L-type Ca$^{2+}$ current and excitation-contraction coupling in single atrial myocytes from rainbow trout

LEIF HOVE-MADSEN AND LLUIS TORT

Department of Physiology and Cell Biology, Faculty of Science, Universitat Autonoma de Barcelona, 08193 Cerdanyola, Barcelona, Spain

Hove-Madsen, Leif, and Luis Tort. L-type Ca$^{2+}$ current and excitation-contraction coupling in single atrial myocytes from rainbow trout. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R2061–R2069, 1998.—We have examined the contribution of L-type Ca$^{2+}$ current (I$_{\text{Ca}}$) to the activation of contraction in trout atrial myocytes under basal and phosphorylating conditions. The average myocyte length was 197 ± 14 µm, width was 5.5 ± 0.2 µm, and cell capacitance was 36.2 ± 2.2 pF. With 25 µM EGTA in the patch pipette and a stimulation frequency of 0.125 Hz, I$_{\text{Ca}}$ was 2.6 ± 0.4 pA/pF and it carried a total charge of 0.10 ± 0.01 pC/pF, giving rise to a contraction of 15.2 ± 2.8% of the resting cell length. With a cell volume of 2.4 ± 0.3 µl, the charge carried by I$_{\text{Ca}}$ corresponded to 14.7 ± 2.2 mmol Ca$^{2+}$/ml nonmitochondrial cell volume (µM). This can account for only 30–40% of the Ca$^{2+}$ release during a contraction. Increasing the stimulation frequency from 0.25 to 2 Hz decreased I$_{\text{Ca}}$ amplitude and charge by 66 ± 5 and 80 ± 3%, respectively. Elevating the pipette EGTA concentration from 25 µM to 5 mM increased I$_{\text{Ca}}$ amplitude and charge by ∼290%. Both isoproterenol and cAMP increased I$_{\text{Ca}}$ by ∼230%. The total charge carried by the isoproterenol- or cAMP-stimulated current was increased by 170%. We conclude that the use of high-EGTA concentration may overestimate the total Ca$^{2+}$ carried by I$_{\text{Ca}}$ under physiological conditions. Furthermore, the results suggest that, in contrast to previous reports from other lower vertebrates, Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels alone is not sufficient to fully activate contraction in trout atrial myocytes at room temperature.

isoproterenol; adenosine 3’5’-cyclic monophosphate; ethyl-ene-glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; contraction

In the amphibian heart, it has been shown that L-type Ca$^{2+}$ current (I$_{\text{Ca}}$) accounts for the major part of the Ca$^{2+}$ that activates contraction (13, 26), and it is often assumed that this is true for other lower vertebrates. This assumption is based on two main observations. First, the cell dimensions from a number of lower vertebrates (28, 33, 34) are similar to cardiac myocytes from the amphibian heart, i.e., cell diameters between 2 and 8 µm (20, 21). This minimizes diffusion distances in the cell and, therefore, facilitates direct activation of the myofilaments by sarcolemmal Ca$^{2+}$ influx. Indeed, it has been shown that I$_{\text{Ca}}$ is sufficient to activate contraction in frog ventricular myocytes (13, 26), which would suggest that the same should be true for cells with similar Ca$^{2+}$ current amplitudes and cell dimensions. Second, inhibition of the sarcoplasmic reticulum (SR) function with ryanodine has a small or no effect on cardiac contraction in situ (22) or in multicellular preparations at physiological temperatures and heart rates (7, 8, 16, 25), suggesting that the SR does not play a dominant role in the activation of contraction under these conditions.

