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Metabolic inhibition induces transient increase of L-type Ca^{2+} current in human and rat cardiac myocytes

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Abstract

Metabolic inhibition is a common condition observed during ischemic heart disease and heart failure. It is usually accompanied by a reduction in L-type Ca^{2+} channel (LTCC) activity. In this study, however, we show that metabolic inhibition results in a biphasic effect on LTCC current (I_{CaL}) in human and rat cardiac myocytes: an initial increase of I_{CaL} is observed in the early phase of metabolic inhibition, which is followed by the more classical and strong inhibition. We studied the mechanism of the initial increase of I_{CaL} in cardiac myocytes during a non-selective β -adrenergic stimulation with isoprenaline. The initial increase of I_{CaL} was induced by a wide range of metabolic inhibitors (FCCP, 2,4-DNP, rotenone, antimycin A). In rat cardiomyocytes, the initial increase of I_{CaL} was eliminated when the cells were pre-treated with thapsigargin leading to the depletion of Ca^{2+} from the sarcoplasmic reticulum (SR). Similar results were obtained when Ca^{2+} release from the SR was blocked with ryanodine. These data suggest that the increase of I_{CaL} in the early phase of metabolic inhibition is due to a reduced calcium dependent inactivation (CDI) of LTCCs. This was further confirmed in human atrial myocytes where FCCP failed to induce the initial stimulation of I_{CaL} when Ca^{2+} was replaced by Ba^{2+} eliminating CDI of LTCCs. We conclude that the initial increase in I_{CaL} observed during metabolic inhibition in human and rat cardiomyocytes is a consequence of an acute reduction of Ca^{2+} release from SR resulting in reduced CDI of LTCCs.

1 Introduction

The L-type Ca^{2+} channels (LTCCs) provide Ca^{2+} for the initiation and regulation of cell contraction and thus play a main role in cardiac muscle contraction. During normal cardiac excitation-contraction coupling a relatively small amount of Ca^{2+} enters the cell via the L-type current (I_{CaL}) and this triggers the release of a larger amount of Ca^{2+} from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs) in a process called Ca^{2+} -induced Ca^{2+} release (Bers, 2006). The increase of intracellular Ca^{2+} concentration in the vicinity of closely associated Ca^{2+} channels and RyRs leads to calcium dependent inactivation (CDI) of LTCCs (Sham, 1997) and reduction of I_{CaL} (Bers, 2002). Hence, the SR participates in the regulation of the activity of plasmalemmal L-type Ca^{2+} channels (Richard et al.,

2006). The close functional interaction between plasmalemma and SR means that LTCCs and RyRs can change the activity of each other and alter the balance of cellular Ca^{2+} , which directly activates contraction of myofilaments. The mechanisms of Ca^{2+} regulation in diseased cardiac cells may be disordered. Malfunction of LTCCs and RyRs interaction may affect contractility of myocardium, and as a consequence the main function of the heart, to pump blood, may be altered.

Metabolic inhibition is commonly observed during ischemic heart disease and heart failure and is associated with biphasic changes in action potential (AP) duration in heart cells and whole heart (Verkerk et al., 1996; Mačianskienė et al., 2018). Numerous studies show a reduction in cardiac LTCC activity and I_{CaL} during metabolic inhibition but the mechanisms involved are not clear (Louch et al., 2002; Fukumoto et al., 2005; Chantawansri et al., 2008; Zima et al., 2013; Kanaporis et al., 2017). In ischemic heart failure, the sympathetic system is activated and the level of catecholamines is increased (Lohse et al., 2003; Marian, 2006). Thus, we decided to investigate the effect of metabolic inhibition on I_{CaL} during β -adrenergic stimulation in human atrial and ventricular myocytes and rat ventricular myocytes. Surprisingly, we found that metabolic inhibition causes an acute transient increase of I_{CaL} amplitude followed by a rapid reduction. This is consistent with the most recent study demonstrating AP prolongation during metabolic inhibition in the rabbit heart (Mačianskienė et al., 2018), however is in contrast with what we reported recently in frog cardiomyocytes, where metabolic inhibition only suppresses I_{CaL} without the initial increase (Kanaporis et al., 2017). We explored the mechanisms responsible for the initial increase in I_{CaL} during metabolic inhibition. Our results point to a modulation of the Ca^{2+} -dependent inactivation of LTCCs by the RyRs mediated Ca^{2+} release.

2 Materials and Methods

2.1 Isolation of cardiomyocytes

2.1.1 Human atrial and ventricular myocytes

This study was carried out in accordance with the recommendations of the principles outlined in the Declaration of Helsinki, Kaunas Regional Bioethics Committee (Lithuania) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Kaunas Regional Bioethics Committee (Lithuania). Specimens of human heart were obtained from 16 patients undergoing heart surgery for coronary artery diseases at the Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania). Most of the patients have received a pharmacological treatment (including Ca^{2+} -channel blockers, digitalis, β -AR antagonists, diuretics, ACE inhibitors, NO-donors, and/or antiarrhythmic drugs) that was stopped 24 h before surgery. In addition, before and during surgery all patients have received sedatives, anesthesia, and antibiotics. Information on patient group and specimens is provided in Supplementary Table 1. Dissociation of the cells was performed as described previously (Kirstein et al., 1995). Briefly, specimens of the atrial or ventricular tissue were washed in an oxygenated Ca^{2+} free Tyrode solution (in mmol/L): 136 NaCl, 5.4 KCl, 1.1 MgCl_2 , 10 HEPES, 20 taurine, 5 sodium pyruvate, 10 D-glucose, 0.3 NaH_2PO_4 , pH 7.3 adjusted with NaOH at room temperature. Later the tissue was cut into pieces of $\sim 1 \text{ mm}^3$ in Tyrode solution with 30 mmol/L 2,3-butanedionemoxime added to the solution. Subsequently, a 30-40 min incubation with collagenase (type V, 200 U/ml) and protease (type XXIV, 5 U/ml) in 10 ml Ca^{2+} free BSA (5 mg/ml) containing Tyrode solution was performed at 37 °C. The solution was then replaced by 10 ml of fresh solution containing collagenase

(400 U/ml) and digestion was repeated for 20-30 min. Every 5 min, a small sample of solution was examined under a microscope to evaluate the stage of cell dissociation.

2.1.2 Rat ventricular myocytes

This study was carried out in accordance with the European Community guiding principles and recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and UK regulations on animal experimentation (Grundy, 2015). The protocol was approved by the State Food and Veterinary Service of the Republic of Lithuania (2015-09-24 No. G2-34). Adult rat ventricular myocytes were obtained by retrograde perfusion from hearts of male Wistar rats (160–180 g) as described previously (Verde et al., 1999). Briefly, rats were anesthetized with intraperitoneal injection of Pentothal and hearts were excised quickly. The hearts were retrogradely perfused at 37 °C with an oxygenated Ca^{2+} free Ringer solution (in mmol/L): 117 NaCl, 5.8 KCl, 4.4 NaHCO_3 , 1.5 KH_2PO_4 , 1.7 MgCl_2 , 11.7 D-glucose, 10 sodium phosphocreatine, 20 taurine, 21 HEPES, pH 7.1 adjusted with NaOH at room temperature. For heart myocyte dissociation collagenase type A (1 mg/ml, Boehringer Mannheim, Germany) was added to the Ca^{2+} free Ringer solution and heart was perfused for 1 h. The ventricles were then separated from atria, cut to small pieces and agitated gently to dissociate the single cells.

2.2 Electrophysiology

For electrophysiological experiments a few drops of cells suspension were placed in a perfusion chamber mounted on an inverted microscope stage. After cells had settled to the bottom, the chamber was superfused with K^+ free control external solution containing (in mmol/L): 127 NaCl; 10 HEPES; 20 CsCl; 4 NaHCO_3 ; 0.8 NaH_2PO_4 ; 1.8 MgCl_2 ; 1.8 CaCl_2 ; 5 D-glucose; 5 sodium pyruvate; pH 7.4 adjusted with NaOH. Patch pipettes were made from glass capillaries (Drummond, Broomall, PA, USA) and had resistances of 0.9-1.2 M Ω when filled with control internal solution. The myocytes were dialysed with control internal solution composed of (in mmol/L): 140 CsCl, 5 EGTA, 4 MgCl_2 , 0.062 CaCl_2 , 5 creatine phosphate disodium salt, 3.1 Na_2ATP , 0.42 Na_2GTP and 10 HEPES; pH to 7.3 was adjusted with CsOH. The whole-cell patch-clamp technique was used to record the I_{CaL} in human and rat cardiac myocytes as described previously (Treinys et al., 2016). Ionic currents through LTCCs were registered every 8 s. I_{CaL} facilitation was generated after 20 seconds of rest applying 4 depolarizing pulses from -80 mV holding potential to 0 mV for 200 ms (frequency 1 Hz). Tetrodotoxin (30 $\mu\text{mol/L}$, Latoxan, Rosans, France) and a 50 ms prepulse to -50 mV before every depolarizing pulse were used to eliminate sodium currents. Inward peak currents were measured as the difference between the maximal inward current amplitude and the current at the end of the pulse. In all the experiments the effect of metabolic inhibitors on I_{CaL} was always determined during the first application of the agent.

