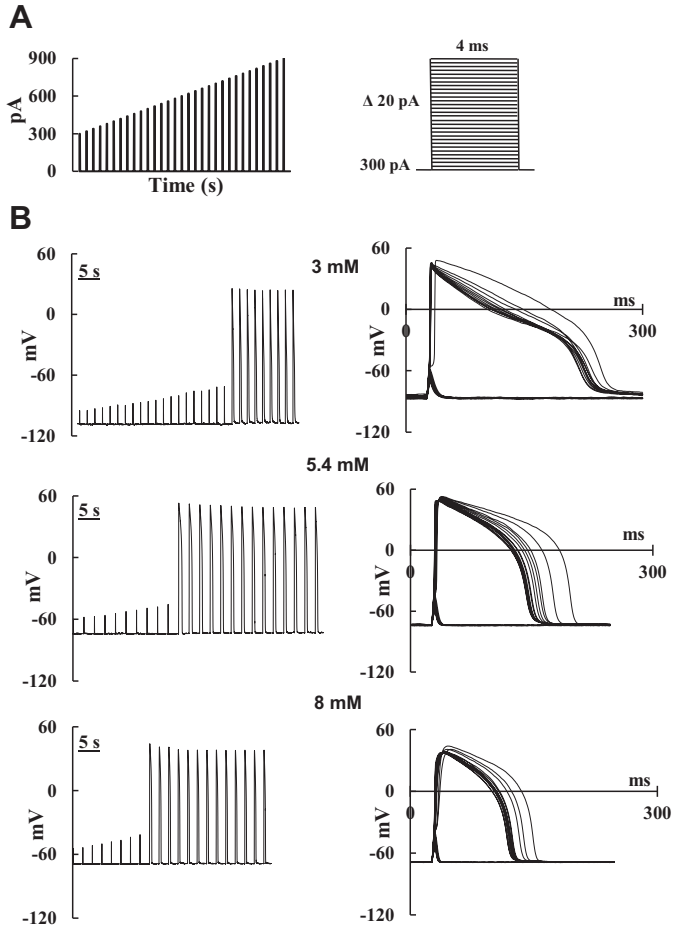

Fig. 3. Effects of extracellular K^+ concentration ($[K^+]_o$) on excitability of roach ventricular action potential, representative experiments. *A*: the stimulus protocol used to trigger action potentials: increasing strength of 4-ms square current pulses. *B*: examples of voltage responses to increasing stimulus strength in the physiological saline solution containing 3, 5.4, and 8 mM K^+. *Left* and *right*: slow and fast time-base recordings of action potential, respectively.

electrical excitability (2–4, 44, 46). However, the heat sensitivity of I_{Na} alone could not explain the temperature-induced deterioration of excitability of the roach heart, suggesting that additional factors are involved (4). The present results show that relatively minor increases in $[K^+]_o$ can severely compromise electrical excitation of roach ventricular myocytes. Therefore, the deterioration of excitability of the roach heart in vivo at high temperatures could be partly due to rate-dependent accumulation of $[K^+]_o$ in the diffusion-limited paracellular space (2, 25). However, putative changes in $[K^+]_o$ need to be verified by direct ion measurements. The present findings are also relevant to other stress situations where changes in $[K^+]_o$ may occur; vigorous exercise, catch-and-release angling, air exposure, and handling stress are known to raise $[K^+]_o$ in fish with potentially depressing effects on cardiac function as in blue shark and yellowfin tuna (11, 41, 47).