However, in the teleost heart there is virtually no information about mechanisms in the excitation-contraction (E-C) coupling at the cellular level (for review see Ref. 31). The density of L-type Ca$^{2+}$ channels in isolated sarcolemmal membranes from trout ventricular tissue has been determined from dihydropyridine-binding studies, and the density is similar to that in rat ventricular tissue (30). Because of the larger surface-to-volume ratio of the trout cells, this again suggests that sarcolemmal Ca$^{2+}$ flux may comprise a significantly larger fraction of the total Ca$^{2+}$ transient in the trout heart. Furthermore, I$_{\text{Ca}}$ has recently been characterized in carp ventricular cells (33). In these cells, I$_{\text{Ca}}$ density appears to be slightly larger than in frog cells and smaller than in mammals, and the author estimates that the I$_{\text{Ca}}$ accounts for an increase in intracellular Ca$^{2+}$ of 35–40 µM during contraction. In the trout heart, however, it has been shown that the SR may also contribute to contraction under some experimental conditions (9, 16, 18). In particular, the contribution of the SR appears to increase with increasing temperature (16). In this respect, we recently published a preliminary report showing that SR Ca$^{2+}$ uptake can be measured in trout ventricular myocytes at physiological temperatures and that significant amounts of Ca$^{2+}$ are accumulated at room temperature (19).

In light of these results, the aim of present study was to characterize I$_{\text{Ca}}$, under basic physiological conditions in trout cardiomyocytes and to quantify the amount of Ca$^{2+}$ carried across the sarcolemma by this current. To do this, we have used the patch-clamp technique combined with measurements of maximal systolic cell shortening.

METHODS

Cell isolation. Rainbow trout were obtained from a commercial trout farm and kept in tanks at 16°C with a 12:12-h light-dark photoperiod. After stunning and decapitation, the heart was rapidly excised from the trout and atrial myocytes were obtained by enzymatic digestion of the heart by use of a modified version of the method described by Fischmeister and Hartzell (12). Briefly, a cannula was inserted into the ventricle, and the heart was rinsed for 5–10 min with nominally Ca$^{2+}$-free Tyrode solution containing (in mM) 125 NaCl, 1.8 MgCl$_2$, 4 NaHCO$_3$, 0.8 Na$_2$HPO$_4$, 10 HEPES, 5 glucose, and 5 pyruvate; pH was adjusted to 7.4 with NaOH. The flow rate during the rinse was gradually increased from <1 to 4
ml/min. The heart was then perfused for 25 min at 20°C with a nominally Ca²⁺-free Tyrode solution containing 50 µM EGTA and 37.5 µM Ca²⁺, 0.1 mg/ml collagenase (Yakult), 0.1 mg/ml trypsin (Sigma Chemical), and 0.5 mg/ml BSA. The collagenase-containing solution was then replaced with a fresh solution, and the heart was perfused for an additional 15 min. At the end of the digestion, the atrial appendices were cut off the ventricle and transferred to a nominally Ca²⁺-free Tyrode solution containing vitamins, amino acids, penicillin, and 1 mg/ml BSA (12). The tissue was gently agitated, and the supernatant was filtered through a nylon filter (100 µm). The remaining tissue was resuspended, agitation was repeated, and the whole suspension was filtered. After filtration, cells were washed once and Ca²⁺ was gradually increased to 750 µM. The cells were then stored at 6°C until use. Cells were used within 48 h after the isolation.

Electrophysiological measurements. I_{Ca} were measured at room temperature (19–23°C) by using a software-driven patch-clamp amplifier (model EPC-9, Heka). After seal formation, the cell was lifted from the bottom of the petri dish and placed in front of one of eight capillaries containing the desired extracellular solution. The pipette solution could be continuously adjusted to 7.4 with NaOH. For the perforated-patch configuration, a width (D), a larger radius (D'), and a smaller radius (d₂ = D/4). Furthermore, when the cell surface area was calculated, it was assumed that the sarcolemma is smooth without t tubules (28, 34).

RESULTS

Measurement of cell contraction. Resting cell length and maximal cell shortening were determined manually from individual video frames. The resolution of the images was limited to ~1 μm. The cell volume was calculated with the assumption that the cells are tubes with an ellipsoidal cross section, a width (D), a larger radius (d₂ = D/2), and a smaller radius (d₃ = D/4). Furthermore, when the cell surface area was calculated, it was assumed that the sarcolemma is smooth without t tubules (28, 34).

