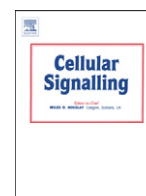


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Removal or masking of phosphatidylinositol(4,5)bisphosphate from the outer mitochondrial membrane causes mitochondrial fragmentation

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ABSTRACT

Mitochondria are central players in programmed cell death and autophagy. While phosphoinositides are well established regulators of membrane traffic, cellular signalling and the destiny of certain organelles, their presence and role for mitochondria remain elusive. In this study we show that removal of PtdIns(4,5)P₂ by phosphatases or masking the lipid with PH domains leads to fission of mitochondria and increased autophagy. Induction of general autophagy by amino acid starvation also coincides with the loss of mitochondrial PtdIns(4,5)P₂, suggesting an important role for this lipid in the processes that govern mitophagy. Our findings reveal that PKC α can rescue the removal or masking of PtdIns(4,5)P₂, indicating that the inositol lipid is upstream of PKC.

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1. Introduction

Mitochondria are not just the powerhouse of the cell, but have also an essential regulatory input into the cell's destiny. Apoptosis, the programmed cell death, involves an increased permeability of the outer mitochondrial membrane and subsequent loss of cytochrome c that in turn will trigger the caspase cascades leading to the cell's suicide [1]. Damaged organelles such as mitochondria can be consumed by autophagy, which can precede apoptosis [2]. Cytosolic components, including mitochondria, are sequestered and enclosed by a double membrane structure of unknown origin, called phagophore, which when closed becomes an autophagosome [3]. Both apoptosis and autophagy of mitochondria (mitophagy) have been indicated in the development of cancer as well as neurodegenerative diseases and have been therefore subject to many investigations [1].

Mitochondria, like the nucleus, are separated from the cytosol by a double membrane system. Most lipids present in other compartments such as phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol (PtdIns) are also found in mitochondria [4]. While there is a wealth of information concerning these lipids and their role in mitochondrial function, much less is known about inositol lipids with respect to this organelle. From

proteomic and lipidomic analyses it is evident that the mitochondria contain PtdIns [4] and the higher phosphorylated phosphoinositides [5] as well as their metabolising enzymes such as kinases, phosphatases and phospholipases [6]. Thus, the mitochondria seem to have all the components to make or remove phosphoinositides such as PtdIns(4,5)P₂, yet nobody investigated whether PtdIns(4,5)P₂ on the outer mitochondrial membrane has a role in mitochondrial functioning, integrity and destiny. In order to elucidate the potential role of this particular lipid on the outer mitochondrial membrane, we employed specific phosphatases and binding proteins to either remove or mask the PtdIns(4,5)P₂. Our data indicate that PtdIns(4,5)P₂ plays a crucial part in determining the destiny of mitochondria, bringing in a novel dimension towards our understanding of mitochondrial destiny and integrity.

2. Results

2.1. Loss of PtdIns(4,5)P₂ on outer mitochondrial membrane leads to fragmentation of mitochondria

In order to elucidate the role of PtdIns(4,5)P₂ on the mitochondria we first constructed a plasmid with an outer mitochondrial localisation sequence that would target any protein exclusively to the mitochondria. Tom20 is a major receptor of the mitochondrial pre-protein translocation system and is bound to the outer mitochondrial membrane (OMM) through the NH₂-terminal transmembrane domain (TMD). It has been demonstrated that this TMD together with positive charges at its proximal COOH-terminal flanking region targets GFP exclusively to the OMM *in vivo* [7]. We made use of this approach to target the PtdIns(4,5)P₂ specific 5-phosphatase Skip [8] to

Abbreviations: PtdIns(4,5)P₂, phosphatidylinositol(4,5)bisphosphate; PtdIns, phosphatidylinositol; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; PLC, phospholipase C; PMA, 12-O-tetradecanoylphorbol 13-acetate; PKC, protein kinase C; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; n.d., not determined.

