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Revealing calcium fluxes by analyzing inhibition dynamics in action potential clamp

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ABSTRACT

In cardiac excitation-contraction coupling (ECC), calcium enters the cytosol via L-type Ca^{2+} channels (LTCC) and reverse Na^+/Ca^{2+}-exchange (NCX_{rev}), or is released from the sarcoplasmic reticulum (SR) by Ca^{2+}-induced Ca^{2+}-release (CICR). The magnitude of Ca^{2+} influx via the different pathways varies with the state of the cell and is difficult to assess quantitatively, because changes in Ca^{2+} influx through one pathway affect the others. In rainbow trout ventricular myocytes, the role of the SR has been uncertain for decades. The aim of this work was therefore two-fold: 1) to develop a method to quantify the Ca^{2+} influx pathways, and 2) to determine the role of CICR from the SR in trout ventricular myocytes. The novelty of our developed method lies in the mathematical analysis of measured transsarcolemmal Ca^{2+} currents and their impact on the corresponding Ca^{2+} transient during gradual inhibition of the currents in action potential (AP) clamp. We tested the developed method using an excitation-contraction model and showed that the method was able to recover calcium fluxes from noisy synthetic data. We applied the approach to trout ventricular myocytes and quantified the relative contributions of different Ca^{2+} influx pathways in ECC and determined the kinetics of these fluxes. Under baseline conditions, NCX_{rev} is the main transmembrane Ca^{2+} influx pathway contributing 29 ± 6% (of the Ca^{2+} influx), LTCC 18 ± 7%, and CICR 53 ± 10% to overall Ca^{2+} transient. Thus, NCX_{rev} is an important regulator of contractility and probably plays a role in the negative force-frequency relationship of trout ventricular preparations. These results demonstrate that trout and neonatal mammalian cardiomyocytes resemble each other not only in terms of morphology and energetics but ECC as well. In summary, the developed method resolves the major problem how to separate highly interconnected fluxes in AP clamp and allows to study Ca^{2+} fluxes in cardiomyocytes under conditions close to *in vivo*.

1. Introduction

In cardiac excitation-contraction coupling (ECC), an action potential (AP) leads to formation of the Ca^{2+} transient by initiating sarcolemmal Ca^{2+} influx via L-type Ca^{2+} channels, LTCC, and the Na^+/Ca^{2+}-exchange running in reverse, NCX_{rev}. This transsarcolemmal Ca^{2+} influx in turn triggers Ca^{2+}-induced Ca^{2+}-release, CICR, from the sarcoplasmic reticulum (SR). In steady state, Ca^{2+} influx via LTCC and NCX_{rev} is pumped out of the cells via the NCX operating in forward mode, and the Ca^{2+} released by CICR is pumped back into the SR via the sarcoendoplasmic reticulum Ca^{2+}-ATPase, SERCA.

The contribution of the three Ca^{2+} influx pathways (LTCC, NCX_{rev} and CICR) to the Ca^{2+} transient is dynamic and varies with species and conditions. The contribution of NCX_{rev} is considered negligible in rabbit cardiomyocytes [1], whereas in mouse cardiomyocytes it participates in the regulation of Ca^{2+} release and contractility [2–4]. Whether triggered by LTCC alone or together with NCX_{rev}, CICR from the SR plays a large role in mammalian ECC. In rat heart, it was estimated to contribute 80% to the Ca^{2+} transient [5]. In contrast, in some fish species, LTCC and NCX_{rev} contribute more or less equally to the Ca^{2+} transient, and CICR does not take place [6]. There is a trend that in more active fish, the SR plays a larger role in cardiac ECC [7]. However, in one of the most studied ectothermic vertebrates, the rainbow trout, the contribution of the three Ca^{2+} influx pathways is still not settled. Some studies suggest that in trout ventricle, CICR from the SR does play a minor role, although this depends on temperature and pacing frequency [8–10]. A more recent study suggested that CICR is only recruited under adrenergic stimulation [11]. The importance of NCX_{rev} under physiological conditions is uncertain,

Abbreviations: AP, action potential; CICR, Ca^{2+}-induced Ca^{2+}-release; ECC, excitation-contraction coupling; LTCC, L-type Ca^{2+} channel; NCX, Na^+/Ca^{2+}-exchange; NCX_{rev}, Na^+/Ca^{2+}-exchange in reverse mode; NIF, nifedipine; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; SR , sarcoplasmic reticulum.

