

Inositol 1,4,5-trisphosphate-mediated sarcoplasmic reticulum–mitochondrial crosstalk influences adenosine triphosphate production via mitochondrial Ca^{2+} uptake through the mitochondrial ryanodine receptor in cardiac myocytes

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Received 25 February 2015; revised 31 May 2016; accepted 21 June 2016; online publish-ahead-of-print 5 August 2016

Time for primary review: 35 days

Aims

Elevated levels of inositol 1,4,5-trisphosphate (IP_3) in adult cardiac myocytes are typically associated with the development of cardiac hypertrophy, arrhythmias, and heart failure. IP_3 enhances intracellular Ca^{2+} release via IP_3 receptors (IP_3Rs) located at the sarcoplasmic reticulum (SR). We aimed to determine whether IP_3 -induced Ca^{2+} release affects mitochondrial function and determine the underlying mechanisms.

Methods and results

We compared the effects of IP_3Rs - and ryanodine receptors (RyRs)-mediated cytosolic Ca^{2+} elevation achieved by endothelin-1 (ET-1) and isoproterenol (ISO) stimulation, respectively, on mitochondrial Ca^{2+} uptake and adenosine triphosphate (ATP) generation. Both ET-1 and isoproterenol induced an increase in mitochondrial Ca^{2+} (Ca^{2+}_m) but only ET-1 led to an increase in ATP concentration. ET-1-induced effects were prevented by cell treatment with the IP_3 antagonist 2-aminoethoxydiphenyl borate and absent in myocytes from transgenic mice expressing an IP_3 chelating protein (IP_3 sponge). Furthermore, ET-1-induced mitochondrial Ca^{2+} uptake was insensitive to the mitochondrial Ca^{2+} uniporter inhibitor Ru360, however was attenuated by RyRs type 1 inhibitor dantrolene. Using real-time polymerase chain reaction, we detected the presence of all three isoforms of IP_3Rs and RyRs in murine ventricular myocytes with a dominant presence of type 2 isoform for both receptors.

Conclusions

Stimulation of IP_3Rs with ET-1 induces Ca^{2+} release from the SR which is tunnelled to mitochondria via mitochondrial RyR leading to stimulation of mitochondrial ATP production.

Keywords

Inositol 1,4,5-trisphosphate • IP_3 sponge • Mitochondrial Ca^{2+} uniporter • Mitochondrial ryanodine receptor • Dantrolene

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1. Introduction

In cardiac myocytes, large amounts of calcium (Ca^{2+}) are released from the sarcoplasmic reticulum (SR) into the cytosol with each heart beat upon activation of ryanodine receptors (RyRs). This Ca^{2+} is directed to sarcomeric structures to initiate muscle contraction. Ca^{2+} is an essential second messenger in cardiac electrical activity and is the direct activator of the myofilaments, which cause contraction. Furthermore, Ca^{2+} within the myocytes is also crucial for signalling purposes as it regulates cell metabolism, cell death, and nuclear gene transcription. The question arises how the myocyte can distinguish between global Ca^{2+} transients required for myocyte contraction and Ca^{2+} signals necessary for signalling purposes.

In addition to the RyRs, inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) are also present on the SR. In contrast to RyRs, these Ca^{2+} release channels are not involved in the rhythmical Ca^{2+} release needed for excitation–contraction (EC) coupling. IP_3Rs are activated through binding of IP_3 which results in Ca^{2+} release from the SR. Although IP_3Rs are less abundant than RyRs,¹ their activation results in measurable Ca^{2+} increase during diastole and systole. It has been previously shown that IP_3 -mediated Ca^{2+} release is an important regulator of nuclear gene transcription.^{2,3} Furthermore, it is a critical player in the pathogenesis of cardiac diseases such as cardiac hypertrophy and heart failure.⁴ Spontaneous Ca^{2+} release by IP_3Rs is one of the factors contributing to the development of arrhythmogenic events in both atrial and ventricular myocytes.^{5,6}

The regulation of cellular metabolism is also Ca^{2+} dependent. By activating key enzymes of the citric acid cycle in the mitochondrial matrix, the mitochondrial matrix Ca^{2+} (together with adenine dinucleotide phosphate and inorganic phosphate) is the key regulator of adenosine triphosphate (ATP) production.⁷ A number of Ca^{2+} uptake pathways have been identified in the inner mitochondrial membrane (IMM).^{8–11} The mitochondrial Ca^{2+} uniporter (MCU) is the most widely studied and considered as the primary Ca^{2+} uptake pathway in mitochondria.^{12–15} The properties of the MCU, however, do not allow mitochondrial Ca^{2+} uptake under physiological conditions (1–2 μM peak cytosolic Ca^{2+} concentrations during systole) because single MCU opening requires about 10–100 μM Ca^{2+} in the cytosol.¹⁶ This is based on two important observations: (i) recent patch-clamp studies on mitochondria have revealed a very high half-activation constant (19 mM) for the MCU,⁸ and (ii) cytosolic Mg^{2+} significantly inhibits the MCU at physiological concentrations (high nanomolar range) of cytosolic Ca^{2+} .^{14,17,18} Recent experimental data obtained on conditional cardiomyocyte-specific mutant mouse lacking Mcu, the pore-forming subunit of the MCU channel, do not support a significant role for the MCU in basal cardiac physiology; however cardiomyocyte-specific deletion did result in a striking inability to increase contractile function in response to the classic beta-adrenergic agonist, isoproterenol (ISO).^{19,20} This raises the important questions if Ca^{2+} influx through the MCU does contribute significantly to ATP production under basal conditions, what drives mitochondrial respiratory rate and which pathway is responsible for Ca^{2+} uptake in mitochondria under normal physiological conditions?

The computational analysis²¹ of Ca^{2+} dynamics in isolated cardiac mitochondria predicts two distinct models of Ca^{2+} uptake: a high affinity fast uptake pathway and a low affinity slow uptake pathway. The low affinity slow Ca^{2+} uptake pathway resembled the characteristics of the MCU; however, the high affinity fast uptake pathway was observed under physiological levels of Ca^{2+} and Mg^{2+} and could be explained only by the properties of the mitochondrial ryanodine receptor [mRyR1 (ryanodine receptor type 1 channel)] located in the IMM. The biochemical and

biophysical properties of the mRyR1 are similar to those of the RyR type 1 expressed in skeletal muscles.²² In contrast to the other RyR subtypes, the mRyR1 serves as a Ca^{2+} uptake channel. The physiological role for the mRyR1 during Ca^{2+} cycling has not been elucidated satisfactorily and remains highly controversial.

Cardiac myocytes have a highly organized structure of sarcolemma, SR, and mitochondria.²³ About 38% of the myocyte volume consists of mitochondria.²⁴ In ventricular myocytes, mitochondria are closely surrounded by the SR with each side neighbouring Ca^{2+} release channels on the junctional SR. In addition to this spatial relationship, dense structures connecting SR and mitochondria to each other could be seen using electron microscopy.²⁵ In non-excitabile cells, the portion of mitochondria taking part in these microdomains was estimated to be 30% of the total number of cellular mitochondria.²⁶ Here, we propose that the close contact of mitochondria and the IP_3R on the SR can create Ca^{2+} fluxes that activate the mRyR1, thereby enabling Ca^{2+} uptake into the mitochondrial matrix. We tested the following hypothesis: IP_3 -mediated Ca^{2+} release from the SR in close contact sites to mitochondria evokes mitochondrial Ca^{2+} uptake. This mitochondrial Ca^{2+} uptake subsequently drives ATP production. We suggest this new concept to be part of the yet unresolved question of how myocytes are able to differentiate between Ca^{2+} signals needed for contraction or signalling purposes.

2. Methods

2.1 Cell isolation

Left ventricular myocytes were isolated from 10- to 16-week-old FVB (Charles River) and IP_3 sponge mice²⁷ and their littermates using a Langendorff perfusion system. Prior to the procedure, mice were anaesthetized by inhalation of isoflurane. When the reflexes were absent, hearts were excised, placed into the Langendorff system and perfused with a Ca^{2+} -free washing solution, followed by an enzyme solution containing Liberase TH (Research Grade, Roche, Basel, Switzerland). Isolated myocytes were kept in Tyrode solution containing 1 mM Ca^{2+} . All protocols were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and approved by the institutional Animal Care and Use Committee.

