SHORT COMMUNICATION

Alteration of Mitochondrial Ca^{2+} Fluxes by Kaempferol and CGP-37157 Regulates Ca^{2+} Oscillations in Human Alveolar Type 2 A549 Cells

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Summary

Mitochondria participate in regulating cytosolic Ca^{2+} signaling by their Ca^{2+} handling *via* mitochondrial Ca^{2+} uniporter (MCU) and mitochondrial Na^+/Ca^{2+} exchanger (mitoNCX). In this study, we examined how agonist-triggered cytosolic Ca^{2+} oscillations in human alveolar type 2 A549 cells were affected by an MCU inhibitor (MCU-i4), MCU activator (kaempferol) and mitoNCX inhibitor (CGP-37157). Whilst inhibition of MCU did not significantly repress Ca^{2+} oscillations, MCU activation by kaempferol considerably dampened oscillatory activities. Inhibition of mitochondrial Ca^{2+} efflux by CGP-37157 also suppressed Ca^{2+} oscillations; the suppressive effects of kaempferol and CGP-37157 were not additive. Both kaempferol and CGP-37157 caused a rise in mitochondrial matrix Ca^{2+} level, but their effects were not additive. Taken together, our results suggest Ca^{2+} oscillations in alveolar type 2 A549 cells were regulated by stimulating Ca^{2+} uptake into, and preventing Ca^{2+} efflux from, the mitochondria, with both cases resulting in disturbed Ca^{2+} traffic and Ca^{2+} accumulation in the mitochondrial matrix.

Key words

Mitochondria • Ca^{2+} oscillations • Mitochondrial Ca^{2+} uniporter • Mitochondrial Na^+/Ca^{2+} exchanger • A549 cells

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One of the functions of mitochondria is its regulation of cytosolic Ca^{2+} signaling by its uptake of Ca^{2+} *via* mitochondrial Ca^{2+} uniporter (MCU) [1]. MCU is a complex comprising the MCU channel and accessory proteins such as mitochondrial Ca^{2+} uniporter regulator 1 and mitochondrial Ca^{2+} uptake proteins (MICU-1, -2 and -3) [1]. In gall bladder smooth muscle, block of mitochondrial Ca^{2+} uptake reduces frequency of Ca^{2+} waves [2]. By contrast, in cardiac myocytes, inhibition of mitochondrial Ca^{2+} uptake by Ru360 increases amplitude and frequency of Ca^{2+} waves [3]. Effects of MCU stimulation on Ca^{2+} signaling are also variable. In rat ventricular myocytes, increased mitochondrial Ca^{2+} uptake by kaempferol promotes Ca^{2+} wave generation under β-adrenergic stimulation [4]. Activation of mitochondrial Ca^{2+} uptake by kaempferol initially enhances but later suppresses histamine-elicited Ca^{2+} oscillations in HeLa cells and human fibroblasts [5].

The modulations of Ca^{2+} waves and oscillations by MCU inhibition and activation are therefore variable and hitherto have not been fully understood. Nonetheless, MCU-modulating drugs are of potential therapeutic values. In a traumatic brain injury rat model, treatment with Ru360, an MCU inhibitor, significantly alleviates oxidative stress and improves sensorimotor behavioral

recovery [6]. MCU activators also offer neuroprotective effects [7].

Lung expansion causes synchronous cytosolic Ca^{2+} oscillations in all alveolar cells and surfactant secretion in type 2 cells; the exocytosis rate is positively correlated to Ca^{2+} oscillation frequency [8]. In this study, we examined how cytosolic Ca^{2+} oscillations in human alveolar type 2 A549 cells were affected by drugs which modulate mitochondrial Ca^{2+} fluxes: MCU inhibition and activation, respectively, by MCU-i4 and kaempferol; and effects of inhibition of mitochondrial Na^{+}/Ca^{2+} exchanger (mitoNCX) by CGP-37157.