Equilibrium for steady-state frequency-dependent facilitation of I_{CaL} is reached after 4 stimulations (Mangoni et al., 2000), therefore I_{CaL} facilitation was assessed comparing the 1st I_{CaL} trace with the 4th trace at 1 Hz. Peak of I_{CaL} and the I_{CaL} area, an integral of I_{CaL} for 200 ms (pA x ms) were registered at the time of 1st and 4th depolarizing pulse. The time integral of I_{CaL} was registered in part of experiments to elucidate the origin of I_{CaL} increase during metabolic inhibition and to compare the initial effect of FCCP with frequency-dependent facilitation and reduced CDI.

To determine current-voltage (I-V) relationship and inactivation of I_{CaL} , a double pulse voltage-clamp protocol was applied every 4 seconds (see insert in Fig. 2C). During the first pulse S1, the membrane

potential was set at membrane potentials ranging from -100 to +100 mV for 200 ms. S1 pulse was followed by a 3 ms repolarization to the -80 mV holding potential and then a depolarizing pulse S2 to 0 mV was applied for 200 ms.

All experiments were performed at room temperature (18–24°C), and the temperature did not change by more than 1°C during an experiment.

2.3 Chemicals and stock solutions

All drugs and chemicals were from Sigma-Aldrich (Schnelldorf, Germany) if not specified otherwise. All drugs tested in patch-clamp experiments were solubilized in experimental solutions just before application onto the cell studied, i.e. only fresh solutions were tested. To block oxidative phosphorylation stock solutions of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 100 µmol/L), 2,4-Dinitrophenol (DNP, 100 mmol/L) in ethanol, rotenone (100 mmol/L) and antimycin A (100 mmol/L) in DMSO were used. The stock solution of the SR Ca²⁺ ATP-ase inhibitor thapsigargin (10 mmol/L) was prepared in ethanol. A stock solution of ryanodine (10 mmol/L) for blocking of RyR channels was prepared in distilled water. An aqueous stock solution of the isoprenaline (ISO, 1 mmol/L) was freshly prepared every day prior experiments. When the stock solutions in DMSO or ethanol were used, the corresponding amount of solvent was also added to the external control solution.

2.4 Data analysis

Inactivation of I_{CaL} traces were best fitted by the sum of two sequential exponentials using the expression:

$$I(t) = I_{CaL,1} \times \exp(-t/\tau_1) + I_{CaL,2} \times \exp(-t/\tau_2),$$

where I is the current at time t; I_{CaL,1} and I_{CaL,2} are the amplitudes and τ₁ and τ₂ are the time constants of the fast and slow components of I_{CaL} respectively. The fitting of I_{CaL} inactivation traces was performed using the modified protocol described by (Richard et al., 1993)) in rat ventricular myocytes.

The fitting time was set from ~1 ms after the peak of inward calcium current until the end of 400 ms pulse. The steady-state current was calculated at the end of 400 ms pulse and this helped to avoid residual leak current influence to the inactivation of I_{CaL}. The quality of the fits were evaluated by correlation coefficients (r) and in all experiments correlation coefficients was ≥0.985.

Steady-state inactivation curves (B₁) and I-V traces (B₂) were fitted with Boltzmann equations:

$$B_1(V) = 1 / (1 + \exp((V_{1/2} - V)/k));$$

$$B_2(V) = g(V - V_{rev}) / (1 + \exp((V_{1/2} - V)/k)),$$

where V_{1/2} – 0.5 of maximal value of steady-state activation and inactivation, V_{rev} – reversal potential of Ca²⁺, g – conductance of LTCC and k – the steepness parameter.

Data is expressed as the means ± standard errors of the mean (SEM). The significance of differences was evaluated using one-way analysis of variance (ANOVA). The significance level was set at p <

0.05. In the text, the “basal I_{CaL} ” refers to the Ca^{2+} current, which was not stimulated by β -adrenergic agonist.

3 Results

3.1 Effect of metabolic inhibition on I_{CaL} in human atrial myocytes

The first series of experiments were set to determine the effect of metabolic inhibition on I_{CaL} in human atrial myocytes. The cardiac myocytes for the first series of experiments were derived from 5 patients. Metabolic inhibition was induced by the application of mitochondrial uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) to isoprenaline (a non-selective agonist of β -adrenergic receptors, ISO) stimulated human atrial cells and changes of I_{CaL} were monitored. Surprisingly, in most of the tested human atrial cells FCCP induced a biphasic effect on I_{CaL} . i.e. a rapid initial stimulation of I_{CaL} that was followed by a strong inhibition of I_{CaL} .

The initial stimulation of I_{CaL} induced by FCCP was recorded in six out of nine (~67%) tested human atrial myocytes. In these cells, application of 30 or 100 nmol/L of FCCP resulted in a transient increase of ISO-stimulated I_{CaL} by $11.4 \pm 2.5\%$ ($n=3$, $p<0.05$, 2 patients) and $9.0 \pm 2.8\%$ ($n=3$, $p<0.05$, 2 patients), respectively. In all human atrial myocytes tested, metabolic inhibition led to a final suppression of I_{CaL} by $65.5 \pm 5.6\%$ ($n=5$, $p<0.05$, 2 patients) and $55.8 \pm 9.8\%$ ($n=4$, $p<0.05$, 3 patients) with 30 and 100 nmol/L FCCP, respectively (Fig. 1A, B).

3.2 Effect of metabolic inhibition on I_{CaL} in human ventricular myocytes

The next series of experiments were set to determine the effect of metabolic inhibition on I_{CaL} in human ventricular myocytes. The ventricular myocytes for these series of experiments were derived from 8 patients. We performed experiments in human ventricle myocytes applying metabolic inhibitors FCCP and 2,4-Dinitrophenol (DNP) on ISO (1 μ mol/L) stimulated cells. We found that FCCP induced a rapid initial stimulation of I_{CaL} in 3 out of 6 ventricular myocytes (5 patients). 100 nmol/L of FCCP increased ISO-stimulated I_{CaL} by $24.8 \pm 5.3\%$ ($n=3$, $p<0.05$). The reduction of I_{CaL} induced by FCCP was registered in all tested cells, i.e. I_{CaL} was reduced by $42.5 \pm 3.5\%$ ($n=6$, $p<0.05$). Another uncoupler, DNP, also evoked a transient increase of ISO-stimulated I_{CaL} in 5 ventricular myocytes from 9 (3 patients). 100 μ mol/L of DNP increased ISO-stimulated I_{CaL} by $5.0 \pm 0.9\%$ ($n=5$, $p<0.05$). The reduction of I_{CaL} by $44.8 \pm 4.4\%$ induced by DNP was registered in all ($n=9$, $p<0.05$) human ventricular myocytes Fig. 1C, D. The initial stimulation of I_{CaL} induced by uncouplers FCCP and DNP was recorded in eight out of fifteen (~53%) tested human ventricular myocytes.

3.3 Transient increase of I_{CaL} is also elicited in rat cardiac myocytes

While I_{CaL} suppression during metabolic inhibition was previously demonstrated by numerous studies (Louch et al., 2002; Fukumoto et al., 2005; Chantawansri et al., 2008; Zima et al., 2013), the transient increase in I_{CaL} amplitude during metabolic inhibition in cardiac myocytes has not previously been reported. To determine if this phenomenon is specific to human cardiomyocytes, we reproduced the same type of experiments in in rat ventricular myocytes under basal and ISO-stimulated conditions. Application of FCCP (100 nmol/L) induced a transient increase in basal I_{CaL} by $4.9 \pm 1.6\%$ that was followed by I_{CaL} reduction by $41.4 \pm 1.7\%$ ($n=3$, $p<0.05$). When I_{CaL} was enhanced by ISO, the

stimulatory effect of FCCP was also registered ($20.2 \pm 2.7\%$, $n=12$, $p < 0.05$, 4 animals). This increase was followed by an inhibition of I_{CaL} by $42.4 \pm 4.6\%$ ($n=12$, $p < 0.05$) (Fig. 1E, F). Higher concentrations of FCCP (300 nmol/L and 1 μ mol/L) also induced a biphasic effect in rat ventricular myocytes (Fig. 1E).

To confirm that the effects of uncouplers on I_{CaL} were due to metabolic inhibition, effects of other metabolic inhibitors were also tested. Rotenone (30 μ mol/L), an inhibitor of the complex I of the mitochondrial respiratory chain, also caused an initial increase of ISO-stimulated I_{CaL} by $11.4 \pm 0.2\%$ ($n=3$, $p < 0.05$, 2 animals) and later reduced I_{CaL} by $28.2 \pm 7.6\%$ ($n=3$, $p < 0.05$). Antimycin A (10 μ mol/L), an inhibitor of the complex III of the mitochondrial respiratory chain, initially increased ISO stimulated I_{CaL} by $8.3 \pm 2.3\%$ ($n=5$, $p < 0.05$) and decreased by $35.5 \pm 1.9\%$ ($n=5$, $p < 0.05$, 3 animals, Fig. 1F, Supplementary Figure S1). The initial transient stimulation of I_{CaL} was recorded in all tested rat cardiomyocytes during the early phase of metabolic inhibition regardless of the metabolic inhibitor used. The effects of metabolic inhibitors in cardiac cells are summarized in Supplementary Table 2.

