Electrophysiological properties of rainbow trout cardiac myocytes in serum-free primary culture

ANTI NURMI AND MATTI VORNANEN
University of Joensuu, Department of Biology, 80101 Joensuu, Finland

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Nurmi, Antti, and Matti Vornanen. Electrophysiological properties of rainbow trout cardiac myocytes in serum-free primary culture. Am J Physiol Regulatory Integrative Comp Physiol 282: R1200–R1209, 2002; 10.1152/ajpregu.00350.2001.—A low-density primary culture of trout ventricular myocytes in serum-free growth medium was established and maintained for up to 10 days at 17°C. The myocytes retained their normal rod shaped morphology, capacitative surface area of the sarcolemma (SL), and contractile quiescence. However, sarcolemmal cation currents changed significantly, some permanently, some transiently, after 8–10 days of culture. TTX-sensitive sodium current ($I_{Na}$) and $Ba^{2+}$-sensitive background inward rectifier potassium current ($I_{K1}$) were permanently depressed to 24–28% of their control density measured in freshly isolated myocytes. In contrast, L-type calcium current ($I_{Ca}$) was only transiently downregulated; after 2–3 days in culture, the density of the current was 32% of the control and recovered to the control value after 8–10 days in culture. The changes in membrane currents were reflected in the shape of the action potential (AP). After 2–3 days in culture, maximal overshoot potential and resting potential were significantly reduced, and the durations of the AP at 50 and 90% repolarization were significantly increased. These changes became significantly more pronounced after 8–10 days of culture, with the exception of AP duration at 50% repolarization level. The shortening of the early plateau phase may reflect an additional current, presumably the rapid component of the delayed rectifier ($I_{K2}$). Although the present findings indicate that fish cardiac myocytes can be maintained in serum-free primary culture for at least 10 days at 17°C, some but not all of the electrophysiological characteristics of the myocytes change markedly during culture. The changes in ion currents were not due to loss of sarcolemmal membrane and therefore are likely to represent altered expression of cation currents as an adaptive response to culture conditions.

cultured cardiac cells; electrophysiology; fish heart; cation currents; action potential

CELL CULTURE OFFERS A SIMPLE environment compared with animal body and therefore a potentially useful in vitro system to examine adaptation mechanisms of the cardiac myocyte to altered environmental conditions. Unlike the complexity of whole animal models, cell culture allows better control of external factors that could help in revealing cause-effect relationships of physiological adaptation processes. Indeed, ventricular myocytes of adult mammals can be maintained in serum-supported culture media for weeks to months (for review, see Refs 13, 16). However, for studying adaptational changes, the presence of serum, which contains a number of growth factors, hormones, and other undetermined components, makes the culture system a nondefined environment and therefore limits its value as a research tool. In contrast, a serum-free cell culture provides a better environment to study the adaption of cardiac myocytes to changing conditions. The goal here was to ascertain, for the first time, whether 1) fish cardiac myocytes could be held in serum-free culture and 2) to examine the electrophysiological stability of fish myocytes held in serum-free conditions.

Mammalian cardiac myocytes have been recently cultured for up to 6 days in serum-free media (15). The adult mammalian cardiac myocytes show several morphological, contractile, and electrophysiological changes during the culture and some of the electrophysiological changes seem to be closely associated with ultrastructural transformations induced by culture conditions (16). For example, the extensive t-tubular system of the adult cardiac myocytes is practically lost during cell culture, resulting in a marked reduction of sarcolemmal surface area (5, 15). The loss of t-tubules may also be responsible for the suppression of inward rectifier K⁺ current ($I_{K1}$) in mammalian cell cultures, as the channels are almost exclusively located in the t-tubular membrane (5, 15, 22). However, fish cardiac myocytes lack t-tubules (18), and therefore the prediction is that the electrophysiological modifications during culture will be smaller. To assess preservation of ion channel currents, action potential (AP) shape and capacitative cell surface area, ventricular myocytes of the rainbow trout heart were cultured in serum-free medium for up to 10 days.

Although there were no changes in capacitative cell surface area during culture, a sequence of changes in sarcolemmal cation currents occurred, suggesting that culture-induced modifications of ion currents are not due to the loss of sarcolemmal membrane area, but rather due to the altered expression of channel proteins under culture stresses.