Effects of high temperature. Cardiac AP is triggered, when the stimulus current is sufficiently large to depolarize myocyte sarcolemma to the voltage, where the inward I_{Na} exceeds the outward K^+ currents, mainly the I_{K1} (42, 44). Furthermore, the propagation of AP requires that in each myocyte the rate of AP upstroke is sufficiently fast and large (positive) to excite the downstream cell(s) of the multicellular cardiac tissue (42). The present results show that the excitation threshold, measured as the minimum current strength, was not affected by increase of temperature from 4 to 14°C. However, at 24°C, which exceeds the break point temperature of heart rate in the winter-acclimatized roach (2), the excitation threshold was ~25% higher than at 4 and 14°C. This change was associated with a significant increase in critical depolarization, i.e., the voltage difference between TP and RMP. Notably, critical depolarization was higher despite the hyperpolarization of the RMP, which increases the availability of Na^+ channels for opening and therefore the density of I_{Na} (21). TP, the take-off voltage of the all-or-none AP, also hyperpolarized at 24°C but less than the RMP, thus increasing the gap between RMP and TP. Since I_{Na}-I_{K1} antagonism is determining for the initiation of AP (17, 42), the temperature-induced elevation of critical depolarization suggests that I_{K1} increases proportionally more than I_{Na} when temperature approaches or exceeds the break point temperature of heart rate (44). From the other AP parameters, $+V_{max}$ strongly increased and APD_{50} decreased with rising temperature, as expected. These changes enable higher rates of AP propagation and therefore higher heart rates and contraction velocities when temperature acutely rises. The increase of $+V_{max}$ between 14 and 24°C may seem contradictory to the earlier findings, which showed that I_{Na} (elicited from a constant holding potential of −120 mV) starts to decline at temperatures above 20.3°C (4). The probability of Na^+ channel opening (steady-state inactivation) is strongly dependent on membrane potential between −100 and −60 mV (21). Therefore, the strong hyperpolarization at 24°C increases the number of Na^+ channels, which can open and compensate to some extent for the temperature-dependent decline of I_{Na}.

Effects of pacing frequency. Pacing rates, which simulated the temperature-specific heart rates in vivo, had no significant effect on excitability. This shows that activation and inactivation kinetics of ion channels do not limit electrical excitability of isolated roach ventricular myocytes, when bathed in the external medium of constant ion composition. However, the situation may be different in the multicellular tissue where cells are tightly packed within the small and diffusion-limited extracellular space. In the intact heart, rate-dependent changes in external and internal ion composition are likely to occur (25). In addition to changes in $[K^+]_o$, the concentration of intracellular Na^+ is expected to change in frequency-dependent manner (10), which might affect APD in fish as in rainbow trout and bluefin tuna (*Thunnus orientalis*) (19, 37).

Effects of high $[K^+]_o$. The tested K^+ concentrations of the external saline (3, 5.4, and 8 mM) cover the $[K^+]_o$ levels of the resting unstressed fish and the exercise-stressed captured fish as in yellowfin tuna (23, 47). The current-clamp experiments showed that $[K^+]_o$ at the concentrations of 5.4 and 8 mM promotes AP generation: the strength of the stimulus current and amplitude of the critical depolarization were significantly decreased in high $[K^+]_o$. The reduced excitation threshold was associated with strong depolarization of both RMP and TP. Since depolarization of RMP from about −90 mV (3 mM $[K^+]_o$) to −60 mV (8 mM $[K^+]_o$) markedly reduces the availability of Na^+ channels for opening, and therefore the density of I_{Na} (21), the reduction in critical depolarization must be due to the shift in the absolute value of the TP. At 3, 5.4, and

