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PAPER

Covalent labeling of mitochondria with a photostable fluorescent thiol-reactive rhodamine-based probe

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Fluorescent imaging of mitochondria is an essential tool for studies of mitochondrial functions. The staining of mitochondria with potential-indicating dyes, *e.g.*, rhodamine 123, readily vanishes upon loss of the transmembrane potential under certain conditions. 1-(Rhodamine B)-4-(2′-chloroacetyl)-piperazine amide (RB-CAP) was shown to be electrophoretically accumulated into mitochondria, forming covalent bioconjugates with intramitochondrial protein sulfhydryls which enabled the mitochondrial staining to endure in subsequent collapse of the transmembrane potentials. RB-CAP is highly photostable and exhibits stringent selectivity in covalent labeling of mitochondria in living cells. Being much less expensive, RB-CAP is a superior substituent for MitoTracker probes in functional studies of mitochondria.

Introduction

As highly dynamic intracellular organelles, mitochondria are critical for a large number of biological activities ranging from oxidative phosphorylation, iron homeostasis, generation of reactive oxygen species, to cell death, *etc.*[1] Functional mitochondria are hallmarked by the presence of electrochemical gradients of protons and the negative electrical potential differences across the mitochondrial membrane.[1d] Mitochondrial dysfunction is associated with multiple acute and chronic diseases such as diabetes and parkinson's diseases.[1c,d,2] Fluorescent staining of mitochondria is highly valuable for assessing mitochondrial functions and parameters in biomedical research.

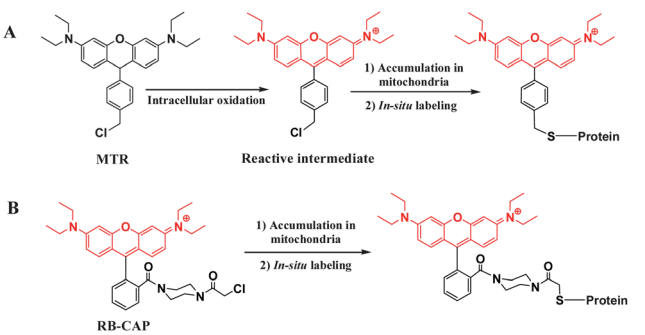
Lipophilic and cationic dyes, including rhodamine 123, JC-1 and TMRM, can be accumulated in mitochondria driven by the negative mitochondrial transmembrane potential. Albeit widely used for visualization of mitochondria in living cells, staining of mitochondria with the aforementioned dyes quickly disappears upon loss of the mitochondrial transmembrane potential which compromises their applications in many conditions, *e.g.*, mitochondrial depolarization.[3] To overcome this limitation, MitoTracker probes with a chloromethyl moiety have been developed. The reactive chemical handle can form covalent bioconjugates with intramitochondrial protein sulfhydryls once accumulated electrophoretically into mitochondria (Scheme 1A). The covalent linkage between MitoTrackers and proteins was shown to be able to prevent the loss of intramitochondrial fluorescence at dissipation of the membrane potentials,[4] which facilitates the studies of mitochondrial permeability transition, and depolarized mitochondria, *etc.*

Herein we report the selective and covalent labeling of mitochondria with 1-(rhodamine B)-4-(2′-chloroacetyl)-piperazine amide (RB-CAP) (Scheme 1B). RB-CAP is much less expensive than MitoTracker Red and yet exhibits superior labeling characteristics.

Experimental procedure

Materials and methods

MitoTracker Red (MTR) and Rhodamine 123 were purchased from Invitrogen. All other chemical reagents were obtained from Alfa Aesar. Column chromatography was performed on silica gel (300–400 mesh). NMR spectra (^{1}H at 400 MHz and ^{13}C at 100 MHz) were recorded on a Bruker instrument using



Scheme 1 Fluorescent labeling of intra-mitochondrial proteins with RB-CAP as compared to MitoTracker Red.

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tetramethyl silane as the internal reference. The mass analysis was performed in Bruker En Apex ultra 7.0T FT-MS. The fluorescence spectra and UV-Vis absorption spectra were performed on a spectrofluorimeter (Spectamax M5, Molecular Device) using the excitation wavelength (λ_{ex}) of 560 nm.