2.2 Confocal measurements

Laser scanning confocal microscopy (Leica SP5 and a Zeiss LSM 780) was used to follow the changes in cytosolic (Ca^{2+}_i) and mitochondrial (Ca^{2+}_m) Ca^{2+} concentration, mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS) generation, and mitochondrial ATP generation using specific fluorescent indicators. All fluorescence signals were recorded from individual quiescent cells incubated in Tyrode's solution containing 1 mM Ca^{2+} at room temperature. Images were recorded every 10 s. Experiments for *Figure 1G* and *H* and *Figure 3C* and *D* were performed on electrically stimulated myocytes using the same solutions and the same recording protocol as was used with the quiescent cells. Changes of fluorescence intensity are presented as background-subtracted normalized fluorescence (F/F_0) where F_0 is the initial fluorescence recorded under steady-state conditions at the beginning of each experiment. All fluorescent indicators were obtained from Molecular Probes/Life Technologies (Waltham, Massachusetts, USA) unless noted otherwise.

2.3 Ca^{2+} measurements

Ca^{2+} was measured in cytoplasm (Ca^{2+}_i) or in mitochondria (Ca^{2+}_m), using different fluorescent dyes and loading protocols. Changes in

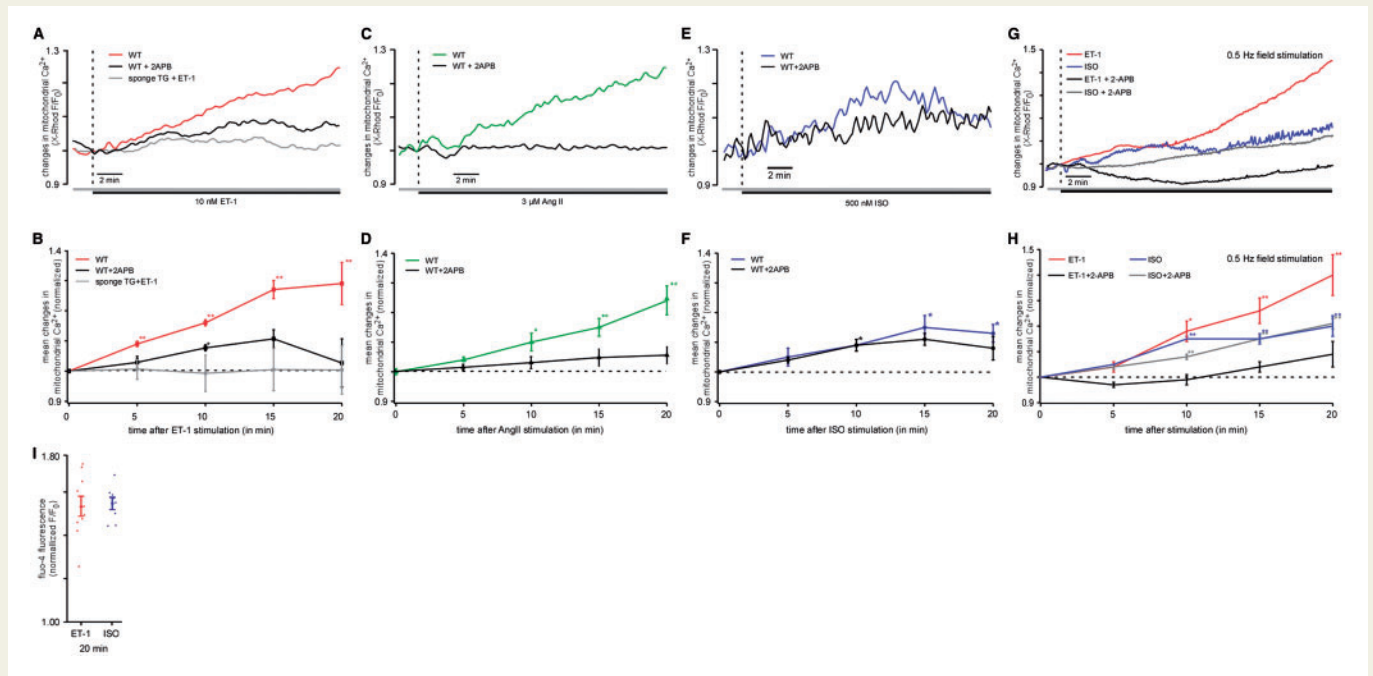


Figure 1 Mitochondrial Ca^{2+} is increased by IP_3R agonists. (A) Original confocal recordings obtained from WT and transgenic (sponge) myocytes stimulated with 10 nM ET-1 \pm 3 μM 2-APB (red: WT, ET-1 alone; black: WT, ET-1 + 2-APB; grey: TG, ET-1 alone) for 20 min. (B) Mean values of resulting mitochondrial Ca^{2+} (Ca^{2+}_m) changes induced by ET-1 in WT mice in the absence ($n = 27$ from seven animals) or presence of 2-APB ($n = 19$ from four animals) and in IP₃ sponge mice [colours same as in (A)]. (C) Original confocal recordings of myocytes stimulated with 3 μM Ang II \pm 3 μM 2-APB (green: Ang II; black: Ang II + 2-APB). (D) Mean values of resulting Ca^{2+}_m changes induced by Ang II in the absence ($n = 10$ from three animals) or presence of 2-APB ($n = 5$ from two animals) [colours same as in (C)]. Both IP₃ agonists evoked a significant increase in Ca^{2+}_m , respectively. This increase could be blocked effectively by 3 μM of the IP₃ receptor blocker 2-APB and was abolished in IP₃-sponge mice. (E) Original confocal recordings of WT and transgenic (sponge) myocytes stimulated with 500 nM ISO \pm 3 μM 2-APB (blue: WT, ISO alone; black: WT, ISO + 2-APB) for 20 min. (F) Mean values of Ca^{2+}_m changes induced by ISO in the absence ($n = 10$ from three animals) or presence of 2-APB ($n = 8$ from two animals) [colours same as in (E)]. The smaller peak and different time course indicate different underlying mechanisms. (G) Original confocal recordings obtained from electrically stimulated (0.5 Hz) WT myocytes stimulated with 10 nM ET-1 \pm 3 μM 2-APB (red: ET-1 alone; black: ET-1 + 2-APB) or ISO \pm 3 μM 2-APB (blue: ISO alone; grey: ISO + 2-APB) for 20 min. (H) Mean values of resulting mitochondrial Ca^{2+} (Ca^{2+}_m) changes induced by ET-1 and ISO in WT mice in the absence (ET-1: $n = 6$ from three animals; ISO: $n = 7$ from two animals) or presence of 2-APB (ET-1 + 2-APB: $n = 5$ from three animals; ISO + 2-APB: $n = 11$ from two animals) [colours same as in (G)]. (I) Mean values of cytosolic Ca^{2+} levels (Ca^{2+}_i) in mouse myocytes measured with the Ca^{2+} -sensitive probe fluo-4. The myocytes were stimulated with ET-1 (red) or ISO (blue), respectively, demonstrating that both agonists induced a stable increase of Ca^{2+}_i . * $P < 0.05$ compared with untreated control using ANOVA Dunnett's test; ** $P < 0.01$ compared with untreated control ANOVA Dunnett's test.