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MATERIALS AND METHODS

Isolation of ventricular myocytes. Female rainbow trout (n = 13, body mass between 101 and 154 g) were obtained from a local fish farm (Kontiolahti) and acclimated to 17–18°C for at least 3 wk before the experiments. Fish were maintained under a constant 15:9-h light-dark photoperiod in 500-liter stainless steel tanks provided with circulating (~0.5 l/min) and aerated tap water. The fish were fed daily with a commercial trout chow (Ewos; Turku, Finland). Ventricular myocytes were obtained by enzymatic isolation procedure described in detail in earlier publications (23, 24). Isolation procedures were conducted with aseptic instruments and equipment to avoid microbial contamination. All solutions were sterilized and filtered before use. Briefly, the fish was stunned with a blow to the head and the spine was cut. The heart was quickly dissected and perfused retrogradely with oxygenated (100% O₂) nominally Ca²⁺-free isolation solution (in mM: 100 NaCl, 10 KCl, 1.2 KH₂PO₄, 4 MgSO₄, 50 taurine, 20 glucose, and 10 HEPEs at pH 6.9) for 8–10 min followed by enzymatic digestion with collagenase IA (Sigma, 0.75 mg/ml) and trypsin III (Sigma, 0.5 mg/ml) for 20 min at room temperature (~20°C). The isolation solution was supplemented with fatty acid-free BSA (Sigma; 0.75 mg/ml) and antibiotics (50 IU/ml of streptomycin and penicillin). The enzymatically digested ventricle was minced with scissors in small pieces, and single myocytes were liberated in fresh and oxygenated isolation solution by trituration of ventricular pieces through the opening of a Pasteur pipette. The number of viable cells, determined by Trypan blue (Sigma-Aldrich) exclusion in a hemocytometer (Bürker, Kebolab), accounted for 50–70% of the whole cell population. Isolated cells were suspended in sterile isolation solution at the density of ~200,000 cells/ml. Cells were punched from the璧 membrane or cultured on glass coverslips (12 mm in diameter) in plastic culture dishes (Cellstar7; 35/10 mm; Greiner Labortechnik). Four coverslips with a total number of 50,000 cells/ml. The isolation solution (in mM) 140 KCl, 5 Na₂ATP, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.6 adjusted with NaOH was used. Nifedipine (10 μmol/l; Sigma) and TTX (1 μmol/l; Alomone Labs, Israel) were included, respectively, when recording INa and ICa-L. APs and K⁺ currents were recorded in K⁺-based external solution (in mM: 150 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPEs, and 10 glucose, pH 7.6 adjusted with NaOH) was used. Nifedipine (10 μmol/l; Sigma) and TTX (1 μmol/l) were added to this solution when K⁺ currents were examined. APs were stimulated at 0.2 Hz with 1-ms current pulses just exceeding the AP threshold (~1 nA).

The pipette solution for INa and ICa-L recordings was as follows (in mM): 130 CsCl, 15 TEA, 5 MgATP, 1 MgCl₂, 5 oxaloacetate, 5 EGTA, pH 7.2 adjusted with CsOH. For the recording of K⁺ currents, the pipette solution contained (in mM) 140 KCl, 4 MgATP, 1 MgCl₂, 5 EGTA, 10 HEPEs, pH 7.2 adjusted with KOH. Current clamp recording of APs was performed with a pipette solution that contained (in mM) 140 KCl, 5 Na₂ATP, 1 MgCl₂, 10 HEPEs at pH 7.2 adjusted with KOH.

Glass pipettes were pulled from borosilicate capillaries (Modulohm) using a two-stage vertical puller (Narishige MF 83). Pipette resistance was 2–3 MΩ when filled with internal solutions. In AP recordings, the potential difference (~7 mV) between the bath solution and the pipette solution was corrected in the final results. Voltage and current clamp experiments were made using an Axopatch 1D amplifier (Axon Instruments) equipped with a CV-4 1/100 head stage (Axon Instruments). Junction potentials were zeroed before formation of the gigaohm seal. The pipette capacitance (4–6 pF) was compensated for after the seal formation. The patch was ruptured by delivering a short (0.1–1 ms) voltage pulse (1.5 V) to the cell. Capacitive transients were eliminated by iteratively adjusting the calibrated series resistance and whole cell capacitance circuits. The cell capacitance was read directly from the dial of the amplifier. APs and INa recordings were low-pass filtered at 10 kHz, inward rectifier potassium current (IK1) and ICa-L at 2 kHz. Current traces were sampled with an analog-to-digital converter (TL-1 DNA, Axon Instruments) and stored on the hard drive of a personal computer for offline analysis using the pClamp 6.04 software (Axon Instruments).

