Research article

Characterization of the stimulus for reactive oxygen species generation in calcium-overloaded mitochondria

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We have used two different probes with distinct detection properties, dichlorodihydrofluorescein diacetate and Amplex Red/horseradish peroxidase, as well as different respiratory substrates and electron transport chain inhibitors, to characterize the reactive oxygen species (ROS) generation by the respiratory chain in calcium-overloaded mitochondria. Regardless of the respiratory substrate, calcium stimulated the mitochondrial generation of ROS, which were released at both the mitochondrial-matrix side and the extra-mitochondrial space, in a way insensitive to the mitochondrial permeability transition pores inhibitor cyclosporine A. In glutamate/malate-energized mitochondria, inhibition at complex I or complex III (ubiquinone cycle) similarly modulated ROS generation at either mitochondrial-matrix side or extra-mitochondrial space; this also occurred when the backflow of electrons to complex I in succinate-energized mitochondria was inhibited. On the other hand, in succinate-energized mitochondria the modulation of ROS generation at mitochondrial-matrix side or extra-mitochondrial space depends on the site of complex III which was inhibited. These results allow a straight comparison between the effects of different respiratory substrates and electron transport chain inhibitors on ROS generation at either mitochondrial-matrix side or extra-mitochondrial space in calcium-overloaded mitochondria.

Keywords: Mitochondria, Calcium, Reactive oxygen species, Amplex red, Dichlorodihydrofluorescein diacetate, Respiratory chain inhibitors

Introduction

Under physiological conditions, mitochondria play an important role in cellular calcium homeostasis. However, mitochondrial calcium overload in pathological situations is associated with the increase in reactive oxygen species (ROS) generation, oxidation of membrane protein thiols, and induction of the mitochondrial permeability transition (MPT) process. MPT is mediated by the opening of membrane permeability transition pores (PTP) in the presence of calcium and evidenced by cyclosporine A (CsA)-sensitive mitochondrial swelling; it is considered to play an important role in cell death by necrosis and apoptosis.[1–5]

Mitochondria are the main intracellular sources of ROS. The primary ROS generated by mitochondria is superoxide anion ($O_2^{\bullet-}$), the product of molecular oxygen reduction by the electrons that leak from the electron transport chain. The two main ROS-generating sites in the electron transport chain are complex I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome c oxidoreductase).[6,7] In complex I, $O_2^{\bullet-}$ is produced by the oxidation of reduced flavin ($FMNH_2$)/mitochondrial-matrix facing flavin semiquinone ($FMNH^\bullet$)[8] or at the iron−sulfur centers.[9] In complex III, the source of $O_2^{\bullet-}$ is the ubisemiquinone radical intermediate ($QH^\bullet$), which is formed during the Q cycle at the Qo site facing the mitochondrial inter-membrane space.[10,11] *In vitro*, electrons entering at complex II (succinate dehydrogenase) can flow backward through complex I to produce ROS at the FMN site.[8] Regardless of the production site, $O_2^{\bullet-}$ quickly undergoes dismutation to H_2O_2, catalyzed by

superoxide dismutase in the mitochondrial-matrix (Mn-SOD) or in the inter-membrane space (Cu,Zn-SOD).[12] H_2O_2 can diffuse across the mitochondrial membranes and be detected in the extra-mitochondrial space.[13] In the presence of metal ions, H_2O_2 can be further transformed into hydroxyl radical (OH•) by Fenton chemistry.[14] Mitochondria are also believed to be primary source of other reactive species such as nitrogen-centered species, which are derived from nitric oxide and carbonate radical.[15,16]

Despite the vast literature addressing the respiratory chain sites involved in mitochondrial ROS generation, the process in calcium-overloaded mitochondria has not yet been completely characterized. Here, we have used two different probes with distinct detection properties, as well as different respiratory substrates and electron transport chain inhibitors, to characterize the ROS generation by the respiratory chain in calcium-overloaded mitochondria. This work continues our previous studies on the potential role of calcium on PTP opening.[17,18]