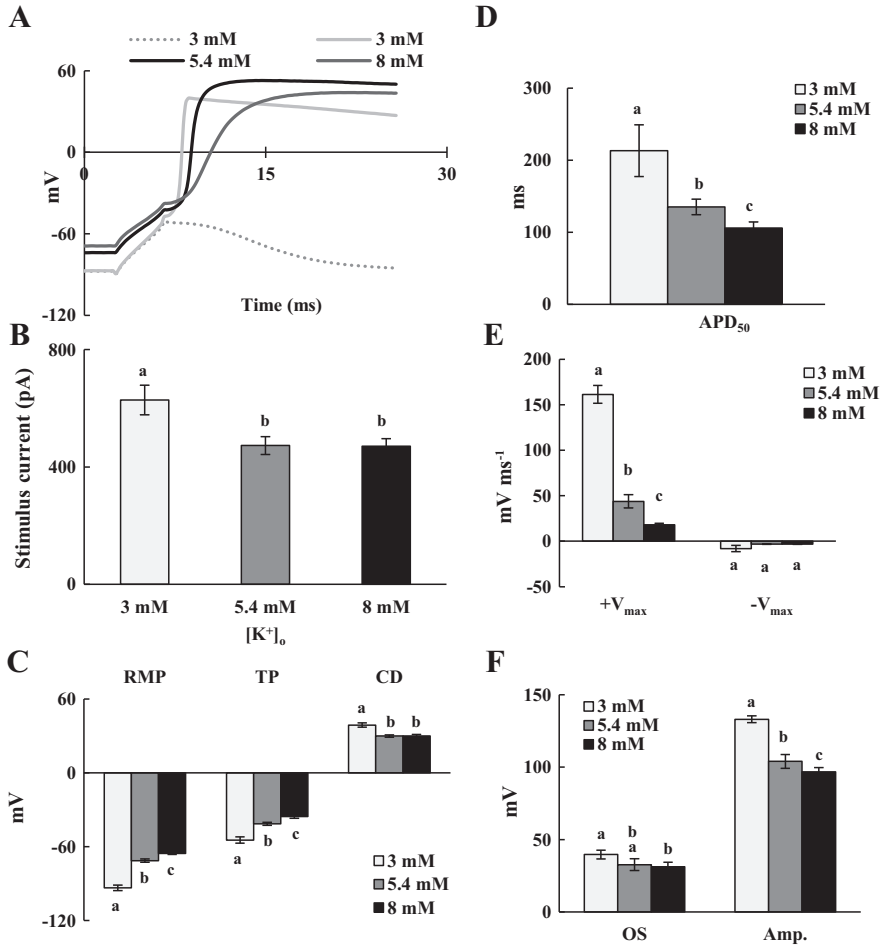

Fig. 4. Effects of extracellular K^+ concentration ($[K^+]_o$) on action potential (AP) parameters of roach ventricular myocytes at 24°C and at the pacing frequency of 1.96 Hz. *A*: representative recordings of ventricular APs in the presence of 3, 5.4, and 8 mM $[K^+]_o$. Dotted line represents a passive membrane response to subminimal stimulus current. *B*: the strength of stimulus current needed to trigger APs in the presence of different K^+ concentrations. *C–F*: resting membrane potential (RMP), threshold potential (TP), and critical depolarization (CD) (*C*); AP duration (APD_{50}) (*D*); maximum rate of AP upstroke ($+V_{max}$) and repolarization ($-V_{max}$) (*E*); and overshoot (OS) and AP amplitude (Amp.) of AP (*F*) in the presence of different K^+ concentrations. Results are means ± SE of 10–17 cells from 4 fish. Dissimilar letters indicate statistically significant differences ($P < 0.05$) between different K^+ concentrations (*t*-test for paired samples).

8 mM $[K^+]_o$, the AP threshold was about −55, −41, and −35 mV, respectively. This depolarizing shift of TP means that at 8 mM $[K^+]_o$ the membrane potential is very close to the value, where I_{Na} reaches its peak amplitude (21). Even though the depolarization of the RMP reduces the availability of Na^+ channels, I_{Na} is still sufficiently large to exceed the density of outward K^+ current and trigger AP, when it is triggered at the optimum voltage of Na^+ channel conductance.

Although the current threshold for AP initiation was lowered by 5.4 and 8 mM $[K^+]_o$, in other respects the electrical excitability of ventricular myocytes was strongly depressed in high $[K^+]_o$. In particular, this appeared in the drastic depression of $+V_{max}$ and to a lesser extent also in reductions of amplitude, overshoot, and duration of AP. $+V_{max}$ was reduced as much as 72.8 and 88.8% at 5.4 and 8 mM $[K^+]_o$, respectively. Indeed, $+V_{max}$ at 8 mM $[K^+]_o$ at 24°C was only ~47%