Modulation of I_{Ca} by EGTA. Figure 2 depicts the current-voltage relation of I_{Ca} in trout atrial myocytes. Figure 2A shows current traces obtained when the cell...
is depolarized from a holding potential of −80 mV to three different test potentials. The current traces were obtained in the absence and presence of a saturating dose of 5 µM nifedipine. Figure 2B shows the voltage dependence of the peak inward current and the current measured at the end of the depolarization. Figure 2C shows the voltage dependence of the difference between the peak inward current and the end pulse current. Nifedipine abolished the inward current (Fig. 2A), which, together with the current-voltage relation, suggests that it is an L-type Ca\(^{2+}\) current. In the following experiments, \(I_{Ca}\) refers to the difference between the peak inward current and the current measured at the end of the stimulation pulse.

It may be expected that the EGTA concentration in the patch pipette affects \(I_{Ca}\), and we therefore examined the effect of changing the pipette EGTA concentration from 25 µM to 5 mM during an experiment. Figure 3A shows the time course of the increase in \(I_{Ca}\) amplitude after an increase in EGTA. Because 5 mM EGTA abolishes contraction, the concomitant disappearance of contraction and increase in Ca\(^{2+}\) current was taken as evidence for a correct intracellular perfusion of the cell (data not shown). Figure 3B shows the current traces obtained before and after the increase in the EGTA concentration corresponding to points a–d in Fig. 3A. Figure 3C shows the time integrals of the Ca\(^{2+}\) currents in Fig. 3B. Figure 4 summarizes the stimulatory effect of 5 mM EGTA on peak \(I_{Ca}\) and the charge carried by it. The two parameters are stimulated to the same extent by EGTA. Furthermore, to eliminate other possible effects of the pipette solution on the Ca\(^{2+}\) current, we also measured \(I_{Ca}\) in the perforated-patch configuration using nystatin. The results obtained with 25 µM EGTA and nystatin are compared in Table 1. Peak \(I_{Ca}\) and total charge were not significantly different in the two configurations.

The influence of the stimulation frequency on \(I_{Ca}\) amplitude and the charge carried by it was also examined. Figure 5A shows that the \(I_{Ca}\) amplitude decreased with increasing stimulation frequency. Current traces corresponding to points a–c in Fig. 5A are shown in Fig. 5B. Figure 5C summarizes the effect of stimulation frequency on \(I_{Ca}\) amplitude and the charge carried by it. Values were normalized to the corresponding value at 0.25 Hz and corrected for rundown of the current. Current amplitude and charge decreased significantly (\(P < 0.5\)) at all stimulation frequencies >0.25 Hz.

Fig. 2. Voltage dependence of Ca\(^{2+}\) current in trout atrial myocytes. A: representative Ca\(^{2+}\) current traces from a trout atrial myocyte in absence (control, Con) and presence of 5 µM nifedipine (Nif). Current traces were obtained by depolarization from holding potential of −80 mV to −40 mV (a), 0 mV (b), and +20 mV (c). B: Ca\(^{2+}\) current amplitude calculated as difference between peak inward current (c) and current at end of stimulation pulse (C). Points a–c correspond to current traces in A. C: dependence of Ca\(^{2+}\) current amplitude on stimulation voltage.

Fig. 3. Effect of low and high exogenous Ca\(^{2+}\)-buffering capacity. Atrial myocytes were initially perfused with 25 µM EGTA through patch pipette. This was followed by a switch to a pipette solution containing 5 mM EGTA. A: representative experiment showing effect of EGTA concentration on current amplitude. Pipette EGTA concentration is given above data points. B: current traces corresponding to points a–d in A. C: time integrals of current traces in B.