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the OMM (pOMML-Skip). Upon expression of pOMML (mock) in three different cell lines (MCF-7, HepG2 and NIH3T3) a green fluorescent mitochondrial network became visible (Fig. 1; EGFP), which is similar to what has been described before as a “normal” mitochondrial network in these types of cells [9,10]. Single mitochondria are elongated and occupy an average area of $1.5 \mu\text{m}^2$. The cells are viable and divide at a normal rate. However, expression of pOMML-Skip leads to a severe phenotype in all three cell lines. 24 h after transfection the mitochondria appear fragmented with a maximum cross-section area of $0.6 \mu\text{m}^2$ (Fig. 1; Skip) and after 48 h only few fluorescent mitochondria are visible and stably expressing cell lines cannot be established (as opposed to mock transfected cells). To test, whether this severe loss of Skip targeted mitochondria occurs specifically due to the lack of PtdIns(4,5)P₂ and/or is due to the accumulation of PtdIns(4)P, we aimed to render PtdIns(4,5)P₂ inaccessible without removing it. Therefore we overexpressed a Pleckstrin Homology (PH) domain that specifically binds PtdIns(4,5)P₂. This approach should trap PtdIns(4,5)P₂ and thus make it unavailable as a signalling molecule without changing the membrane composition. The PH domain of PLC δ 1(PLC δ PHd) is well known for its high affinity to PtdIns(4,5)P₂ [11] and can be used to mask available PtdIns(4,5)P₂ when overexpressed in cells [12]. When targeted to the mitochondria (pOMML-PLC δ PHd) transfected cells display punctiform, small, fragmented fluorescent mitochondria and a complete loss of the mitochondrial network (Fig. 1; PLC δ PHd). After 48 h only few fluorescent mitochondria remain and the cells are eventually detach (data not shown). Furthermore, expression of a mutated PH domain, that was shown not to bind the InsP₃ headgroup anymore (pOMML-R37D) [13], does not produce such a phenotype and the mitochondrial network looks similar to mock transfected cells (Fig. 1; PLC δ PHd-R37D). Interestingly, targeting of the PLC δ PHd to the inner mitochondrial membrane with the commercially available pMito Vector does not interfere much with mitochondrial integrity (Fig. 1; pMitoPLC δ PHd) and did not cause the severe loss of cells observed with OMM targeting (data not shown). The effects have been most pronounced in the MCF 7 cells, which were subsequently employed to quantify the amount and size of mitochondria with or without PtdIns(4,5)P₂ capture or removal. As shown in Fig. 2, introducing the PtdIns(4,5)P₂ 5-phosphatase SKIP or the PtdIns(4,5)P₂ capturing PH domain at the OMM drastically and significantly reduced the amount of mitochondria per cell, whereas the IMM localisation of these tools had no significant effect (Student’s t-test). The reduction in mitochondrial numbers per cell was accompanied with

an equally drastic and significant reduction in the size of the mitochondria, an effect that became more severe with time.

Taken together, these data (i) confirm the importance of the OMM localisation of PtdIns(4,5)P₂, (ii) validate the distinct localisation of the two mitochondrial targeting vectors employed here, (iii) demonstrate that PtdIns(4,5)P₂ removal (Skip) and masking (PH domain) are equally effective and finally (iv) imply an important role for PtdIns(4,5)P₂ on the outer mitochondrial membrane in maintaining a healthy mitochondrial network.

