



Article

# Metabolic Inhibition Induces Transient Increase of L-type $\text{Ca}^{2+}$ 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  $\text{Ca}^{2+}$  channel (LTCC) activity. In this study, however, we show that metabolic inhibition results in a biphasic effect on LTCC current ( $I_{\text{CaL}}$ ) in human and rat cardiac myocytes: an initial increase of  $I_{\text{CaL}}$  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  $I_{\text{CaL}}$  in cardiac myocytes during  $\beta$ -adrenergic stimulation by isoprenaline, a non-selective agonist of  $\beta$ -adrenergic receptors. The whole-cell patch-clamp technique was used to record the  $I_{\text{CaL}}$  in single cardiac myocytes. The initial increase of  $I_{\text{CaL}}$  was induced by a wide range of metabolic inhibitors (FCCP, 2,4-DNP, rotenone, antimycin A). In rat cardiomyocytes, the initial increase of  $I_{\text{CaL}}$  was eliminated when the cells were pre-treated with thapsigargin leading to the depletion of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR). Similar results were obtained when  $\text{Ca}^{2+}$  release from the SR was blocked with ryanodine. These data suggest that the increase of  $I_{\text{CaL}}$  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  $I_{\text{CaL}}$  when  $\text{Ca}^{2+}$  was replaced by  $\text{Ba}^{2+}$ , eliminating CDI of LTCCs. We conclude that the initial increase in  $I_{\text{CaL}}$  observed during the metabolic inhibition in human and rat cardiomyocytes is a consequence of an acute reduction of  $\text{Ca}^{2+}$  release from SR resulting in reduced CDI of LTCCs.

**Keywords:** L-type  $\text{Ca}^{2+}$  current; metabolic inhibition; FCCP; heart; cardiac myocytes; sarcoplasmic reticulum; calcium dependent inactivation

## 1. Introduction

The L-type  $\text{Ca}^{2+}$  channels (LTCCs) provide  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  enters the cell via the L-type current ( $I_{\text{CaL}}$ ) and this triggers the release of a larger amount of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs) in a process called  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release [1]. The increase of intracellular  $\text{Ca}^{2+}$  concentration in the vicinity of closely associated  $\text{Ca}^{2+}$  channels and RyRs leads to calcium dependent inactivation (CDI) of LTCCs [2] and reduction of  $I_{\text{CaL}}$  [3]. Hence, the SR participates in regulation of the activity of plasmalemmal L-type  $\text{Ca}^{2+}$  channels [4]. 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  $\text{Ca}^{2+}$ , which directly activates the contraction of myofilaments. The mechanisms

of  $\text{Ca}^{2+}$  regulation in diseased cardiac cells may be disordered. Malfunction of LTCCs and RyRs interaction may affect the 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 [5,6]. Numerous studies show a reduction in cardiac LTCC activity and  $I_{\text{CaL}}$  during metabolic inhibition, however the mechanisms involved are not clear [7–11]. In ischemic heart failure, the cardiac  $\beta$ -adrenergic receptors are downregulated, partly because of the decreased number of the receptors and partly because of internalization and desensitization of the receptors, however also because the sympathetic system is activated and the level of catecholamines is increased [12,13]. Thus, we decided to investigate the effect of metabolic inhibition on  $I_{\text{CaL}}$  during  $\beta$ -adrenergic stimulation in human atrial and ventricular myocytes and rat ventricular myocytes. Surprisingly, we found that metabolic inhibition causes an acute transient increase of  $I_{\text{CaL}}$  amplitude followed by a rapid reduction. This is consistent with the most recent study demonstrating AP prolongation during metabolic inhibition in the rabbit heart [6], however is in contrast with what we reported recently in frog cardiomyocytes, where metabolic inhibition only suppresses  $I_{\text{CaL}}$  without the initial increase [11]. We explored the mechanisms responsible for the initial increase in  $I_{\text{CaL}}$  during metabolic inhibition. Our results point to a modulation of the  $\text{Ca}^{2+}$ -dependent inactivation of LTCCs by the RyRs mediated  $\text{Ca}^{2+}$  release.

## 2. Results

### 2.1. Effect of Metabolic Inhibition on $I_{\text{CaL}}$ in Human Atrial Myocytes

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

The initial stimulation of  $I_{\text{CaL}}$  induced by FCCP was recorded in six out of nine (~67%) tested human atrial myocytes. In these cells, the application of 30 or 100 nmol/L of FCCP resulted in a transient increase of ISO-stimulated  $I_{\text{CaL}}$  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  $I_{\text{CaL}}$  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 (Figure 1a,b).