Fig. 4. Summary of effects of low and high EGTA on Ca\(^{2+}\) current amplitude and charge. L-type Ca\(^{2+}\) current (\(I_{Ca}\)) amplitude (A) and its time integral (B) were normalized to value with 25 µM EGTA (n = 5). Con, control; \(Q_{Ca}\), charge carried by \(I_{Ca}\).
Because the duration of the stimulation pulse may affect the degree of $I_{\text{Ca}}$ inactivation at the end of the stimulation pulse (and thereby the calculated time integral of the current), we also examined the influence of the length of the stimulation pulse on the charge carried by $I_{\text{Ca}}$. Figure 6A shows superimposed Ca\(^{2+}\) currents elicited by stimulation pulses of increasing duration. The vertical lines indicate the termination of a current trace. Figure 6C shows the corresponding time integral of the currents. The current measured at the end of the 300-ms stimulation was considered to represent complete inactivation of the Ca\(^{2+}\) channels. The charge carried by $I_{\text{Ca}}$ increased by only $38 \pm 6\%$ when the duration was increased from 100 to 300 ms.

Figure 6B shows the corresponding tail currents elicited by repolarization to $-80$ mV. The solid line in Fig. 6B represents the steady-state holding current at $-80$ mV, and the charge carried by the tail current shown in Fig. 6D was calculated as the time integral of the difference between the tail and the holding current. Figure 6E summarizes the influence of the duration of the stimulation pulse on the charge carried by $I_{\text{Ca}}$ and the corresponding tail currents from 12 experiments. Significant tail currents were elicited, even with a stimulation pulse duration of 3 ms, where the charge carried by $I_{\text{Ca}}$ is insignificant.

$\beta$-Adrenergic stimulation of $I_{\text{Ca}}$. To examine the $\beta$-adrenergic regulation of the Ca\(^{2+}\) current we first applied a saturating dose of isoproterenol (1 or 10 µM) with 5 mM EGTA in the pipette. Figure 7A shows the time course of the stimulatory effect of isoproterenol, and the original current traces corresponding to points a and b are shown in Fig. 7B. The average maximal stimulation

### Table 1. Comparison of $I_{\text{Ca}}$ with low EGTA and nystatin

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>Capacitance, pF</th>
<th>$I_{\text{Ca}}$</th>
<th>$pA$</th>
<th>pA/pF</th>
<th>$Q_{\text{Ca}}$</th>
<th>pC</th>
<th>pC/pF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nystatin</td>
<td>8</td>
<td>$37.9 \pm 2.6$</td>
<td>102</td>
<td>2.81</td>
<td>0.71</td>
<td>4.46</td>
<td>0.124</td>
<td>0.124</td>
</tr>
<tr>
<td>EGTA (25 µM)</td>
<td>11</td>
<td>$36.2 \pm 2.2$</td>
<td>95.8</td>
<td>16.2</td>
<td>2.63</td>
<td>3.76</td>
<td>0.57</td>
<td>0.101</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, number of cells. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$. L-type Ca\(^{2+}\) current ($I_{\text{Ca}}$) characteristics in trout atrial myocytes with use of nystatin perforated patch and whole cell patch configuration with 25 µM EGTA in patch pipette are shown. $Q_{\text{Ca}}$, charge carried by $I_{\text{Ca}}$.
of $I_{Ca}$ by isoproterenol in eight cells is summarized in Fig. 7C. Figure 7D shows the stimulatory effect of 10 µM cAMP on $I_{Ca}$ in 15 cells. The stimulatory effect of the two compounds was similar. To verify that the stimulatory effect of isoproterenol occurs through a cAMP-mediated pathway, we furthermore examined the effect of 10 µM isoproterenol after stimulation of $I_{Ca}$ with 10 µM internal cAMP. In four experiments, isoproterenol had no additional effect on a cAMP-stimulated $I_{Ca}$ (data not shown).