Data analysis. Kinetic parameters of steady-state activation as well as current-voltage relationships for both INa, and ICa-L, were obtained from square pulse recordings (see RESULTS). Activation voltage-dependence, dV(V), was determined as normalized Na⁺ or Ca²⁺ conductance, dV(V) = GNa or Ca-L /Gmax where Gmax is the maximum value of INa or ICa-L conductance. The voltage dependence of the peak conductance for both currents was calculated using the equation

\[ G_{Na or Ca-L} = I_{Na or Ca-L}/(V - V_{rev}) \]

where GNa or Ca-L is Na⁺ or Ca²⁺ conductance, INa or Ca-L is the peak Na⁺ or Ca²⁺ current at a given potential (V), and Vrev is the apparent reversal potential obtained by extrapolating the ascending portion of the current-voltage (I-V) relationship of Na⁺ or Ca²⁺ current to zero current. Steady-state activation parameters were obtained by fitting the obtained data to Boltzmann equation

\[ d_a(V) = 1/(1 + \exp[(V_{0.5} - V)/k]) \]

where V0.5 is the half-activation potential and k is the slope factor. Statistical analysis. Statistical comparisons between freshly isolated trout ventricular cells (control) and from both...
groups of cultured cells were made by using a one-way ANOVA followed by Dunnet’s post hoc analysis. In the case of unequal variances between groups, a two-tailed Student’s t-test was used. A P value of ≤0.05 was considered as statistically significant.

RESULTS

Cell morphology during culture. Trout ventricular cells adhered firmly on clean, uncoated glass coverslips. After 1–2 h of plating, the myocytes were so firmly attached to the glass that removal of an individual cell was impossible without breaking it. The majority of the ventricular cells maintained their rod shape morphology for 8–10 days of culture, even though the cell endings became slightly rounded in some cells (Fig. 1). Extensive branching or spreading of the cells on the glass was rare. No cell-to-cell contacts were observed, and the myocytes did not beat spontaneously.

Culture conditions did not significantly affect sarcolemmal surface area as whole cell capacitance was unchanged during the 10-day culture period (Fig. 2).

Changes in resting membrane potential and APs during culture. APs recorded from freshly isolated cells displayed a rapid upstroke, a large overshoot (OS) with a maximum value of $59.9 \pm 3.79 \text{ mV (n = 15)}$ and a long plateau phase between +40 to +10 mV before repolarization (Fig. 3). The duration of the AP (APD) at 50 and 90% repolarization was 262.6 ± 21.2 and 306.6 ± 28.7 ms (n = 15), respectively. The mean resting membrane potential (RP) of the freshly isolated myocytes was $-75.2 \pm 0.82 \text{ mV}$. After 2–3 days of culture, changes in RPs and APs were evident in trout ventricular myocytes (Fig. 3). RP was less negative, OS was reduced, and APD was markedly increased. Subsequent recordings from cells cultured for 8–10 days (Fig. 3) revealed progressively more depolarized RPs, decreased OSs, and increased APD at 90% repolarization level. There was, however, no statistical difference in APD at 50% repolarization level between freshly isolated cells and cells cultured for 8–10 days (Table 1).

