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Regulation of superoxide flashes by transient and steady mitochondrial calcium elevations

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The mitochondria play essential roles in both intracellular calcium and reactive oxygen species signaling. As a newly discovered universal and fundamental mitochondrial phenomenon, superoxide flashes reflect transient bursts of superoxide production in the matrix of single mitochondria. Whether and how the superoxide flash activity is regulated by mitochondrial calcium remain largely unknown. Here we demonstrate that elevating mitochondrial calcium either by the calcium ionophore ionomycin or by increasing the bathing calcium in permeabilized HeLa cells increases superoxide flash incidence, and inhibition of the mitochondrial calcium uniporter activity abolishes the flash response. Quantitatively, the superoxide flash incidence is correlated to the steady-state mitochondrial calcium elevation with 1.7-fold increase per 1.0 $\Delta F/F_0$ of Rhod-2 signal. In contrast, large mitochondrial calcium transients (e.g., peak $\triangle F/F_0$ ~2.8, duration ~2 min) in the absence of steady-state elevations failed to alter the flash activity. These results indicate that physiological levels of sustained, but not transient, mitochondrial calcium elevation acts as a potent regulator of superoxide flashes, but its mechanism of action likely involves a multi-step, slow-onset process.

superoxide flash, calcium signaling, reactive oxygen species (ROS), mitochondrion

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Calcium (Ca^{2+}) and reactive oxygen species (ROS) are important intracellular signaling molecules. The mitochondria play pivotal roles in both Ca^{2+} handling and redox homeostasis. It also provides a stage for Ca^{2+} and ROS to tango on. Ca^{2+} modulates ROS homeostasis by regulating the ROS-generating and antioxidant systems, while ROS modify the components of the Ca^{2+} signaling toolkit and reshape local and global Ca^{2+} signal amplitudes and kinetics [1].

With the aid of a novel, reversible superoxide biosensor, mt-cpYFP, we recently discovered stochastic, discrete and transient superoxide production events, named superoxide flashes, in single mitochondria [2]. As a novel ROS-generating activity, superoxide flash exists in isolated single mitochondria, cultured cells, *ex vivo* beating hearts and even living animals [2–4]. A superoxide flash event is initiated with transient opening of putative mitochondrial permeability transition pore (mPTP) and requires an intact mitochondrial electron transport chain (ETC) [2]. Functionally, superoxide flashes participate in the regulation of metabolism, cell proliferation and differentiation, hyperosmotic, inflammatory and oxidative stress responses, and even in the process of aging [5–10].

Whether and how is superoxide flash activity intertwined with mitochondrial Ca^{2+} signaling? Wang et al. [2] explored the relationship between Ca^{2+} and superoxide flash in cardiac cells and found that superoxide flash frequency is unaltered by either a 2-fold increase or an abolition of Ca^{2+} sparks. Hou et al. [5] found that elevating Ca^{2+} or ROS

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production alone was inefficacious in triggering superoxide flash in HeLa cells. But when the two components act together, a flurry of flashes occur due to synergistic effect. However, the exact regulatory mechanism of Ca^{2+} on superoxide flash production remains incompletely understood. Given that extreme mitochondrial Ca^{2+} elevation, verging on apoptosis, suffices to trigger irreversible mPTP openings [11], it is also of interest to delineate the mode with which Ca^{2+} regulates transient mPTP openings as reflected by discrete superoxide flashes. In this study, we used several ways to selectively manipulate intracellular Ca^{2+} and found that superoxide flash frequency is tightly correlated with the steady mitochondrial Ca^{2+} level but appears to be insensitive to transient mitochondrial Ca^{2+} elevations (duration ~2 min). These results indicate that mitochondrial Ca^{2+} is a potent regulator of superoxide flashes, but its mechanism of action likely involves a multi-step, slow-onset process.

1 Materials and methods

1.1 Reagents

Cyclosporine A, saponin, ionomycin and ruthenium red were from Sigma, USA. Rhod-2 AM, Fluo-4 AM, Lipofectamine RNAiMax, Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were from Invitrogen, USA. Fetal bovine serum (FBS) was from Hyclone, USA.