Figure 8 compares the effects of 10 µM isoproterenol and 5 mM EGTA on the current-voltage relation. Isoproterenol increased the maximal current amplitude and caused a typical 10-mV negative shift in the current-voltage relation while EGTA increased the maximal current amplitude without affecting the current-voltage relation.

**DISCUSSION**

$I_{Ca}$ and contraction in teleost heart. In lower vertebrates, activation of contraction is considered to depend strongly on transsarcolemmal Ca$^{2+}$ fluxes. This is, to a large extent, based on the cardiac myocyte dimensions and ultrastructural studies in a number of species. These data show that the myocytes are thin long cells without t tubules and a relatively poorly developed SR (10, 20, 28, 33, 34). Furthermore, in the frog heart, experimental data (26) and a model of diffusion of the sarcolemmal Ca$^{2+}$ flux to the cell interior have shown that Ca$^{2+}$ entering through L-type Ca$^{2+}$ channels is sufficient to activate contraction (13). In agreement with this, a recent report shows that the $I_{Ca}$ may contribute with a significant fraction of the total Ca$^{2+}$ transient in carp ventricular myocytes (33). Determination of the density of dihydropyridine receptors in sarcolemmal vesicles from trout heart suggests that this may also be true for the trout heart (30). Furthermore, the current density obtained in trout atrial
myocytes in the presence of 5 mM EGTA in the patch pipette is comparable to frog and values published recently for trout ventricular cells (34) but smaller than that found in carp cardiomyocytes (Table 1). However, when we used nystatin perforated patch or changed the intracellular EGTA concentration from 5 mM to 25 µM to mimic more closely intracellular conditions, the total Ca$^{2+}$ carried by $I_{\text{Ca}}$ in trout atrial cells was relatively small. Under these conditions, the charge carried by $I_{\text{Ca}}$ corresponded to an increase in the total Ca$^{2+}$ concentration of ~15 µM.

Table 2 compares cell dimensions, current densities, time integral of $I_{\text{Ca}}$, and the expected increase in total intracellular Ca$^{2+}$ due to $I_{\text{Ca}}$. Values obtained in heart cells from three species that have thin elongated cells expected to depend strongly on transsarcolemal Ca$^{2+}$ fluxes are compared with data from the rat, which has been shown to depend strongly on SR Ca$^{2+}$ release. Data for trout are from the present study, whereas data for carp (33), frog (2), and rat (5) have been published elsewhere. Although the cells from the three lower vertebrates are thin and elongated with low current densities compared with rat, there are significant differences when the time integrals of $I_{\text{Ca}}$ and the expected increase in intracellular Ca$^{2+}$ are compared. Thus the charge density and increase in intracellular Ca$^{2+}$ found in trout fell between values obtained in rat and values obtained in carp and frog.

$I_{\text{Ca}}$ and passive Ca$^{2+}$ buffering. Although the total passive buffering capacity is not known for trout cardiomyocytes, Ca$^{2+}$ binding to the contractile proteins and the Ca$^{2+}$-ATPase activity have been determined in trout heart (6), and the myofibrillar volume reported in trout ventricular myocytes (34) is similar to mammalian values (4). With use of the dissociation constant for the trout Ca$^{2+}$-ATPase and the assumption that the maximal binding capacity is similar to that in mammals (4), calculation of the Ca$^{2+}$ bound to the myofilaments between 0.1 and 1.0 µM Ca$^{2+}$ gives a difference of 36 µM. This is already two to three times larger than the calculated increase in total Ca$^{2+}$ due to $I_{\text{Ca}}$. Considering that Ca$^{2+}$ binding to the myofilaments probably accounts for only about one-half of the total passive Ca$^{2+}$ buffering (17) or that a total increase of ~50 µM Ca$^{2+}$ is needed (4), this would suggest that $I_{\text{Ca}}$ alone contributes with a minor fraction of the total Ca$^{2+}$ transient in trout atrial myocytes. In agreement with this, the ratio of the total Ca$^{2+}$ from $I_{\text{Ca}}$ to the total Ca$^{2+}$ needed to activate a normal contraction is ~1:5 in trout ventricular myocytes (19a).