Changes in $I_{Na}$ during culture. In freshly isolated myocytes, 10-ms depolarizing pulses from a holding potential (HP) of $-80 \text{ mV}$ to various potentials between −120 and +60 mV generated a fast, transient,
and large (6–10 μA) inward current (Fig. 4). This current was completely blocked by a relatively low concentration (1 μM) of TTX and, therefore, represent typical $I_{\text{Na}}$ of fish cardiac myocytes (23). The I-V relationship was bell shaped, with a threshold around −50 mV, the peak current density at −10 mV, and the apparent reversal potential at +90 mV (Fig. 4; Table 2). The potential for half-maximal activation ($V_{0.5}$) occurred at $-25.97 \pm 1.24$ mV with a slope (k) of 5.38 ± 0.15 (n = 5). The inactivation rate of current at peak $I_{\text{Na}}$ (−10 mV) was fitted to a double exponential function, which gave mean time constants of 0.177 ± 0.01 ms and 0.867 ± 0.01 ms for the fast ($\tau_f$) and slow component ($\tau_s$) of current decay, respectively (Fig. 5).

In myocytes cultured for 2–3 days, the density of $I_{\text{Na}}$ was only 53% of that measured in freshly isolated cells, and the peak current density was shifted to more positive voltages (Fig. 4; Table 2). The slope factor of the steady-state activation curve was also increased, but the $V_{0.5}$ was not significantly different from the value of freshly isolated cells. Although the time constants of current inactivation at the peak density of $I_{\text{Na}}$ (0 mV) were somewhat slower than in control cells, the differences were not statistically significant at this phase of culture (Table 2).

After 8–10 days in culture, the peak density of $I_{\text{Na}}$ was further shifted to +30 mV, and the current density was further reduced to 24% of the value in freshly isolated myocytes (Fig. 4). The voltage-dependence of steady-state activation ($V_{0.5}$) was now significantly shifted to the right, and the slope remained less steep than in freshly isolated myocytes (Fig. 4). The rate of current inactivation in myocytes cultured for 8–10 days was also much slower than in freshly isolated cells (Table 2).

The progressive decrease in density of $I_{\text{Na}}$ correlated strongly with the diminishing OS of the AP during the culture (Fig. 6).

Changes in $I_{\text{K1}}$ during culture. K$^+$ currents were elicited in the presence of TTX and nifedipine by square-wave voltage steps (500 ms) from the HP of −80 mV to various potentials between −120 and +20 mV. Hyperpolarizing voltage steps between −20 and −80 mV elicited a noninactivating, Ba$^{2+}$-sensitive inward current with nearly linear I-V relationship (Fig. 7). The slope conductance of the current in freshly isolated myocytes was 0.626 ± 0.046 nS/pF. Extrapolation of

Table 1. Characteristics of action potentials in freshly isolated and cultured ventricular myocytes of the rainbow trout heart

<table>
<thead>
<tr>
<th>AP Parameter</th>
<th>Control</th>
<th>2–3 day</th>
<th>8–10 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>APA, mV</td>
<td>135.1 ± 3.35</td>
<td>99.8 ± 2.96$^\circ$</td>
<td>84.7 ± 5.96$^{+\dagger}$</td>
</tr>
<tr>
<td>$APD_{200}$, ms</td>
<td>262.6 ± 21.2</td>
<td>471.2 ± 55.7$^\circ$</td>
<td>283.3 ± 59.4$^\circ$</td>
</tr>
<tr>
<td>$APD_{90}$, ms</td>
<td>306.6 ± 28.7</td>
<td>577.1 ± 68.1$^\circ$</td>
<td>785.7 ± 159.7$^\circ$</td>
</tr>
<tr>
<td>RP, mV</td>
<td>−75.2 ± 0.82</td>
<td>−63.4 ± 1.96$^\circ$</td>
<td>−57.1 ± 1.46$^\circ$</td>
</tr>
<tr>
<td>OS, mV</td>
<td>59.9 ± 3.79</td>
<td>36.3 ± 3.55$^\circ$</td>
<td>27.6 ± 6.3$^\circ$</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE. $n$, no. of cells studied. APA, amplitude of action potential; $APD_{200}$, duration of action potential at 50% of repolarization; $APD_{90}$, duration of action potential at 90% of repolarization; RP, resting potential; OS, the maximum overshoot potential. *Statistically significantly differences ($P < 0.05$) from the value of freshly isolated cells (control) and †between 2 and 3 day cultured and 8 and 10 day cultured cells.