1.2 Cell culture and RNA interference assay

The HeLa cells stably expressing mitochondria-targeted cpYFP (mt-cpYFP) were established as previously [8]. Cells were grown in DMEM medium supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C under 5% CO_2. For RNA interference of human MCU (CCDC-109a), two double-stranded small RNAs and a negative control (NC) RNA were used as follows: MCU-1 sense: CCAGCAACUAUACACCACACUdTdT; MCU-1 antisense: AGUGUGGUGUAUAGUUGCUGGdTdT; MCU-2 sense: GCAAGGAGUUUCUUUCUCUUUdTdT; MCU-2 antisense: AAAGAGAAAGAAACUCCUUGCdTdT; NC sense: UUCUCCGAACGUGUCACGUdTdT; NC antisense: ACGUGACACGUUCGGAGAAdTdT.

For transfection, 100 nmol L^{-1} siRNA was transiently transfected into the mt-cpYFP-expressing HeLa cells with Lipofectamine RNAiMax according to the manufacturer's instructions. Confocal imaging and Western blotting were performed 60–72 h after transfection.

1.3 Ca^{2+} measurement

Fluo-4 AM and Rhod-2 AM were used to measure the cytosolic and mitochondrial Ca^{2+} signal respectively according to the manufacturer's instructions. Briefly, HeLa cells were loaded with Fluo-4 AM (5 μmol L^{-1}) or Rhod-2 AM (5 μmol L^{-1}) at 37°C for 15–30 min. After washing with Tyrode's solution consisting (in mmol L^{-1}) 137 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 D-glucose, and 20 HEPES (pH 7.35, adjusted with NaOH), the Fluo-4 and Rhod-2 signals were monitored on an inverted Zeiss LSM 710 confocal microscope equipped with a 40×, 1.3 NA oil-immersion objective. For Fluo-4 fluorescence, images were acquired by exciting at 488 nm and collecting the emission at 505–530 nm. For Rhod-2 fluorescence, images were taken by exciting at 543 nm and collecting the emission at >560 nm. Usually, 450 frames of 512×512 pixels were collected at 2 s/frame in bidirectional scanning mode.

1.4 Cell permeabilization

The cells were permeabilized with saponin treatment. Briefly, cells were washed in Ca^{2+}-free Tyrode's solution for five times to remove intracellular Ca^{2+} before permeabilization and then permeabilized with 50 μg mL^{-1} saponin in the intracellular solution (in mmol L^{-1}, 100 KOH, 100 aspartic acid, 20 KCl, 0.81 $MgCl_2$, 3 Mg-ATP, 0.5 EGTA, 5 phosphocreatine·ditris, 10 phosphocreatine·Na, 5 creatine phosphokinase, 10 glutathione, 8% dextran and 20 HEPES (pH 7.2, adjusted with KOH)) for 30 s. After permeabilization, cells were bathed in intracellular solution without saponin for further experiments.

1.5 Imaging and analysis of superoxide flashes

A Zeiss LSM 710 inverted confocal microscope with a 40×, 1.3 NA oil-immersion objective was used for detecting superoxide flashes. Mt-cpYFP was excited at 488 and 405 nm, and the emission was collected at 505–530 nm. The axial resolution was set to 2.0 μm and the size of the imaging region was 70.71 μm×70.71 μm. In a typical time series recording, 100 frames of 512×512 pixels were collected at 1 s/frame in bidirectional scanning mode. Images were usually acquired within 40 min after different treatments. All experiments were performed at room temperature (24–26°C) unless specified otherwise.

Superoxide flashes were analyzed using custom-developed programs written in Interactive Data Language (IDL, Research Systems) as described previously [12]. Individual superoxide flashes were identified with the aid of custom-devised computer algorithms, and their morphological and kinetic properties were measured automatically.

1.6 Statistics

Data are expressed as mean±SEM. Student's *t*-test was applied to determine the statistical significance. $P<0.05$ was considered statistically significant.