There is no clear explanation for the differences between the examined ectothermic species. Some of the differences may, however, be due to differences in experimental conditions and assumptions. Thus the present data were obtained with relatively short stimulation pulses, which may lead to an underestimation of the total Ca$^{2+}$ carried by $I_{\text{Ca}}$ compared with a 500-ms pulse used in the carp cells. A pulse duration of 200 ms, however, is the same as that used in frog ventricular myocytes and is physiologically relevant at room temperature for the trout heart (25). Furthermore, our results suggest that the differences cannot be explained by the different pulse durations used. Thus, when judging from the current traces, $I_{\text{Ca}}$ appears fully inactivated at the end of a 200-ms pulse (Figs. 2, 3, and 6) and overlaps in the absence and presence of a saturating dose of nifedipine (Fig. 2). Finally, the charge carried by $I_{\text{Ca}}$ increases by only 38% when the pulse duration is increased from 100 to 300 ms (Fig. 6).

Rundown of the Ca$^{2+}$ current during an experiment could also lead to an underestimation of the Ca$^{2+}$ current and its time integral, and we did indeed see some rundown of Ca$^{2+}$ current and contraction in trout atrial cells. However, the average time integral of the Ca$^{2+}$ current given in Table 2 was measured at the beginning of each experiment to avoid this problem. Furthermore, values obtained with the perforated-patch configuration, where rundown is expected to be small, were similar to those obtained with 25 µM EGTA in the whole cell configuration.

Finally, physiologically important parameters such as experimental temperature and stimulation frequency have been shown to affect the inhibitory effect of ryanodine (16, 18, 22, 25, 29), suggesting that these parameters may be crucial when examining the relative contribution of different Ca$^{2+}$ sources to the activation of contraction. With respect to the stimulation frequency, it has previously been shown that the trout heart exhibits a negative force-frequency relation (16, 18, 29). Furthermore, the inhibitory effect of ryanodine diminishes with increasing stimulation frequency (25, 29), and this has led to the suggestion that the SR does not contribute significantly to the activation of contraction at physiological heart rates (25, 29). The present

Table 2. Comparison of trout atrial myocytes with data in literature

<table>
<thead>
<tr>
<th></th>
<th>[EGTA], µM</th>
<th>Length, µm</th>
<th>Depth, µm</th>
<th>Width, µm</th>
<th>Volume, pl</th>
<th>Cell Capacitance</th>
<th>[Ca], pA/pF</th>
<th>Q Ca, pC/pF</th>
<th>Total [Ca], µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog</td>
<td>5,000</td>
<td>290</td>
<td>2.5</td>
<td>5</td>
<td>2.85*</td>
<td>50</td>
<td>11.1</td>
<td>17.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Carp</td>
<td>5,000</td>
<td>110</td>
<td>2.9</td>
<td>5.8</td>
<td>1.38*</td>
<td>26</td>
<td>1.6</td>
<td>19.1</td>
<td>7</td>
</tr>
<tr>
<td>Trout</td>
<td>25</td>
<td>197</td>
<td>2.7</td>
<td>5.5</td>
<td>2.4*</td>
<td>36</td>
<td>1.3</td>
<td>15.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Rat</td>
<td>100</td>
<td>110</td>
<td>7</td>
<td>21</td>
<td>16.00</td>
<td>99</td>
<td>1.7</td>
<td>6.2</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Cell dimensions, $I_{\text{Ca}}$, and expected increase in total Ca$^{2+}$ due to $I_{\text{Ca}}$ in myocytes are compared in 3 ectothermic species and rat. [EGTA], EGTA concentration; [Ca], Ca$^{2+}$ concentration. Data for frog ventricle are from Argibay et al. (2), data for carp ventricle are from Vornanen (33), data for trout are from present study, and data for rat ventricle are from Bouchard et al. (5). * Cell cross section is assumed to be elliptical (33, 34). † Unpublished data.
results do not, however, provide direct evidence for a larger contribution of ICa to the activation of contraction at physiological stimulation frequencies. Thus the amplitude and the charge carried by ICa decreased as the stimulation frequency was increased.