2.2. Fragmentation of PtdIns(4,5)P₂ deprived mitochondria does not involve apoptotic cell death

As mitochondria are at the centre of programmed cell death control, we investigated whether the fragmentation and elimination of targeted mitochondria were caused by apoptosis. The release of cytochrome c from the mitochondrial intermembrane space is known to be a pro-apoptotic event and thus cytochrome c release from the mitochondria is widely used as a marker for the loss of membrane integrity due to apoptosis [14–19]. In order to investigate this cells (MCF7) were depleted of their OMM PtdIns(4,5)P₂ by targeting the PLC δ PH domain to this compartment, fixed and then tested for cytochrome c release by immunofluorescence with an anti-cytochrome c antibody. This approach reveals that the mitochondrial membrane is intact in both, mock (empty vector) and pOMML-PLC δ PHd transfected cells, as cytochrome c is localised to the mitochondrial interspace (Fig. 3 inset), appearing in clearly defined spots of mitochondrial shape (Fig. 3 in red), but clearly absent in the cytosol. For comparison, cells were treated with Ionomycin to induce apoptosis (Fig. 3; 18 h/Ionomycin), which indeed resulted in a diffuse cytosolic cytochrome c staining. In contrast, mock transfected cells were devoid of cytosolic cytochrome c staining, with all cytochrome c “spots” being surrounded by green fluorescence from the mitochondrial targeted EGFP (Fig. 3 inset). Interestingly, upon targeting of green fluorescent PLC δ PHd to the mitochondria, a significant number of red-labelled intact mitochondria, which do not display any green fluorescence, appear (Fig. 3; 24 h/PLC δ PHd) and over time seem to prevail (Fig. 3; 48 h/PLC δ PHd). However, these cells will eventually die, implying that the appearance of “wildtype” mitochondria is not sufficient to overcome the process initiated by the removal of PtdIns(4,5)P₂. Nevertheless, due to the absence of cytochrome c in the cytosol, it is clear that PtdIns(4,5)P₂ deprived mitochondria fragment without losing their outer membrane

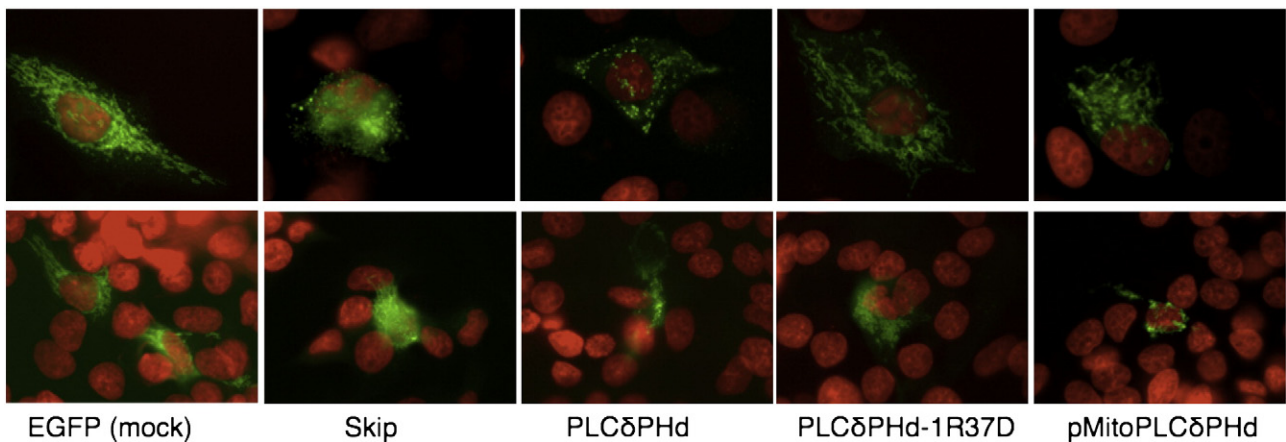


Fig. 1. Removal of PtdIns(4,5)P₂ at the OMM causes fragmentation in mitochondria. MCF-7 cells (top) and HepG2 cells (bottom) were fixed and immunostained 24 h after transfection of the indicated constructs. Cells expressing pOMML-EGFP (mock), pOMML-PLC δ PHd1R37D or pMito-PLC δ PHd display a healthy, tubular mitochondrial network (green). Transfection of pOMML-Skip or pOMML-PLC δ PHd causes fragmentation of mitochondria. The nuclei were counterstained with DAPI (false colour red). Images are representative of at least 3 independent experiments. NIH fibroblasts showed similar responses to the expression of these constructs (not shown).