Materials and methods
Isolation of rat liver mitochondria
Male Wistar rats weighting approximately 200 g were used, according to research protocols approved by the local ethics committee (Comissão de Ética no Uso de Animais do Campus de Ribeirão Preto – USP, CEUA-USP). Mitochondria were isolated by standard differential centrifugation, at 4°C, according to Pedersen *et al.*[19] with minor modifications.[17] Mitochondrial protein content was determined by the biuret reaction. Rat liver-isolated mitochondria energized with 5 mM succinate (plus 2 µM rotenone) presented respiratory control ratio and ADP/O in the 4.5–5.5 and 1.3–1.7 ranges, respectively; the mitochondrial membrane potential was ~160 mV. We have used freshly prepared mitochondria (used within 3 hours) incubated in a medium devoid of inorganic phosphate in order to prevent non-specific sensitization of PTP opening. At 100 µM, but not at 10 µM, calcium significantly increased state-4 respiration rate.

Mitochondrial assays
The standard medium for the mitochondrial assays consisted of 125 mM sucrose, 65 mM KCl, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH pH 7.4, at 30°C. Mitochondrial ROS were monitored spectrofluorimetrically using as probes 5 µM dichlorodihydrofluorescein diacetate (H_2DCFDA)[20] or 2 µM Amplex Red plus 1 U/ml horseradish peroxidase (HRP)[21] at 503/529 nm (slits 5/10 nm), and 563/587 nm (slits 5/5 nm) excitation/emission wavelength pairs, respectively, in a Model F-4500 Hitachi fluorescence spectrophotometer (Tokyo, Japan). The fluorescence levels of probes

assayed in the absence of mitochondria were not altered in the presence of the respiratory substrates or inhibitors used in the study. Mitochondrial calcium uptake was assessed in the standard medium by monitoring the changes in the absorbance of arsenazo III (25 µM) at 675–685 nm. The calcium concentration was determined based on a standard curve under the assay conditions. The concentration of calcium in the medium with mitochondria was ~2 µM. Mitochondrial membrane potential was assessed spectrofluorimetrically using 10 µM safranine *O* as a probe at the 495/586 nm excitation/emission wavelength pair. Mitochondrial swelling was estimated spectrophotometrically from the decrease in apparent absorbance at 540 nm, using a Model U-2910 Hitachi spectrophotometer (Japan).

Statistical analysis
Data are represented as average ± standard error of mean of 3–6 repetitions using different mitochondrial preparations. Multiple comparisons were performed using one-way analysis of variance followed by Tukey test. $P < 0.05$ was considered significant.

Results and discussion
In order to characterize the ROS generation by the respiratory chain in calcium-overloaded mitochondria, we first evaluated the saturation of calcium uptake by the organelles: calcium uptake increased from 10 to 100 µM calcium and no further increase was observed at 250 µM (Fig. 1A). Therefore, we chose to work with 100 µM calcium, a condition which significantly increased the basal rate of mitochondrial H_2O_2 generation (as demonstrated with the Amplex red probe, Fig. 1B). Then we assessed ROS generation in the presence of calcium plus different respiratory chain substrates and inhibitors by means of two different probes, H_2DCFDA and Amplex Red, regarding their specificity for different ROS types and location. It is worth considering that calcium overload as addressed in the present study may be found under specific pathological situations such as excitotoxin-induced neuronal cell death and ischemia/reperfusion injury, as well as during local calcium spikes mediated by IP3 receptors at the endoplasmic reticulum–mitochondrion interface.[22] It has been proposed that ROS generation induced by calcium involves either: (1) stimulation of TCA cycle/oxidative phosphorylation enhancing electron transport through the respiratory chain, (2) stimulation of mtNOS generating NO which inhibits complex IV, or (3) displacement of cytochrome *c* from mitochondrial inner membrane either by competing for cardiolipin binding sites, or through the PTP pore.[23]