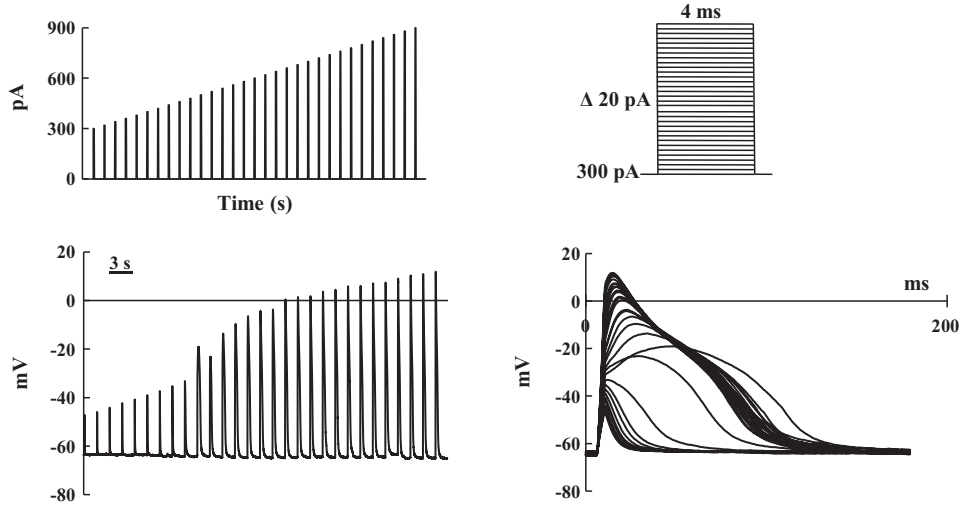

Fig. 5. An example of ventricular myocyte that did not respond to current stimuli with an all-or-none action potential at 24°C, pacing frequency of 1.96 Hz, and 8 mM extracellular K^+. The amplitude of action potential was graded with the strength of the current pulse, and the rate of action potential upstroke was slow. This type of response was found in 3 cells out of 17.