2 Results

2.1 Superoxide flash incidence positively correlated with intracellular steady-state Ca^{2+} level

First, we sought to determine whether Ca^{2+} transient elicited by Ca^{2+} ionophore ionomycin is able to activate superoxide flashes. When cells were perfused with Tyrode's solution containing 1.8 mmol L^{-1} Ca^{2+}, application of ionomycin (1 μmol L^{-1}) immediately elicited robust cytosolic and mitochondrial Ca^{2+} transients that displayed a 7.5- and 3.8-fold peak increase of Fluo-4 and Rhod-2 fluorescence, respectively. Then cytosolic and mitochondrial Ca^{2+} gradually declined toward an elevated steady level (Fluo-4: F/F_0=1.31±0.03; Rhod-2: F/F_0=1.30±0.02, n=61–70 cells) (Figure 1A). Ionomycin was equally efficient in eliciting Ca^{2+} transients in cells perfused with higher extracellular Ca^{2+} ($[Ca^{2+}]_{ex}$=3.6, 5 and 10 mmol L^{-1}) (Figure 1B–D). Interestingly, the steady Ca^{2+} level after ionomycin stimulation increased dose-dependently with the extracellular Ca^{2+} concentration (Fluo-4: F/F_0=2.02±0.06, 3.03±0.10 and 2.88±0.08; Rhod-2: F/F_0=1.62±0.04, 2.59±0.0 and 2.70±0.07; at 3.6, 5 and 10 mmol L^{-1} $[Ca^{2+}]_{ex}$ respectively, n=61–70 cells) (Figure 1F), while the peak amplitudes remained unaffected (Fluo-4: F/F_0=7.75±0.20, 8.18±0.19, 6.63±0.16; Rhod-2: F/F_0=4.42±0.12, 5.31±0.12, 4.11±0.11 at 3.6, 5 and 10 mmol L^{-1} $[Ca^{2+}]_{ex}$ respectively, n=61–70 cells) (Figure 1E). One possible explanation is that the Ca^{2+} peak reflects the Ca^{2+} store capacity of the endoplasmic reticulum (ER), while the steady-state Ca^{2+} level is the result of Ca^{2+} ionophone ionomycin equilibrating the extra- and intracellular Ca^{2+}.

Parallel experiments were performed to measure the superoxide flash response. At $[Ca^{2+}]_{ex}$ of 3.6 mmol L^{-1}, superoxide flash incidence was slightly but significantly increased after ionomycin stimulation compared with ionomycin treatment at 1.8 mmol L^{-1} $[Ca^{2+}]_{ex}$. With further increase of $[Ca^{2+}]_{ex}$, the flash incidence reached 1.45±0.15 and 2.03±0.29 events/100 s/cell in groups at 5 and 10 mmol L^{-1} $[Ca^{2+}]_{ex}$, which was 1.2- and 2.1-fold greater than that in control cells (0.65±0.10 flashes/100 s/cell) (Figure 2A). Moreover, linear regression showed a trend of positive correlation between the superoxide flash incidence and the steady level of mitochondrial Ca^{2+} (R^2=0.82, r=0.91, P=0.092) (Figure 2B), revealing a high sensitivity of superoxide flashes to the steady-state mitochondrial Ca^{2+} elevation.