The truncated protein of Ras homolog enriched in brain (Rheb) was prepared following a published procedure.[8] The cytosolic asparaginase from *E. coli.* was prepared following literature.[9] Mammalian cells, Hela, L929, Raw 264.7 and MEF were obtained from American Type Culture Collection. Cells were analyzed using a fluorescence microscope (Ti–S; Nikon eclipse) equipped with a 100 W mercury lamp (C-SHG1, Nikon). Confocal fluorescence microscopic images were obtained on LeicaSP2 using the following filters: λ_{ex}@543 nm and λ_{em}@565–625 nm for RB-CAP; λ_{ex}@488 nm and λ_{em}@500–530 nm for rhodamine 123. The fluorescence of RB-CAP is shown in red while that of rhodamine 123 is shown in green (Fig. 2). The fluorescence of RB-CAP and that of rhodamine 123 inside cells was merged using Photoshop CS 3.0. Graph by GraphPad Prism5 software. Flow cytometry data were obtained on Beckman Coulter. The fluorescence emission of RB-CAP and MTR was recorded with filter FL1 (562–588 nm) while that of rhodamine 123 was recorded by filter FL2 (520–540 nm). 10 000 cells were analyzed and the data were analyzed with Origin 8.0.

Synthesis of RB-CAP

Hexachloroethane (5 g) was added in portions into a flask containing dichloromethane (100 ml), rhodamine B (5 g), triphenylphosphine (5 g), 1-boc-piperazine (2 g) and triethylamine (10 ml). The mixture was stirred at room temperature for 40 min and then concentrated by rotary evaporation. The residue was purified by column chromatography on silica gel using ethyl acetate as the eluent to afford crude solid (**1**) as a colored solid (4.5 g). The crude compound **1** (4.5 g) was dissolved in dichloromethane containing trifluoroacetic acid (80 ml, 50%, v/v). The solution was stirred at r.t. for 3 h and then concentrated to remove the solvent and trifluoroacetic acid. The residue was dissolved in dichloromethane (80 ml) containing TEA (10 ml) and 2-chloroacetic anhydride (2 g). The solution was extensively washed with saturated aqueous solution of sodium carbonate (200 ml). The organic layer was collected, dried over anhydrous sodium sulfate, and then concentrated. The residue was purified by silica gel column chromatography using a gradient elution from ethyl acetate to methanol to afford RB-CAP (3.0 g) in 70% yield. ^{1}H-NMR (400 MHz, CD3OD), δ: 7.80 (m, 2H), 7.73 (m, 1H), 7.54 (m, 1H), 7.30 (d, 2H, J = 9.48 Hz), 7.09 (dd, 2H, $J1$ = 9.48 Hz, $J2$ = 2.36 Hz), 6.99 (d, 2H, J = 2.24 Hz), 4.19 (s, 2H), 3.71 (q, 8H, J = 7.10 Hz), 3.43 (br, 8H), 1.33 (t, 12H, J = 7.10 Hz) HRMS ($C_{34}H_{40}ClN_4O_3^+$): calculated (MH^+): 587.28, found: 587.3.

Fluorescence spectra of RB-CAP

The fluorescence emission spectra of RB-CAP or rhodamine B (1 μg ml^{-1}) in PBS buffer (100 mM, pH 7.4) were recorded using with λ_{ex}@560 nm. The fluorescence excitation spectra were scanned by monitoring the fluorescence intensity using λ_{em}@580 nm.

Photobleaching of RB-CAP and rhodamine 123

Hela cells were grown on 24-well plate, then incubated with RB-CAP (1 μg ml^{-1}) or MTR (1 μg ml^{-1}) for 30 minutes at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Invitrogen) supplemented with 10% fetal bovine serum. The culturing media were removed and replaced with fresh medium. The two specimens were both exposed to constant laser illumination with a 100 W mercury lamp (C-SHG1, Nikon) and imaged at indicated time points (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 minutes) with a fluorescence microscope. The images were analyzed using Image J (1.43; NIH) software.