The H_2O_2 in the extra-mitochondrial (inter-membrane) space can be measured by the reaction with
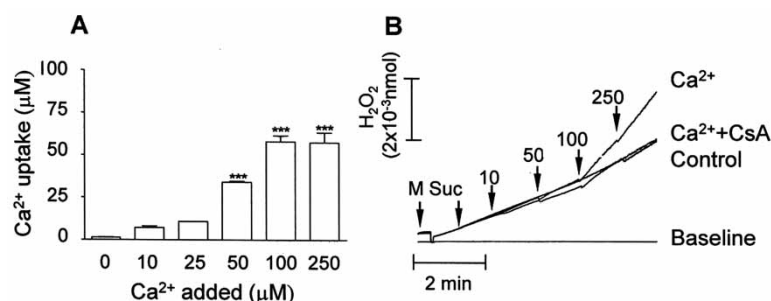
Figure 1 Calcium uptake (A) and calcium-stimulated ROS (H_2O_2) generation (B) in mitochondria isolated from rat liver. (A) Mitochondria (0.5 mg protein/ml) were energized with 5 mM succinate ($+2\ \mu M$ rotenone) in a standard medium consisting of 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH pH 7.4, at 30°C; calcium (Ca^{2+}) was determined with arsenazo III as described in Materials and methods. (B) Mitochondria were incubated in the standard medium at 30°C and H_2O_2 was determined with the Amplex Red probe/HRP as described in Materials and methods. Additions: standard medium + Amplex Red probe/HRP (base line); Mitochondria (M) $+ 2\ \mu M$ rotenone $+ 100\ \mu M$ EGTA $+ 5$ mM succinate (Suc) (Control); M $+ 2\ \mu M$ rotenone $+$ Suc $+$ 10–250 μM calcium (Ca^{2+}). Bars are average $\pm$ SEM and traces are representative experiments.

the non-fluorescent probe Amplex Red in conjunction with HRP, with formation of the fluorescent product resorufin.[21] In ROS assays with H_2DCFDA, the probe crosses the mitochondrial membranes and is enzymatically hydrolyzed by the matrix esterase forming the non-fluorescent H_2DCF product.[24] Once in the mitochondrial matrix and in the presence of hydroxyl, carbonate, or nitrogen dioxide radicals, H_2DCF is rapidly oxidized to the highly fluorescent dichlorofluorescein (DCF) product.[25] In virtue of the wide spectrum of detection, data from DCF indicate the overall ROS status rather than any particular species. These different characteristics of detection allowed us to assess both overall ROS status in the matrix side and H_2O_2 in the extra-mitochondrial space of calcium-overloaded organelles.

Under the control conditions (presence of ethylene glycol tetra-acetate, EGTA), only the Amplex Red/ HRP assay showed a significant difference between ROS generated in mitochondria energized with gluta-mate/malate (Fig. 2A) or succinate (Fig. 2B), being higher for succinate. Since mitochondria energized with succinate, in the absence of rotenone, generates ROS by the backflow of electrons to complex I,[8] this result suggests that H_2O_2 was released at the extra-mitochondrial space (detection by Amplex Red/ HRP), without significant ROS release at the mito-chondrial-matrix side (lack of detection by H_2DCFDA). Calcium, in its turn, stimulated ROS release in mitochondria energized with either gluta-mate/malate, or succinate, which was detected by both Amplex Red (Fig. 2) and H_2DCFDA (Fig. 3),
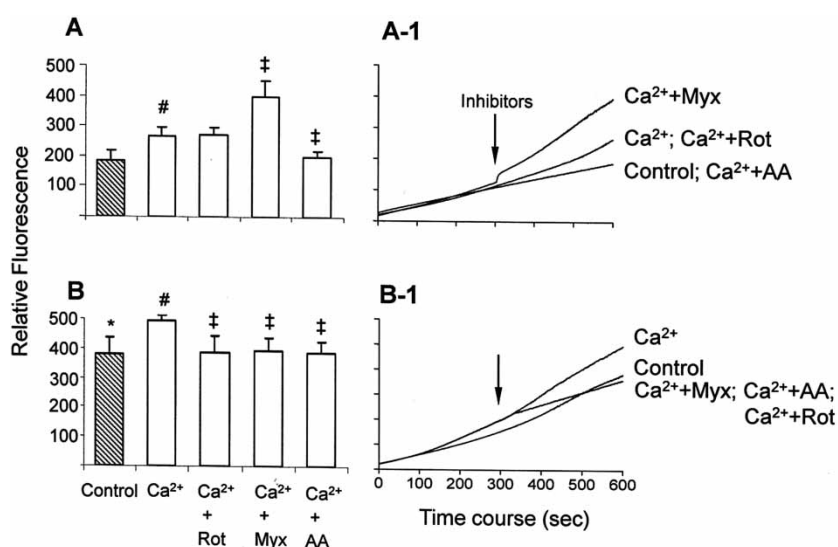