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). ATP and cyclopiazonic acid (CPA) were from Sigma-Aldrich chemical Co. (St. Louis, MO, USA). MCU-i4, kaempferol and CGP-37157 were from Tocris Bioscience (Bristol, U.K.). Except for ATP (dissolved in distilled water; 100 mM as stock), all other chemicals mentioned above were dissolved in DMSO as stock solutions (30 or 50 mM). All other chemicals of reagent grade were obtained from Sigma-Aldrich. A549 cells were cultured in RPMI-1640 medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C with humidified 5 % CO_2.

Measurement of cytosolic Ca^{2+} concentration was conducted as previously described [9,10]. Cells were incubated with 5 µM fura-2 AM (Invitrogen) for 1 h at 37 °C and subsequently washed in bath solution (mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (NaOH was used to adjust pH to 7.4). Cells were excited with 340 nm and 380 nm alternately (frequency of switching = 1 Hz) with the aid of an optical filter changer (Lambda 10-2, Sutter Instruments, Novato, CA). Emission was collected at 500 nm and data were captured with a CCD camera (CoolSnap HQ2, Photometrics, Tucson, AZ, USA) connected to a Nikon TE2000-U microscope. Microscope magnification was 400×. ATP (10 µM) was used to trigger cytosolic Ca^{2+} oscillations. Results were analyzed by an MAG Biosystems Software (Sante Fe, MN). Experiments were carried out at 25 °C. 340/380 ratio changes were measured and analyzed at a region of interest of single cells in one experiment; the same experimental protocols were repeated a few more times to obtain the mean.

Cells were incubated with 5 µM Rhod-2 AM (Invitrogen, Carlsbad, CA) for 1 h at 25 °C and then washed. Cells were permeabilized and washed with a digitonin (30 µM)-containing intracellular solution which contained (mM): 140 KCl, 8 NaCl, 1 $MgCl_2$, 1.85 EGTA, 1 $CaCl_2$, 10 HEPES, and 8 MgATP (pH 7.25 adjusted with KOH; free $[Ca^{2+}]$ was calculated to be 114 nM using the "Ca-EGTA Calculator TS version 1.3", a free online program provided by University of California, Davis). Cells were trypsinized, dispersed and washed in intracellular solutions. The cells were then treated with different agents for 5 min before subject to fluorescence-activated cell sorting and then analyzed using a FACS Canto flow cytometer system (BD Biosciences, San Jose, CA, USA). Excitation and emission wavelengths were set at 488 and 576 nm, respectively. Data were analyzed by BD FACSDIVA™ software (BD Biosciences).

Results were presented as means ± S.E.M. When 2 groups were compared, paired or unpaired Student's *t*-test was used when appropriate. Multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD *post hoc* test. Statistical significance is reached when P is <0.05.

Continuous Ca^{2+} transfer from endoplasmic reticulum (ER) *via* IP_3 receptor (IP_3R) and ryanodine receptor (RYR) to mitochondria (*via* MCU) is necessary for mitochondrial functions [11]. As reported in our previous study [9], addition of ATP to A549 cells triggered Ca^{2+} oscillations in the majority of cells. Inhibition of MCU by MCU-i4 did not significantly affect the percentage of oscillating cells (Fig. 1A, B), but moderately affected Ca^{2+} oscillation frequency: the latter was reduced by 21.4 % by 10 µM MCU-i4 ($P=0.028$; Fig. 1C).

By contrast, pretreatment with kaempferol (MCU activator), strongly suppressed Ca^{2+} oscillations (Fig. 1D). Kaempferol reduced % oscillating cells and oscillation frequencies ($P<0.001$; Fig. 1E, F). Intriguingly, with kaempferol treatment, the first peaks of ATP-triggered Ca^{2+} signal were not smaller but indeed slightly higher than those of the control group ($P<0.01$; Fig. 1G).

Emptiness of ER Ca^{2+} stores triggers Ca^{2+} entry from the extracellular space into the cell, a term coined "store-operated Ca^{2+} entry" (SOCE) [12]. We showed earlier that SOCE was necessary to support Ca^{2+} oscillations in A549 cells [9]. Therefore, we examined if kaempferol affected SOCE. After Ca^{2+} pool discharge by cyclopiazonic acid, Ca^{2+} influx upon Ca^{2+} replenishment was not affected by kaempferol, suggesting kaempferol did not suppress Ca^{2+} oscillations by blocking SOCE (data not shown).