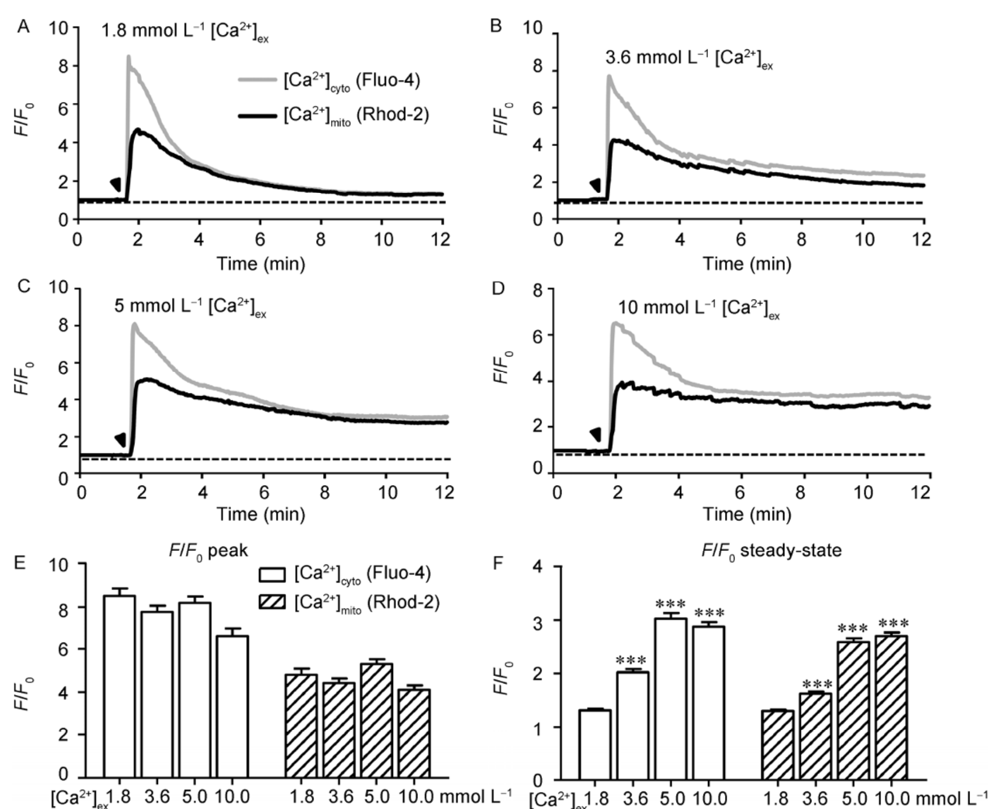

Figure 1 Cytosolic and mitochondrial Ca^{2+} transients induced by ionomycin. A–D, Response of cytosolic and mitochondrial Ca^{2+} measured with Fluo-4 and Rhod-2 respectively upon ionomycin (1 μmol L^{-1}) treatment in the presence of 1.8 (A), 3.6 (B), 5 (C) and 10 (D) mmol L^{-1} extracellular Ca^{2+} ($[Ca^{2+}]_{ex}$). Arrowheads indicate the time of adding ionomycin. E, Averaged peak value of the cytosolic and mitochondrial Ca^{2+} transients. Note that there are no significant difference with altering $[Ca^{2+}]_{ex}$. F, Cytosolic and mitochondrial steady state Ca^{2+} level after ionomycin treatment with varying $[Ca^{2+}]_{ex}$. Data are expressed as the mean±SEM. n=61–70 cells per group. ***, P<0.001 vs. 1.8 mmol L^{-1} $[Ca^{2+}]_{ex}$ group.

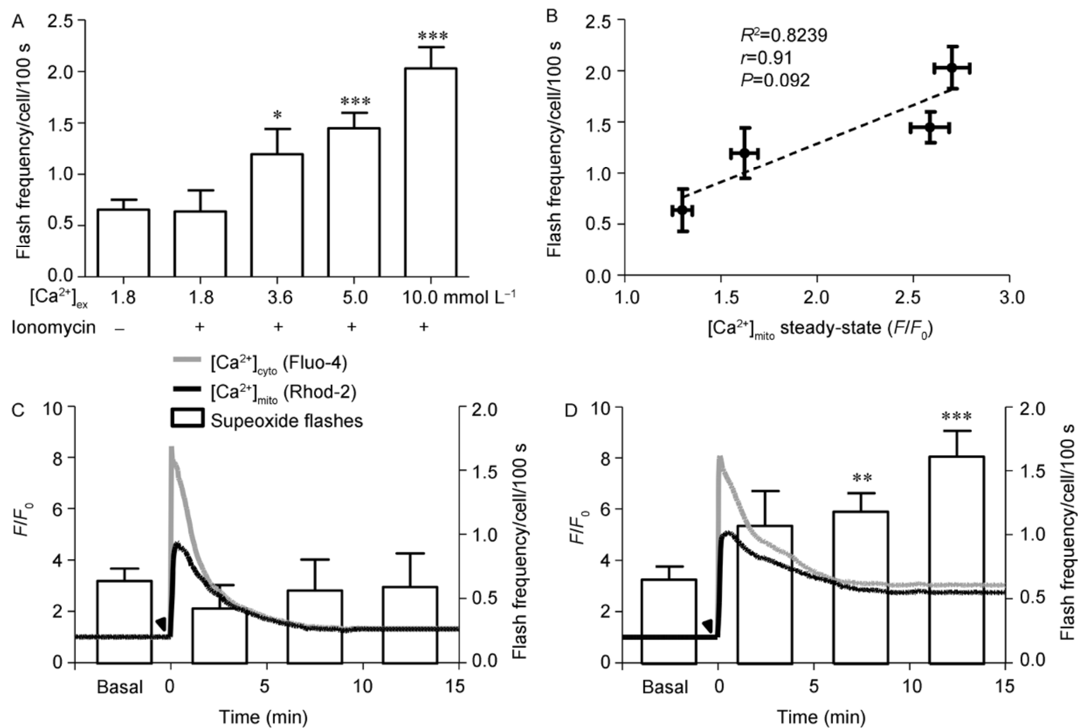

Figure 2 Induction of superoxide flashes by steady-state Ca^{2+} elevation. A, Superoxide flash frequency after ionomycin treatment in the presence of varying $[Ca^{2+}]_{ex}$. Data are expressed as the mean±SEM. *n*=51–98 cells per group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. 1.8mmol L^{-1} $[Ca^{2+}]_{ex}$ without ionomycin treatment group. B, Trend of positive correlation between steady-state mitochondrial Ca^{2+} level and superoxide flash incidence. Dashed line shows the linear regression result (R^2=0.82, *r*=0.91, *P*=0.092). C and D, Time-course of superoxide flash activity induced by ionomycin (1 μmol L^{-1}) treatment in the presence of 1.8 (C) and 5 (D) mmol L^{-1} $[Ca^{2+}]_{ex}$. The gray and black traces show cytosolic and mitochondrial Ca^{2+} transients respectively. Arrowheads indicate the time of adding ionomycin. Data are expressed as mean±SEM. *n*=14–84 cells per group. **, $P<0.01$; ***, $P<0.001$ vs. basal group.