3.4 Effect of metabolic inhibition on I_{CaL} kinetics

To get an insight on the possible mechanisms of I_{CaL} increase, we analyzed the effect of metabolic inhibition on time dependent inactivation of I_{CaL} in ISO stimulated cells. The time dependent inactivation of I_{CaL} in isolated adult rat ventricular myocytes was determined by bi-exponential fitting to the decay of current (see Methods) and by characterizing two kinetically distinct fast ($I_{CaL,1}$) and slow ($I_{CaL,2}$) current components (Tiaho et al., 1994). Double exponential fit to the decay phases of I_{CaL} revealed that during the transient increase of I_{CaL} with FCCP (100 nmol/L) in ISO stimulated cells the time constant of the fast component τ_1 increased from 5.3 ± 0.3 ms to 7.9 ± 1.1 ms ($n=12$, $p < 0.05$), while the time constant of the slow component τ_2 decreased from 48.7 ± 2.9 ms to 42.6 ± 2.5 ms ($n=12$, $p < 0.05$). The changes in the amplitude of fast and slow components of I_{CaL} were relatively small. During the inhibitory phase when the effect of FCCP reached steady-state, τ_1 and τ_2 were 13.8 ± 2.1 ms ($n=12$, $p < 0.05$) and 42.7 ± 3.6 ms ($n=12$), respectively, meanwhile the amplitude of both components of I_{CaL} were reduced. The I_{CaL} curves are presented in Supplementary Figure S2. These data show that in the presence of FCCP there is a significant increase in τ_1 suggesting a diminished calcium dependent inactivation (CDI) of the channels. Values of the time constants are provided in Supplementary Table 3 and Supplementary Table 4.

We also measured the current-voltage relationship (I-V) and the steady-state inactivation of I_{CaL} in rat ventricular cells (Fig. 2). Averaged data of I_{CaL} I-V curves (Fig. 2A and normalized data in Fig. 2B) and inactivation (Fig. 2C) in control conditions, in the presence of ISO and in the presence of ISO+FCCP are shown. The points in the figure represent experimental recorded data and the I-V curves were fitted to the Boltzmann equation (see Methods). Derived values for $V_{1/2}$ in control, ISO and FCCP were -13.26 ± 0.87 mV, -20.71 ± 0.81 mV and -18.80 ± 0.99 mV and k was 6.9 ± 0.4 , 7.1 ± 0.6 and 6.5 ± 0.2 ($n=3$), respectively. As expected, ISO significantly shifted I-V curve to the left (Tiaho et al., 1991) by >7 mV ($p < 0.05$) while FCCP had no significant effect on I_{CaL} I-V relationship. Fit of the steady-state inactivation curves for control, ISO and FCCP led to $V_{1/2}$ values of -29.20 ± 1.15 mV, -32.05 ± 1.60 mV and -32.75 ± 1.42 mV, respectively, and k was 6.2 ± 0.1 , 5.9 ± 0.3 and 5.7 ± 0.1 ($n=3$), respectively. Again, ISO significantly shifted the inactivation of I_{CaL} to the left by ≈ 3 mV ($p < 0.05$) but the effect of FCCP was negligible. The changes of curve steepness k under the influence of ISO or FCCP were insignificant both for I-V and for steady-state inactivation curves.

3.5 Suppression of cytosolic Ca^{2+} release abolish transient increase of I_{CaL}

The above results showed that in human and rat cardiac cells metabolic inhibition induced initial transient increase of I_{CaL} . In our previous study we found no such stimulation during metabolic inhibition in frog cardiomyocytes (Kanaporis et al., 2017) where SR is scarce and lacks ryanodine channels (Tijskens et al., 2003), and, in contrast to mammalian cells, LTCCs don't have such strong functional relationship with RyRs. Consequently, we hypothesized that the observed increase of I_{CaL} in mammalian cells may involve metabolic sensitivity of SR and RyRs in regulation of calcium dependent inactivation (CDI) of LTCCs. To test this hypothesis, additional experiments were performed in rat cardiomyocytes in which Ca^{2+} release from SR was suppressed prior to metabolic inhibition.

First, metabolic inhibition was induced after the pretreatment of myocytes with thapsigargin (2 $\mu\text{mol/L}$). Thapsigargin inhibits SR Ca^{2+} ATPase (SERCA) and impairs Ca^{2+} uptake by the SR and thus leads to the depletion of Ca^{2+} in the SR (Janczewski and Lakatta, 1993). As expected, thapsigargin diminished CDI of LTCCs which led to the increase in ISO-stimulated I_{CaL} in rat ventricular myocytes to $142.1 \pm 8.3\%$, i.e. from 17.7 ± 0.5 pA/pF to 25.1 ± 0.9 pA/pF ($n=5$, $p<0.05$). In the presence of thapsigargin the initial I_{CaL} increase was completely eliminated and only inhibitory effect of FCCP on ISO stimulated I_{CaL} was observed ($n=5$, $p<0.05$) (Fig. 3A, B). Double exponential fit of the decay of I_{CaL} revealed that application of thapsigargin on ISO stimulated rat cardiac cells resulted in an increase of all I_{CaL} parameters. The time constants τ_1 and τ_2 increased from 5.5 ± 0.5 ms to 26.2 ± 2.6 ms and from 70.3 ± 4.2 ms to 88.2 ± 3.0 ms ($n=4$, $p<0.05$), respectively. In the presence of thapsigargin, the effect of FCCP on τ_1 was abolished. The time constant τ_1 in such conditions remained similar to thapsigargin treated cells and was 26.4 ± 0.5 ms, while τ_2 was similar to ISO stimulated I_{CaL} and was 69.1 ± 3.7 ms ($n=4$). The representative I_{CaL} traces and their fits are shown in Fig. 3C. Additionally, we used an alternative approach to eliminate the CDI of LTCCs prior the metabolic inhibition by blocking RyR channels with ryanodine (10 $\mu\text{mol/L}$) in rat ventricular cells. Similarly to experiments with thapsigargin, ryanodine increased ISO stimulated I_{CaL} and application of FCCP failed to induce the initial I_{CaL} increase and, thus, only an inhibition of ISO stimulated I_{CaL} was observed (Supplementary Figure S3).

The results obtained with thapsigargin and ryanodine in rat ventricular cells point to a possible role of CDI in the I_{CaL} increase during the early phase of metabolic inhibition. To further confirm this hypothesis, we measured the changes of I_{CaL} facilitation in rat cardiac cells in the absence and presence of FCCP. Increased frequency (to 1 Hz) of depolarization after a 20 s pause was used in these series of experiments. The peak amplitude of I_{CaL} and the I_{CaL} area were compared at the time of 1st and 4th depolarizing pulse. In control conditions, increased stimulation frequency enhanced I_{CaL} amplitude by $8.3 \pm 2.1\%$ and area by $46.9 \pm 6.3\%$ ($n=12$, $p<0.05$) during the 4th depolarizing pulse. In ISO stimulated rat cardiac cells, increasing stimulation frequency to 1 Hz also induced I_{CaL} facilitation and increased I_{CaL} peak amplitude and area by $22.2 \pm 5.3\%$ and $42.0 \pm 8.6\%$ ($n=7$, $p<0.05$), respectively (Fig. 4A, B). Double exponential fits of I_{CaL} decay during the 1st and 4th depolarizing pulses revealed that the time constant of the fast component τ_1 increased from 5.8 ± 0.5 ms to 7.3 ± 0.7 ms ($n=8$, $p<0.01$), while the time constant of the slow component τ_2 decreased from 62.1 ± 4.8 ms to 51.3 ± 2.6 ms ($n=8$, $p<0.01$). The I_{CaL} curves are presented in Supplementary Figure S4. After treatment of rat ventricular myocytes with FCCP, I_{CaL} facilitation was no longer observed and I_{CaL} amplitude and area were reduced by $23.6 \pm 4.1\%$ and $23.1 \pm 3.3\%$ ($n=6$, $p<0.05$, Fig. 4A, B) during 4th depolarizing pulse, respectively.

These data indicate, that pharmacological or frequency-dependent depletion of Ca^{2+} from the cytosolic Ca^{2+} store results in an increased τ_1 and thus reduced CDI, followed by an increase of Ca^{2+} influx through LTCCs, a process which is possibly observed during the early phase of metabolic inhibition.

Depletion of Ca^{2+} from the SR by thapsigargin eliminated I_{CaL} facilitation (Fig. 4C) and, during application of I_{CaL} facilitation protocol, the amplitude and area of I_{CaL} measured during 4th depolarizing pulse were reduced by $20.0 \pm 0.6\%$ and $22.8 \pm 2.4\%$, respectively ($n=4$, $p < 0.05$) when compared with the 1st depolarizing pulse. After application of FCCP in ISO-stimulated and thapsigargin treated rat cells the I_{CaL} frequency-dependent facilitation remained abolished and the amplitude and area of I_{CaL} were reduced during 4th depolarizing pulse by $22.7 \pm 1.8\%$ and $20.0 \pm 1.4\%$, respectively ($n=4$, $p < 0.05$). The observation that application of FCCP abolishes facilitation of I_{CaL} in the similar manner as depletion of Ca^{2+} from the SR, indicates that FCCP affects CDI of LTCCs.