Ca^{2+} concentration are presented as relative changes in fluorescence because the calibration of non-ratiometric dyes used in our study in terms of Ca^{2+} concentration is not possible. For easier understanding of the manuscript, the relative changes in fluorescence measured by us will be termed 'Ca²⁺ concentration' throughout the manuscript. For Ca^{2+}_i measurements, cells were loaded with 20 μM of the Ca^{2+} sensitive dye fluo-4 ($\lambda_{\text{ex}} = 488$ nm and $\lambda_{\text{em}} = 565$ –605 nm) for 20 min at room temperature. Ca^{2+}_m measurements were performed in intact cells loaded with 5 μM X-Rhod-1/AM ($\lambda_{\text{ex}} = 543$ nm and $\lambda_{\text{em}} = 552$ –617 nm) for 30 min at 37 °C followed by 10 min incubation in 1 mM CoCl_2 -containing Tyrode to quench cytosolic X-Rhod-1 fluorescence.^{28,29} To rule out incomplete quenching, only myocytes with fluorescence-free nuclei and mitochondrial staining pattern were used for experiments. To block the MCU, intact myocytes were incubated in 10 μM Ru360 for 1 h at room temperature. One micromolar of Ru360 was present during the experiment. To block the mRyR1 cells were incubated in 1 μM dantrolene for 10 min, and 1 μM dantrolene was present during the experiment. To correct for photobleaching, all data obtained were normalized to an untreated control.

2.4 ROS measurements

Reactive oxygen species measurements were performed in intact cells loaded with 0.5 μM of the fluorescent probe MitoSOX Red ($\lambda_{\text{ex}} = 543$ nm and $\lambda_{\text{em}} = 555$ –617 nm) which preferentially detects superoxide (O_2^-). Cells were loaded for 30 min at 37 °C. To account for MitoSOX Red fluorescence changes due to laser radiation and autofluorescence, control experiments without any treatment were performed. All data were normalized to these control data.

2.5 Mitochondrial membrane potential ($\Delta\Psi_m$) measurements

Intact myocytes were loaded using 10 nM tetramethylrhodamine methyl ester (TMRM) ($\lambda_{\text{ex}} = 543$ nm and $\lambda_{\text{em}} = 565$ –605 nm) for 15 min at 37 °C. To prevent TMRM wash out, all solutions used during the experiments contained 10 nM TMRM. In the end of each experiment, 5 μM carbonyl cyanide p-(tri-fluoromethoxy)phenylhydrazone was added for calibration. To correct for photobleaching and autofluorescence, all data were normalized to the fluorescence levels recorded before treatment.

2.6 ATP measurements

ATP measurements were performed directly utilizing a commercially available luciferine–luciferase ATP detection assay (Invitrogen, Waltham, Massachusetts, USA, #A22066) and indirectly via the free magnesium (Mg^{2+}) concentration using the fluorescent dye mag-fluo-4 (Invitrogen) as described before.³⁰ This method is based on the fact that a large portion of the intracellular Mg^{2+} pool is bound to ATP and present as MgATP. As free $[Mg^{2+}]_i$ is kept constant within a rather narrow range, ATP hydrolysis leads to concomitant increase in free $[Mg^{2+}]_i$ as measured with fluorescent Mg^{2+} indicators such as mag-fluo-4.³¹ Therefore, an increase in mag-fluo-4 fluorescence indicates a decrease in ATP concentration. Vice versa, a decrease in mag-fluo-4 fluorescence indicates an increase in ATP concentration. For ATP measurements, myocytes were loaded with 10 μM mag-fluo-4 ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 565$ – 605 nm) for 30 min at 37 °C. All data from these measurements are expressed as $R = 1 - F/F_0$.

2.7 Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted using a RNA fibrous tissue mini kit (Qiagen, Venlo, Netherlands) and cDNA synthesized from 1 μg of RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with commercial and customized TaqMan probes (Life Technologies™, Waltham, Massachusetts, USA). Target gene mRNA levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which served as housekeeping gene for comparison.

2.8 IP₃-inhibited transgenic mice

As a model for functional IP₃ knockout transgenic mice were used.²³ The animals express an IP₃ chelating protein which absorbs free IP₃ in the cells (IP₃-sponge). The result is a functional IP₃ knockout model.

2.9 Statistics

All data are presented as mean \pm standard error of the mean for the indicated number (n) of experiments. For comparisons of multiple groups, ANOVA was used. After a significant overall F -test, multi-group comparisons were done via Dunnett's test (including correction for multiple testing) for treatment vs. control. The real-time PCR data (Figure 5) were analysed using Student's t -test. Data were considered significant at $P < 0.05$.

3. Results

3.1 IP₃-mediated Ca^{2+} release mediates mitochondrial Ca^{2+} uptake

To test whether IP₃-mediated Ca^{2+} release results in mitochondrial Ca^{2+} (Ca^{2+}_m) changes, isolated murine cardiac myocytes were loaded with the Ca^{2+} sensitive fluorescent dye X-Rhod-1 under conditions which allow preferential dye loading in mitochondria (see Section 2). Therefore, observed changes in X-Rhod-1 fluorescence reflect changes in the mitochondrial Ca^{2+} concentration. To induce IP₃-mediated Ca^{2+} release, the IP₃ receptor agonist endothelin-1 (ET-1) was used to stimulate the cells. Following the addition of 10 nM ET-1 mitochondrial Ca^{2+} was significantly increased during 20 min of observation ($+29 \pm 3\%$) (Figure 1). To test whether this effect was specific for ET-1-mediated IP₃ release, the experiment was repeated with angiotensin II (Ang II), another IP₃R agonist. As shown in Figure 1C and D, 3 μM Ang II also

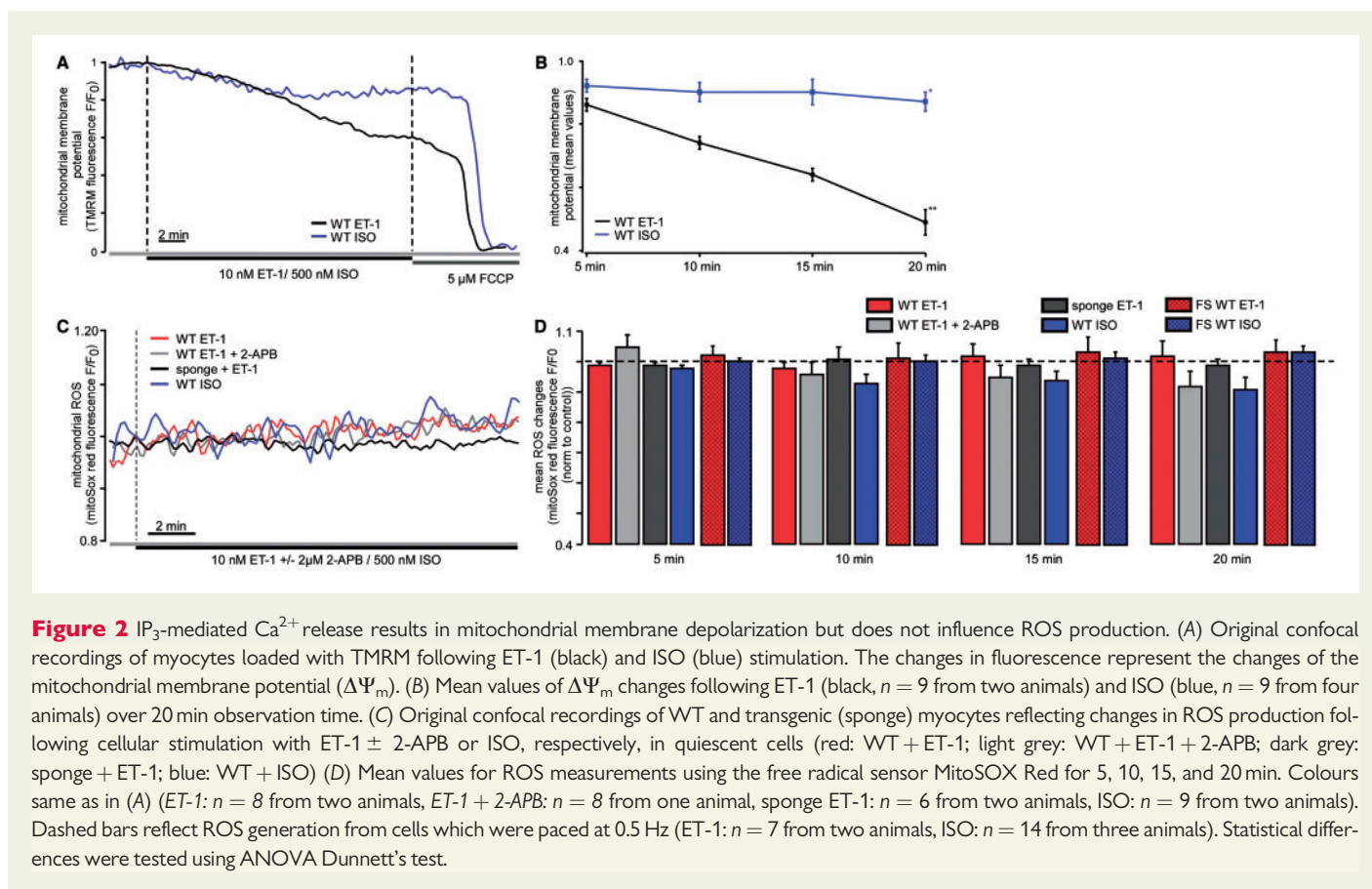
significantly increased the mitochondrial Ca^{2+} concentration ($+24 \pm 5\%$). Cell treatment with 3 μM 2-aminoethoxydiphenyl borate (2-APB, IP₃R antagonist) prevented mitochondrial Ca^{2+} elevation in both ET-1- (ET-1 + 2-APB: $+3 \pm 8\%$) and Ang II-treated myocytes (Ang II + 2-APB: $+6 \pm 3\%$), demonstrating that the observed increase in mitochondrial Ca^{2+} was depending on IP₃ receptor stimulation. Furthermore, in myocytes isolated from IP₃ sponge mice (functional IP₃ knockout mice, see Section 2), the addition of ET-1 did not induce any measurable increase in mitochondrial Ca^{2+} (Figure 1A and B). These results further prove the IP₃ dependency of the observed ET-1 effects on mitochondrial Ca^{2+} . During the observation time of 20 min, a maximum in mitochondrial Ca^{2+} uptake was not reached. To exclude motion artefacts, the experiments described were performed in quiescent myocytes. As quiescent conditions in cardiac myocytes do not reflect the physiological situation, the same experimental protocol was also performed in field stimulated cells (Figure 1G and H). Here, an even more pronounced effect of ET-1 ($+40 \pm 8\%$) on mitochondrial Ca^{2+} could be seen (Figure 1G and H) which could again be blocked by 2-APB (ET-1 + 2-APB: $+6 \pm 5\%$). These data confirm that the effect observed is also relevant in contracting myocytes under physiological conditions.