Co-staining of mitochondria with RB-CAP and rhodamine 123 in live cells

Mammalian cells, Hela, L929, Raw 264.7 and MEF were grown at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. Cells were seeded on 35 mm glass-bottom dishes (NEST) and incubated for 24 hours, followed by addition of Rhodamine 123 (1 μg ml^{-1}) and RB-CAP (1 μg ml^{-1}). The cells were further incubated for 30 minutes. The medium was removed and replaced with fresh medium. Cells were then analyzed with confocal fluorescence microscope.

Cytotoxicity of RB-CAP

The cytotoxicity of RB-CAP was evaluated on L929 cells. The cells were cultured with DMEM medium containing RB-CAP (0, 0.5, 1, 2, 4, 8 μg ml^{-1}) for various amounts of time (0–24 h) at 37 °C with 5% CO_2. The cells before and after incubation were stained with trypan blue. Cell number and cell viability were determined using the trypan blue exclusion test.

Staining of Hela with RB-CAP, rhodamine 123 and MTR

Hela cells were respectively incubated with RB-CAP (1 μg ml^{-1}), rhodamine 123 (1 μg ml^{-1}) or MTR (1 μg ml^{-1}) at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum for 45 minutes. A portion of the three specimen cells were washed with PBS buffer, fixed with formaldehyde and then washed with PBS to remove unlabeled reagent. The fluorescence intensities of the treated or untreated specimens were respectively analyzed by flow cytometry.

Labeling of proteins with RB-CAP

Chemical modifications were respectively performed in degassed Tris-HCl buffer (200 mM, pH 8.0) containing tris(2-carboxyethyl)phosphine (1 mM), RB-CAP (20 μg ml^{-1}) and the Rheb mutant or asparagenase (2.0 mg ml^{-1}). The reaction solutions were respectively incubated at 4 °C for 5 h with gentle mixing. Modified protein samples were analyzed by SDS-PAGE in parallel with protein markers. The resultant gels were first imaged on Tanon 2500R (Gel Image System), and then stained by Coomassie blue.

Influence of mitochondrial transmembrane potential on labeling of Hela with RB-CAP or MTR

Hela cells were first incubated at 37 °C under 5% CO_2 in DMEM supplemented with sodium azide (10 mM) and 10% fetal bovine serum. The cells were then respectively incubated with RB-CAP (1 μg ml^{-1}) or MTR (1 μg ml^{-1}) for 40 minutes. The cells were collected, washed with PBS buffer, and then analyzed using flow cytometry.

In a parallel control experiment, Hela cells were respectively incubated with RB-CAP (1 μg ml^{-1}) or MTR (1 μg ml^{-1}) for 40 minutes at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. The cells were collected, washed with PBS buffer, and then respectively analyzed with flow cytometry.

Results and discussion

Synthesis of RB-CAP

Given the high cost of MitoTracker probes (>$100 per mg), it is valuable to develop probes from rhodamine B (<$1 per g) that are much less expensive and yet still could stain mitochondria with high efficiency and selectivity. RB-CAP, the lipophilic and cationic dye bearing a chemically reactive chloroacetyl moiety, was synthesized from rhodamine B in bulky quantities (in grams scale) *via* a facile 3-step synthetic procedure (Scheme 2). Efficient amidation of rhodamine B with *tert*-butyl-*N*-piperazine was achieved to afford compound **1** in the presence of hexachloroethane and triphenylphosphine in dichloromethane containing triethylamine (TEA). Deprotection of the *tert*-butyl moiety of **1** with trifluorescetic acid yielded compound **2** in nearly quantitative yields. Amidation of **2** with 2-chloroacetic anhydride gave RB-CAP in an overall yield of 70%. Previously RB-CAP has been synthesized by trimethylaluminum ($AlMe_3$) promoted amidation of rhodamine B with piperazine.[5] In contrast to $AlMe_3$ which is highly reactive and flammable, the herein reported synthesis of RB-CAP was achieved under very mild conditions (Scheme 2).