Table 2. Characteristics of $I_{\text{Na}}$ in freshly isolated and culture cardiac cells of the rainbow trout heart

<table>
<thead>
<tr>
<th>$I_{\text{Na}}$ Parameter</th>
<th>Control</th>
<th>2–3 day</th>
<th>8–10 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>density, pA/pF</td>
<td>$-191 \pm 24$</td>
<td>$-103 \pm 10^*$</td>
<td>$-46 \pm 3^*\dagger$</td>
</tr>
<tr>
<td>$V_{0.5}$, mV</td>
<td>$-25.9 \pm 1.2$</td>
<td>$-20.7 \pm 2.1$</td>
<td>$-11.7 \pm 2.63^*\dagger$</td>
</tr>
<tr>
<td>$d(V_{0.5})$, mV</td>
<td>$5.38 \pm 0.14$</td>
<td>$7.56 \pm 0.58^*$</td>
<td>$7.74 \pm 0.55^*\dagger$</td>
</tr>
<tr>
<td>$d(t)$, ms</td>
<td>0.17 ± 0.01</td>
<td>0.49 ± 0.10$^*$</td>
<td>0.52 ± 0.06$^*\dagger$</td>
</tr>
<tr>
<td>$\tau_f$, ms</td>
<td>0.86 ± 0.06</td>
<td>1.77 ± 0.31</td>
<td>2.59 ± 0.43$^*\dagger$</td>
</tr>
<tr>
<td>$\tau_s$, ms</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE. $d(V_{0.5})$, voltage for half-maximal steady-state activation; $d(t)$, the slope factor of steady-state activation; $\tau_f$, and $\tau_s$, fast and slow time constant of the current decay, respectively. *Statistically significantly difference ($P < 0.05$) from the value of freshly isolated cells (control) and †between 2 and 3 day cultured and 8 and 10 day cultured cells.
this linear current to zero current level gave a reversal potential of about $-73 \text{ mV}$. At the positive side of the reversal potential, the current exhibited strong inward rectification. On the basis of the inward rectification and sensitivity to $\text{Ba}^{2+}$, this current represents the background inward rectifier ($I_{K1}$).

Subsequent recordings in cultured myocytes revealed a dramatic reduction of the $I_{K1}$ density (Fig. 7). Compared with freshly isolated cells, after 2–3 days in culture the slope conductance was <36% (0.228 ± 0.056 pS/pF; $P < 0.05$, $n = 9$) and after 8–10 days in culture only 28% (0.175 ± 0.066 pS/pF; $P < 0.05$, $n =$ 10) of the value in freshly isolated cells. The changes in slope conductance of $I_{K1}$ and RP were strongly correlated (Fig. 8).

There was a small but significant increase in outward $\text{K}^+$ current density between 0 and +20 mV in myocytes cultured for 8–10 days compared with freshly isolated ventricular myocytes. This was probably due to a time-dependent outward current that had developed after 8–10 days in culture (Fig. 9). The time-dependent outward current was only slightly reduced by 0.5 mM $\text{Ba}^{2+}$ and it was not blocked by either $\text{Cd}^{2+}$ (25 μmol/l) or verapamil (10 μmol/l) (data not shown).

**Changes in $I_{\text{Ca,L}}$ during culture.** $\text{Ca}^{2+}$ currents were recorded by clamping the membrane potential for 500 ms from the HP of $-50 \text{ mV}$ to various potentials between $-50$ and $+60 \text{ mV}$. In freshly isolated trout ventricular myocytes, a small inward current with inverted bell-shape $I-V$ relationship was recorded. The current activated at around $-40 \text{ mV}$, attained peak density at $+10 \text{ mV}$, and finally approached zero current level at $+60 \text{ mV}$ (Fig. 10). The current was blocked
by 10 μmol/l nifedipine, 25 μmol/l Cd²⁺, and 10 μmol/l verapamil (data not shown). Analysis of steady-state activation parameters gave $V_{0.5} = -6.63 \pm 0.53$ and $k$ of $6.22 \pm 0.07$ ($n = 5$). The rate of current inactivation was biexponential with fast and slow time constants of $27.7 \pm 2.3$ and $95.6 \pm 7.3$ ms, respectively, $n = 8$) (Fig. 11).

During the initial 2–3 days of serum-free culture, the peak density of $I_{Ca-L}$ was reduced by 36%, and the $I-V$ relationship was shifted to the right. Voltage parameter of steady-state activation was also shifted to more positive potentials, but the slope factor was not changed (Table 3). The fast component of current inactivation ($\tau_f$) was unchanged during the first 2–3 days in culture, but the slow component ($\tau_s$) increased significantly during this period (Table 3).