3.2 Cytoplasmic Ca^{2+} increase alone is not able to mimic the effect seen after IP₃ stimulation

To test whether a cytoplasmic Ca^{2+} increase by IP₃-independent mechanisms alone is sufficient to increase mitochondrial Ca^{2+} in the same way as induced by IP₃-mediated Ca^{2+} release, the myocytes were treated with the β -adrenergic agonist ISO. We used the concentration of ISO (500 nM) which in electrically stimulated myocytes induced a stable increase in cytosolic Ca^{2+} levels as ET-1 did without evoking spontaneous activity often seen after stimulation with ISO (Figure 1I). This effect on cytosolic Ca^{2+} was also seen under depolarizing conditions as shown in the Supplementary material online (Figure S1). We determined that ISO also induced an increase in mitochondrial Ca^{2+} (Figure 1E and F). The maximum amplitude of Ca^{2+}_m increase induced by ISO was about 50% smaller compared with the changes induced by ET-1, and this increase was not sensitive to 2-APB. The average Ca^{2+} increase following 20 min ISO stimulation was $13 \pm 3\%$ ($+20 \pm 4\%$ in electrically stimulated myocytes (Figure 1G and H)) compared with $+29 \pm 3\%$ Ca^{2+}_m increase induced by ET-1. Furthermore, the kinetics of ISO-induced Ca^{2+}_m response were clearly different from the effect on mitochondrial Ca^{2+} following ET-1 stimulation (Figure 1F). The maximum Ca^{2+} uptake induced by ISO was reached after 15 min then Ca^{2+}_m slowly declined during the next 5 min of observation. When the cells were treated with ET-1, Ca^{2+}_m continued to rise during the entire 20 min of treatment. This indicates different underlying mechanisms of the described effects on mitochondrial Ca^{2+} .

3.3 IP₃-mediated mitochondrial Ca^{2+} uptake results in mitochondrial depolarization

When mitochondria take up Ca^{2+} into their matrix in significant amounts, this Ca^{2+} uptake is accompanied by a depolarization of the mitochondrial membrane. Therefore, we measured mitochondrial membrane potential ($\Delta\Psi_m$) using the potentiometric dye TMRM. Cell treatment with 10 nM ET-1 induced $\Delta\Psi_m$ depolarization which corresponded to the increase in Ca^{2+}_m (Figure 2A and B). Similarly to the described Ca^{2+}_m changes, ET-1-induced $\Delta\Psi_m$ depolarization did not



reach a maximum over the observation time of 20 min (20 min ET-1: $-51 \pm 4\%$). In contrast, no significant changes in $\Delta\Psi_m$ were observed during the first 15 min of ISO (500 nM) treatment. Only after 20 min, ISO induced a significant $\Delta\Psi_m$ depolarization of $-13 \pm 3\%$ (Figure 2A and B).

3.4 IP₃-mediated mitochondrial Ca²⁺ uptake does not stimulate mitochondrial ROS production

As the Ca²⁺_m concentration regulates the activity of the respiratory chain, it is likely that an increase in Ca²⁺_m and therefore an activation of respiration is accompanied by an increase in ROS production. Thus, we measured changes in ROS concentration using the fluorescent probe MitoSOX Red following cellular stimulation with ET-1. To correct for laser radiation-induced ROS, the fluorescence signals were normalized to an untreated control. Interestingly, no detectable ROS generation was detected following ET-1 (10 nM) stimulation in both quiescent cells (Figures 2C and D, open bars) and cells paced at 0.5 Hz (Figure 2D, dashed bars) despite the observed increase in Ca²⁺_m (20 min ET-1: $1 \pm 5\%$) (Figure 2C). As expected, cell treatment with 3 μ M 2-APB showed no significant effect on ROS levels upon ET-1 stimulation (20 min ET-1 + 2-APB: $-2 \pm 2\%$) and no increase in ROS generation was observed in cardiac myocytes from IP₃R-sponge mice (20 min ET-1: $-9 \pm 5\%$). Furthermore, in the model used by us, the cellular stimulation with 500 nM ISO was not followed by changes in ROS generation measured using the same fluorescent probe ($-10 \pm 4\%$) in both quiescent and paced cells (Figure 2C and D).

3.5 IP₃-mediated mitochondrial Ca²⁺ uptake increases mitochondrial ATP content in both quiescent and electrically stimulated cells

As the mitochondrial matrix Ca²⁺ can regulate oxidative phosphorylation at several sites, including the F₁F₀-ATPase and several dehydrogenases,³² the impact of IP₃-mediated Ca²⁺ uptake on mitochondrial ATP production was examined. To measure the ATP concentration, two different approaches were used: (i) an indirect measurement of ATP using the magnesium (Mg²⁺) sensitive fluorescent probe mag-fluo-4 and (ii) a commercially available luciferase assay for ATP measurements (see Section 2). The mag-fluo-4 approach is based on the fact that nearly all Mg²⁺ is bound to ATP inside the cell, and only a small fraction is free.³¹ As free [Mg²⁺]_i is kept constant within a rather narrow range, any change in cellular ATP levels leads to a concomitant change in free [Mg²⁺]_i. Thus, changes in [Mg²⁺]_i can be interpreted as reciprocal changes of [ATP]_i. In these measurements, the stimulation of myocytes with 10 nM ET-1 resulted in a significant increase ($25 \pm 2\%$) of mitochondrial ATP concentration after 20 min of stimulation (Figure 3A and B). Cell treatment with the IP₃R antagonist 2-APB prevented ET-1-induced increase in ATP (ET-1 + 2-APB: $10 \pm 3\%$ after 20 min) (Figure 3A and B). These data were confirmed in electrically stimulated myocytes in which ET-1 evoked about the same increase in ATP concentration as in quiescent myocytes (ET-1: $+30 \pm 5\%$). Similar results were obtained using a luciferase assay for ATP measurements. As shown in Figure 3C, IP₃R stimulation with ET-1 induced an increase in ATP content ($+12 \pm 14\%$) which was absent in myocytes from IP₃-sponge mice ($-18 \pm 8\%$).