Figure 2 H_2O_2 generation assessed with the Amplex Red probe/HRP in mitochondria energized with glutamate/malate (A) or succinate (B). Mitochondria were incubated in the standard medium described in Fig. 1, in the presence of 5 mM glutamate/ malate (A) or 5 mM succinate (B) as described in Materials and methods; 100 μM EGTA (control) or 100 μM calcium (Ca^{2+}) were added at the initial time (0 seconds). The inhibitors rotenone (Rot, 2 μM), antimycin A (AA, 1 μM), or myxothiazol (Myx, 2 μM) were added after 300 seconds. Bars (A, B) are average $\pm$ SEM of the relative fluorescence at 600 seconds from the traces represented in A-1 and B-1. $^*P < 0.05$, succinate vs. glutamate/malate; $^\#P < 0.05$ vs. Control; $^\ddagger P < 0.05$ vs. Ca^{2+}.
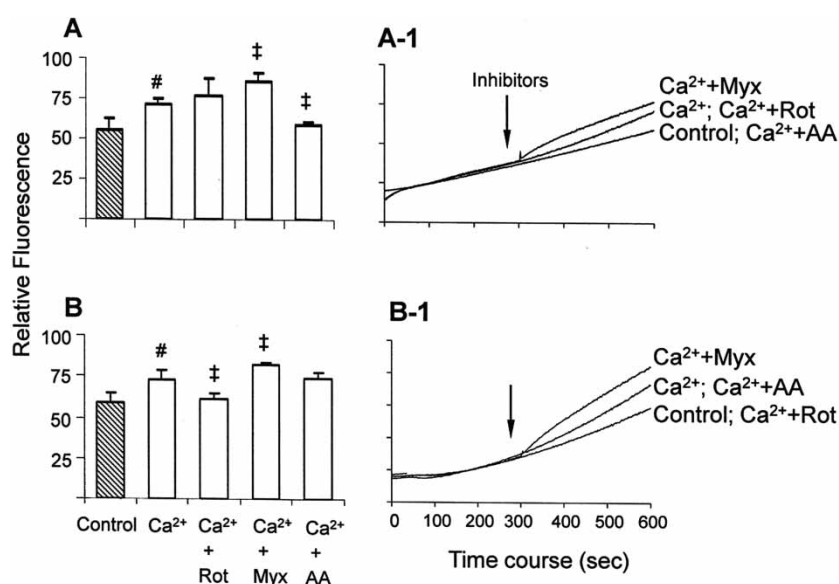
Figure 3   ROS generation assessed with the H_2DCFDA probe in mitochondria energized with glutamate/malate (A) or succinate (B). The assays were performed as described in Fig. 2. [#]$P < 0.05$ vs. control; [‡]$P < 0.05$ vs. Ca^{2+}.

indicating ROS release at both the mitochondrial-matrix side and extra-mitochondrial space.

In mitochondria energized with glutamate/malate and in the presence of respiratory chain inhibitors, Amplex Red (Fig. 2A) and H_2DCFDA (Fig. 3A) assays showed similar results. Rotenone, which blocks electron transfer from Fe–S center to ubiquinone and increases ROS generation at complex I,[9,26] had no significant effect on calcium-stimulated ROS generation. Under this condition, ROS generation is supported only by complex I, since the blockage of electrons flow to ubiquinone renders it in an oxidized state unable to donate electrons to oxygen, thus reducing ROS generation at complex III.[27] This suggests an interference of rotenone at the site where calcium-stimulated ROS is generated, i.e. it decreases at complex III and increases at complex I. On the other hand, the complex III inhibitors myxothiazol and antimycin A had opposite effects as compared with each other: myxothiazol increased, while antimycin A decreased, calcium-stimulated ROS generation (Figs 2A and 3A). The effects of complex III inhibitors on mitochondrial ROS generation, in the absence of calcium, have already been described: antimycin A inhibits the transfer of an electron from cytochrome *b* to ubiquinone at the Qi site, thus accumulating semiquinone at the Qo site, which favors $O_2^{\bullet-}$ generation.[28] However, our results with both probes showed that in mitochondria energized with glutamate/malate, antimycin A prevents calcium-stimulated ROS generation. Myxothiazol inhibits $O_2^{\bullet-}$ generation by preventing the transfer of an electron from ubiquinol to the Rieske iron–sulfur protein in the respiratory chain, thereby inhibiting semiquinone formation.[29] Its effect has been reported either alone[30] or in association[31] with antimycin A, which enhances ROS generation.