Photophysical properties of RB-CAP

RB-CAP was first characterized for its photophysical properties. Fig. 1A shows that the optimal fluorescence excitation of RB-CAP is obtained at 560 nm while the maximal fluorescence emission intensity is located at 580 nm. The fluorescence emission and excitation spectra of RB-CAP is almost identical to that of rhodamine B. Photo-bleaching is a common problem for many organic dyes, often compromising the temporal monitoring of dynamic events inside cells. For example, the fluorescence of rhodamine 123 is highly susceptible to photobleaching.[6] MTR was later reported to be more photostable than rhodamine 123.[7] Hela cells pre-cultured with RB-CAP or MTR were respectively exposed to constant laser illumination, the intracellular fluorescence intensity was recorded over time by fluorescence microscopy. It was shown that the fluorescence of MTR quickly decayed upon laser illumination while RB-CAP exhibited relatively constant fluorescence emission, suggesting the superior photostability of RB-CAP over MTR inside cells (Fig. 1B).

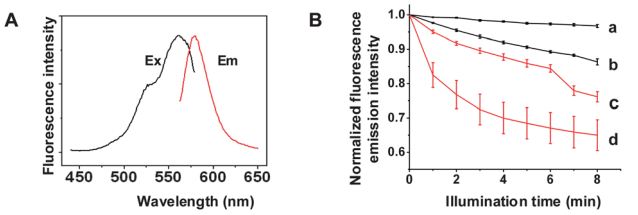
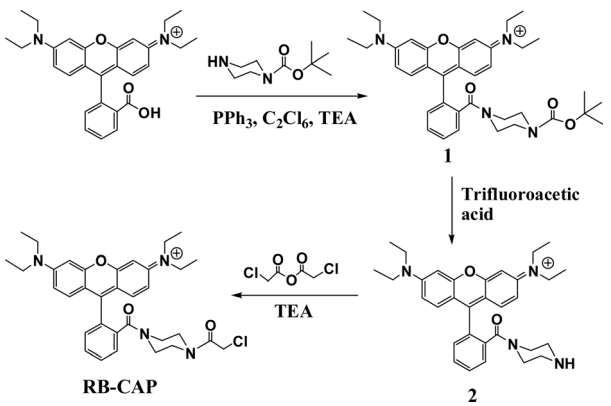


Fig. 1 Photophysical properties of RB-CAP. (A) Fluorescence excitation and emission spectra of RB-CAP in PBS buffer. The fluorescence emission spectrum was obtained using an excitation wavelength of 560 nm, and the fluorescence excitation spectrum was scanned using a fixed emission wavelength of 580 nm; (B) stability of the fluorescenec of RB-CAP in Hela cells. Cells preincubated with RB-CAP (1 μg ml^{-1}) remained in dark (a) or exposed to constant laser illumination with a 100 W mercury lamp for various periods of time as indicated (b), and then analyzed with a fluorescence microscope for the intracellular fluorescence intensities. As the control, cells precultured with MTR (1 μg ml^{-1}) were kept in dark (c) or exposed to constant laser illumination (d) and then analyzed with a fluorescence microscope for the intracellular fluorescence intensities as a function of time.

Mitochondrial staining with RB-CAP

To ascertain the specificity of mitochondrial staining with RB-CAP, cells of Hela, L929, mouse embryonic fibroblast (MEF) and mouse leukaemia monocyte macrophage (Raw 264.7) were respectively co-cultured with rhodamine 123 and RB-CAP for 30 minutes at 37 °C. The cells were harvested, washed with PBS, and then visualized by confocal fluorescence microscopy to probe the intracellular distribution of RB-CAP relative to rhodamine 123 which is an established fluorescent indicator for mitochondria.[3a] The double staining experiments revealed that RB-CAP stained mitochondria in all cell lines tested, exhibited patterns that were identical to that of rhodamine 123 (Fig. 2). The exact colocalization of RB-CAP signal with that of rhodamine 123 showed that RB-CAP could be selectively accumulated in mitochondria in various types of cells (Fig. 2). The cytotoxicity of RB-CAP was evaluated in L929 cells by trypan blue exclusion test. No toxic effects were observed on cell viability after incubation with RB-CAP for 24 h at doses up to 4 μg ml^{-1} (Fig. 3), which was 2 fold higher than the concentration used in mitochondria labeling.

Demonstrated to be selectively accumulated in mitochondria in live cells, RB-CAP was further evaluated for its capability to



Scheme 2 Synthesis of RB-CAP.