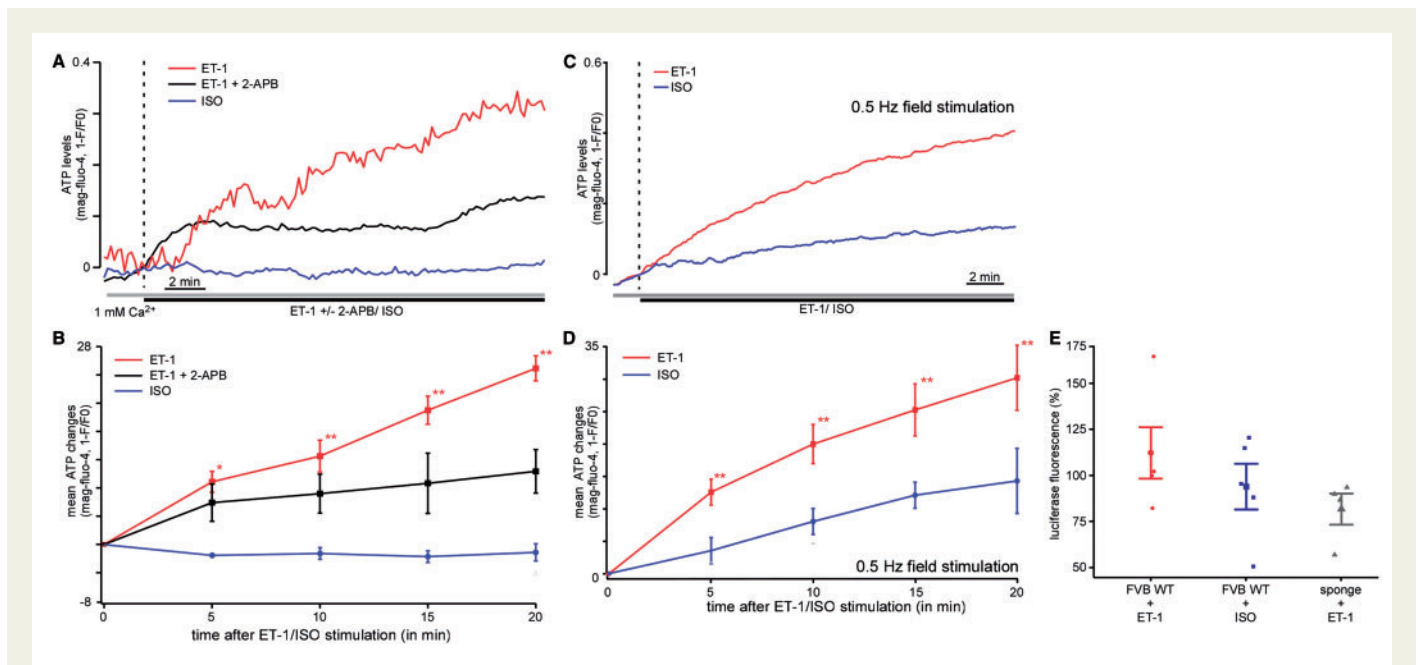


Figure 3 ATP content is increased by ET-1 but not by ISO stimulation. (A) Original confocal recordings of myocytes loaded with the dye mag-fluo-4. The myocytes were treated with 10 nM ET-1 (red, $n = 10$ from two animals), ET-1 plus 2-APB (black, $n = 5$ from two animals), or 500 nM ISO (blue, $n = 4$ from two animals). The changes in fluorescence represent the changes in the mitochondrial ATP concentration. (B) Mean values for the experiment described in (A). The stimulation of the cells with ET-1 induced a significant increase in mitochondrial ATP concentration after 20 min, whereas ISO had no effect. (C) Original confocal recordings of field stimulated myocytes (0.5 Hz) loaded with the dye mag-fluo-4. The myocytes were treated with 10 nM ET-1 (red, $n = 4$ from two animals), or 500 nM ISO (blue, $n = 8$ from two animals). (D) Mean values for the experiment described in (C). (E) Percentage of changes of luciferase fluorescence as measurement of the cellular ATP content following ET-1 (red, $n = 5$ from five animals) and ISO (blue, $n = 5$ from four animals) in WT mouse and following ET-1 stimulation in IP₃ sponge mice (grey, $n = 4$ from four animals) measured using a luciferase assay. * $P < 0.05$ compared with untreated control using ANOVA Dunnett's test; ** $P < 0.01$ compared with untreated control using ANOVA Dunnett's test.

3.6 β -adrenergic receptor activation does not result in a significant increase in mitochondrial ATP levels

As the mitochondrial Ca^{2+} increase following ET-1 stimulation markedly increased mitochondrial ATP content, we also tested the effect of β -adrenergic receptor stimulation on the mitochondrial ATP concentration using ISO. Although ISO application was associated with an increase in Ca^{2+}_m (although less compared with ET-1 stimulation, as described above), the mitochondrial ATP content was not increased. In fact, we observed a tendency for ATP levels to decline following exposure to ISO but it was not statistically significant. Using mag-fluo-4 for indirect determination of the ATP concentration, we found no detectable changes in mitochondrial ATP concentration after 15 min exposure to 500 nM ISO in quiescent cells ($-1 \pm 1\%$ at 20 min) (Figure 3A and B). These findings were confirmed using a luciferase kit for the determination of ATP concentration ($-6 \pm 12\%$ ATP after 20 min ISO) (Figure 3C). However, in electrically stimulated myocytes (Figure 3C and D), an increase of $+14 \pm 3\%$ was observed which was statistically not significant but is about half the increase observed following ET-1 indicating an increased respiratory activity in contracting myocytes compared with quiescent myocytes.

3.7 IP₃-mediated Ca^{2+}_m uptake depends on the mitochondrial RyR1

Based on the findings described above, two different mechanisms of Ca^{2+}_m uptake following ET-1 and ISO could be suggested. To elucidate

the exact mechanism involved, we first blocked the MCU using 1 μM Ru360 (For effect of Ru360 on intact myocytes please see Figure S2 in the Supplementary material online). Following this treatment, we stimulated the myocytes with ET-1 (Figure 4A) or ISO (Figure 4B), and measured Ca^{2+}_m uptake using the Ca^{2+} sensitive dye X-Rhod-1 loaded into mitochondria. Interestingly, blocking the MCU did not affect ET-1-mediated Ca^{2+}_m uptake (ET-1 alone: $+29 \pm 3\%$, ET-1 + Ru360: $+31 \pm 7\%$) but completely prevented ISO-induced Ca^{2+}_m increase (ISO alone: $+13 \pm 3\%$ and ISO + Ru360: $+1 \pm 2\%$). Therefore, the MCU clearly mediates ISO-induced Ca^{2+}_m uptake; however, ET-1-mediated Ca^{2+}_m uptake is independent of the MCU. The second candidate for the mediation of IP₃-induced Ca^{2+}_m uptake was the mRyR1.³³ We, therefore, blocked the mRyR1 by cellular treatment with 1 μM of dantrolene for 10 min.³⁴ Following pre-incubation with dantrolene, the addition of ET-1 resulted in a significantly reduced Ca^{2+}_m increase (ET-1 alone: $+29 \pm 3\%$, ET-1 + dantrolene: $+20 \pm 4\%$ after 20 min), whereas the Ca^{2+}_m increase induced by ISO was not affected by dantrolene (ISO alone $13 \pm 3\%$, ISO + dantrolene: $+19 \pm 3\%$). To test whether the described effect induced by dantrolene was mediated by an unspecific effect on the RyR type 2 (RyR2) located in the SR, myocytes were loaded with the Ca^{2+} -sensitive fluorescent probe fluo-4-AM and electrically stimulated. As demonstrated in Figure 4C, dantrolene had no effect on electrically induced cytosolic Ca^{2+} elevations mediated by RyR2 activation.