After 8–10 days of culture, the density of $I_{Ca-L}$ had recovered to the level of freshly isolated myocytes. However, the steady-state activation remained shifted to the right, the slope had also increased significantly, and inactivation rate constants ($\tau_f$ and $\tau_s$) were increased significantly compared with the freshly isolated myocytes.

DISCUSSION

Myocyte culture offers a homogenous population of single cells that can be examined in a controlled environment. In principle, the experimenter can change a single factor at a time and record its influence on electrical activity of the myocyte. This requires that the cells are grown in a defined culture medium in a constant physical environment and that the electrophysiological changes associated with myocyte adaptation to culture environment are known. In an ideal case, after an adaptation phase the electrophysiological properties of the cell will achieve a steady state, which can be used as a control for the experimental manipulation.

Two basic methods have been used previously for culturing mammalian cardiac myocytes (see Refs 13 and 16). In the redifferentiation method, the myocytes lose their normal rod shaped morphology after a short time in culture and begin to contract spontaneously in the absence of external stimulation. First, the cells quickly dedifferentiate to a fetal phenotype and then begin to differentiate back toward an adult phenotype. Spontaneous activity and altered morphology indicate that myocytes have lost their native in vivo properties.
In contrast, in the rapid-attachment method the cells start to grow in the adult differentiated state and therefore retain their normal gross morphology and contractile quiescence for a longer time before starting a gradual transformation (dedifferentiation). Another major advantage of the rapid-attachment method is that cells can be grown in de...
The high density of $I_{K1}$ in freshly isolated cells is consistent with our own observations in ventricular myocytes of the rainbow trout acclimated to 17°C (26). The background inward rectifier $K^+$ current is severely and quickly depressed during serum-free culture of the trout myocytes, which is a routine finding in mammalian cardiac cell culture preparations (3, 5, 15, 19, 23). The depression in the density of $I_{K1}$ in cultured mammalian cardiac myocyte is the result of a reduction in the number of active channels (23). On the basis of the delay of block and unblock of $I_{K1}$ by extracellular Ba$^{2+}$ applied rapidly to the cells, it is suggested that the inward rectifier $K^+$ channels of the mammalian ventricular myocytes are located mainly in t-tubules (5). Thus the loss of a greater part of the t-tubes during primary culture could explain the culture-induced depression of $I_{K1}$ in mammalian myocytes (14, 15). The same conclusion cannot, however, be made for the depression of $I_{K1}$ in trout ventricular myocytes that are devoid of t-tubes (18). It is possible that some dedifferentiation toward the phenotype of embryonic myocytes occurred during culture of trout ventricular myocytes. Dedifferentiation could in principle cause the depression of $I_{K1}$ as the expression of the inward rectifier channel is developmentally regulated and less well developed in early developmental stages (3, 28, 29). It is also possible that culture conditions decrease the rate of protein synthesis and/or increase the rate of protein degradation with subsequent depression in the number of functional channels.

There was also a significant increase in outward $K^+$ current at positive voltages (0 to +20 mV) in trout ventricular myocytes cultured for 8 to 10 days. The increase in outward current is accounted for by the time-dependent and Ba$^{2+}$-resistant current that appears in later phases of culture. The time and voltage dependence of this current when superimposed on the background inward rectifier $I_{K1}$ gave a superficial impression of a Ca$^{2+}$ current and therefore we assumed that it might be a nifedipine-insensitive Ca$^{2+}$ current. The resistance of this current to inhibition by verapamil and Cd$^{2+}$ suggests that it is not carried by Ca$^{2+}$ channels. Voltage dependence and time course of activation of this current were similar to those of the rapid component of the delayed rectifier $K^+$ current ($I_{Kd}$), which we have characterized in trout atrial and ventricular myocytes (26). The density of this current is small in ventricular myocytes of trout acclimated to 17°C but it is upregulated when the fish are exposed for longer periods to the cold (4°C) (25). Therefore, it is possible that in serum-free culture the ventricular myocytes undergo a similar physiological change as the ventricular myocytes of the rainbow trout during cold acclimation.