### 2.2. Effect of Metabolic Inhibition on $I_{\text{CaL}}$ in Human Ventricular Myocytes

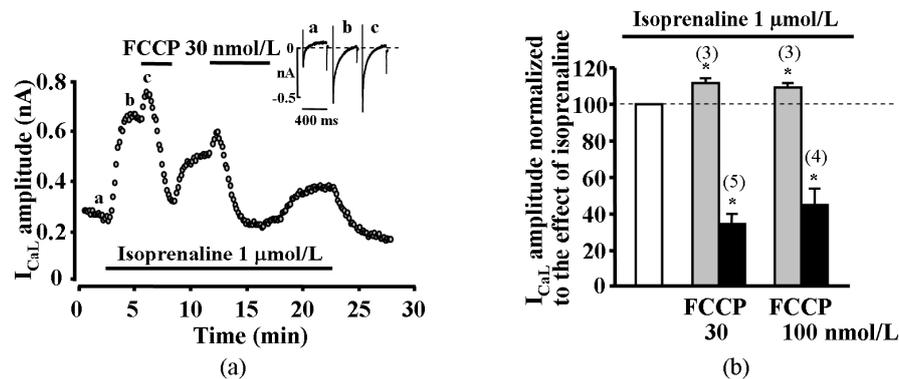
The next series of experiments were set to determine the effect of metabolic inhibition on  $I_{\text{CaL}}$  in human ventricular myocytes. The ventricular myocytes for these series of experiments were derived from eight patients. We performed experiments in human ventricle myocytes, applying metabolic inhibitors FCCP and 2,4-Dinitrophenol (DNP) on ISO (1  $\mu\text{mol/L}$ ) stimulated cells. We found that FCCP induced a rapid initial stimulation of  $I_{\text{CaL}}$  in three out of six ventricular myocytes (five patients). Further, 100 nmol/L of FCCP increased ISO-stimulated  $I_{\text{CaL}}$  by  $24.8 \pm 5.3\%$  ( $n = 3$ ,  $p < 0.05$ ). The reduction of  $I_{\text{CaL}}$  induced by FCCP was registered in all tested cells, i.e.,  $I_{\text{CaL}}$  was reduced by  $42.5 \pm 3.5\%$  ( $n = 6$ ,  $p < 0.05$ ). Another uncoupler, DNP, also evoked a transient increase of ISO-stimulated  $I_{\text{CaL}}$  in five ventricular myocytes from nine (three patients). Further, 100  $\mu\text{mol/L}$  of DNP increased ISO-stimulated  $I_{\text{CaL}}$  by  $5.0 \pm 0.9\%$  ( $n = 5$ ,  $p < 0.05$ ). The reduction of  $I_{\text{CaL}}$  by  $44.8 \pm 4.4\%$  induced by DNP was registered in all ( $n = 9$ ,  $p < 0.05$ ) human ventricular myocytes (Figure 1c,d). The initial stimulation of  $I_{\text{CaL}}$  induced by uncouplers FCCP and DNP was recorded in eight out of 15 (~53%) tested human ventricular myocytes.

### 2.3. 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 [7–10], 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 rat ventricular myocytes. Application of FCCP (100 nmol/L) in ISO-stimulated cells induced a transient increase in  $I_{CaL}$  by  $20.2 \pm 2.7\%$  ( $n = 12, p < 0.05$ , four animals) that was followed by  $I_{CaL}$  reduction by  $42.4 \pm 4.6\%$  ( $n = 12, p < 0.05$ ) (Figure 1e,f). Higher concentrations of FCCP like 300 nmol/L (not shown) and 1  $\mu\text{mol/L}$  (Figure 1e) also induced a biphasic effect in rat ventricular myocytes.

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  $\mu\text{mol/L}$ ), 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  $\mu\text{mol/L}$ ), 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, Figure 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 S1.