3.6 Effect of metabolic inhibition on Ba^{2+} current (I_{Ba}) through L-type calcium channels

As an alternative approach to test if reduced CDI plays a key role in the transient potentiation of I_{CaL} during metabolic inhibition we performed experiments in human atrial myocytes in which CaCl_2 in the external solution was replaced by the equimolar BaCl_2 . LTCCs are permeable to Ba^{2+} but Ba^{2+} ions do not induce Ca^{2+} release from the SR, and thus abolish CDI of LTCCs. As shown in Fig. 5, metabolic inhibition induced only suppression of Ba^{2+} current (I_{Ba}) without the transient increase observed in I_{CaL} . At 100 nmol/L and 1 $\mu\text{mol/L}$ concentrations, FCCP suppressed the ISO-stimulated I_{Ba} by $83.3 \pm 1.6\%$ ($n=7$, $p < 0.05$, 3 patients) and $84.5 \pm 3.5\%$ ($n=3$, $p < 0.05$, 2 patients), respectively (Fig. 5B).

4 Discussion

In the present study we demonstrate that metabolic inhibition has a biphasic effect on I_{CaL} in mammalian cardiac myocytes. In most human atrial and ventricular myocytes and in all tested rat ventricular myocytes which were stimulated by isoprenaline metabolic inhibition induced an initial transient increase of I_{CaL} which was followed by a strong inhibition of I_{CaL} . The transient increase in ISO stimulated I_{CaL} was induced by various metabolic inhibitors (FCCP, DNP, rotenone, antimycin A) demonstrating that the observed effect is not dependent on the method used to induce metabolic inhibition. In control experiments without metabolic inhibition we have observed only run-down of the I_{CaL} , and no spontaneous increase in I_{CaL} was detected (Supplementary Figure S5). We propose that the transient I_{CaL} increase during the initial phase of metabolic inhibition is due to impaired intracellular calcium cycling and suggest that this increase in I_{CaL} is mediated by weak CDI due to metabolic failure of Ca^{2+} release from SR via RyRs.

Presumably, during metabolic inhibition the stimulation of I_{CaL} and suppression of I_{CaL} happen at the same or almost at the same time and therefore it is difficult to discriminate between these two processes.

To our knowledge, I_{CaL} stimulation by metabolic inhibitors has not been previously reported in cardiac cells. McHugh and Beech (1996) (McHugh and Beech, 1996) found a similar effect in single smooth muscle cells isolated from basilar artery of the guinea pig where the mitochondrial uncoupler 2,4-dinitrophenol induced an initial stimulation of I_{CaL} in some of the cells, which was followed by a pronounced current inhibition. The authors have attributed this effect to a leftward shift of the Ca^{2+} channel activation curve. In our experiments, the leftward shift in the I_{CaL} I-V relationship was induced only by application of ISO which is consistent with β -adrenergic stimulation (Tiaho et al., 1991).

However, there was no additional shift in the I_{CaL} I-V curve during the application of FCCP (Fig. 2). Therefore, the transient increase in I_{CaL} during metabolic inhibition in cardiac myocytes can not be attributed to the changes in LTCCs voltage-dependent activation.

Our recent study (Kanaporis et al., 2017) has revealed that metabolic inhibition only exerts a strong inhibitory effect on I_{CaL} in amphibian heart cells without the initial transient increase observed here. In frog cardiomyocytes SR is scarce and lacks ryanodine channels (Tijskens et al., 2003). Thus LTCCs are the primary source of Ca^{2+} for contraction in amphibian cardiomyocytes and Ca^{2+} release from the SR plays only a minor role, while in mammalian heart the SR serves as the main Ca^{2+} store and plays an important role in the regulation of the activity of LTCCs (Cens et al., 2006; Richard et al., 2006). Therefore, we have hypothesized that impairment of Ca^{2+} induced Ca^{2+} release (CICR) leading to diminished Ca^{2+} dependent inactivation (CDI) of LTCCs might underlie the transient increase in I_{CaL} amplitude. It has previously been demonstrated that metabolic inhibition affects properties of intracellular Ca^{2+} release and decreases frequency of spontaneous Ca^{2+} waves (Overend et al., 2001; Zima et al., 2013). The reports on the changes in SR Ca^{2+} load during metabolic inhibition are rather inconsistent as some studies have reported no change or decrease in SR Ca^{2+} content (Fukumoto et al., 2005; Hanninen et al., 2010) while others demonstrated an increase in SR Ca^{2+} load (Overend et al., 2001). Such discrepancies in the results can be at least partially explained by the reported biphasic effect of metabolic inhibition on intracellular Ca^{2+} signaling, consisting of an initial inhibition followed by stimulation of SR Ca^{2+} release (Zima et al., 2013). Recently, it was demonstrated that during metabolic inhibition calcium transients (CaTs) are decreased and AP is prolonged (Mačianskienė et al., 2018). This reduction of CaT can result in the slowdown of LTCCs inactivation and may be involved in the prolongation of AP in rabbit heart (Mačianskienė et al., 2018). In our study double exponential fit to I_{CaL} curves showed significant increase of the fast component τ_1 during the transient increase of I_{CaL} in the presence of FCCP. It is known that the fast component of I_{CaL} inactivation is dependent on the magnitude of Ca^{2+} release from SR (Sham, 1997). Thus, this also points to the possible connection between transient increase of I_{CaL} and intracellular Ca^{2+} release during metabolic inhibition. In order to test the hypothesis that the changes of Ca^{2+} release from SR result in potentiation of I_{CaL} we have performed a series of experiments where, prior to the application of metabolic inhibitors, SR Ca^{2+} release was suppressed. Suppression of SR Ca^{2+} release in cardiac cells reduces CICR and therefore leads to decreased CDI of LTCCs. Consequently, a significant augmentation of I_{CaL} was observed in the presence of thapsigargin or ryanodine (Fig. 3 and Supplementary Figure S3). This is consistent with observations that elimination of the CICR-dependent CDI, by depletion of Ca^{2+} from the SR using thapsigargin or ryanodine, significantly prolongs AP duration in rat ventricular myocytes (Fauconnier et al., 2003; Takamatsu et al., 2003). When thapsigargin and ryanodine treated rat ventricular myocytes were exposed to FCCP the initial increase of I_{CaL} was completely abolished and only pronounced suppression of I_{CaL} was observed. In addition, in the presence of thapsigargin the effect of FCCP on the I_{CaL} inactivation fast time constant τ_1 (a parameter that is greatly affected by CDI) was eliminated. We suggest that under these conditions CDI of I_{CaL} is abolished and therefore increase in I_{CaL} is not observed. For the same reason there is a lack of I_{CaL} facilitation in thapsigargin treated rat cardiac cells, where the decay characteristics of fast and slow components of I_{CaL} were significantly augmented and subsequent metabolic inhibition resulted only in a rapid decrease of I_{CaL} without any initial I_{CaL} increase. The finding that CDI plays a crucial role in the initial potentiation of I_{CaL} during metabolic inhibition was also supported by the experiments where CDI was eliminated by equimolar substitution of extracellular Ca^{2+} for Ba^{2+} (Fig. 5).

The double exponential fits and analysis of ISO stimulated I_{CaL} decay traces showed that transient I_{CaL} increase during metabolic inhibition mimics what is observed during rapid pacing induced facilitation of I_{CaL} , which is well known to be related to CDI (Richard et al., 2006). Increased frequency of stimulation resulted in a significant augmentation of the time constant of the fast component τ_1 , while τ_2 was significantly diminished. Exactly the same changes in I_{CaL} kinetics were revealed at the stimulation phase of I_{CaL} during metabolic inhibition (Supplementary Table 3 and Supplementary Table 4). These results are consistent with Tiaho et al (1994), where they describe the decay phases of I_{CaL} at the stimulation frequency of 1 Hz.

We were not able to find a relation between the transient increase in I_{CaL} (or its absence) and any pathology of the human heart. Moreover, we found that even myocytes derived from the same patient showed a different response to metabolic inhibition, i.e. in some cardiomyocytes the transient increase of I_{CaL} was registered, while in others – not. We suggest that the observation, that transient I_{CaL} increase is induced just in some of human atrial and ventricular myocytes while all rat ventricular myocytes exhibit increase in I_{CaL} during metabolic, can be attributed to the pathophysiological state of the cells as well as to differences between species. Rat ventricular myocytes were isolated from the healthy animals, while human cardiomyocytes were obtained from the patients of different age and various cardiac pathologies (Supplementary Table 1). Disorganization and degradation of T-tubule network during pathophysiological remodeling of the heart is known to disrupt LTCC and RyR coupling (Zhang et al., 2013) and contribute to the variability of response in myocytes isolated from the patients. In addition, it was demonstrated that even in the healthy hearts there are substantial differences between small rodents and large mammals in T-tubule organization and RyR distribution, with T-tubule network being much denser and Ca^{2+} release from SR more homogeneous in rodents ventricular myocytes (Heinzel et al., 2002).

In conclusion, our data demonstrate that metabolic inhibition in mammalian cardiomyocytes causes an initial stimulation of I_{CaL} which is due to a reduction in SR function and an alleviation of the Ca^{2+} -dependent inactivation of the L-type Ca^{2+} channels. These mechanisms may contribute in formation of arrhythmias in the ischemic heart and may be targets for new treatment methods of heart pathologies in the future.