An explanation is that (1) myxothiazol-induced ROS originate from an electropositive site of complex III, and (2) an increase in $O_2^{\bullet-}$ production occurs from the accumulation of QH_2, resulting in auto-oxidation of ubiquinol with leakage of electrons to oxygen.[32] Since our study focused on the inhibitors' effects on calcium-stimulated ROS generation, these results suggest that in mitochondria energized with glutamate/malate, complex I, and ubiquinone cycle at complex III are the main sources of ROS, which are released both at the mitochondrial-matrix side and the extra-mitochondrial space. Moreover, the modulation of complex III at Qo and Qi produces opposite effects on calcium-stimulated ROS generation.

In succinate/rotenone-energized mitochondria both probes Amplex Red (Fig. 2B) and H_2DCFDA (Fig. 3B) showed a decrease in calcium-stimulated ROS generation. Since rotenone prevents the generation of ROS via backflow of electrons to complex I,[8] this is a probable site of calcium-stimulated ROS generation in succinate-energized mitochondria. On the other hand, the effects of complex III inhibitors myxothiazol and antimycin A in succinate-energized mitochondria, similar to the observations with glutamate/malate, were detected differently by Amplex Red (Fig. 2B) and H_2DCFDA (Fig. 3B). In fact, in succinate-energized mitochondria in the absence of calcium, complex III inhibitors lead to the entry of electrons through complex II, generating ROS at complex III.[32] In the presence of calcium, the increase of ROS generation induced by myxothiazol in succinate-energized mitochondria was significantly detected only by H_2DCFDA. This suggests that when the Qo site of complex III is inhibited, higher ROS levels are released at the mitochondrial-matrix side, but H_2O_2 is not generated/released at the extra-mitochondrial

space. Moreover, in succinate-energized mitochondria, the effect of antimycin A was also detected differently by Amplex Red and H_2DCFDA. The decrease of calcium-stimulated ROS generation promoted by antimycin A was only detected by Amplex Red (not by H_2DCFDA); this result suggests that blockage at the Qi site of complex III attenuates the calcium-stimulated generation of H_2O_2 at the extra-mitochondrial space. Therefore, in succinate-energized mitochondria both backflow of electrons to complex I and the ubiquinone cycle at complex III may be sources of calcium-stimulated ROS generation. Moreover, the complex III inhibition at Qo or Qi sites promotes distinct effects on ROS release concerning mitochondrial-matrix side or extra-mitochondrial space.

Mitochondria-generated ROS favor PTP-opening, which in turn generates more ROS.[33] In order to verify whether ROS release in calcium-overloaded mitochondria results from PTP opening, we assessed ROS in the presence of the classical PTP opening inhibitor CsA. H_2DCFDA detection showed that ROS release in the presence of calcium and/or myxothiazol either in glutamate/malate- (Fig. 4A) or succinate-energized mitochondria (Fig. 4B) was only weakly inhibited by CsA, indicating no significant contribution of PTP opening. In this context, we also examined whether ROS release in the presence of