Next, using quantitative real-time PCR, we demonstrated the presence of all three subtypes of the RyR in wild-type (WT) murine

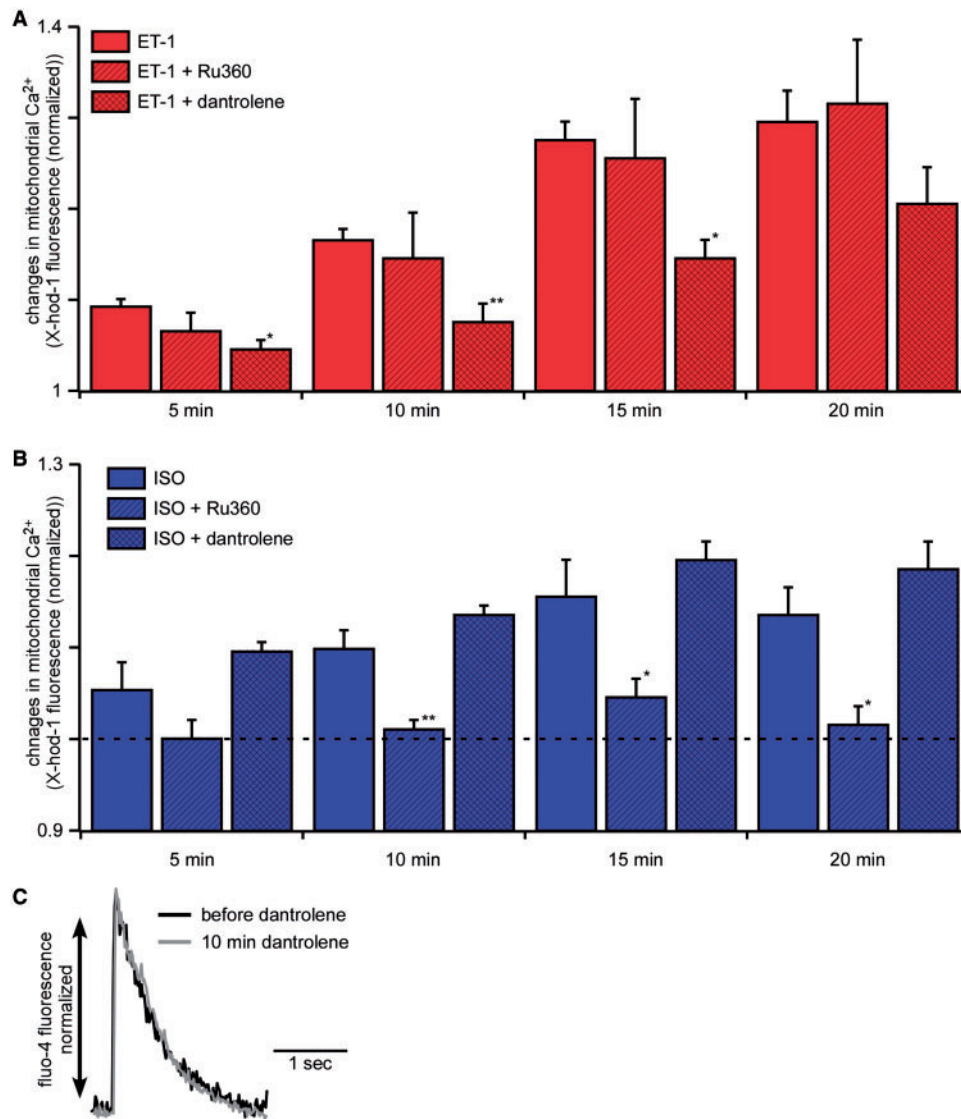


Figure 4 IP₃-mediated increase in mitochondrial Ca²⁺ is depending on the mRyR1 whereas ISO-induced mitochondrial Ca²⁺ increase is depending on the MCU. (A) Mean values of changes in the mitochondrial Ca²⁺ concentration following the stimulation with ET-1 (red, *n* = 27 from seven animals), ET-1 plus Ru360 (red, diagonally hatched, *n* = 11 from three animals), and ET-1 plus dantrolene (red, cross hatched, *n* = 13 from two animals). Mitochondrial Ca²⁺ uptake following the cellular stimulation with ET-1 was significantly blunted by the mitochondrial RyR blocker dantrolene. (B) Mean values of changes in the mitochondrial Ca²⁺ concentration following the stimulation with ISO alone (blue, *n* = 10 from three animals), ISO plus Ru360 (blue, diagonally hatched, *n* = 8 from two animals), and ISO plus dantrolene (blue, cross hatched, *n* = 9 from two animals). Mitochondrial Ca²⁺ uptake following the cellular stimulation with ISO was prevented by the MCU blocker Ru360. (C) Field stimulated (0.5 Hz) Ca²⁺-transients of a murine cardiac myocyte before (black) and after 10 min of dantrolene treatment (grey). The traces are normalized and fitted to the same amplitude for the comparison of kinetics (*n* = 2 from one animal). **P* < 0.05 compared with ET-1/ISO alone using ANOVA Dunnett's test; ***P* < 0.01 compared with ET-1/ISO alone using ANOVA Dunnett's test, respectively.

ventricular myocytes (Figure 5A). As expected the expression of type RyR2 was dominant in ventricular myocytes (0.3 ± 0.02). However, we also detected the presence of both RyR subtype 1 (0.008 ± 0.001) and type 3 (0.004 ± 0.0003). Furthermore, we found that the expression levels of RyR type 1 were unchanged ($\pm 0 \pm 0.1\%$) whereas type 2 ($-37 \pm 7\%$) and type 3 ($-6 \pm 9\%$) were decreased in IP₃ sponge mice (Figure 5B) compared with expression levels in WT mice.

3.8 IP₃ receptor types 1 and 3 are up-regulated in cardiac myocytes from sponge mice

To elucidate which IP₃ receptor subtype is responsible for the observed Ca²⁺_m uptake following cellular stimulation with IP₃, we performed real-time PCR of the subtypes. Here, we could demonstrate that all three subtypes were expressed in murine cardiac myocytes with a