#### Human atrial myocytes



#### Human ventricular myocytes

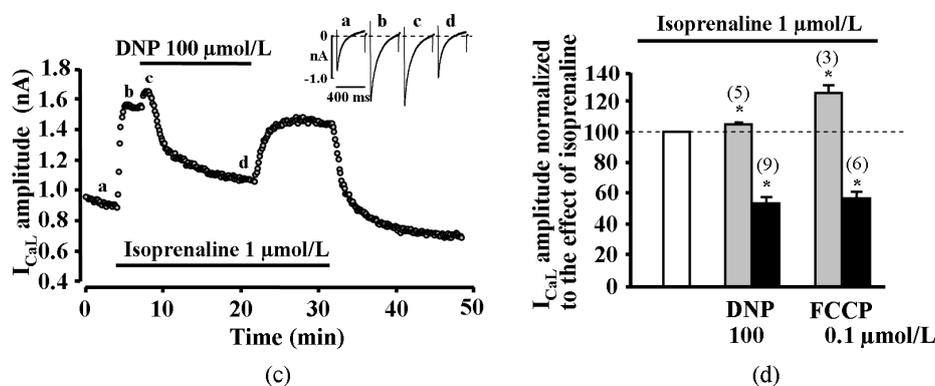
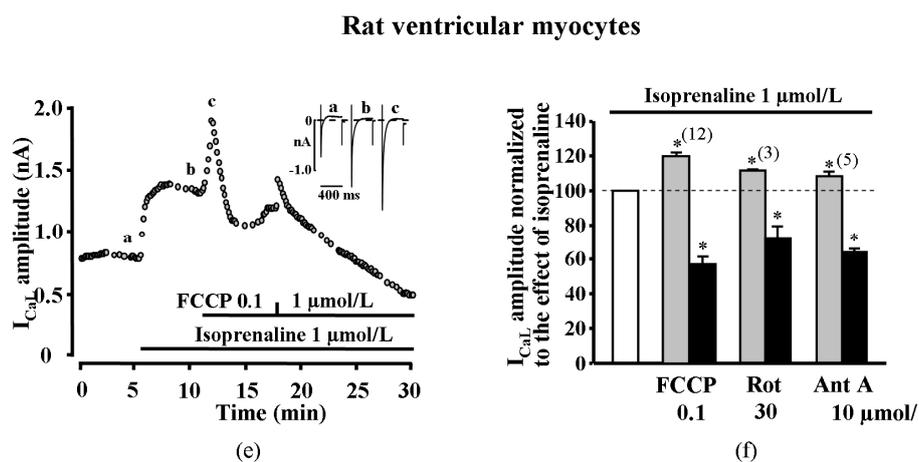


Figure 1. Cont.



**Figure 1.** Effect of metabolic inhibition on isoprenaline stimulated  $I_{CaL}$  in human and rat cardiomyocytes. (a) Effect of FCCP on isoprenaline (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. (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. (e) Effect of FCCP on ISO-stimulated  $I_{CaL}$  in rat ventricular cells. 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. White bars in panels (b), (d) and (f) represent 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. \*  $p < 0.05$  versus ISO alone. DNP—2,4-Dinitrophenol, Rot—rotenone, Ant A—antimycin A.

#### 2.4. 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 [14]. 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  $\tau_1$  increased from  $5.3 \pm 0.3$  ms to  $7.9 \pm 1.1$  ms ( $n = 12$ ,  $p < 0.05$ ), while the time constant of the slow component  $\tau_2$  decreased from  $48.7 \pm 2.9$  ms to  $42.6 \pm 2.5$  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,  $\tau_1$  and  $\tau_2$  were  $13.8 \pm 2.1$  ms ( $n = 12$ ,  $p < 0.05$ ) and  $42.7 \pm 3.6$  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  $\tau_1$ , suggesting a diminished calcium dependent inactivation (CDI) of the channels.

We also measured the current–voltage relationship (I–V) and the steady–state inactivation of  $I_{CaL}$  in rat ventricular cells (Figure 2). Averaged data of  $I_{CaL}$  I–V curves (Figure 2a and normalized data in Figure 2b) and inactivation (Figure 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  $-13.26 \pm 0.87$  mV,  $-20.71 \pm 0.81$  mV and  $-18.80 \pm 0.99$  mV and  $k$  was  $6.9 \pm 0.4$ ,  $7.1 \pm 0.6$  and  $6.5 \pm 0.2$  ( $n = 3$ ), respectively. As expected, ISO significantly shifted I–V curve to the