5 Abbreviations

AP, action potential; I_{CaL} , L-type calcium current; LTCC, L-type calcium channel; SR, sarcoplasmic reticulum; τ_1 , time constant of the fast component of I_{CaL} ; τ_2 , time constant of slow component of I_{CaL} ; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; ISO, isoprenaline.

6 Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author contributions

RF and JJ designed the research. RT, GK and JJ performed the experiments. RT and JJ analyzed the data and created the figures. RT, GK, RF and JJ wrote the article. All authors reviewed and approved the final version of the manuscript.

8 Funding

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Figure legends

Figure 1. Effect of metabolic inhibition on isoprenaline stimulated I_{CaL} in human and rat cardiomyocytes. (A) Effect of FCCP on ISO-stimulated I_{CaL} in human atrial cell. Traces of I_{CaL} shown in the panel were recorded at the times indicated by the corresponding letters on the main graph. (B) Peak amplitude of I_{CaL} during exposure of ISO-stimulated human atrial cells to FCCP. White bar represents 100% of ISO stimulation, grey bars represent transient stimulation of I_{CaL} and black bars represent suppression of I_{CaL} during application of FCCP. (C) Effect of 2,4-Dinitrophenol (DNP) on ISO-stimulated I_{CaL} in human ventricular cell. Traces of I_{CaL} shown in the panel were recorded at the times indicated by the corresponding letters on the main graph. (D) Peak amplitude of I_{CaL} during exposure of ISO-stimulated human ventricular cells to DNP and FCCP. White bar represents 100% of ISO stimulation, grey bars represent transient stimulation of I_{CaL} and black bars represent suppression of I_{CaL} during application of DNP and FCCP. (E) Effect of FCCP on ISO-stimulated I_{CaL} in rat ventricular cell. Traces of I_{CaL} shown in the panel were recorded at the times indicated by the corresponding letters on the main graph. (F) Peak amplitude of ISO-stimulated I_{CaL} during exposure of rat ventricular cells to various inhibitors of oxidative phosphorylation. DNP – 2,4-Dinitrophenol, Rot – rotenone, Ant A – antimycin A, white bar represents 100% of ISO stimulation, grey bars represent transient stimulation of I_{CaL} and black bars represent suppression of I_{CaL} during metabolic inhibition. Values are presented as means \pm SEM for the number of cells indicated in parentheses. * $P < 0.05$ vs. ISO alone.

Figure 2. Current-voltage relationship and inactivation of I_{CaL} in rat ventricular cells.

(A) Current-voltage (I-V) relationship, (B) normalized I-V relationship. Insets on the right: I-V measurements (representative traces of I_{CaL} registered during S1 in control, in ISO stimulated cell and in the presence of FCCP). (C) inactivation curves of I_{CaL} under basal conditions, during ISO (1 $\mu\text{mol/L}$) stimulation and during exposure to FCCP (100 nmol/L) in the presence of ISO in rat ventricular cells. Inset: Double-pulse protocol used for the inactivation curves (see Methods for details). I_{CaL} peak amplitude during S2 pulse is expressed as a percentage of I_{CaL} measured at 0 mV without conditioning pulse S1 and plotted as a function of S1 pulse potential. Points represent experimentally measured values; lines represent values calculated with Boltzmann equation. Insets below: Inactivation of I_{CaL} (representative traces of I_{CaL} registered during S2 in control, in ISO stimulated cell and in the presence of FCCP).

Figure 3. Effect of FCCP on isoprenaline stimulated I_{CaL} in rat cardiomyocytes after suppression of cytosolic Ca^{2+} release. (A) A typical experiment representing the effect of FCCP on I_{CaL} in ISO-stimulated cell during exposure to thapsigargin. Traces of I_{CaL} shown in the panel were recorded at the times indicated by the corresponding letters on the main graph. (B) Peak amplitude of I_{CaL} during exposure of ISO-stimulated rat ventricular cells to FCCP in the presence of thapsigargin. Values are presented as means \pm SEM for the number of cells indicated in parentheses. * $P < 0.05$ vs. ISO alone. (C) The time dependent inactivation of LTCCs currents. Traces of I_{CaL} in isoprenaline (ISO, 1 $\mu\text{mol/L}$) stimulated (black line) rat cell during application of thapsigargin (Thapsig, 2 $\mu\text{mol/L}$) (red line) and metabolic inhibition by FCCP (0.1 $\mu\text{mol/L}$) (blue line). Dashed lines represent double exponential fits of I_{CaL} s decay.

Figure 4. Fast facilitation of I_{CaL} in rat ventricular cells is abolished by metabolic inhibition and by suppression of cytosolic Ca^{2+} release. (A) Depolarization protocol for induction of fast facilitation of I_{CaL} shown on top. In ISO-stimulated rat ventricular cells and in control conditions increased frequency (to 1 Hz) depolarization induced increase of I_{CaL} amplitude and delayed the inactivation; FCCP abolished this effect in ISO-stimulated cells. (B) Peak amplitude and area (time integral) of I_{CaL} during the 4th depolarizing pulse in control conditions, in ISO stimulated cells and during exposure of 0.1 μ mol/L of FCCP. Values are presented as means \pm SEM for the number of cells indicated in parentheses. * $P < 0.05$ vs I_{CaL} of the first pulse. (C) Suppression of cytosolic Ca^{2+} release by thapsigargin abolished the increase of I_{CaL} amplitude itself during increased frequency depolarization and FCCP additionally reduced I_{CaL} amplitude.

Figure 5. Effect of FCCP on isoprenaline stimulated Ba^{2+} current through L-type calcium channels in human atrial myocytes. Only suppression of Ca^{2+} channel current was registered during metabolic inhibition after equimolar substitution of extracellular Ca^{2+} for Ba^{2+} . (A) A typical experiment representing the effect of FCCP on I_{Ba} in ISO-stimulated cell. (B) Peak amplitude of I_{Ba} during exposure of ISO-stimulated human atrial cells to FCCP. Values are presented as means \pm SEM for the number of cells indicated in parentheses. * $P < 0.05$ vs. ISO alone.

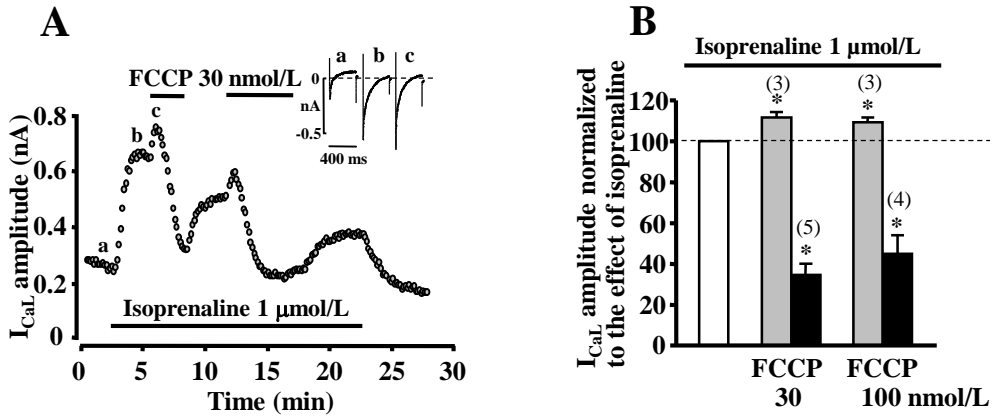
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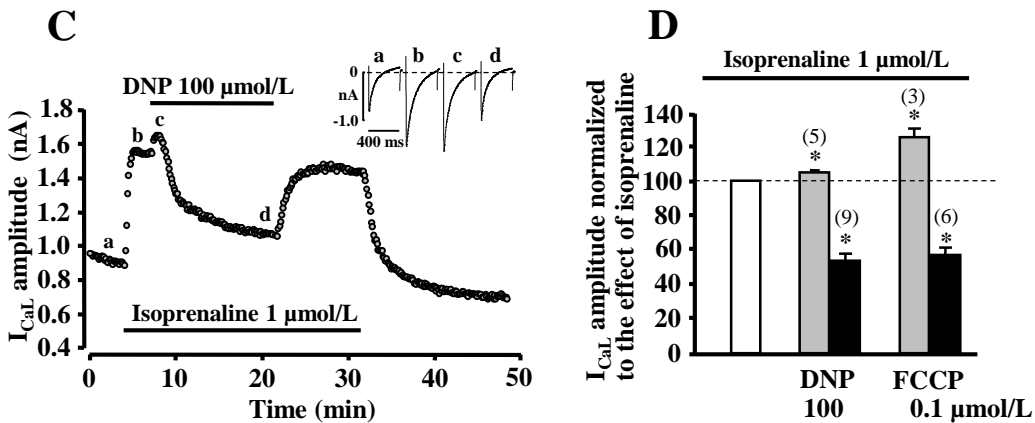
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Human atrial myocytes