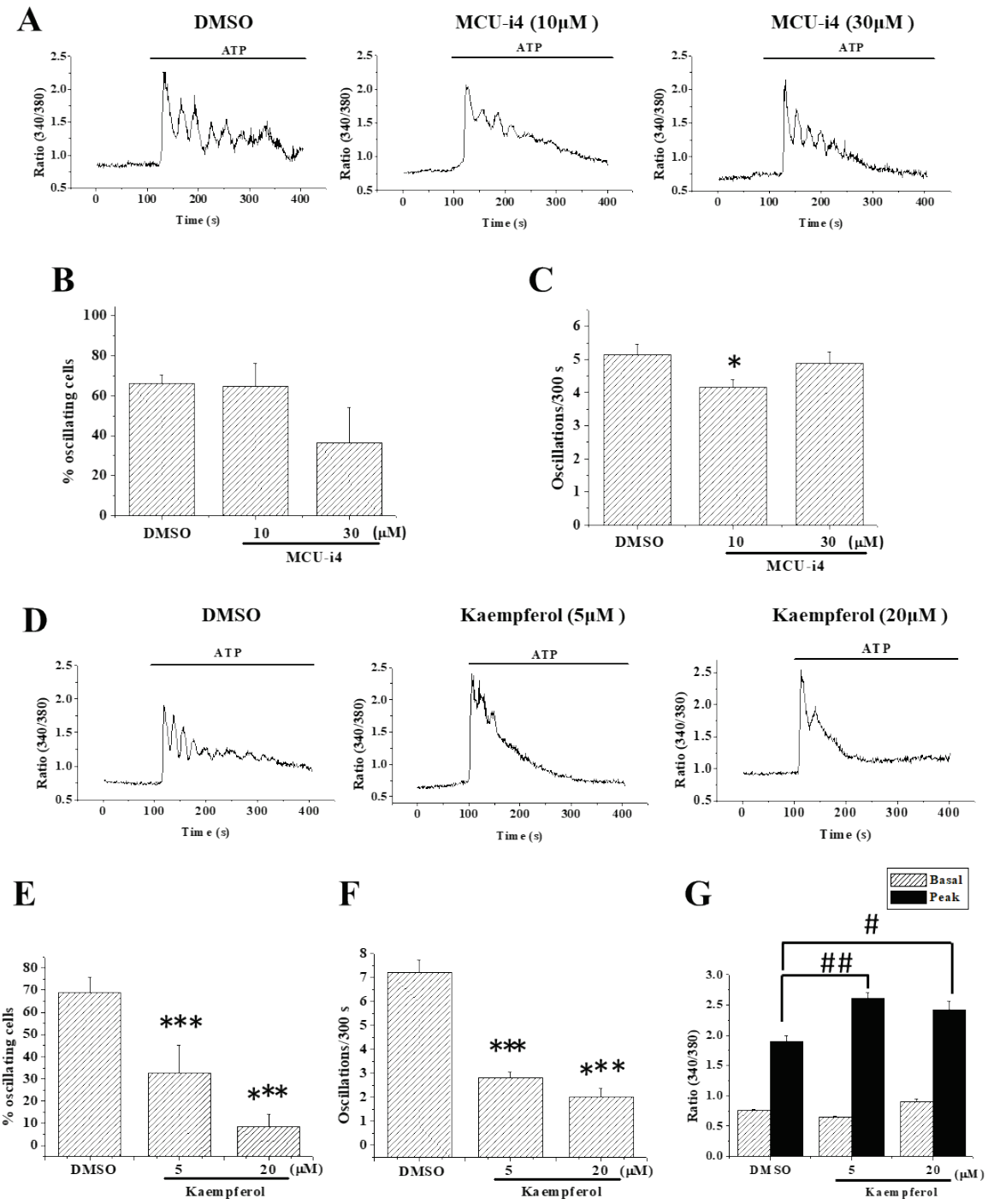

Fig. 1. Effects of MCU-i4 and kaempferol on ATP-triggered Ca^{2+} oscillations. (**A**) A549 cells in Ca^{2+}-containing bath solution were pre-treated with DMSO, 10 or 30 μM MCU-i4 for 8 min and then stimulated by 10 μM ATP. (**B-C**) * significantly different from the control (P=0.028). (**D**) A549 cells in Ca^{2+}-containing bath solution were pre-treated with DMSO or kaempferol for 8 min and then stimulated by 10 μM ATP. (**E-F**) *** different from control (P<0.001). (**G**) shows averaged basal/peak values of first ATP-triggered Ca^{2+} signal. # P<0.05 and ## P<0.01 indicate significant difference from control. Results are mean ± S.E.M.; each group had 49-98 cells from 5-11 separate experiments.

If kaempferol inhibited Ca^{2+} oscillations by increasing mitochondrial Ca^{2+} level, then prevention of Ca^{2+} efflux *via* inhibiting mitoNCX would be expected to suppress Ca^{2+} oscillations. The effects of kaempferol, CGP-37157 (selective mitoNCX inhibitor), or a combination of both agents, were examined (Fig. 2A). Kaempferol suppressed both % oscillating cells and oscillation frequency whilst CGP-37157 only reduced oscillation frequency (P<0.01; Fig. 2B, C). Unexpectedly, suppressive effects of kaempferol and CGP-37157 were not additive. There were also no additive effects of kaempferol and CGP-37157 in enhancing the first peak of