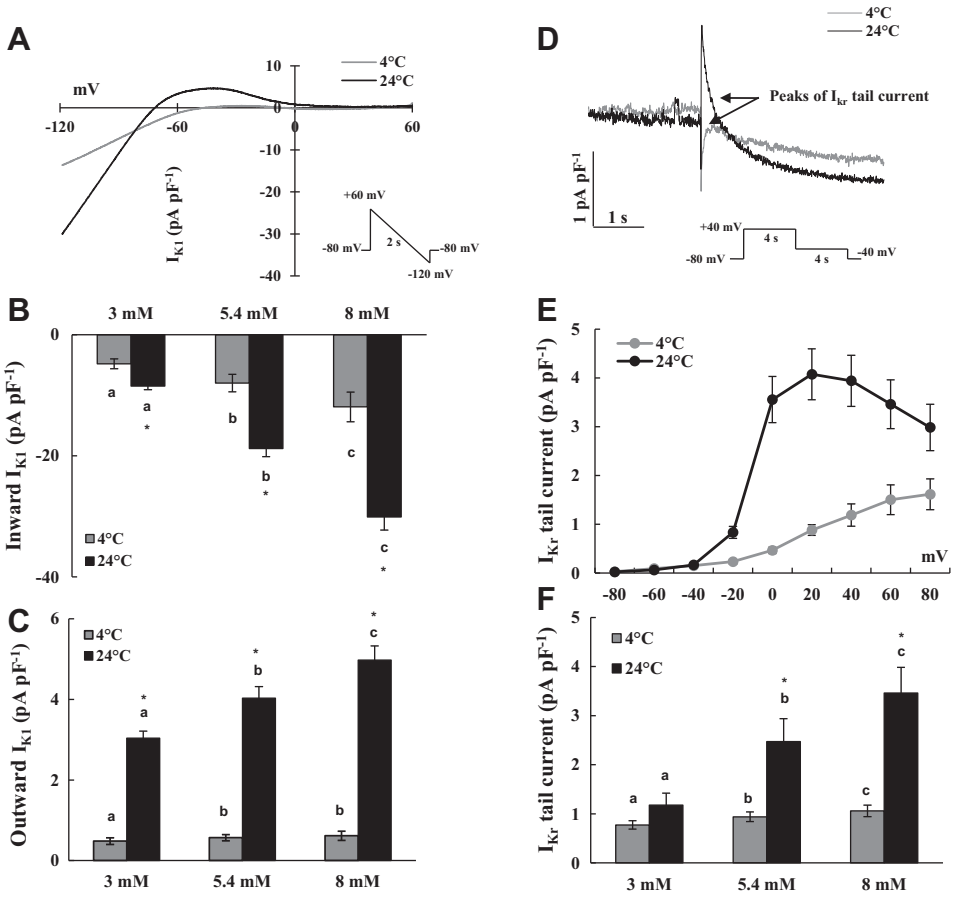

Fig. 6. Effects of extracellular K^+ concentration ($[K^+]_o$) on the density of ventricular inward rectifier K^+ current (I_{K1}) and delayed rectifier K^+ current (I_{Kr}) at 4°C and 24°C. *A*: representative I_{K1} recordings in physiological saline solution containing 8 mM K^+ at 4 and 24°C. *Inset*: voltage ramp used to trigger I_{K1}. *B* and *C*: the density of inward (*B*) and outward (*C*) I_{K1} at 3, 5.4, and 8 mM $[K^+]_o$. The results are means ± SE of 10–12 cells from 3 to 5 fish. *D*: representative recordings of I_{Kr} tail current in physiological saline solution containing 8 mM K^+ at 4°C and 24°C. *Inset*: voltage ramp used to trigger I_{Kr}. The peak tail current was measured at the beginning of the 4 s pulse to −40 mV. *E*: mean (±SE) tail current-voltage relationships of I_{Kr} at 4°C and 24°C at 8 mM $[K^+]_o$. *F*: the peak outward tail current (means ± SE) at 4 and 24°C in the presence of 3, 5.4, and 8 mM $[K^+]_o$. Results in *E* and *F* are means of 13–15 myocytes from 3 to 4 fish. Dissimilar letters indicate statistically significant differences ($P < 0.05$) between different $[K^+]_o$ (*t*-test for paired samples). *$P < 0.05$, statistically significant differences between the 2 temperatures: 4 and 24°C (one-way ANOVA).

of the $+V_{max}$ at 3 mM $[K^+]_o$ at 4°C. $+V_{max}$ is the main determinant for the rate of AP propagation over the heart and therefore one of the factors that can limit heart rate. The precipitous depression of $+V_{max}$ at 8 mM $[K^+]_o$, if occurring in stressed fish, would probably prevent attaining the heart rate of 116 beats/min measured for the nonexercising roach at 24°C (2). Hanson et al. (18) have shown that strong β-adrenergic stimulation (0.5 μM epinephrine) can counteract the negative chronotropic effects of hyperkalemia in rainbow trout in vivo. However, I_{Na} and I_{K1} are not markedly modified by the adrenergic system, and therefore, it remains to be shown whether epinephrine can ameliorate the depressive effects of high $[K^+]_o$ on cardiac excitability.

In the most extreme case (3 cells) 8 mM $[K^+]_o$ totally prevented the generation of propagating APs. This is understandable based on activation and inactivation kinetics of Na^+ channels. Since activation is ~10 times faster than inactivation (21), during a normal fast-rising AP the inactivation process is unable to prevent the regenerative and rapid increase of Na^+ channel opening. However, 8 mM $[K^+]_o$ depolarize RMP and reduce I_{Na} (via steady-state inactivation or reduced availability) making $+V_{max}$ much slower. The slow rise of AP (depolarization) allows more time for steady-state inactivation, which further reduces I_{Na}. This phenomenon is known as accommodation (5, 13). It is obvious that complete abolition of the all-or-none APs, even if present in a limited number of ventricular myocyte, would cause conduction blocks and unexcitable tissue areas in the ventricle. In mammalian cardiac preparations, high $[K^+]_o$ (7 mM) depolarizes membrane potential, reduces AP overshoot, and depresses conduction velocity of AP (12, 39).

The strong effects of increased $[K^+]_o$ on the excitability of ventricular myocytes were associated with prominent increases in the density of I_{K1} and I_{Kr}. Increases in outward I_{K1} and I_{Kr} explain the shortening of AP in high $[K^+]_o$. It should be noted that in the heat-stressed fish heart as in yellowfin tuna the putative heart rate-dependent increases in $[K^+]_o$ (and thence in I_{K1} and I_{Kr}) would be additive to the temperature-induced increases in these currents (4). The balance between I_{Na} and I_{K1} would be further distorted by $[K^+]_o$. Increases in I_{K1} also affect passive membrane properties; specific membrane resistance and space constant decrease, and I_{Na} is less able to depolarize the membrane of the downstream myocytes, because of the current sink of the downstream cells increases.

Importance of $[K^+]_o$ in thermal stress. I_{Na} is the most heat-sensitive ion current in roach and brown trout cardiac myocytes (4, 46). It is depressed at temperatures where the densities of the outward K^+ currents I_{K1} and I_{Kr} are still increasing. This may cause an imbalance between inward and outward currents and potentially impair excitability of the fish heart as in roach and brown trout (2, 44).

Rising temperature increases heart rate, which may increase K^+ leakage from myocytes due to the frequent recruitment of the repolarizing K^+ currents (25). Accumulation of $[K^+]_o$ in the paracellular space depolarizes RMP and reduces the availability of Na^+ channel for opening and hence I_{Na}. Furthermore, the high $[K^+]_o$ will increase the density of I_{K1} and I_{Kr}. This means that the temperature-dependent mismatch between de-

polarizing and repolarizing currents will get worse, if the temperature rise is associated with an increase in $[K^+]_o$. Indeed, the rate-dependent increase in the paracellular $[K^+]_o$ could be one of the critical factors that contributes to the temperature-dependent deterioration of heart rate in roach and other fish (2, 44, 46).