Human ventricular myocytes



Rat ventricular myocytes

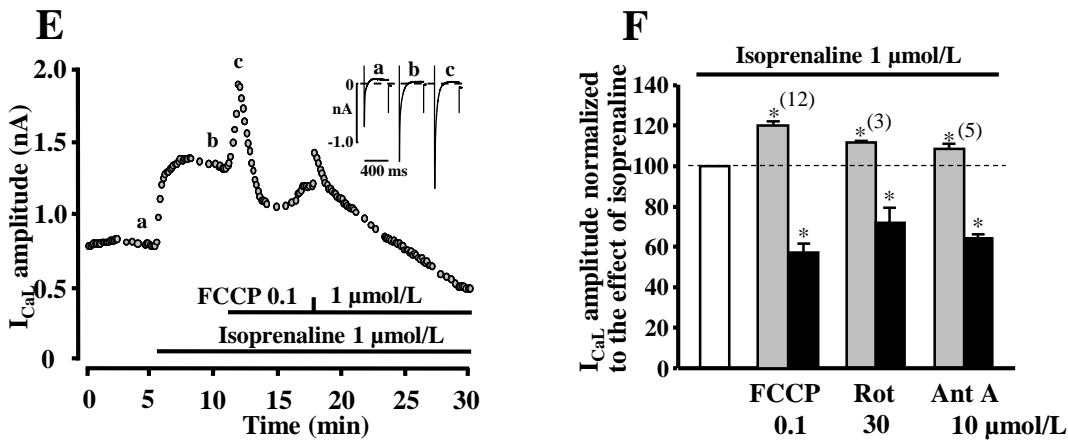


Figure 1

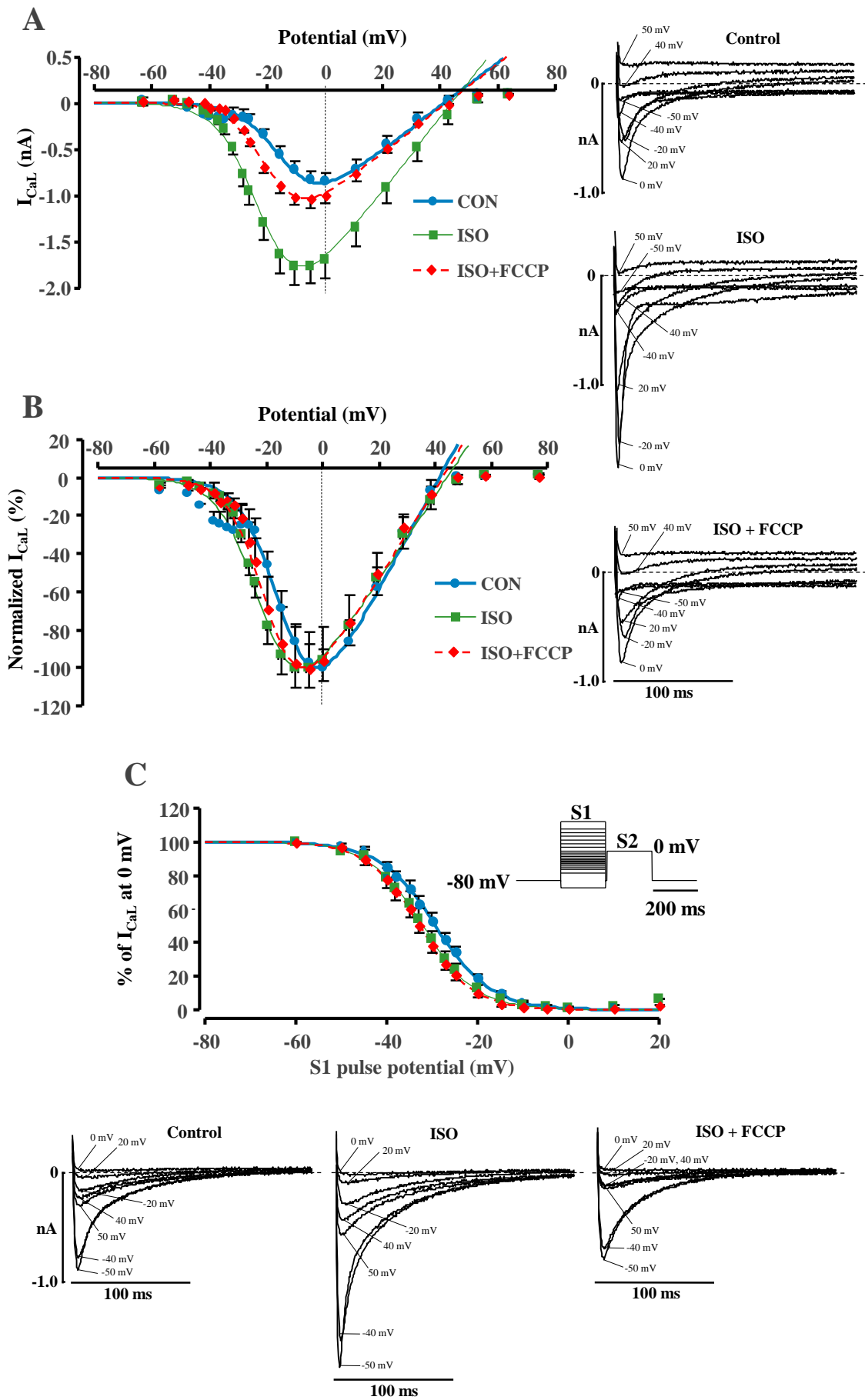


Figure 2

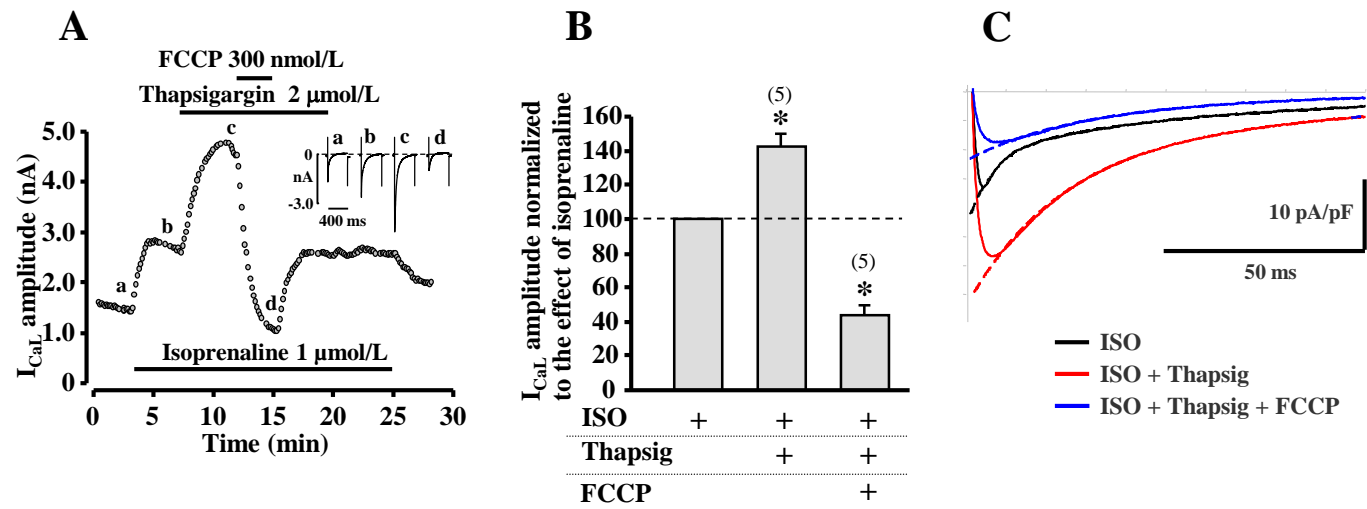


Figure 3

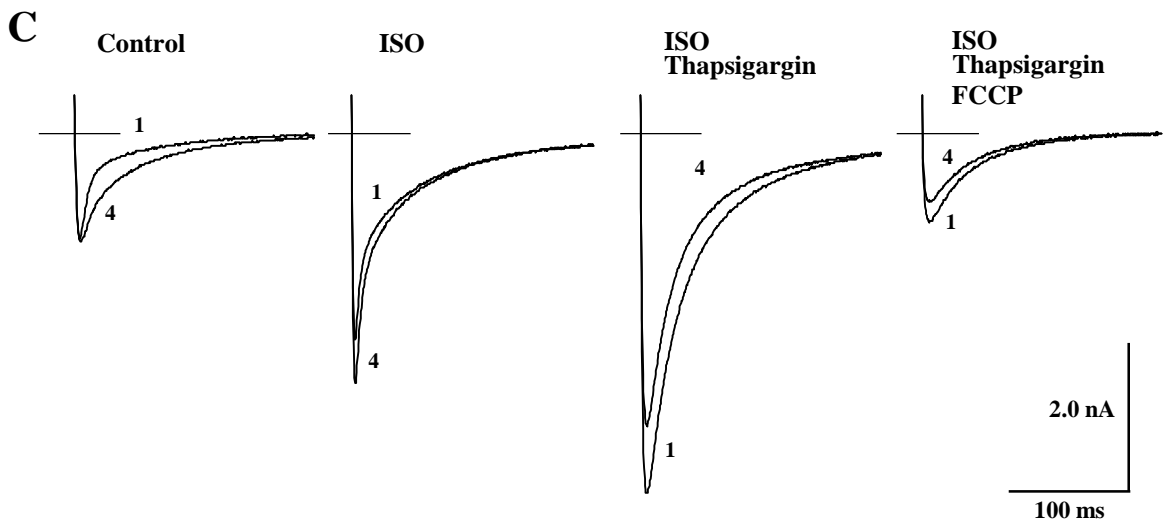
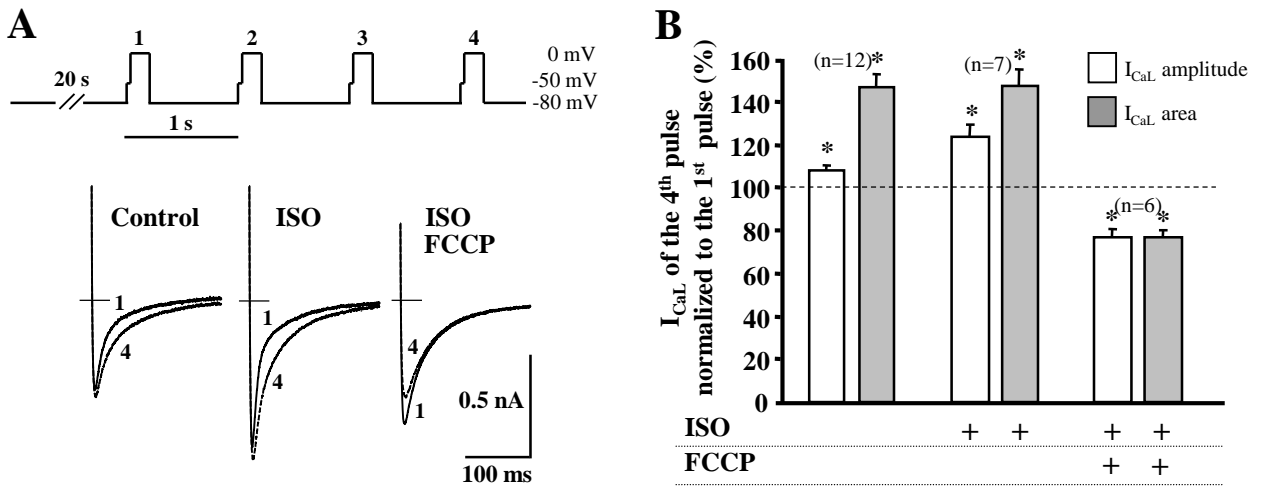


Figure 4

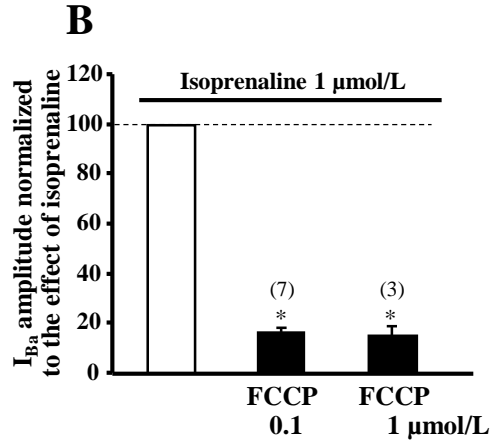
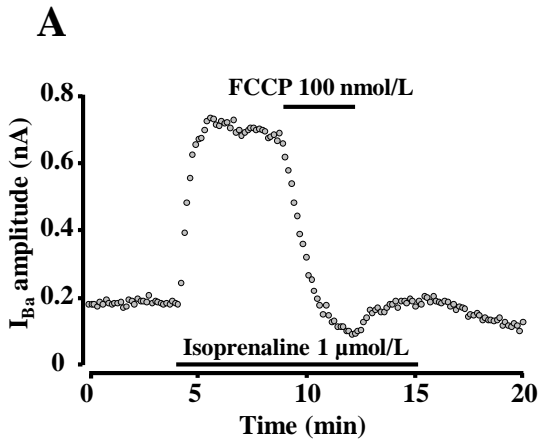


Figure 5

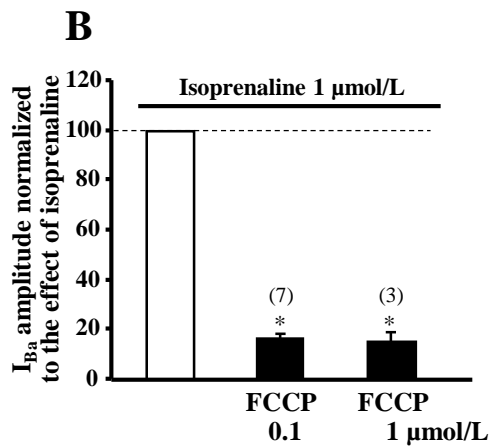
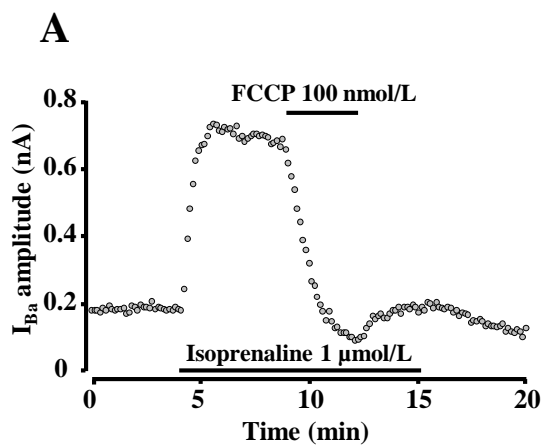


Figure 6

Supplementary Material

Metabolic inhibition induces transient increase of L-type Ca²⁺ current in human and rat cardiac myocytes

Rimantas Treinys, Giedrius Kanaporis, Rodolphe Fischmeister and Jonas Jurevičius*

* Correspondence: Jonas Jurevičius: jonas.jurevicius@lsmuni.lt

Table 1

Clinical characteristics of patients

Patient data

Age range (years)	14 – 81
Mean age (years)	63
Female, <i>n</i>	9
Male, <i>n</i>	7
Total, <i>n</i>	16

Surgical intervention

Aortic valve surgery ^a , <i>n</i>	5/16
Mitral valve surgery ^a , <i>n</i>	8/16
CABG surgery ^a , <i>n</i>	9/16
Bentall operation, <i>n</i>	1/16

Origin of specimen

Right atrial appendage, <i>n</i>	7/16
Left atrial appendage, <i>n</i>	1/16
Left ventricle apex, <i>n</i>	5/16
Interventricular septum, <i>n</i>	2/16
Left ventricle papillary muscle, <i>n</i>	1/16

^a Some patients underwent both valve surgery and coronary bypass graft surgery. One patient underwent aortic valve, mitral valve and tricuspid valve surgery.

Supplementary Material

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Table 2

The effect of metabolic inhibitors on isoprenaline stimulated I_{CaL}