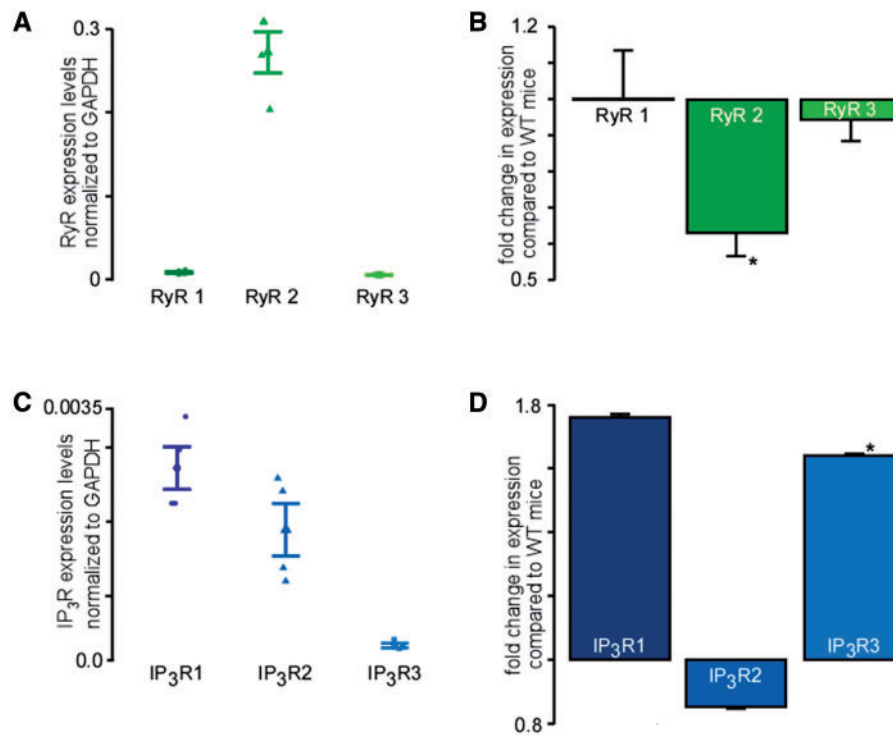


Figure 5 All three subtypes of the RyR and the IP₃R are expressed in murine cardiac myocytes. (A) Expression levels of three RyR subtypes normalized to GAPDH (four experiments with cells from four animals) as revealed by real-time PCR in WT murine ventricular myocytes. (B) Fold changes in expression levels of the three RyR subtypes in IP₃ sponge mice compared with expression levels in WT mice (four experiments with cells from four animals). (C) IP₃R expression levels normalized to GAPDH measured by real-time qRT-PCR (three experiments with cells from three animals). (D) Relative changes of IP₃R expression in cardiac myocytes of sponge mice compared with WT mice measured by real-time qRT-PCR. (three experiments with cells from three animals). **P* < 0.05 calculated using Student's *t*-test.

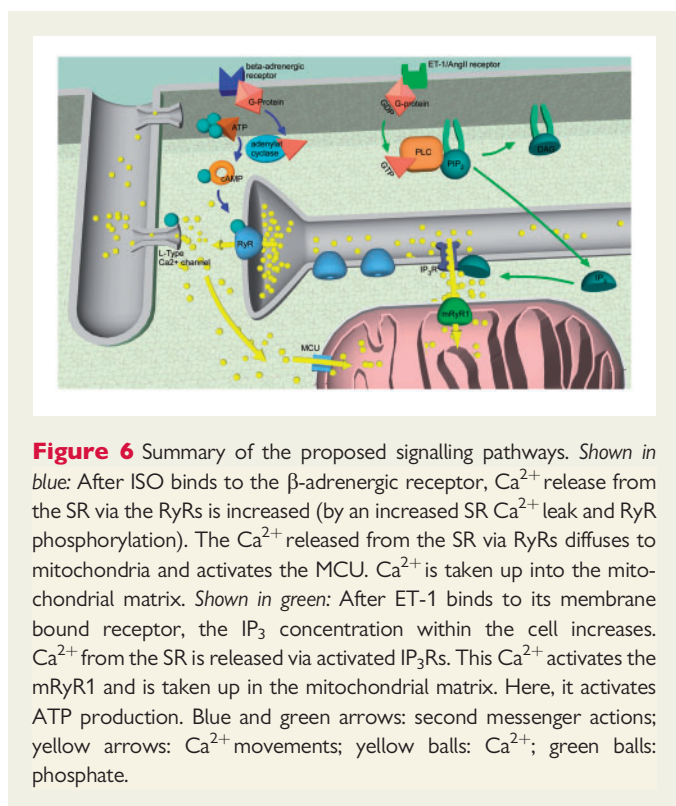
predominance of IP₃R subtypes 1 and 2 (IP₃R subtype 1: 0.003 ± 0 ; IP₃R subtype 2: 0.002 ± 0 ; IP₃R subtype 3: 0.0003 ± 0) (Figure 5C), so that either of them could be involved in IP₃R agonist-induced increase in mitochondrial Ca²⁺ uptake and ATP generation. Interestingly, we found a significant higher expression of IP₃R subtypes 3 and 1 in IP₃ sponge mice compared with WT mice (Figure 5D). The expression levels of IP₃R subtype 2 were actually decreased in ventricular myocytes from IP₃ sponge mouse [IP₃R expression levels in IP₃ sponge mice compared with WT mice: IP₃R subtype 1: $+76 \pm 0.3\%$, IP₃R subtype 2: $-15 \pm 0.1\%$, IP₃R subtype 3: $+65 \pm 0.2\%$ (Figure 5D)]. These data imply different (patho)physiological roles for each IP₃R subtypes which need to be further investigated.

4. Discussion

The most significant finding of our studies is that agonist-induced IP₃R-mediated Ca²⁺ release is essential for the maintenance of optimal cellular bioenergetics in cardiac myocytes under normal physiological conditions in both quiescent and field-stimulated cells. In contrast, beta-adrenergic stimulation was not able to influence mitochondrial metabolism in quiescent cells, but did enhance both mitochondrial Ca²⁺ uptake and ATP levels in field-stimulated cells (Figures 1 and 3). However, the levels of ATP production following ISO stimulation were about 50% lower compared with the levels measured following IP₃R stimulation.

Furthermore, the kinetics of mitochondrial Ca²⁺ uptake following the treatment with IP₃R and beta-receptor agonists were different as well. IP₃-mediated mitochondrial Ca²⁺ uptake induced a sustained increase in Ca²⁺_m levels without reaching a maximum, whereas beta-receptor stimulation was associated with a transient increase in mitochondrial Ca²⁺ concentration with a maximum reached at 15 min. Thus, we found that despite a similar increase in cytosolic Ca²⁺_i levels, only IP₃ agonists were able to induce a sustained increase in Ca²⁺_m and ATP generation, suggesting the existence of a close inter-organelle Ca²⁺ signalling. The different kinetics and effects on ATP levels imply an enduring effect of IP₃R agonists and suggest an acute effect of beta-receptor stimulation (for summary see Figure 6).

Our data are in agreement with the recent findings obtained on cardio-specific Mcu-deleted mice^{19,20} which demonstrated that MCU matches energetic supply with cardiac workload during stress conditions. Beta-receptor stimulation with ISO induced MCU-dependent mitochondrial Ca²⁺ uptake and an acute increase in ATP production. The loss of MCU from the heart resulted in a selective inability to acutely respond to beta-adrenergic receptor stimulation or maximal forced exercise by augmenting cardiac metabolic capacity when the cardiomyocyte's work was enhanced.²⁰ Relative oxygen consumption rates were lower following ISO exposure in adult cardiac myocytes in Mcu-deleted cells. However, the effect was lost after chronic exposure to ISO. Importantly, MCU-deficient cardiomyocytes or deficient mice eventually catch up to controls in their contractile or metabolic performance, as



did total Ca^{2+} load in the mitochondria. Altogether, these data suggest the existence of an alternative mechanism required to maintain mitochondrial respiration and ATP generation under basal physiological conditions, and the IP_3 -mRyR1 pathway could be a candidate for this role.

Furthermore, under basal conditions total cardiomyocyte cytosolic Ca^{2+} -transient kinetics and amplitude were unaltered in myocytes from cardio-specific Mcu-deleted mice ($Mcu^{fl/fl-MCM}$) compared to $Mcu^{fl/fl}$ controls. On top, neither Ca^{2+} spark frequency and characteristics (amplitude, duration) or SR Ca^{2+} load was different in $Mcu^{fl/fl-MCM}$ myocytes compared with controls. Taken together, these results demonstrate that MCU-mediated mitochondrial Ca^{2+} import neither contributes to overall cardiac Ca^{2+} cycling in the heart nor is required to support normal cardiac function and adaptation with aging.²⁰ Also, mitochondrial respiration and ATP generation under basal conditions were not affected by Mcu deletion; however, when mitochondrial respiration was measured in the presence of high Ca^{2+} (100 μM external Ca^{2+} , i.e. imitating conditions of the mitochondrial Ca^{2+} overload during acute stress) both Mcu deletion and inhibition of the MCU with Ru360 led to the inhibition of the mitochondrial respiration and ATP generation.