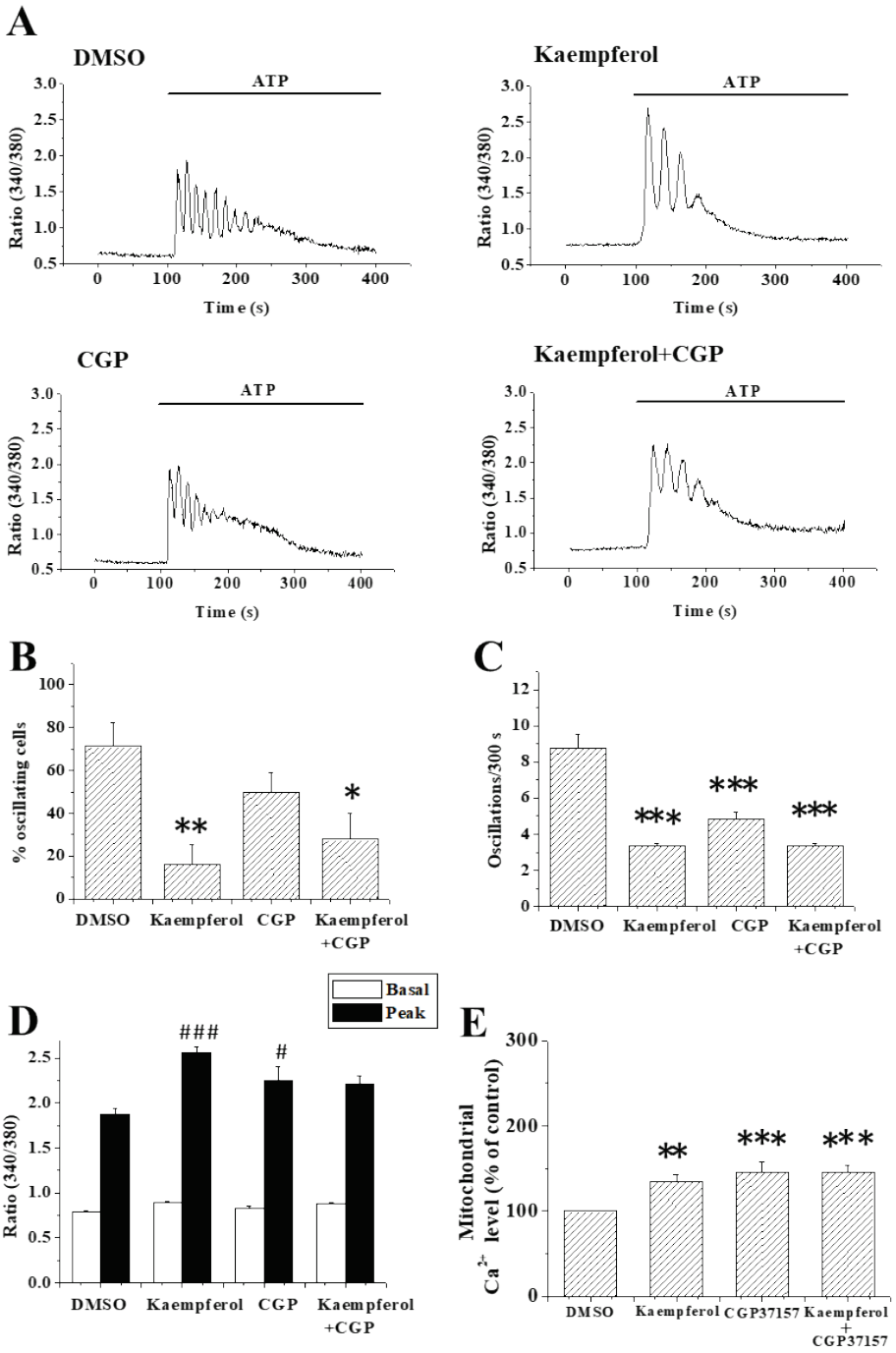

Fig. 2. Effects of kaempferol and CGP-37157 on Ca^{2+} oscillations and mitochondrial Ca^{2+} concentration. (**A**) A549 cells in Ca^{2+}-containing bath solution were pre-treated with DMSO, 20 μM kaempferol, 10 μM CGP-37157 (CGP) or 20 μM kaempferol plus 10 μM CGP-37157 for 8 min before stimulated by 10 μM ATP. (**B-C**) * P<0.05, ** P<0.01 and *** P<0.001 indicate significant differences compared to the control. (**D**) shows averaged basal/peak values of first ATP-triggered Ca^{2+} signals. # P<0.05 and ### P<0.001 indicate significant differences from control. Results are mean ± S.E.M.; each group had 38-66 cells from 3-6 separate experiments. (**E**) A549 cells were treated with DMSO, 20 μM kaempferol (kaemp), 10 μM CGP-37157 (CGP), or a combination of these two agents for 5 min and then subject to mitochondrial Ca^{2+} level measurement. ** P<0.01 and *** P<0.001 are significantly different from the control. Results are mean ± SEM from 3 independent experiments.

ATP-triggered Ca^{2+} signal (Fig. 2D). We also examined whether kaempferol and CGP-37157 affected mitochondrial Ca^{2+} levels (Fig. 2E). These drugs, either alone or in combination, caused a significant rise in mitochondrial Ca^{2+} level, but their effects were not additive.