With respect to the experimental temperature, the present data were obtained at room temperature, where inhibition of the SR function has been reported to affect contraction more strongly (16, 25). Furthermore, this temperature is near the upper lethal temperature limit for trout, and it is possible that this could alter the properties of the Ca2+ channels. The experimental temperature may, however, not be expected to affect the present results strongly. First, we did not see consistent differences between the lowest and highest experimental temperature (19 and 23°C). Second, experiments could be done for >1 h without serious rundown of ICa. Together this would suggest that room temperature is not deleterious to the myocytes. Third, the trout heart Na+/Ca2+ exchanger is not strongly affected by the experimental temperature (32), and results from the mammalian heart show that a 10° change in the experimental temperature does not alter the charge carried by ICa significantly (27). Finally, it has also been shown that the acclimation temperature of the trout does not affect ICa density strongly (34). This would, therefore, suggest that although the present data are obtained at room temperature, the conclusions may not be drastically altered at more physiological temperatures.

ICa and E-C coupling. In light of these results, it appears that factors other than ICa are necessary to fully activate contraction in trout myocytes at room temperature. If this is the case, it might be expected that under nonequilibrium conditions the Ca2+ entering the cell is not equal to the Ca2+ extruded from the cell. Indeed, if Ca2+ is released from the SR, it would be expected that more Ca2+ is extruded from the cell than enters across the sarcolemma. Figure 6 confirms that SR Ca2+ release takes place when short stimulation pulses (3–30 ms) are used, since much more Ca2+ is extruded from the cell than enters the cell. On the other hand, with longer stimulation pulses (100 and 300 ms), the charge carried by the Ca2+ current is similar to that carried by the tail current. Thus, even with these longer pulses, Ca2+ influx through L-type Ca2+ channels (2 charges/Ca2+) is still only one-half of the Ca2+ extruded by the Na+/Ca2+ exchanger (1 net charge/Ca2+). This suggests that 1) even with long stimulation pulses, an additional Ca2+ influx across the sarcolemma may occur through reverse Na+/Ca2+ exchange or other Ca2+ channels, 2) SR Ca2+ release is less important with long stimulation pulses, or 3) the major part of the Ca2+ released by the SR is taken up again during these long stimulation pulses.

Our results do not support Ca2+ influx through other types of Ca2+ channels, and the current-voltage relations do not support Ca2+ influx through T-type Ca2+ channels, which has been shown to be prominent in the shark heart (24). Although we cannot determine precisely the total amount of Ca2+ released from the SR, the difference between Ca2+ efflux and influx with 3- and 10-ms stimulation pulses suggests that at least twice as much Ca2+ is released from the SR as is carried by ICa at steady state. Thus, if ICa contributes with ~15 µM Ca2+ to the total Ca2+ transient (Table 1), the SR contributes with at least twice that amount, and if there is an additional Ca2+ influx across the sarcolemma of the same magnitude as ICa, the total Ca2+ transient will be ~60 µM. This would indeed be sufficient to account for passive Ca2+ binding to the myofilaments and other Ca2+ buffers during a contraction. The assumption that the SR contributes significantly to the activation of contraction would also agree with measurements of SR Ca2+ uptake in isolated trout ventricular myocytes (19) and a recent report on the effect of β-adrenergic stimulation on contraction in multicellular preparations from the trout atrium and ventricle (14), where it was found that in atrial myocytes Ryanodine reduces steady-state contraction as well as the recovery of contraction with and without β-adrenergic stimulation.