Pharmacol. agent ($\mu\text{mol/L}$)	Increase of I_{CaL} in HAM	Suppression of I_{CaL} in HAM	Increase of I_{CaL} in HVM	Suppression of I_{CaL} in HVM	Increase of I_{CaL} in RVM	Suppression of I_{CaL} in RVM
FCCP (0.1)	9.0±2.8% (n=3)	55.8±9.8% (n=4)	24.8±5.3% (n=3)	42.5±3.5% (n=6)	20.2±2.7% (n=12)	42.4±4.6% (n=12)
DNP (100)	–	–	5.0±0.9% (n=5)	44.8±4.4% (n=9)	–	–
Ant A (10)	–	–	–	–	8.3±2.3% (n=5)	35.5±1.9% (n=5)
Roten (30)	–	–	–	–	11.4±0.2% (n=3)	28.2±7.6% (n=3)

HAM – human atrial myocytes, HVM – human ventricular myocytes,

RVM – rat ventricular myocytes, Ant A – antimycin A, Roten – rotenone.

Supplementary Material

Metabolic inhibition induces transient increase of L-type Ca^{2+} current in human and rat cardiac myocytes

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* Correspondence: Jonas Jurevičius: jonas.jurevicius@ismuni.lt

Table 3

The effect of FCCP and thapsigargin-induced depletion of Ca^{2+} from SR on isoprenaline stimulated I_{CaL} inactivation in rat ventricular myocytes

Parameter	ISO [#]	ISO + FCCP [#] Increase of I_{CaL}	ISO + FCCP Suppr. of I_{CaL}	ISO	ISO + Thapsig.	ISO + Thapsig + FCCP
τ_1	5.3±0.3 ms (n=12)	7.9±1.1 ms (n=12)	13.8±2.1 ms (n=12)	5.5±0.5 ms (n=4)	26.2±2.6 ms (n=4)	26.4±0.5 ms (n=4)
τ_2	48.7±2.9 ms (n=12)	42.6±2.5 ms (n=12)	42.7±3.6 ms (n=12)	70.3±4.2 ms (n=4)	88.2±3.0 ms (n=4)	69.1±3.7 ms (n=4)

Table 4

The effect of increased frequency of depolarization on isoprenaline stimulated I_{CaL} inactivation in rat ventricular myocytes

Parameter	1 st pulse [#]	4 th pulse [#]
τ_1	5.8±0.5 ms (n=8)	7.3±0.7 ms (n=8)
τ_2	62.1±4.8 ms (n=8)	51.3±2.6 ms (n=8)

Note the tendency of τ_1 and τ_2 changes during the increase of I_{CaL} because of the effect of FCCP (Table 3) and increased depolarization frequency (Table 4).