The mechanisms of Ca^{2+} oscillations have been extensively studied (for a review see [12]). Agonist-triggered generation of IP_3 activates IP_3R on ER to release Ca^{2+}; the resultant elevated concentration of cytosolic Ca^{2+} acts as a co-agonist of IP_3R and promotes

IP_3-induced Ca^{2+} release (IICR). Meanwhile, the empty state of ER induces SOCE, causing further elevation in cytosolic Ca^{2+}. The latter subsequently inactivates IICR, stimulates ER Ca^{2+} pump (to re-sequester Ca^{2+}) and plasma membrane Ca^{2+} pump to extrude Ca^{2+}. The decline of cytosolic Ca^{2+} back to resting level removes inhibition of IP_3R and allows the latter to be reactivated by IP_3 to begin the cycle anew.

CGP-37157 was effective in reducing oscillation frequency (Fig. 2), suggesting Ca^{2+} efflux from mitochondria is important in maintaining Ca^{2+} oscillations. This is in agreement with a previous report that mitochondrial Ca^{2+} release *via* mitoNCX sustains Ca^{2+} oscillations [13]. The effect of MCU-i4 on Ca^{2+} oscillations, however, was only minimal (Fig. 1), suggesting reduced mitochondrial Ca^{2+} uptake was much less pivotal than reduced mitochondrial Ca^{2+} efflux in regulating oscillations.