In our study, ISO also activated acute mitochondrial Ca^{2+} uptake via the MCU. However, in contrast to ET-1 stimulation, ISO stimulation was not followed by a significant increase in ATP levels which indicates that, despite an increase in ATP generation to match the increased cellular energetic needs, the rate of ATP hydrolysis induced by ISO was higher than ATP generation. This implies that despite the activation of respiratory chain activity described by Kwong *et al.*,²⁰ the total amount of ATP cannot be maintained. This is in line with the finding of O-Uchi *et al.*³⁵ who also did not find an increase in ATP concentration following MCU stimulation but demonstrated an increase in mitochondrial ATP concentration in mRyR1 overexpressing cardiac H9c2 myoblasts. Additionally, a recent study from Andrew Marks' lab demonstrated that diastolic SR

Ca^{2+} leak via type 2 RyRs causes mitochondrial Ca^{2+} overload and dysfunction in a murine model of post-myocardial infarction heart failure.³⁶ The exact mechanism how the enhanced type 2 RyR leak results in mitochondrial Ca^{2+} overload was not determined but the enhanced SR Ca^{2+} leak was associated with a decline in mitochondrial ATP levels. Importantly, the Marks' study found that type 2 IP_3Rs did not contribute to mitochondrial dysfunction. The study of Kwong *et al.*²⁰ confirmed that MCU mediates acute mitochondrial Ca^{2+} overload *in vivo* following cardiac ischaemia-reperfusion. Under these conditions, mitochondrial Ca^{2+} uptake, mitochondrial permeability transition pore (mPTP) opening, and cell death were decreased by Mcu deletion resulting in 50% decrease in infarct size.

The different actions following the activation of P_3R and beta-adrenergic receptors imply the activation of different molecular pathways. In our study, the second major finding was that Ca^{2+} released via P_3R from the SR was taken up into mitochondria by the RyR type 1 (mRyR1) whereas Ca^{2+} released via beta-adrenergic stimulation was taken up into mitochondria via the MCU (Figure 4; see Figure 6 for summary). So far, the physiological function of the mRyR1 has not been elucidated and after its detection in the mitochondrial inner membrane, the role of the mRyR1 in Ca^{2+}_m cycling remains unclear.^{22,33} The existence of all three types of the RyRs in cardiac myocytes was demonstrated previously with RyR type 1 located on the IMM.²² Now, our data suggest a crucial role of the mRyR1 together with the IP_3R in the adaptation of the mitochondrial metabolism to the cell's energetics needs. This finding is based on experiments in which the presence of dantrolene, a known blocker of the RyR type 1 and the mRyR1 which are structurally related, suppressed Ca^{2+}_m uptake following ET-1. As shown by us (Figure 4C) and others,³⁷ dantrolene had no effect on cardiac EC-coupling in young mice. This excludes significant contribution of RyR2 blocking by dantrolene as a confounder to our results.

Our data demonstrate that the MCU is not involved in IP_3 -mediated Ca^{2+}_m uptake; however, it mediates Ca^{2+}_m uptake triggered by ISO. This is in line with other publications demonstrating that Ca^{2+} is taken up into the mitochondrial matrix via the MCU during the EC-coupling cycle.^{38,39} Hajnoczky and his group⁴⁰ demonstrated that Ca^{2+} released from the endoplasmic reticulum via IP_3R increased the Ca^{2+}_m concentration in permeabilized hepatocytes which is in accordance to our findings in intact cardiac myocytes. The activation of mRyR1 by IP_3 -mediated signals and not by global cytosolic Ca^{2+} indicates that cellular bioenergetics in cardiac myocytes are precisely controlled by local targeted Ca^{2+} transfer from the SR. It was shown before that mitochondria and the SR are linked physically.²⁵ These structures may shield IP_3R release sites from the cytosol. Furthermore, the mRyR1 is regulated by Ca^{2+} in a similar fashion as the RyR1 from skeletal muscles.^{3,41} Both mRyR1 and the RyR1 are activated by low Ca^{2+} concentrations (1–10 μM) and inactivated by high Ca^{2+} concentrations (1–10 mM) which would prevent mitochondria from Ca^{2+} overload and enable localized Ca^{2+} signalling.⁴¹ The mRyR1 shows a bell-shaped Ca^{2+} -dependent activation in a physiological range of Ca^{2+}_i , from 0.1 to ~ 10 –30 μM Ca^{2+} . This unique property makes mRyR1 an ideal candidate for sequestering Ca^{2+} quickly and transiently under physiological Ca^{2+}_i oscillations observed during each heartbeat. The molecular identity of RyR1 in cardiac mitochondria was carefully analysed and confirmed using not only native cardiomyocytes but also those obtained from RyR1 knockout mouse hearts.²² Interestingly, in RyR1 knockout mouse heart mitochondria, the respiratory control index was very low ($\cong 1$). An increase in Ca^{2+}_i did not stimulate O_2 consumption, suggesting that mRyR1 is required for both the basal metabolic state and Ca^{2+} -dependent acceleration of the TCA cycle in cardiomyocytes²² even though its expression level was much lower

compared with the RyR2, which is the main RyR isoform in the cardiac SR.⁴² Furthermore, plotting the normalized currents of MCU and mRyR1 as a function of Ca^{2+} concentration revealed that the open probability of mRyR1 peaks in the presence of 10–30 μM Ca^{2+} , whereas the MCU is fully activated at much higher concentrations of Ca^{2+} (above 100 μM Ca^{2+}).²² These data suggest that the MCU with its low affinity and high capacity for mitochondrial Ca^{2+} uptake is better suited for reducing a possible cytosolic Ca^{2+} overload to prevent necrotic or apoptotic cell death.⁴³

Last, the question arose which IP₃R subtype was involved. In accordance with the literature, we were able to demonstrate via real-time PCR, that all three IP₃R subtypes were expressed in murine ventricular myocytes. The Molkenkin laboratory,²³ which generated the IP₃-sponge transgenic animals, described a general down-regulation of the IP₃R without differentiating between the different subtypes. In this study, we demonstrated that IP₃R type 2 expression (the most abundant type in cardiac myocytes¹) was decreased whereas IP₃R type 1 and 3 expression levels in IP₃-sponge mice were significantly increased compared with myocytes from WT mice. These data are in line with the findings of Drawnel et al.⁴⁴ who also found decreased IP₃R type 2 expression in these animals. As in the transgenic mouse model used by us, the produced IP₃-molecules are bound within the cytosol and cannot bind to their receptor, this up-regulation can represent a compensatory mechanism underlining the importance of IP₃R type 1 and 3 mediated signalling for the cell. Although, increased expression of IP₃R has been shown under pathological conditions, particularly in heart failure,⁴⁵ valvular heart disease,⁴⁶ and atrial fibrillation.⁴⁷ Our study provides the evidence, however, that under normal physiological conditions, IP₃R-mediated Ca^{2+} transfer to mitochondria leads to increased ATP generation in cardiac myocytes.

Altogether, our data indicate that constitutive activity of IP₃R is important for modulation of cellular energetics in cardiac myocytes. Furthermore, our result that IP₃R–mRyR1 crosstalk is responsible for the maintenance of ATP levels in cardiac myocytes could actually explain the recent puzzling discoveries why MCU knockout mice do not reveal any significant bioenergetic problems in their hearts.⁴⁸ To conclude, we were able to demonstrate a new signalling pathway through which mitochondria of cardiac myocytes can decode Ca^{2+} signals of the cell and thus are able to adapt their function to the whole cell's energetic needs.

4.1 Study limitations

Ca^{2+} indicators used in this study are non-ratiometric fluorescent dyes, and therefore were not calibrated. With non-ratiometric dyes, we could not account for the possible dye leakage from the cells and bleaching, thus it is not appropriate to convert the fluorescent signal into Ca^{2+} concentration in terms of millimolar. Therefore, changes in Ca^{2+} are reported as background corrected fluorescence levels normalized to basal levels.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

Funding

This project was funded by the Interdisciplinary Center for Clinical Research (IZKF) Würzburg (project number Z3-19), the Comprehensive Heart Failure Centre (CHFC), the American Heart Association

Grant-in-Aid 15GRNT25090220 (to E.N.D.), and the Rudolph Virchow Center for Experimental Biomedicine.

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