Time-course analysis revealed that the aforementioned strong transient Ca^{2+} elevation within the first 5 min was ineffective in augmenting superoxide flash frequency when cells were perfused with 1.8 mmol L^{-1} $[Ca^{2+}]_{ex}$ (Figure 2C), in agreement with Hou et al. [5]. In the presence of 5 mmol L^{-1} $[Ca^{2+}]_{ex}$, superoxide flash activity was activated gradually, displaying a significant increase only after 5 min of ionomycin addition when mitochondrial and cytosolic Ca^{2+} levels were at plateau, while a prominent early cytosolic and mitochondrial Ca^{2+} peak occurred at 48 and 72 s (Figure 2D). These results indicate that steady-state rather than transient elevation of intracellular Ca^{2+} induces superoxide flashes.

2.2 Activation of superoxide flashes in permeabilized cells by increasing bathing Ca^{2+}

To further elucidate the regulation of superoxide flash by sustained Ca^{2+} elevation, we directly manipulated the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) by permeabilizing cells with saponin (1 μg mL^{-1}) and then bathing the cells in intracellular solution with varying Ca^{2+} concentrations (0.1, 0.3, 0.5, 1, 3 and 10 μmol L^{-1}). Consistent with aforementioned strong dependence of superoxide flash on steady Ca^{2+} level, superoxide flashes responded to $[Ca^{2+}]_c$ manipulations in a dose-dependent manner. Flurries of superoxide flash activity were visualized upon challenge with $[Ca^{2+}]_c$ greater than 1 μmol L^{-1}, displaying 2.2-, 2.7- and 3-fold increase at 1, 3 and 10 μmol L^{-1} $[Ca^{2+}]_c$, respectively (Figure 3A).

Mitochondrial targeting cpYFP also enabled us to monitor mitochondrial morphology changes. We noticed that the flash response at supra-micromolar $[Ca^{2+}]_c$ was accompanied by mitochondrial fragmentation and swelling (Figure 3C), typical features of mPTP opening. Pretreating cells with cyclosporine A (CsA, 1 μmol L^{-1}), which inhibits the mPTP opening by targeting to cyclophilin D [13], blocked the morphological change together with Ca^{2+}-induced flashes (Figure 3B and C). These data substantiate the notion that superoxide flashes in permeabilized cells are likely triggered by transient and reversible opening of mPTP.

2.3 Roles of mitochondrial Ca^{2+} uniport in activation of superoxide flashes by Ca^{2+}

To determine whether cytosolic or mitochondrial Ca^{2+} is responsible for activating superoxide flashes, we investigated the effect of inhibiting the mitochondrial Ca^{2+} transport. By taking advantage of the recent molecular identification of MCU [14,15], the channel pore element of mitochondrial Ca^{2+} uniporter, we showed that siRNA knock-