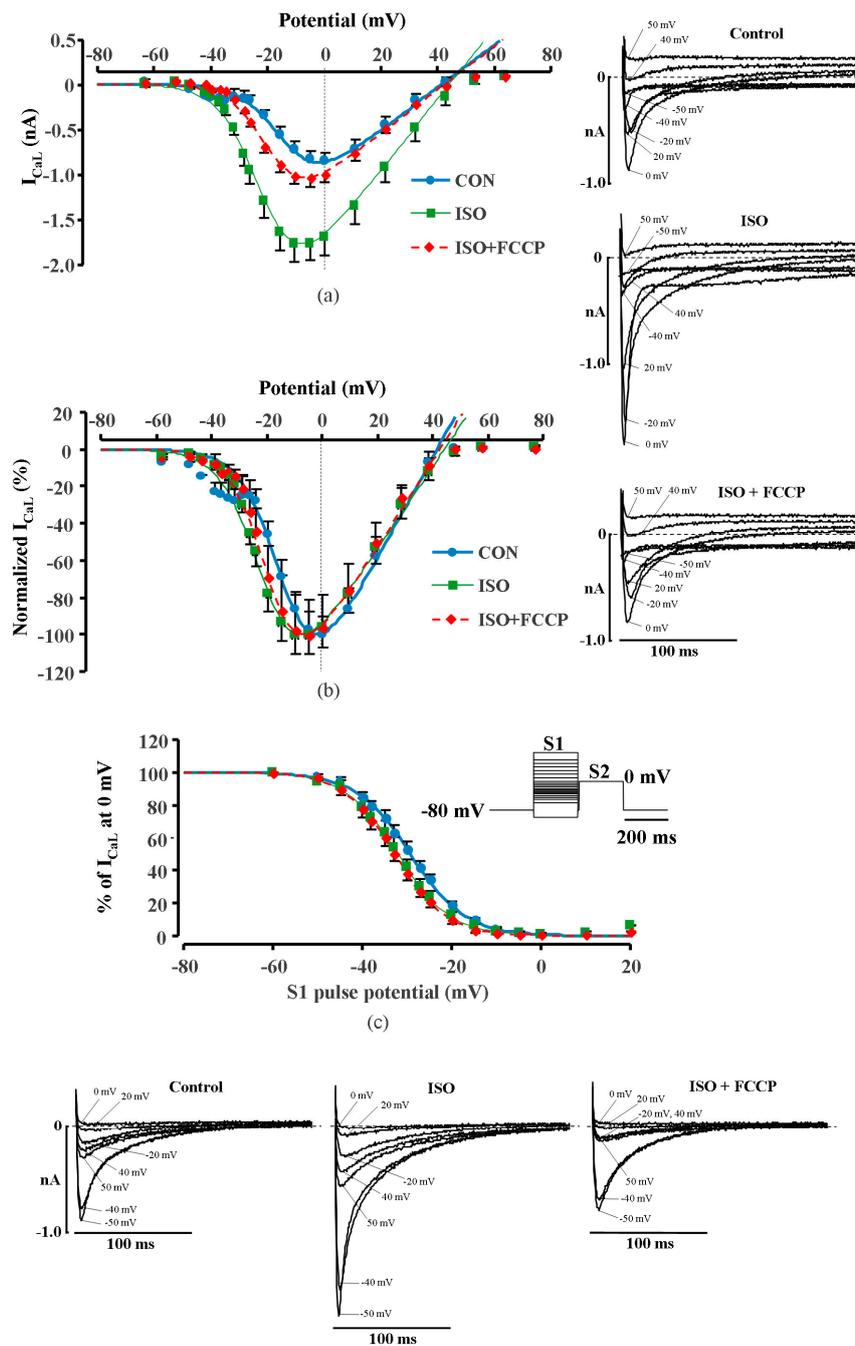
left [15] by  $> 7$  mV ( $p < 0.05$ ) while FCCP had no significant effect on the  $I_{CaL}$  I-V relationship. The fit of the steady-state inactivation curves for control, ISO and FCCP led to  $V_{1/2}$  values of  $-29.20 \pm 1.15$  mV,  $-32.05 \pm 1.60$  mV and  $-32.75 \pm 1.42$  mV, respectively, and  $k$  was  $6.2 \pm 0.1$ ,  $5.9 \pm 0.3$  and  $5.7 \pm 0.1$  ( $n = 3$ ), respectively. Again, ISO significantly shifted the inactivation of  $I_{CaL}$  to the left by  $\approx 3$  mV ( $p < 0.05$ ), however 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.