calcium and/or myxothiazol favors calcium-induced PTP opening as evidenced by mitochondrial swelling, as well as a possible involvement of changes in mitochondrial membrane potential. As expected, calcium induced a slight swelling in either glutamate/malate- (Fig. 4C) or succinate-energized (Fig. 4D) mitochondria, both inhibited by CsA. The rate and extent of swelling were higher in succinate-energized mitochondria compared to glutamate/malate-energized organelles; in the former case, the rate was higher in the absence of rotenone (Fig. 4D). Antimycin A induced mitochondrial swelling regardless of the respiratory substrate, being larger with glutamate/malate, whereas myxothiazol only induced swelling in succinate-energized mitochondria. Under all conditions, swelling was significantly prevented by the antioxidant Trolox (50 µM, data not shown), suggesting that oxidative stress provided by the presence of calcium is required. Remarkably, the patterns of swelling and ROS generation in the presence of calcium and/or respiratory chain inhibitors were quite similar. Concerning mitochondrial membrane potential, antimycin A and myxothiazol promoted its disruption in calcium-overloaded mitochondria energized with either glutamate/malate (Fig. 4E), or succinate (Fig. 4F), apparent consequence of the respiratory chain inhibition. These results show that PTP
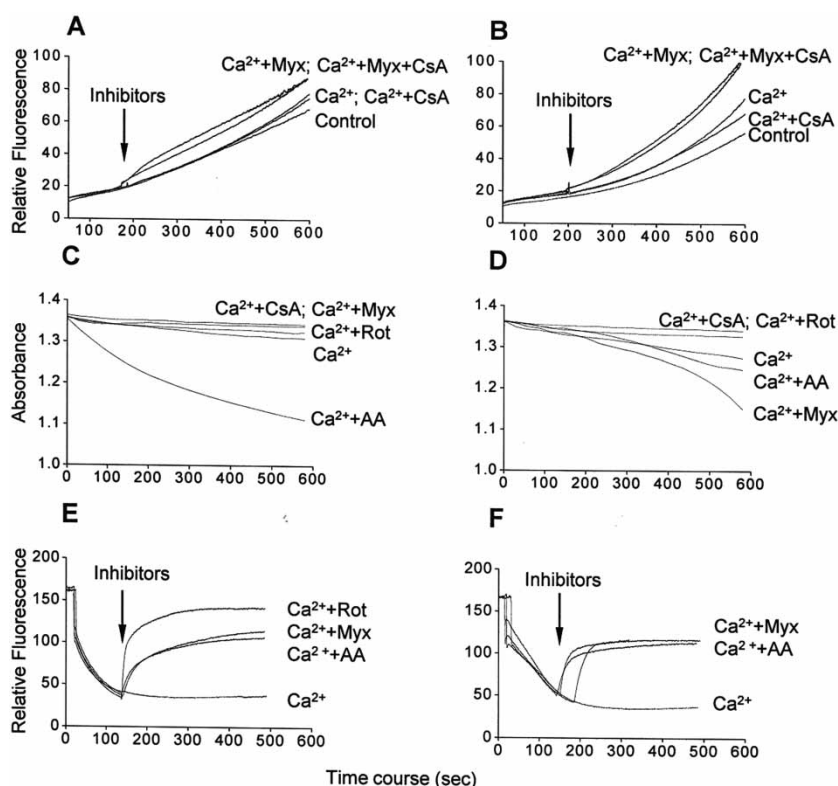

Figure 4   ROS generation (A and B), mitochondrial swelling (C and D), and membrane potential dissipation (E and F) in the presence of calcium/respiratory chain inhibitors in glutamate/malate-energized (A, C, and E) or succinate-energized (B, D, and F) mitochondria. Mitochondria were incubated in the standard medium with 100 µM calcium, as described in Fig. 1 and Materials and methods. Additions: only Ca²⁺ (Ca²⁺), 1 µM cyclosporine A (CsA), 2 µM rotenone (Rot), 1 µM antimycin A (AA), and 2 µM myxothiazol (Myx). Traces are representative of three independent preparations.

opening and mitochondrial membrane potential dissipation do not have any significant role on calcium-stimulated ROS generation and suggest a calcium action at the respiratory chain level.

Conclusion

Regardless of the respiratory substrate, calcium stimulates the mitochondrial generation of ROS, which are released at both the mitochondrial-matrix side and extra-mitochondrial space. In glutamate/malate-energized mitochondria, inhibition at complex I or complex III (ubiquinone cycle) similarly modulates ROS generation at the either the mitochondrial-matrix side or the extra-mitochondrial space; this also occurs when the backflow of electrons to complex I in succinate-energized mitochondria is inhibited. In succinate-energized mitochondria, the modulation of ROS generation at the mitochondrial-matrix side or extra-mitochondrial space relies on the site of complex III which is inhibited. These results allow a straight comparison between the effects of different respiratory substrates and electron transport chain inhibitors on ROS generation at either the mitochondrial-matrix side or extra-mitochondrial space in calcium-overloaded mitochondria.

Acknowledgements

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