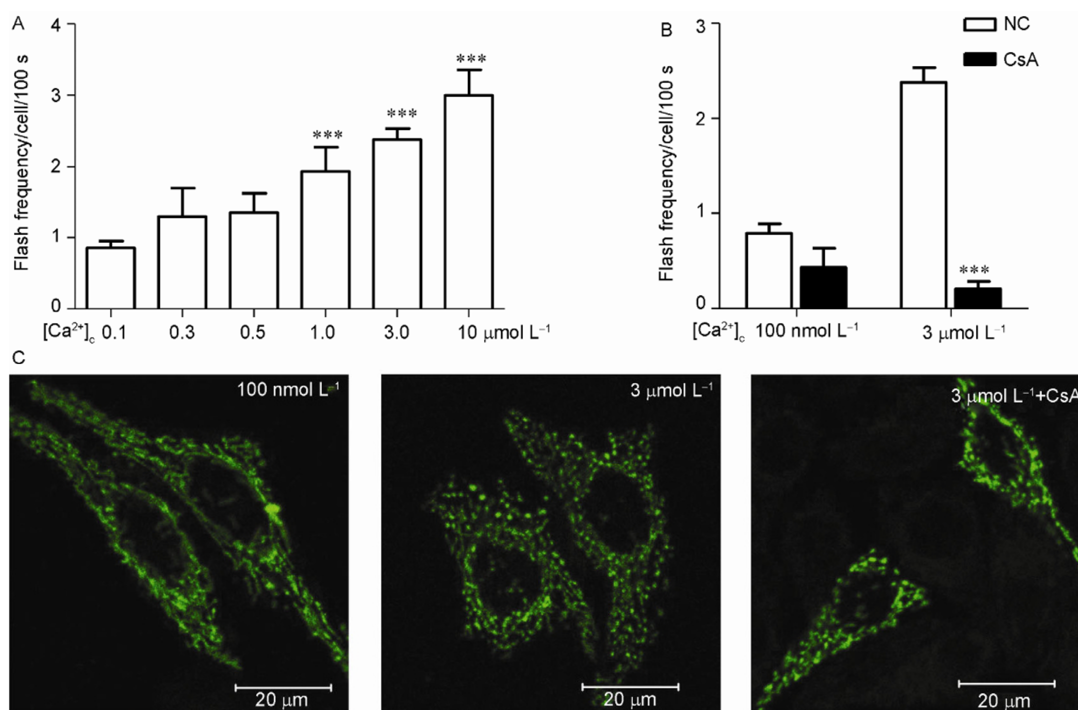

Figure 3 Increasing bathing Ca^{2+} in permeabilized cells augments superoxide flash incidence. A, Superoxide flash incidence in permeabilized HeLa cells with altering cytosolic Ca^{2+} ($[Ca^{2+}]_c$). The data are expressed as the mean±SEM. n=17–195 cells per group. ***, P<0.001 vs. 0.1 μmol L^{-1} $[Ca^{2+}]_c$ group. B, Inhibition of superoxide flashes by cyclosporine A (CsA, 2 μmol L^{-1}). The data are expressed as the mean±SEM. n=14–195 cells per group. ***, P<0.001 vs. negative control (NC). C, Representative confocal images of mitochondrial morphology under 0.1 and 3 μmol L^{-1} $[Ca^{2+}]_c$ with or without CsA treatment.

down of MCU with two independent double-strand small RNAs (siMCU-1 and siMCU-2) decreased MCU protein expression (Figure 4A). As a result, transient mitochondrial Ca^{2+} elevation responding to ionomycin was significantly decreased but transient cytosolic Ca^{2+} elevation and the steady cytosolic and mitochondrial Ca^{2+} levels remained unchanged (Figure 4B and C). Concomitantly, superoxide flashes induced by ionomycin under 5 mmol L^{-1} $[Ca^{2+}]_{ex}$ was not affected upon MCU knockdown (Figure 4D), consistent with the idea that steady-state but not transient Ca^{2+} elevation activates superoxide flashes.

Since ionomycin permits Ca^{2+} entry into the mitochondria independent of mitochondrial Ca^{2+} uniport, this explains the insensitivity of mitochondrial steady-state Ca^{2+} and superoxide flash to knockdown of MCU. However, in permeabilized cells in the absence of ionomycin, MCU provides the major route to couple cytosolic Ca^{2+} with mitochondrial matrix Ca^{2+} elevation. Inhibition of MCU activity by ruthenium red (RR, 500 nmol L^{-1}), a blocker of MCU, or by genetic disruption with siRNAs both significantly depressed superoxide flashes induced by micromolar $[Ca^{2+}]_c$ in permeabilized cells (Figure 5). Specifically, RR decreased flash frequency by 73.8%, siMCU-1 by 73.2% and siMCU-2 by 49.1%. These results showed that superoxide flashes are activated by the steady-state mitochondrial Ca^{2+} elevation, but not the cytosolic one.