It seems plausible that the changes in the expression of these two functionally similar $K^+$ currents were coupled so that depression of one current results in upregulation of another, thereby maintaining a balance between repolarizing and depolarizing currents in cardiac myocytes. The two currents are not, however, functionally identical, and the mutual replacement will probably alter excitability of the myocytes. Clearly, more studies are needed to resolve the physiological consequences of this altered balance in repolarizing $K^+$ currents in trout ventricular myocytes and the factors that regulate their expression.

Changes in $I_{Na}$. TTX-sensitive $I_{Na}$ causes the fast upstroke and prominent OS of the cardiac AP. The properties of $I_{Na}$ in trout ventricular myocytes are in some respects different from those of mammalian cardiac myocytes. The Na$^+$ channels of the fish heart are more TTX sensitive than their mammalian counterparts as 1 μM TTX completely blocks $I_{Na}$ in fish cardiac myocytes (24) but not in mammalian cardiac cells. Voltage dependence of $I_{Na}$ is also different; the threshold voltage of $I_{Na}$ activation and $V_{0.5}$ of steady-state activation are more positive in trout ventricular myocytes than in mammalian cardiac myocytes.

In trout ventricular myocytes, $I_{Na}$ was dramatically decreased during culture. The correlation between the density of the $I_{Na}$ and the OS of AP suggests that progressive depression of OS is due to the diminished $I_{Na}$. In addition to the decreased $I_{Na}$, the low RP of cultured cardiac cells may also diminish the OS because depolarization reduces the number of Na$^+$ channels that are available for opening.

In mammals, the cardiac Na$^+$ channels seem to be sensitive to culture environment, although the response depends on the culture conditions (20). In trout myocytes, $I_{Na}$ was severely depressed even though the capacitive area of the SL stayed constant for the whole culture period. Therefore, the reduction in $I_{Na}$ must be due to changes in the number of functional Na$^+$ channels or in the conductance of individual channels. Similar for K$^+$ channels, the number of Na$^+$ channels might be reduced due to the depression of protein synthesis or increase in the rate of degradation of the existing channels.

In addition to the depression of $I_{Na}$ density, there were also very prominent changes in the kinetics of $I_{Na}$. $I-V$ relationship was shifted to the right, and the activation and inactivation kinetics became slower when the cells were grown in culture medium. On the extracellular side of the membrane, Na$^+$ channel proteins are conjugated with carbohydrate moieties, which may have a role in ion channel function (see Ref. 4). Sialic acids, which form terminal components of oligosaccharide chains of glycolipids and glycoproteins, are considered to be especially important (1, 21). Indeed, a decrease in the amount of sialic acid on the Na$^+$ channel shifts all voltage-dependent gating parameters such that channels require larger depolarizations to gate (2). Enzymes used for myocyte isolation might also shift the voltage dependence of $I_{Na}$ (7). Therefore, we cannot exclude the possibility that our method of myocyte isolation or culture conditions might have affected sarcolemmal glycolipids and glycoproteins and caused the positive shift in $I_{Na}$ voltage dependence during culture, e.g., by changing the surface charge.

Activation and inactivation rate of $I_{Na}$ decreased during the whole culture period in rainbow trout ventricular myocytes. An increase in the rate of activation
and inactivation kinetics of $I_{Na}$ has been found in cultured ventricular myocytes of the cat heart (20), whereas no changes were evident in the kinetics of $I_{Na}$ in human atrial myocytes during a 5-day culture period (9). In regard to the kinetics of $I_{Na}$, cultured trout myocytes clearly differ from mammalian cardiac myocytes. This might be due to the fact that fish cardiac myocytes express a different (TTX sensitive) isof orm of Na$^+$ channel (24). The mechanisms, which cause the observed alterations in $I_{Na}$ voltage dependence, conductance, time to peak activation and inactivation, remain unknown and must be resolved in further studies.