### 2.5. Suppression of Cytosolic $Ca^{2+}$ Release Abolish Transient Increase of $I_{CaL}$

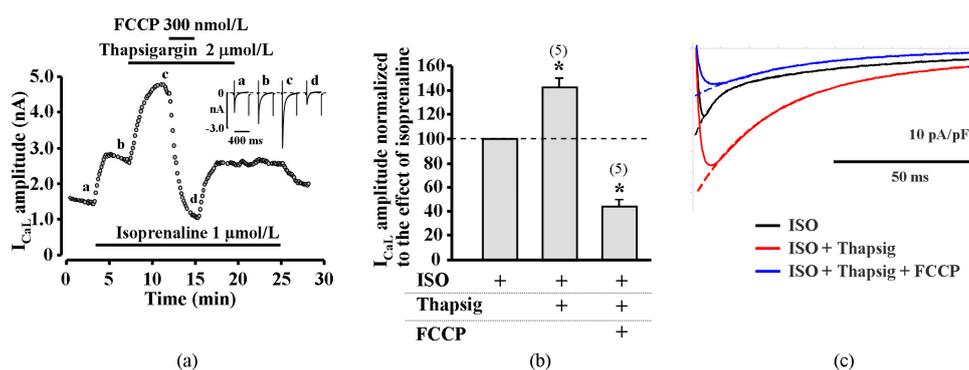
The above results showed that in human and rat cardiac cells, metabolic inhibition induced initial transient increase of  $I_{CaL}$ . In our previous study, we found no such stimulation during metabolic inhibition in frog cardiomyocytes [11] where SR is scarce and lacks ryanodine channels [16] and, in contrast to mammalian cells, LTCCs do not have such a 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 [17]. 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  $17.7 \pm 0.5$  pA/pF to  $25.1 \pm 0.9$  pA/pF ( $n = 5$ ,  $p < 0.05$ ). In the presence of thapsigargin, the initial  $I_{CaL}$  increase was completely eliminated and only an inhibitory effect of FCCP on ISO stimulated  $I_{CaL}$  was observed ( $n = 5$ ,  $p < 0.05$ ) (Figure 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  $\tau_1$  and  $\tau_2$  increased from  $5.5 \pm 0.5$  ms to  $26.2 \pm 2.6$  ms and from  $70.3 \pm 4.2$  ms to  $88.2 \pm 3.0$  ms ( $n = 4$ ,  $p < 0.05$ ), respectively. In the presence of thapsigargin, the effect of FCCP on  $\tau_1$  was abolished. The time constant  $\tau_1$  in such conditions remained similar to thapsigargin treated cells and was  $26.4 \pm 0.5$  ms, while  $\tau_2$  was similar to ISO stimulated  $I_{CaL}$  and was  $69.1 \pm 3.7$  ms ( $n = 4$ ). The representative  $I_{CaL}$  traces and their fits are shown in Figure 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. Similar to experiments with thapsigargin, ryanodine increased ISO stimulated  $I_{CaL}$  and the 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 the first and fourth depolarizing pulse. In control conditions, increased stimulation frequency enhanced  $I_{CaL}$  amplitude by  $8.3 \pm 2.1\%$  and the area by  $46.9 \pm 6.3\%$  ( $n = 12$ ,  $p < 0.05$ ) during the fourth 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 (Figure 4a,b). Double exponential fits of  $I_{CaL}$  decay during the first and fourth depolarizing pulses revealed that the time constant of the fast component  $\tau_1$  increased from  $5.8 \pm 0.5$  ms to  $7.3 \pm 0.7$  ms ( $n = 8$ ,  $p < 0.01$ ), while the time constant of the slow component  $\tau_2$  decreased from  $62.1 \pm 4.8$  ms to  $51.3 \pm 2.6$  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  $23.1 \pm 3.3\%$  ( $n = 6$ ,  $p < 0.05$ , Figure 4a,b) during the fourth depolarizing pulse, respectively.



**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 of 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  $\mu$ 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).



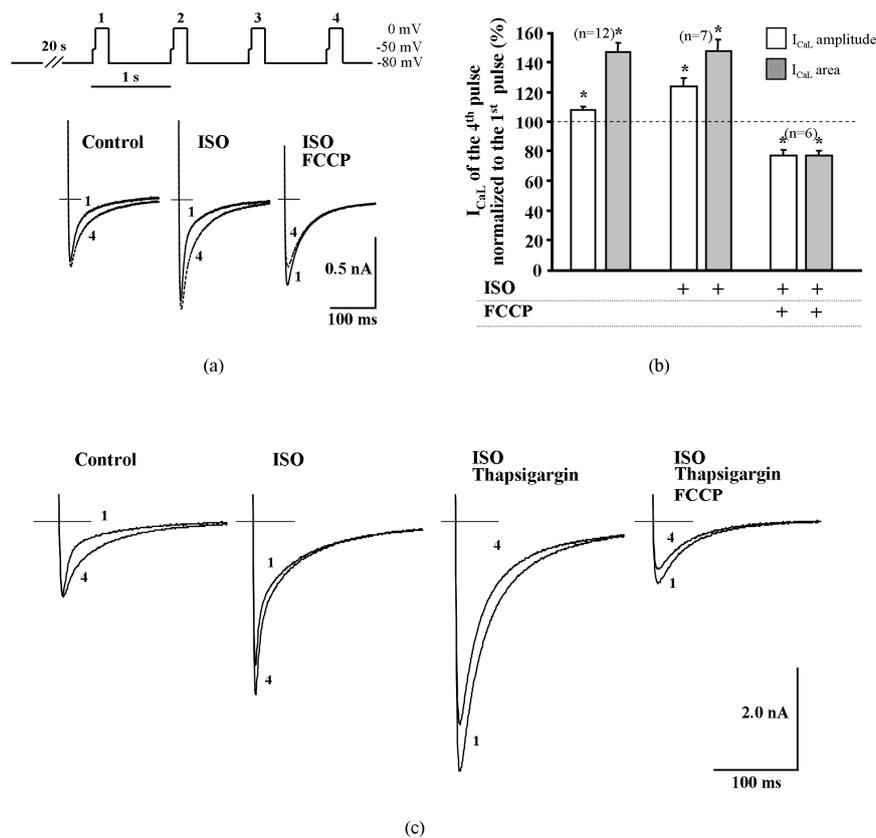
**Figure 3.** Effect of FCCP on isoprenaline stimulated  $I_{CaL}$  in rat cardiomyocytes after the suppression of cytosolic  $Ca^{2+}$  release. (a) A typical experiment representing the effect of FCCP on  $I_{CaL}$  in ISO-stimulated cells during exposure to thapsigargin. Traces of  $I_{CaL}$  shown 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. \*  $p < 0.05$  versus ISO alone. (c) The time dependent inactivation of LTCCs currents. Traces of  $I_{CaL}$  in isoprenaline (ISO, 1  $\mu$ mol/L) stimulated (black line) rat cell during application of thapsigargin (Thapsig, 2  $\mu$ mol/L) (red line) and metabolic inhibition by FCCP (0.1  $\mu$ mol/L) (blue line). Dashed lines represent double exponential fits of  $I_{CaL}$ s decay.