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but it is expected to be significant due to the high intracellular sodium concentration [12].

Within an individual, the contribution of the different Ca^{2+} pathways also varies with life stage and fitness of the animal. For example, NCX_{rev} contributes to the Ca^{2+} transient in cardiomyocytes of neonatal rabbits. As the animals mature, the contribution of NCX_{rev} decreases, and the contribution of CICR increases [13,14]. Failing cardiomyocytes of adult mammals return to an "immature ECC" with diminished CICR and enhanced flow through NCX_{rev} [15]. Thus, a quantitative assessment of the Ca^{2+} influx pathways can give important information about cardiac health.

Quantification of the Ca^{2+} influx pathways is, however, far from trivial. Currents through LTCC, NCX and CICR can be measured using a combination of patch clamp and fluorescence microscopy on cells loaded with a Ca^{2+} indicator. It is common to assess the importance of one pathway by inhibiting it and recording the effect on the Ca^{2+} transient. Unfortunately, the inhibition of one pathway will affect the contribution of the others. For example, CICR is proportional to the size of the trigger [16] and will diminish if LTCC or NCX_{rev} current is inhibited. Inhibition of LTCC and/or CICR affects the Ca^{2+} balance across the sarcolemma and the shape of the AP, which in turn affects the NCX current. Therefore, quantification of the Ca^{2+} influx pathways requires an integrated approach where these relationships are taken into account.

The present study was undertaken with the aim to develop a method to quantify the Ca^{2+} influxes in rainbow trout cardiomyocytes. For this, we used AP clamp of individual cells and recorded how inhibition of LTCC and NCX elicited a compensatory current and reduced the Ca^{2+} transient. The recorded data was analyzed by a simple mathematical model of Ca^{2+} dynamics to reveal Ca^{2+} fluxes through LTCC, NCX, and SR.

2. Methods

2.1. Animals

Female rainbow trout (270 ± 54 g, n = 12) (*Oncorhynchus mykiss*) were purchased from a local fish farm (SK Trade OÜ, Staadioni 11-4, Juuru Parish, Rapla County, 79401, Reg: 11187349). Before experiments, the fish were acclimated at least three weeks in a 1600 liter fresh water tank at 15 ± 1 °C under 12:12 h light-dark photoperiod. They were fed commercial fish food on a daily basis. All procedures were approved by the Estonian National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture).

2.2. Cell isolation

Trout ventricular cardiomyocytes were isolated using a standard enzymatic technique as in [17–19]. In brief, the animal was killed with a single blow to the head followed by a cut of the spine. The heart was removed and placed in ice cold isolation solution containing (mM): 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose, 10 HEPES and pH adjusted to 6.9 with NaOH. The heart was cannulated through the bulbus arteriosus and perfused 8–10 min with cell isolation solution at a flow rate of 0.6–0.8 ml/min. After this, 0.5 mg/ml trypsin (Type IX-S), 0.75 mg/ml collagenase (Type IA), and 0.75 mg/ml fatty-acid-free bovine serum albumin (BSA) was added to the isolation solution and perfusion was continued with the same flow rate for 16–20 min. After the perfusion, the heart was taken off the cannula and cut into a few pieces. The cells were suspended with a Pasteur pipette and the suspension was then filtered through nylon tissue. The cells were left to sediment for 5 min, after which the supernatant was replaced with fresh isolation solution. This washing procedure was repeated 3 times. All this was carried out at room temperature. Isolated cells were kept in isolation solution at 4 °C and used for experiments within 5 h after the cell isolation. Before each experiment, the cells were incubated 20 min in external solution (see below) at 4 °C with 1 μM of the calcium indicator Fluo-4 AM (Invitrogen, OR, USA). In the experiments with inhibited SR, ryanodine receptors (RyRs) and SERCA were inhibited by pre-incubating cells for 20 min with 10 μM ryanodine (Ascent) and 2 μM thapsigargin (Ascent), respectively, as in [9].