Kaempferol was much more efficacious than MCU-i4 and CGP-37157 in suppressing Ca^{2+} oscillations. Our previous study suggests blockade of SOCE sufficed to suppress Ca^{2+} oscillations in A549 cells [9]. However, the inability of kaempferol to block SOCE (not shown) suggests it did not suppress Ca^{2+} oscillations by blocking SOCE. Furthermore, kaempferol suppression of Ca^{2+} oscillations was unlikely attributed to inhibition of purinergic signaling, as the first peak of ATP-triggered Ca^{2+} signal was even slightly higher in the presence of kaempferol (and CGP-37157 as well) (Fig. 1 and Fig. 2). The reason was unclear. Kaempferol- and CGP-37157-treated cells had mitochondrial Ca^{2+} overload (Fig. 2E). The higher first peaks of Ca^{2+} oscillations of kaempferol- and CGP-37157-treated cells may be due to Ca^{2+} efflux from over-loaded mitochondria *via* the Ca^{2+}/H^+ exchanger [14].

A tantalizing explanation to kaempferol suppression is that it increased Ca^{2+} loading in the mitochondrial matrix; if it is true, CGP-37157 should be able to amplify this effect by preventing mitochondrial Ca^{2+} efflux. However, CGP-37157 and kaempferol did not add up in their enhancing effect on mitochondrial matrix Ca^{2+} accumulation (Fig. 2E); the reason was uncertain but could be due to near-saturation of matrix Ca^{2+} level. The observation that a combination of CGP-37157 and kaempferol did not have an additive effect in suppressing Ca^{2+} oscillations was in concordance with the non-additive effects of these two agents on mitochondrial matrix Ca^{2+} accumulation.

As Ca^{2+} is a co-agonist of IP_3R and Ca^{2+} dependence of IICR has a bell-shaped relationship [15], one possible hypothesis is that kaempferol, by activating MCU, accelerated the Ca^{2+} fluxes in mitochondria-associated membranes (MAM; region of ER tethering to mitochondria), reducing the local Ca^{2+} accumulation which would otherwise provide a positive feedback on IICR [16]. MCU knockdown, however, reduces Ca^{2+} buffering, resulting in Ca^{2+}-dependent inactivation of IP_3R and eventually run down of oscillations [17]. These contradictory results of MCU manipulation on Ca^{2+} oscillations (including those mentioned in the Introduction) is difficult to explain. One possible explanation is the variable thickness of the MAM (which determines the distance between IP_3R and MCU): such thickness may vary in different cell types and is modulated by metabolic or disease states [18]. Given the variable distance between IP_3R and MCU, and the bell-shaped Ca^{2+} dependence of IICR, changes of micro-domain Ca^{2+} concentration (at IP_3R) due to MCU manipulation (activation or block) could be either stimulatory or inhibitory. Accurate measurement of Ca^{2+} changes at such MAM micro-domains is needed to gain further insight into how Ca^{2+} oscillations are modulated by MCU manipulation.

The implication of kaempferol-induced inhibition of Ca^{2+} oscillations is two-fold. First, pharmacological activation of MCU offers beneficial effects [7]. Further development of MCU activators as therapeutic agents should be cautious in view of their suppression of Ca^{2+} oscillations. Second, kaempferol-induced inhibition of Ca^{2+} oscillations suggests any increased intrinsic MCU activity may affect Ca^{2+} oscillations; these situations may arise due to MCU overexpression [19] or MICU-1 loss-of-function [20].

In conclusion, ATP-triggered Ca^{2+} oscillations in A549 cells were regulated by stimulating Ca^{2+} uptake into, and preventing Ca^{2+} efflux from, the mitochondria, with both cases resulting in disturbed Ca^{2+} traffic and Ca^{2+} accumulation in the mitochondrial matrix.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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