These data indicate that pharmacological or frequency-dependent depletion of  $Ca^{2+}$  from the cytosolic  $Ca^{2+}$  store results in an increased  $\tau_1$  and thus reduced CDI, followed by an increase of  $Ca^{2+}$  influx through LTCCs, a process that is possibly observed during the early phase of metabolic inhibition.

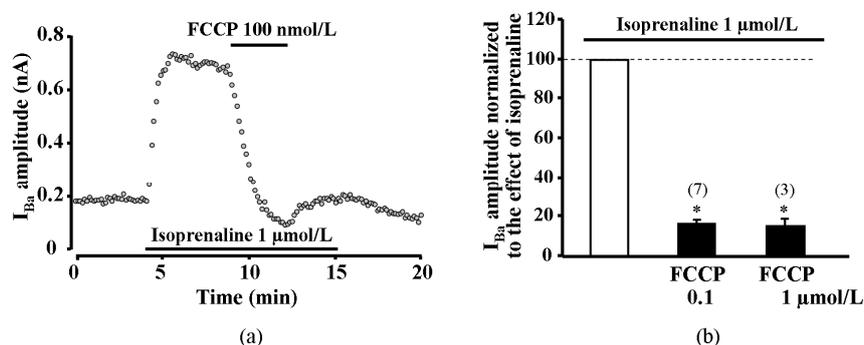
Depletion of  $Ca^{2+}$  from the SR by thapsigargin eliminated  $I_{CaL}$  facilitation (Figure 4c) and, during the application of  $I_{CaL}$  facilitation protocol, the amplitude and area of  $I_{CaL}$  measured during the fourth depolarizing pulse were reduced by  $20.0 \pm 0.6\%$  and  $22.8 \pm 2.4\%$ , respectively ( $n = 4$ ,  $p < 0.05$ ) when compared with the first depolarizing pulse. After the 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 the fourth depolarizing pulse by  $22.7 \pm 1.8\%$  and  $20.0 \pm 1.4\%$ , respectively ( $n = 4$ ,  $p < 0.05$ ). The observation that the application of FCCP abolishes the facilitation of  $I_{CaL}$  in a similar manner as the depletion of  $Ca^{2+}$  from the SR, indicates that FCCP affects CDI of LTCCs.

### 2.6. 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  $Ba^{2+}$ , however  $Ba^{2+}$  ions do not induce  $Ca^{2+}$  release from the SR and, thus, abolish CDI of LTCCs. As shown in Figure 5, metabolic inhibition induced only the suppression of  $Ba^{2+}$  current ( $I_{Ba}$ ) without the transient increase observed in  $I_{CaL}$ . At 100 nmol/L and 1  $\mu$ 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 (Figure 5b).



**Figure 4.** Fast facilitation of  $I_{CaL}$  in rat ventricular cells is abolished by metabolic inhibition and by the suppression of cytosolic  $Ca^{2+}$  release. (a) Depolarization protocol for the 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 an 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 fourth depolarizing pulse in control conditions, in ISO stimulated cells and during exposure of 0.1  $\mu\text{mol/L}$  of FCCP. Values are presented as means  $\pm$  SEM for the number of cells indicated in parentheses. \*  $p < 0.05$  versus  $I_{CaL}$  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 cells. (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. \*  $p < 0.05$  versus ISO alone.

### 3. 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 the tested rat ventricular myocytes that were stimulated by isoprenaline metabolic inhibition, this 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 the control experiments without metabolic inhibition, we 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. The suppression becomes dominant during exposure to the metabolic inhibitors and therefore makes evaluation of dose-response difficult and not reliable. In this study, we seek to reveal the mechanism of the transient increase of  $I_{CaL}$  during metabolic inhibition and did not conduct concentration-dependence experiments.