β-Adrenergic stimulation of ICa. Neurohormonal regulation of cardiac contraction is well described in teleosts in situ and in multicellular preparations. In particular, a bulk of work has focused on the regulation of heart rate (1, 11). Furthermore, work has been done on the inotropic effect of β-adrenergic stimulation (14, 15, 29), showing a two- to threefold stimulation of peak contraction and an acceleration of contraction and relaxation (14). However, at the cellular level, there is little information. Recent studies (33, 34) report a two- to threefold stimulation of ICa by isoproterenol in carp and trout ventricular myocytes, and we report a similar stimulation of ICa in trout atrial myocytes. Furthermore, we have found that the effect of isoproterenol occurs through a cAMP-dependent stimulation of ICa and that this stimulation is independent of the pipette EGTA concentration. This stimulation is much smaller than that seen in the frog heart, where a β-adrenergic stimulation causes a 10-fold increase in ICa (2, 12, 21) but it is similar to that observed in mammalian cardio-myocytes (3).

Thus the β-adrenergic stimulation of Ca2+ current in trout atrial myocytes differs substantially from that in frog cells: it is three to five times smaller. This, in turn, would suggest that under phosphorylating conditions the relative contribution of the Ca2+ current to the total transient is smaller in carp ventricular and trout atrial and ventricular myocytes than in the frog. This, however, does not necessarily imply that the contribution of ICa to the total Ca2+ transient is smaller in trout and carp under phosphorylating than basal conditions. On the contrary, it has been shown that at room temperature β-adrenergic stimulation can abolish the inhibitory effect of Ryanodine on contraction in trout ventricle (29). This, together with the increase in ICa, could suggest that SR Ca2+ release plays a minor role in the activation of contraction under phosphorylating conditions.

It should, however, be kept in mind that no information about the relative contribution of the SR to the
contraction is obtained when ryanodine is without effect. Thus it may indicate that the SR does not contribute to the activation of contraction or that other Ca$^{2+}$ sources are capable of compensating for the impairment of SR Ca$^{2+}$ release by ryanodine. Furthermore, it has recently been shown that although ryanodine does not affect contraction under phosphorylating conditions in trout ventricular tissue, contraction is reduced by ryanodine in trout atrial tissue under basal and phosphorylating conditions (14). Finally, phosphorylation not only increases $I_{\text{Ca}}$ but also decreases myofilament Ca$^{2+}$ sensitivity and enhances Ca$^{2+}$ uptake and release from the SR (23). Therefore, although phosphorylation clearly stimulates $I_{\text{Ca}}$, it appears premature to address the relative contribution of different mechanisms to the activation of contraction under phosphorylating conditions.

Perspectives

The present results provide a basic characterization and quantification of the Ca$^{2+}$ carried across the sarcolemma through L-type Ca$^{2+}$ channels in trout atrial myocytes at room temperature. The data are at odds with the general idea that transsarcolemmal Ca$^{2+}$ flux through Ca$^{2+}$ channels dominates the regulation of contraction in hearts from lower vertebrates (13, 24, 26, 31, 33, 34), since we find that at room temperature the $I_{\text{Ca}}$ can account for only a relatively small part of the total Ca$^{2+}$ needed to activate a contraction. The results, however, are in line with recent results on SR Ca$^{2+}$ uptake in isolated myocytes (19, 19a) and inhibition of SR function with ryanodine in trout atrial tissue (14). Thus it appears that under our experimental conditions the trout represents a first exception to the general picture of E-C coupling in the lower vertebrate heart. In the light of the fact that this general picture is largely based on extrapolations from studies in frog cardiac myocytes, it would seem important to examine carefully the mechanisms involved in the E-C coupling and their regulation by environmental factors at the cellular level in other lower vertebrates.

This work was supported by Generalitat de Catalunya Grants P0EC 94-77 (L. Hove-Madsen) and SGR95-00594 (L. Tort).


Received 13 March 1998; accepted in final form 7 August 1998.

REFERENCES