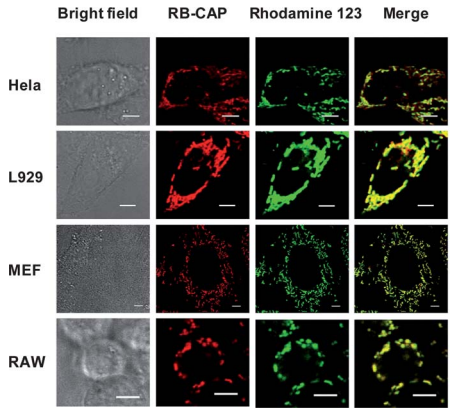


Fig. 2 Intracellular distribution of RB-CAP as compared to rhodamine 123. Hela, L929, MEF, and Raw cells were respectively co-cultured with RB-CAP (1 μg ml^{-1}) and rhodamine 123 (1 μg ml^{-1}) for 30 minutes. The cells were collected, washed with PBS buffer, and then analyzed with confocal fluorescence microscopy to pinpoint the locations of RB-CAP and rhodamine 123 inside cells. Merging of the fluorescence of RB-CAP (shown in red) and rhodamine 123 (in green) was shown in yellow. Bars, 5 μm.

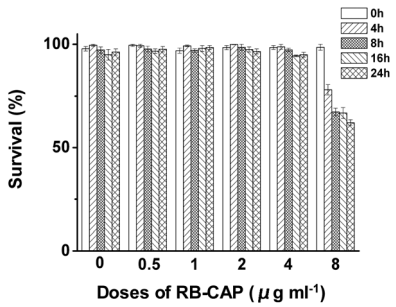


Fig. 3 Cytotoxicity of RB-CAP on L929 cells. Cells were incubated with various doses of RB-CAP (0–8 μg ml^{-1}) for 0–24 hours. The cell number and cell viability were determined by trypan blue exclusion test as a function of time.

form covalent bioconjugates with intra-mitochondrial proteins. Hela cells were respectively cultured with rhodamine 123, MitoTracker Red (MTR), or RB-CAP for 45 minutes at 37 °C. A portion of the cells were fixed with 3.7% formaldehyde and then washed twice with PBS buffer.[7] The treated cells were resuspended in PBS and then analyzed by flow cytometry (Fig. 4). It was shown that the intracellular rhodamine 123 significantly vanished after fixation indicating that mitochondrial membrane potential driven retention of rhodamine 123 was washed away under the fixing conditions. In contrast with rhodamine 123, MTR has been documented to form covalent linkage with protein sulfhydryls inside mitochondria which enabled the retention of the staining after loss of mitochondrial membrane potential.[4] It was shown that 70% of MTR remained in the fixed cells relative to the control cells, which confirmed the covalent labeling of the intramitochondrial proteins. As we expected, Fig. 4 shows that more than 80% of the intramitochondrial RB-CAP endured after fixation and washes, which indicates the formation of covalent conjugates between RB-CAP and intramitochondrial proteins.

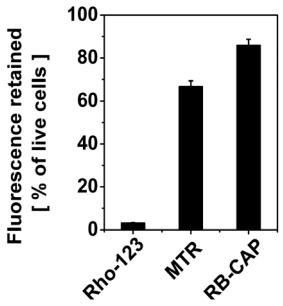


Fig. 4 Comparison of the retention rates of RB-CAP, rhodamine 123 and MTR in the mitochondria in Hela cells after fixation with formaldehyde and washing with acetone. The fluorescence intensities are the mean of three independent measurements; standard deviations are <5% of the respective mean values. All fluorescence intensities are normalized to the fluorescence of stained cells without fixation.

Labeling of cysteine-containing proteins with RB-CAP

To further probe the nature of *in situ* covalent labeling of intramitochondrial proteins with RB-CAP in L929 cells, asparaginase and Rheb protein were respectively modified with RB-CAP in borate buffer (200 mM, pH 8.0). The truncated Rheb contains no cysteine residues[8] while the cytoplasmic asparaginase from *E. coli.* contains one cysteine at position 273 which is solvent-exposed and accessible to alkylating reagents.[9] Analysis of the chemically modified proteins with SDS-PAGE showed that the fluorescence of RB-CAP colocalized with asparaginase (Fig. 5B, lane 5) while no significant fluorescence was observed in Rheb (Fig. 5B, lane 2). The differential labeling pattern of asparaginase relative to Rheb supported that RB-CAP was thiol specific, which suggested that the staining of intramitochondrial protein with RB-CAP was achieved *via* protein thiol alkylation in a manner similar to MTR.