To our knowledge,  $I_{CaL}$  stimulation by metabolic inhibitors has not been previously reported in cardiac cells. McHugh and Beech (1996) [18] found a similar effect in single smooth muscle cells isolated from the 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  $\beta$ -adrenergic stimulation [15]. However, there was no additional shift in the  $I_{CaL}$  I-V curve during the application of FCCP (Figure 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 [11] 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 [16]. 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 hearts, the SR serves as the main  $Ca^{2+}$  store and plays an important role in the regulation of the activity of LTCCs [4,19]. 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 [10,20]. The reports on the changes in SR  $Ca^{2+}$  load during metabolic inhibition are rather inconsistent as some studies have reported no change or a decrease in SR  $Ca^{2+}$  content [8,21], while others demonstrated an increase in SR  $Ca^{2+}$  load [20]. 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 [10]. The effect of metabolic inhibition on SR may arise due to the changes in high-energy phosphate compounds and/or the increase in intracellular acidosis. Recently, we have shown that acidosis may play a key role in the suppression of LTCCs activity during metabolic inhibition [11]. The intracellular acidification may also inhibit the release of  $Ca^{2+}$  from SR as well as the reuptake of  $Ca^{2+}$  to the SR by SR  $Ca^{2+}$  ATP-ase, the activity of which may also be inhibited by deficiency of energy compounds. During metabolic inhibition, both  $Ca^{2+}$  release (RyRs) and reuptake ( $Ca^{2+}$  ATP-ase) mechanisms are inhibited to a different extent [20]. Recently, it was demonstrated that during metabolic inhibition, calcium transients (CaTs) are decreased and AP is prolonged [6]. This reduction of CaT can result in the slowdown of LTCCs inactivation and may be involved in the prolongation of AP in rabbit hearts [6].

The transient increase of  $I_{CaL}$  during ischemia may lead to early afterdepolarisation and long QT which can be followed by arrhythmias. In our study, double exponential fit to  $I_{CaL}$  curves showed a significant increase of the fast component  $\tau_1$  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 [2]. Thus, this also points to the possible connection between the 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 the 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 (Figure 3 and Supplementary Figure S3). This is consistent with observations that the elimination of the CICR-dependent CDI using thapsigargin or ryanodine, significantly prolongs AP duration in rat ventricular myocytes [22,23]. 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  $\tau_1$  (a parameter that is greatly affected by CDI) was eliminated. We suggest that under these conditions, CDI of  $I_{CaL}$  is abolished and, therefore, an 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  $Ba^{2+}$  (Figure 5). There are also evidences that mitochondria can control the local  $Ca^{2+}$  level in the micro-domain near SR ryanodine receptors and play an important role in the regulation of intracellular  $Ca^{2+}$  waves and arrhythmogenesis.

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 [4]. Increased frequency of stimulation resulted in a significant augmentation of the time constant of the fast component  $\tau_1$ , while  $\tau_2$  was significantly diminished. Exactly the same changes in  $I_{CaL}$  kinetics were revealed at the stimulation phase of  $I_{CaL}$  during metabolic inhibition. 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.

The increase in  $I_{CaL}$  amplitude was registered not in all human cardiac myocytes and this may point to possible impairment in cells derived from pathological cardiac specimens. However, 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 it was 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 an increase in  $I_{CaL}$  during metabolic inhibition, can be attributed to the pathophysiological state of the particular cell as well as to differences between species. The pathophysiological state of the cell and thus the different response to metabolic inhibition may be related with attenuation or downregulation of  $\beta$ -adrenergic signaling in human heart cells [12,13]. Rat ventricular myocytes were isolated from the healthy animals, while human cardiomyocytes were obtained from patients of different ages and various cardiac pathologies (Supplementary Table S2). Disorganization and degradation of T-tubule network during pathophysiological remodeling of the heart is known to disrupt LTCC and RyR coupling [24] and to 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 the T-tubule network being much denser and  $Ca^{2+}$  release from SR more

homogeneous in rodents' ventricular myocytes [25]. There is also evidence that mitochondria are involved in the control of the local  $\text{Ca}^{2+}$  level in the micro-domain near SR RyRs and that they play an important role in the regulation of intracellular  $\text{Ca}^{2+}$  waves and arrhythmogenesis [26]. The initial increase of  $I_{\text{CaL}}$  shows the abnormalities of  $\text{Ca}^{2+}$  cycling in heart cells during ischemia (metabolic inhibition) which may lead to the arrhythmogenic processes of the heart.

## 4. Materials and Methods

### 4.1. Isolation of Cardiomyocytes

#### 4.1.1. 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 (16 November 2015 No. BE-2-40). 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 had received a pharmacological treatment (including  $\text{Ca}^{2+}$ -channel blockers, digitalis,  $\beta$ -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 received sedatives, anesthesia and antibiotics. Information on patient group and specimens is provided in Supplementary Table S2. Dissociation of the cells was performed as described previously [27]. Briefly, the specimens of the atrial or ventricular tissue were washed in an oxygenated  $\text{Ca}^{2+}$  free Tyrode solution (in mmol/L): 136 NaCl, 5.4 KCl, 1.1  $\text{MgCl}_2$ , 10 HEPES, 20 taurine, 5 sodium pyruvate, 10 D-glucose, 0.3  $\text{NaH}_2\text{PO}_4$ , 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  $\text{Ca}^{2+}$  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 five minutes, a small sample of solution was examined under a microscope to evaluate the stage of cell dissociation.