Changes in $I_{Ca-L}$. L-type Ca$^{2+}$ current provides inward current that is necessary to maintain the long plateau phase of the fish ventricular cells, and it is also indispensable for the excitation-contraction coupling. Density, voltage dependence of activation, kinetics of activation, and decay of the $I_{Ca-L}$ in freshly isolated myocytes are similar as recorded previously in trout ventricular and atrial myocytes (22, 25). After 2–3 days of culture, the density of $I_{Ca-L}$ was decreased, the voltage dependence was shifted to more positive potentials, and current decay was slower compared with freshly isolated trout ventricular myocytes. The decrease in $I_{Ca-L}$ density was only transient as it recovered to the initial control level after 8–10 days in culture. In contrast, the kinetic changes persisted or increased after 8–10 days of culture.

Significant changes in $I_{Ca-L}$ during culture have been observed in a number of mammalian culture preparations. $I_{Ca-L}$ density shows great variability (increase, decrease, or no change) between studies, which might indicate species specificity, but could also be due to differences in culture methods or recording conditions (see Ref. 16). A decrease in the early phase of culture and the subsequent recovery of $I_{Ca-L}$ density to the initial control level of freshly isolated myocytes has been observed in rat and rabbit ventricular myocytes cultured in defined (6) or nonsupplemented media (15). The behavior of $I_{Ca-L}$ in trout ventricular myocytes therefore resembles, at least qualitatively, the $I_{Ca-L}$ density changes of mammalian cardiac myocytes in serum-free cultures, suggesting that regulation of L-type Ca$^{2+}$ channels may be mediated by similar mechanisms in fish and mammalian hearts.

The changes in voltage dependence of steady-state activation and in the rate of activation and decay are very similar for $I_{Ca-L}$ and $I_{Na}$, which suggest that they might have a common origin in the membrane environment of these channels or in the channels themselves, e.g., due to a change in surface charge. The voltage dependence of activation for both $I_{Ca-L}$ and $I_{Na}$, was shifted to more depolarized voltages, which could be due a net decrease in extracellular or a net increase in intracellular negative surface charge. As sialic acids are intrinsic components in the carbohydrate moieties of the ion channel proteins and important anionic binding sites in the external surface of the SL, culture-dependent reduction in the amount of sialic acids or other anionic binding sites could have caused the changes in I-V relationship and current kinetics of $I_{Ca-L}$ and $I_{Na}$. Experiments where surface charges of the SL are screened with different concentrations of external Ca$^{2+}$ could possibly resolve this issue. It should be noted, however, that changes in voltage dependence of $I_{Na}$ and $I_{Ca}$ cannot explain the large depression of $I_{Na}$ and $I_{Ca}$ during culture, as both currents are much smaller in cultured than freshly isolated cells both at strongly positive voltages and at peak current.

**Perspectives**

In contrast to mammalian cardiac myocytes, the depression of K$^+$ and Na$^+$ currents in trout ventricular myocytes was not associated with any changes in capacitive membrane area. Therefore, it seems unlikely that depression of these cation currents is due to the specific loss of surface membrane in areas where the channels are located. Transient downregulation of $I_{Ca-L}$ and appearance of another K$^+$ conductance, probably $I_{K,R}$, during later phases of culture are also inconsistent with the “membrane-loss” hypothesis. Rather, the present results suggest that culture conditions alter the expression of different cation channels without any major changes in the morphology of the myocytes. SL of fish cardiac myocytes have variable amounts of membrane invaginations in the form of caveolae that are thought to be precursors for t-tubules. The caveolae are enriched in a variety of membrane-signaling molecules like G protein-coupled receptors, inositol trisphosphate (IP$_3$) receptors, Ca$^{2+}$-ATPase, and protein kinase C (for review, see Ref. 12). Although there is currently no direct evidence for the presence of ion channels in caveolae of cardiac myocytes in either fish or mammals, the possibility remains that the reduction in the density of $I_{K1}$ in this study was due to reduction in number of functional channels or channel regulators in the caveolae of cultured trout ventricular myocytes. Localization and quantitation of K$^+$ channels in the SL are needed to test this possibility.

The present findings indicate that fish cardiac myocytes can be kept in serum-free primary culture for at least 10 days at 17°C. The electrophysiological characteristics of the myocytes change markedly but in a well-predicted and consistent manner during culture. The serum-free culture of the fish cardiac myocytes is a promising preparation that can be used in the future to clarify how the expression of sarcoplasmic cation currents is regulated by temperature and other environmental stresses.

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