Importance of $[K^+]_o$ in other stresses. Elevation of $[K^+]_o$ is a regular finding in exercise and capture-stressed fish as in yellowfin tuna (41, 47). This rise is due to the increased activity of skeletal muscles; frequent contractions of large skeletal muscle masses are associated with extensive recruitment of repolarizing K^+ currents resulting in K^+ leakage from muscle fibers. In mammals, the leak occurs through the delayed rectifier K^+ channels or via ATP-sensitive K^+ channels (6, 26, 27). K^+ accumulate in the blood because the clearance rate of K^+ from the plasma does not match the rate of K^+ release. Indeed, the plasma K^+ remains significantly elevated for several hours after the cessation of the exercise in fish (38, 41). $[K^+]_o$ may also increase at the tissue level in the heart due to exercise-induced increase in heart rate, although the increase may not be as prominent as in response to temperature rise (8, 40). Because of the systemic and tissue level increases in K^+ leakage, the heart of exercised-stressed fish is exposed to high $[K^+]_o$ which may impair cardiac excitability. The risk for cardiac malfunction is expected to be particularly high, when strenuous exercise occurs at high water temperature. Indeed, studies on catch-and-release angling have shown that the mortality of released fish is especially high if fish exercise at elevated temperatures (1, 7, 11). For example, the recovery of adult Atlantic salmon from angling stress was practically complete at 8 and 16°C (survival rate 100%) but poor at 20°C (survival rate 20%). Interestingly, immediately after angling the heart rate of the salmon at 20°C dropped below the resting level and was very irregular (1). In a recent study on the white marlin (*Kajikia albida*), Schlenker et al. (35) measured plasma ions and lactate immediately after angling stress and found the only factor that predicted postrelease mortality of the fish was the elevated plasma K^+ concentration. The same conclusion seems to be valid for several highly active marine fish like yellowfin tuna (47). Thus it seems likely that high temperature and elevated plasma $[K^+]_o$ synergistically contribute to the postexercise mortality of fish. We believe that our current results provide a mechanistic explanation for the postexercise depression of heart rate and poor survival of fish after the release.

Summary of the findings. The effects of temperature and high $[K^+]_o$ on the excitability of roach ventricular myocytes are partly antagonistic and in some respects synergistic. High temperature hyperpolarizes RMP, increases $+V_{max}$, and elevates excitation threshold, while high $[K^+]_o$ depolarizes RMP, depresses $+V_{max}$, and reduces excitation threshold. APD_{50} is strongly shortened, and densities of I_{K1} and I_{Kr} are increased by both high temperature and high $[K^+]_o$. However, the depressing effects of high $[K^+]_o$ are so strong that they override the positive effects of high temperature on RMP and $+V_{max}$. Therefore, the effects of high $[K^+]_o$ predominate. Indeed, the electrophysiological properties of roach ventricular AP are very sensitive to small changes in $[K^+]_o$. All effects of $[K^+]_o$ are already strongly expressed with the shift from 3 to 5.4 mM and further to 8 mM $[K^+]_o$. Eight millimoles of $[K^+]_o$ are most probably cardiotoxic to roach, since $+V_{max}$ is severely depressed and some ventricular myocytes become unexcitable and cannot generate propagating APs.

Perspectives and Significance

The present findings clearly indicate that small changes in $[K^+]_o$ have a major impact on electrical excitability of roach ventricular myocytes. Because basic features of electrical excitation are common to all excitable cells, the present findings are probably valid for neurons and muscle cells. Therefore, future studies should examine the combined effects of $[K^+]_o$ and temperature on muscular and neuronal excitability. Those studies could reveal the impact of environmental and physiological stresses on locomotion, sensory function, behavior, and fitness of ectotherms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.B., E.-S.A.-A., M.F.E.-S., and M.V. conceived and designed research; A.B. performed experiments; A.B. analyzed data; A.B. and M.V. interpreted results of experiments; A.B. prepared figures; A.B. and M.V. drafted manuscript; A.B. and M.V. edited and revised manuscript; A.B., E.-S.A.-A., M.F.E.-S., and M.V. approved final version of manuscript.

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