3 Discussion

Superoxide flashes represent quantal bursts of superoxide production in single mitochondria. The present study aims to determine how this newly discovered, dynamic, fundamental, physiological mitochondrial activity is regulated by the prominent Ca^{2+} signaling. We have demonstrated that elevating mitochondrial steady Ca^{2+} level either by ionomycin (in intact cells) or by increasing the bathing Ca^{2+} (in saponin-permeabilized cells) effectively augmented the superoxide flash incidence. Surprisingly, we found that transient mitochondrial Ca^{2+} elevation, even when the mitochondrial Ca^{2+} level reached several μmol L^{-1} within the first 5 min after ionomycin addition (Rhod-2 peak: $\Delta F/F_0$ ~3, K_d=570 nmol L^{-1}), failed to stimulate any detectable increase in superoxide flash activity. In sharp contrast, a small fractional increase of mitochondrial steady-state Ca^{2+} greatly enhanced the superoxide flash activity (1.7-fold increase in flash incidence per 1.0 $\Delta F/F_0$ of Rhod-2 fluorescence). Linear regression showed a trend of positive correlation between the flash activity and the steady-state mitochondrial Ca^{2+} level, suggesting that the apparent Ca^{2+} affinity for the regulation of flashes is in the same range with K_d of Rhod-2. These results show an important feature for Ca^{2+} regulation of superoxide flashes in HeLa cells: steady mitochondrial Ca^{2+} is a powerful and potent regulator of superoxide flashes, but the transient mitochondrial Ca^{2+}

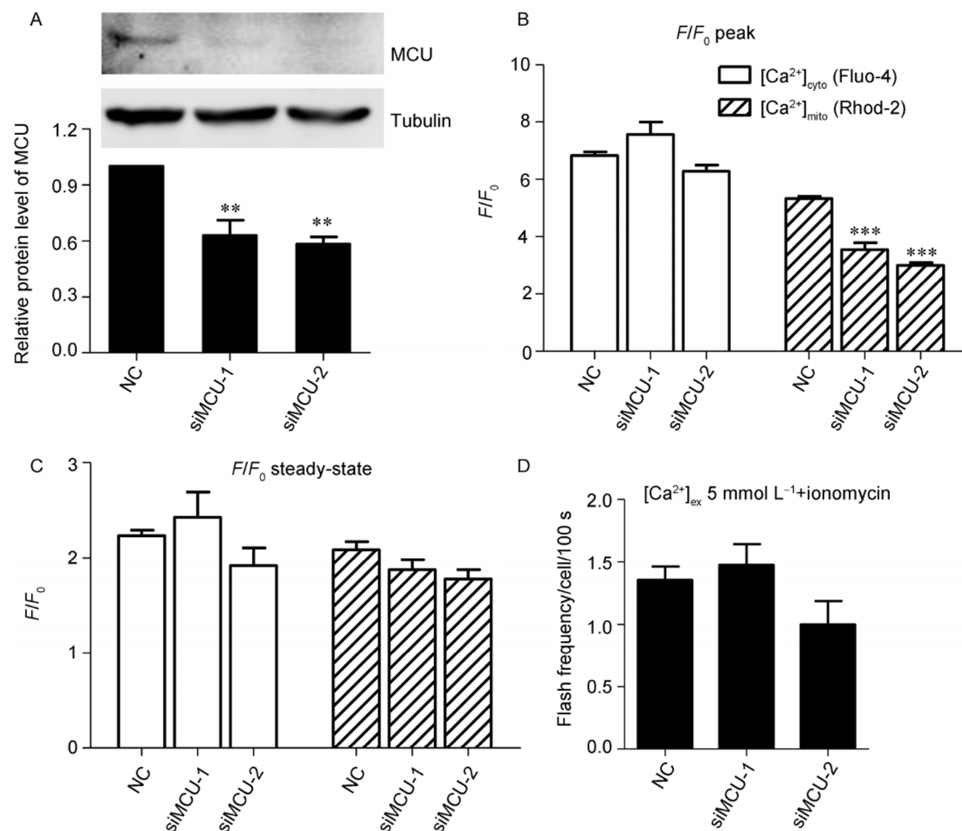

Figure 4 MCU is dispensable for ionomycin induced superoxide flashes in intact cells. A, Western-blot assessing the knockdown efficiency of MCU. Two double-strand siRNAs (siRNA-1, siRNA-2) and one negative control RNA (NC) were used. Data are expressed mean±SEM, n=3 experiments. **, P<0.01 vs. NC. B and C, MCU knockdown decreased the peak of cytosolic and mitochondrial Ca^{2+} transient (B), but showed little effect on the steady Ca^{2+} level (C). The data are expressed as the mean±SEM. n=37–76 cells per group. D, Effect of MCU knockdown on superoxide flash frequency induced by ionomycin in the presence of 5 mmol L^{-1} $[Ca^{2+}]_{ex}$. The data are expressed as the mean±SEM. n=49–177 cells per group. ***, P<0.001 vs. NC.