Given the ubiquitousness of cysteine-containing proteins inside cells, the demonstrated of selectivity of RB-CAP in staining might be ascribed to the unique features of mitochondria. The mitochondrial transmembrane potential drives the

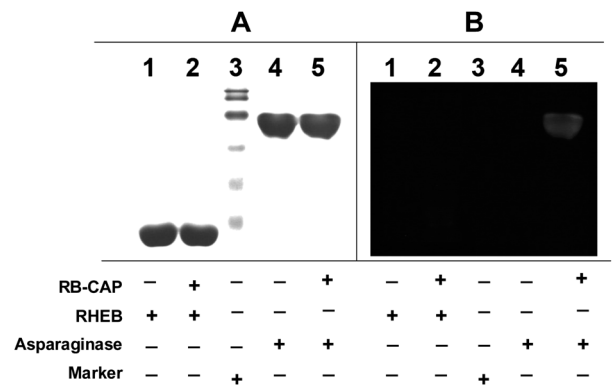


Fig. 5 SDS-PAGE analysis of the labeling of proteins with RB-CAP. Asparaginase and Rheb were respectively incubated with or without RB-CAP in borate buffer (200 mM, pH 8.0), and were then resolved by SDS-PAGE. The gel was first imaged by Tanon 2500R (Gel Image System) for fluorescence emission (B) and then stained with Coomassie-blue (A).

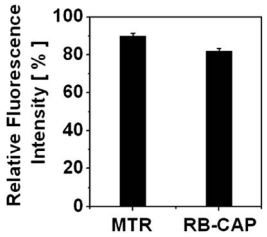


Fig. 6 Effects of sodium azide on the labeling efficiency of mitochondria in L929 cells. The percentage of fluorescence intensity retained in mitochondria of L929 cells that have been pretreated with sodium azide were quantitated by flow cytometry using L929 cells that have not been treated with sodium azide as the control as compared to untreated cells. Results are the mean of three independent experiments; standard deviations are <5% of the respective mean values.

accumulation of RB-CAP into mitochondria from cytosol, which effectively increases the labeling of extramitochondrial proteins. In addition, the intramitocondrial pH is much more alkaline (pH 8.2) than cytosolic pH (pH 7.2) due to the presence of transmembrane potential and proton gradient,[10] which increases the nucleophilicity of the intramitochondrial sulfhydryl group (pK_a 8–9) and thus further enhances the selectivity of mitochondrial labeling.

Influence of mitochondrial membrane potential on labeling with RB-CAP

The influence of mitochondrial membrane potential on the staining efficiency of RB-CAP was further investigated. Sodium azide inhibits mitochondrial electron transportation, and thus could affect the mitochondrial transmembrane potential.[11] Hela cells pre-incubated with or without sodium azide (10 mM) were respectively stained with RB-CAP or MTR. The cells were collected and analyzed by flow cytometry. Fig. 6 shows that the staining efficiency of cells pretreated with sodium azide with RB-CAP or MTR both decreased to roughly 80–90% that of the control cells. This indicates that interference of mitochondrial transmembrane potential could affect the mitochondrial staining with RB-CAP which is in good consistency with the reported behavior of MTR (Fig. 4 and 6).[7]

Conclusions

RB-CAP was shown to be able to selectively accumulate into mitochondria and form stable and covalent linkages with intramitochondrial protein, which leads to retention of dyes in mitochondria upon subsequent loss of mitochondrial transmembrane potential. RB-CAP can be readily prepared through a 3-step procedure under mild conditions. Being much less expensive, RB-CAP exhibits superior photostability relative to MTR and high selectivity in covalent staining of mitochondria. The aforementioned advantageous properties of RB-CAP suggest its broad utility in fluorescent tracking of mitochondria parameters or functions in living cells under certain conditions, *e.g.*, mitochondrial depolarization.

Acknowledgements

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