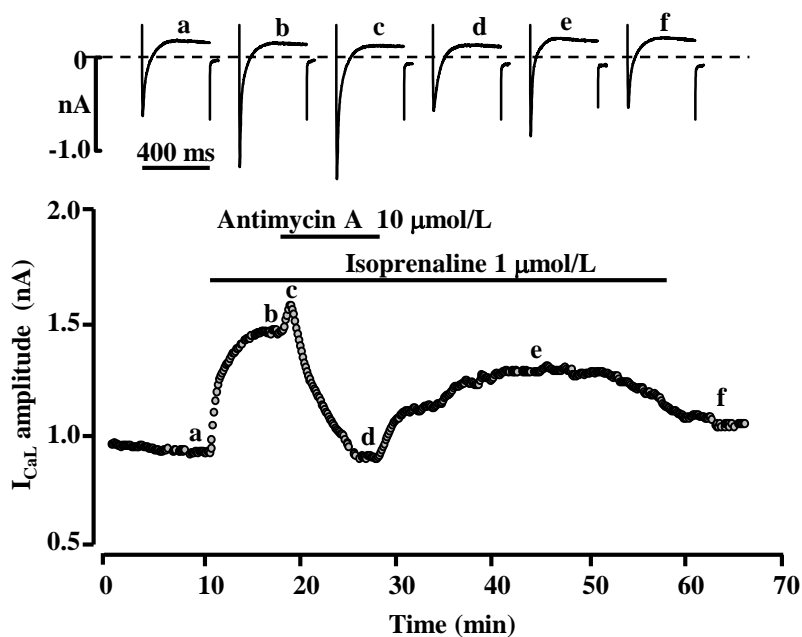
Supplementary Material

Metabolic inhibition induces transient increase of L-type Ca^{2+} current in human and rat cardiac myocytes

Rimantas Treinys, Giedrius Kanaporis, Rodolphe Fischmeister and Jonas Jurevičius*

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Transient increase of LTCCs current in isoprenaline stimulated rat cardiac myocyte during metabolic inhibition



Supplementary Figure S1. Effect of antimycin A on ISO-stimulated I_{CaL} in rat ventricular cell. Traces of I_{CaL} shown on top were recorded at the times indicated by the corresponding letters on the main graph.

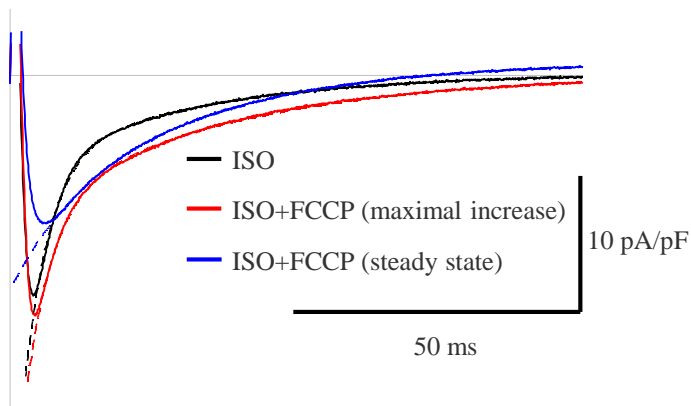
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The time dependent inactivation of LTCCs current in isoprenaline stimulated rat cardiac myocyte during metabolic inhibition



Supplementary Figure S2. Traces of I_{CaL} in isoprenaline (ISO, 1 $\mu\text{mol/L}$) stimulated (black line) rat cell during FCCP (0.1 $\mu\text{mol/L}$) induced maximal stimulation (red line) and suppression (blue line). Dashed lines represent double exponential fits of I_{CaL} 's decay.

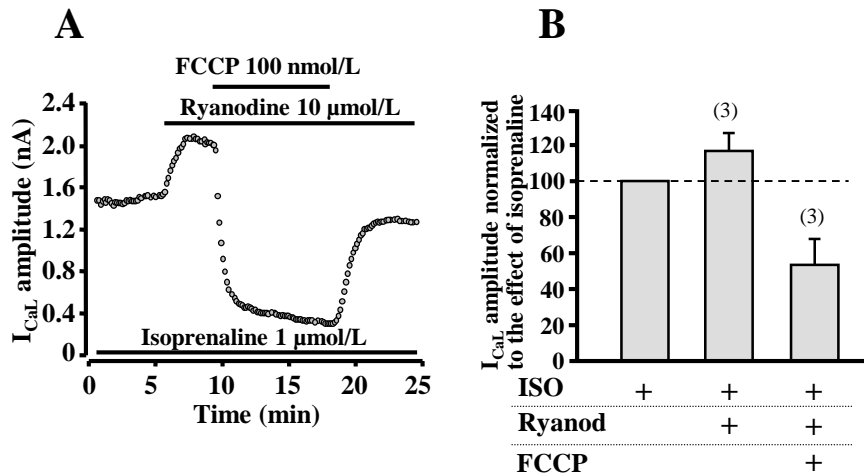
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Suppression of cytosolic Ca^{2+} release



Supplementary Figure S3. Effect of FCCP on isoprenaline stimulated I_{CaL} in rat cardiomyocytes after suppression of cytosolic Ca^{2+} release. (A) A typical experiment representing the effect of FCCP on I_{CaL} in ISO-stimulated cell during exposure to ryanodine. (B) Peak amplitude of I_{CaL} during exposure of ISO-stimulated rat ventricular cells to FCCP in the presence of ryanodine. Values are presented as means \pm SEM for the number of cells indicated in parentheses.

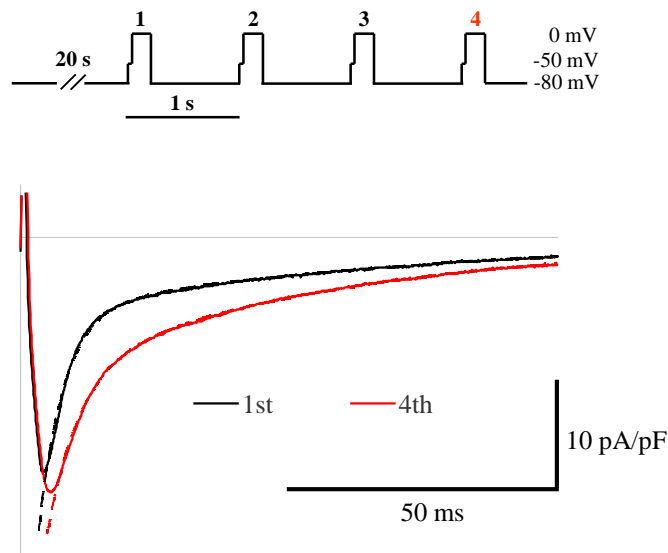
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Facilitation of I_{CaL} in isoprenaline stimulated rat cardiac myocyte



Supplementary Figure S4. Depolarization protocol for induction of I_{CaL} facilitation shown on top. Traces of I_{CaL} in isoprenaline ($1 \mu\text{mol/L}$) stimulated rat cell during application of 1 Hz stimulus. Superimposed are currents evoked at the 1st (black line) and 4th (red line) stimulations. Dashed lines represent double exponential fits of I_{CaL} 's decay.

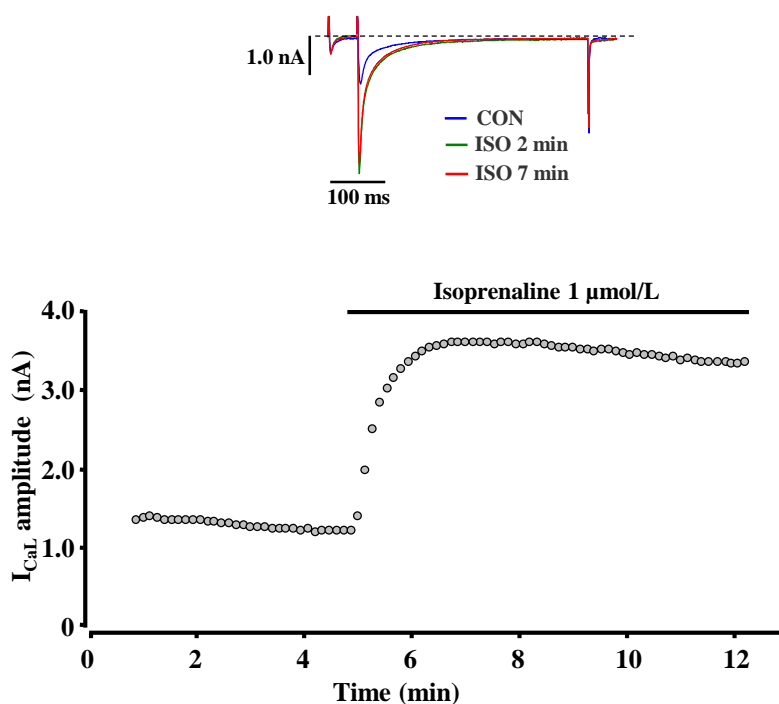
Supplementary Material

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Stability of I_{CaL} over time in rat ventricular myocyte



Supplementary Figure S5. Effect of isoprenaline on I_{CaL} in rat ventricular cell. Only run-down of the I_{CaL} was registered in control conditions and during β -adrenergic stimulation by isoprenaline (ISO), and no spontaneous increase in I_{CaL} was detected.

Traces of I_{CaL} shown on top were recorded in control conditions and during stimulation by ISO.