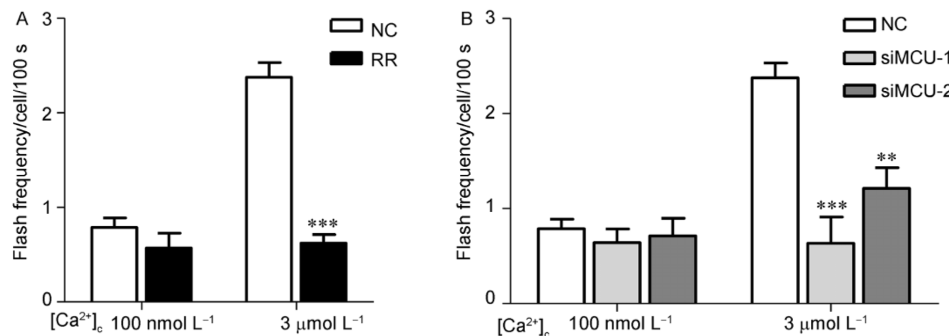

Figure 5 Requirement of MCU for superoxide flashes induced by high cytosolic Ca^{2+} in permeabilized cells. Inhibition of MCU with ruthenium red (RR, 500 nmol L^{-1}) (A) or by RNAi (B) both depressed superoxide flash incidence in permeabilized cells. The data are expressed as the mean±SEM. n=28–195 cells per group. **, P<0.01; ***, P<0.001 vs. NC.

signal is not. It can be inferred that the Ca^{2+}-dependent regulatory mechanism likely involves a multi-step, slow-onset, but high-affinity process.

Blockage of mitochondrial Ca^{2+} uniport in permeabilized cells significantly decreased the hyperactive superoxide flashes induced by micromolar cytosolic Ca^{2+}, indicating that it is the high steady Ca^{2+} level within mitochondria, but not cytosol, that activates superoxide flashes. Interestingly, the peak of transient mitochondrial Ca^{2+} induced by ionomycin is MCU-dependent, while the steady-state mitochondrial Ca^{2+} level and superoxide flash frequency are not. This further emphasizes the close relationship between superoxide flash and steady mitochondrial Ca^{2+} level.

Possible mechanism of superoxide flashes induced by elevated steady-state mitochondrial Ca^{2+} might be (i) increase of ROS originating from ETC accelerated by steady-

state mitochondrial Ca^{2+} elevation. Mitochondrial Ca^{2+} is a potent regulator of tricarboxylic acid (TCA) cycle and oxidative phosphorylation. It activates three dehydrogenases from TCA cycle, pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, thus increases the reaction rate of many steps in the cycle [16]. Adenine nucleotide translocator (ANT) and ATP synthase have also been reported to be activated by mitochondrial Ca^{2+} [17,18]. Hence, mitochondrial Ca^{2+} might increase ROS production by enhancing mitochondrial respiration through the so-called parallel activation mechanism. As a result, mitochondrial Ca^{2+} and basal ROS elevation synergistically trigger superoxide flashes [5]. (ii) Involvement of Ca^{2+} downstream signaling cascades that regulate flash activity. Slow response of superoxide flashes to mitochondrial Ca^{2+} elevation suggests that, rather than being a direct trigger, mitochondrial Ca^{2+} might have evoked cumulative activation of some enzymes such as protein kinase C (PKC) and calmodulin-dependent protein kinase II (CaMK II), resulting in phosphorylation of downstream proteins. In this regard, whether CaMK II, PKC or other Ca^{2+} regulated enzymes are present within mitochondria, and if so, how they regulate superoxide flashes warrant further investigation.

In summary, steady-state elevation of mitochondrial Ca^{2+} dose-dependently augments flash activity, while transient elevation alone fails to induce any detectable flash response. The mechanism of action likely involves a multi-step, slow-onset process. It should also be appreciated that Ca^{2+}-dependent regulation of transient mPTP opening (reflected by the flash incidence) operates at physiological Ca^{2+} range whereas irreversible mPTP opening (as seen in cells committing to apoptosis) requires much higher mitochondrial Ca^{2+} elevations. Nevertheless, both transient and irreversible mPTP activities can be activated by Ca^{2+} or ROS acting alone [11,19,20] (and this study) and, more importantly, share the property that Ca^{2+} and ROS act as powerful synergistic regulators [5,20]. It is thus tempting to speculate that the same or similar core mechanism, operating in distinctly different modes, may underlie the flash genesis and the irreversible mitochondrial permeability transition.

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