2.3. Electrophysiological measurements

All electrophysiological recordings were conducted in the perforated patch clamp configuration. We used an Optopatch patch clamp amplifier (Cairn Research, UK), connected to a computer via an NI PCI-6221 data acquisition board (National Instruments, USA). The amplifier was controlled and data were recorded at a frequency of 10 kHz by custom-made software. All experiments were performed at room temperature (22 °C, temperature controlled by air conditioner).

The pipette solution contained (mM): 6 KCl, 124 K-glutamate, 1 $MgCl_2$, 14 NaCl, 10 $CaCl_2$ and pH adjusted to 7.2 with KOH. Perforation was achieved by front-filling with pipette solution containing additional 240 μ g/ml amphotericin B (Sigma-Aldrich, MO, USA). Having 10 mM $CaCl_2$ in pipette solution ensured that amphotericin perforated patch was permeable only for monovalent ions, otherwise Ca^{2+} in pipette induced hypercontraction of the cell and experiment was discarded. In practice, about 20% of cells were discarded due to the faulty perforation or rupture of the patch. In the used cells, no hypercontraction was observed nor slow increase of Ca^{2+} fluorescence suggesting a leak from pipette to the cell. The external solution for control measurements contained (mM): 130 NaCl, 5.4 KCl, 1.5 $MgSO_4$, 0.4 NaH_2PO_4, 2 $CaCl_2$, 10 glucose, 10 HEPES and pH was adjusted to 7.6 with NaOH. The total Ca^{2+} concentration in trout plasma is around 2.4 mM [20]. It is, however, highly buffered, and the free Ca^{2+} concentration is ~1.4 mM [20]. For our experiments, we used 2 mM Ca^{2+} in the extracellular solution in order to compare with previous studies [11,21-23].

A batch of cells was placed in a diamond-shaped fast-exchange chamber (15 × 6 mm, RC-24N; Warner Instruments, Harvard Apparatus, March-Hugstetten, Germany) under a Nikon Eclipse Ti-U microscope (Nikon, Japan) equipped with a 40× objective (CFI Super Plan Fluor ELWD 40xC NA 0.60, Nikon, Japan). The cells were allowed to attach to the glass for 5 min before starting the perfusion of the chamber with extracellular solution at a flow rate of ~0.5 ml/min. A cell in the middle of the chamber was approached with a patch pipette (resistance 2–3 MΩ). After gigaseal formation (>3 GΩ) in voltage clamp, the pore formation in the patch was monitored by following the current response to a square voltage pulse with baseline −80 mV, width of 10 ms, height 5 mV and pulse period 25 ms. When the current response had reached steady state, cell capacitance and series resistance was noted.

For later analysis, a short series of transmission images were acquired with a CCD camera (IPX-VGA210-LMCN, Imperx Inc., FL, USA). From these images, cell width and length was measured, and cell volume was calculated as in [24]. Namely, cells were considered as a cylinder with an elliptical cross section, where the semi-major and semi-minor axes of the ellipse are taken as 1/2 and 1/4 of cell's width, respectively. The cell volume was found using following equation: $V = \pi \cdot w/2 \cdot w/4 \cdot l$, where w is width and l is length of a cell. To estimate cytosolic volume, we assumed that non-mitochondrial cell volume in trout cardiomyocytes is 55% of total cell volume [19].

After acquisition of the transmission images, the configuration was switched to current clamp, and the minimal current square pulse triggering an AP was found. This pulse had a width of 1–2 ms and height of 600–900 pA, depending on the cell. The minimum pulse was used to record the cell-specific AP at 1.1 Hz for 90 s. This corresponds to 66 bpm, which is close to the routine heart rate at the