#### 4.1.2. 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 [28]. The protocol was approved by the State Food and Veterinary Service of the Republic of Lithuania (24 September 2015 No. G2-34). Adult rat ventricular myocytes were obtained by retrograde perfusion from hearts of male Wistar rats (160–180 g) as described previously [29]. 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  $\text{Ca}^{2+}$  free Ringer solution (in mmol/L): 117 NaCl, 5.8 KCl, 4.4  $\text{NaHCO}_3$ , 1.5  $\text{KH}_2\text{PO}_4$ , 1.7  $\text{MgCl}_2$ , 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  $\text{Ca}^{2+}$  free Ringer solution and the 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.

#### 4.1.3. 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<sup>+</sup> free control external solution containing (in mmol/L): 127 NaCl; 10 HEPES; 20 CsCl; 4 NaHCO<sub>3</sub>; 0.8 NaH<sub>2</sub>PO<sub>4</sub>; 1.8 MgCl<sub>2</sub>; 1.8 CaCl<sub>2</sub>; 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 MΩ 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<sub>2</sub>, 0.062 CaCl<sub>2</sub>, 5 creatine phosphate disodium salt, 3.1 Na<sub>2</sub>ATP, 0.42 Na<sub>2</sub>GTP and 10 HEPES; pH to 7.3 was adjusted with CsOH. The whole-cell patch-clamp technique was used to record the I<sub>CaL</sub> in human and rat cardiac myocytes as described previously [30]. Ionic currents through LTCCs were registered every 8 s. I<sub>CaL</sub> facilitation was generated after 20 s of rest applying four depolarizing pulses from –80 mV holding potential to 0 mV for 200 ms (frequency 1 Hz). Tetrodotoxin (30 μmol/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 test pulse [27].

Equilibrium for steady-state frequency-dependent facilitation of I<sub>CaL</sub> is reached after four stimulations [31], therefore I<sub>CaL</sub> facilitation was assessed comparing the first I<sub>CaL</sub> trace with the fourth trace at 1 Hz. The peak of I<sub>CaL</sub> and the I<sub>CaL</sub> area, an integral of I<sub>CaL</sub> for 200 ms (pA × ms), were registered at the time of the first and fourth depolarizing pulse. The time integral of I<sub>CaL</sub> was registered in part of the experiments to elucidate the origin of I<sub>CaL</sub> increase during metabolic inhibition and to compare the initial effect of FCCP with frequency-dependent facilitation and reduced CDI.

To determine the current–voltage (I–V) relationship and inactivation of I<sub>CaL</sub>, a double pulse voltage-clamp protocol was applied every 4 s (see insert in Figure 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.

#### 4.1.4. 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 Ca<sup>2+</sup> 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 to the experiments. When the stock solutions in DMSO or ethanol were used, the corresponding amount of solvent was also added to the external control solution.

#### 4.1.5. Data Analysis

Inactivation of I<sub>CaL</sub> traces were best fitted by the sum of two sequential exponentials using the expression:

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

where I is the current at time t; I<sub>CaL,1</sub> and I<sub>CaL,2</sub> are the amplitudes and τ<sub>1</sub> and τ<sub>2</sub> are the time constants of the fast and slow components of I<sub>CaL</sub>, respectively. The fitting of I<sub>CaL</sub> inactivation traces was performed using the modified protocol described by [32] 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 of the experiments, the correlation coefficient was  $\geq 0.985$ .

Steady-state inactivation curves ( $B_1$ ) and I–V traces ( $B_2$ ) were fitted with Boltzmann equations:

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

$$B_2(V) = g(V - V_{rev})/(1 + \exp((V_{1/2} - V)/k)), \quad (3)$$

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

Data is expressed as the means  $\pm$  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  $\beta$ -adrenergic agonist.

## 5. Conclusions

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 to the formation of arrhythmias in the ischemic heart and may be targets for new treatment methods of heart pathologies in the future.

**Supplementary Materials:** Supplementary materials can be found at <http://www.mdpi.com/1422-0067/20/6/1501/s1>.

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## Abbreviations

LTCC	L-type $Ca^{2+}$ channel
SR	Sarcoplasmic reticulum
CDI	Calcium dependent inactivation
RyRs	Ryanodine receptors
ISO	Isoprenaline
FCCP	Carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazone

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