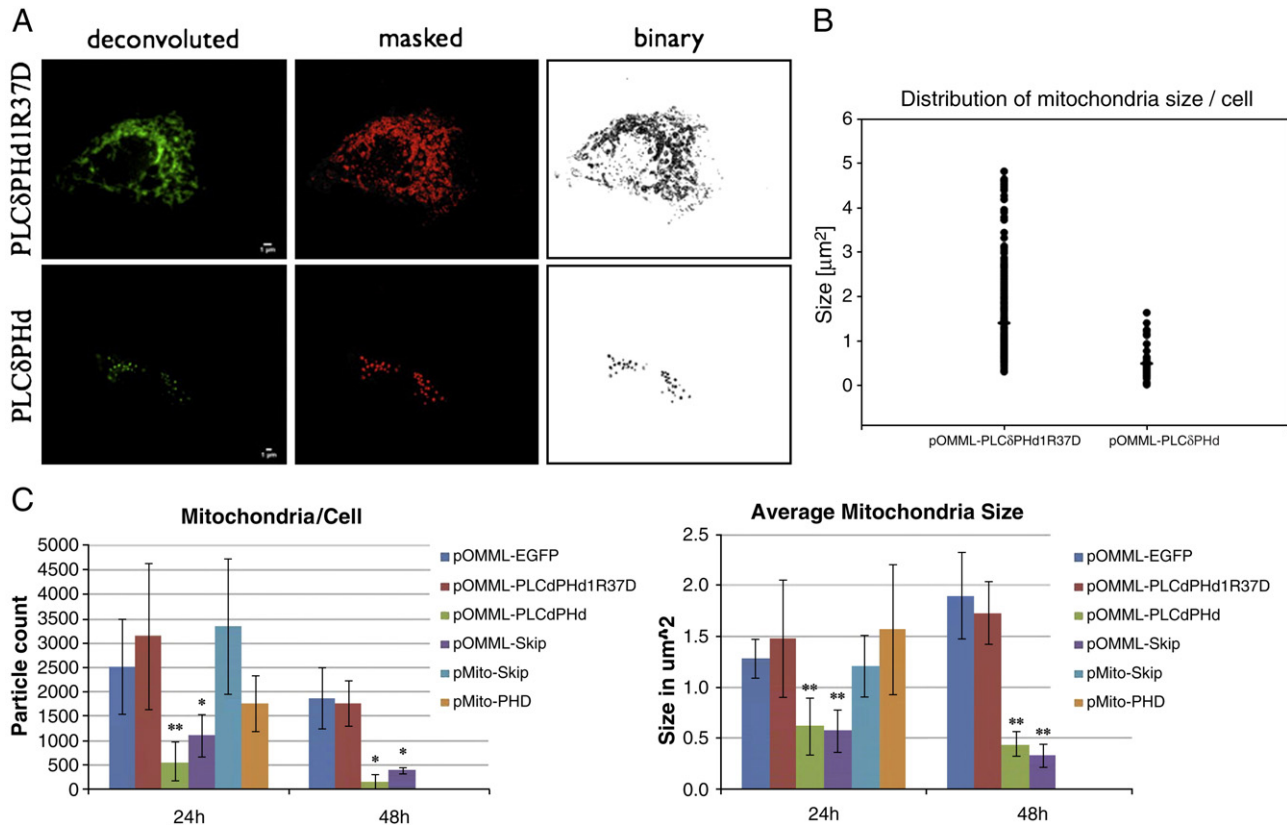


Fig. 2. Measurement of mitochondria size and count. Cells were transiently transfected with specific mitochondrial targeted domains as described in [Material and methods](#). Deconvoluted fluorescence microscopy images of mitochondria were masked and converted into a binary image. Particle size and count were measured using ImageJ software (public domain, NIH Bethesda, Maryland, USA). **A:** An example of masks (middle) overlaying the mitochondria and binary image (right) of a control pOMML-PLCδPHd1R37D (top row) and pOMML-PLCδPHd (bottom row) over-expressing human MCF-7 cell is shown. **B:** Particle size distribution of the shown example cells from (A). The horizontal mark denotes the mean of all sizes (average size). **C:** Statistical analysis of mitochondria particle size and count measurements in randomly chosen fluorescence positive cells MCF-7 cells. Number of experiments ($N=4-6$); data are presented as means \pm SD. Statistical significance of difference to the control (pOMML-EGFP) was assessed using Student's t-test (* denotes $p < 0.05$ and ** denotes $p < 0.01$). 48 h measurements with pMito constructs n.d.

integrity, the latter being a prerequisite for apoptotic cell death. Thus, we conclude that PtdIns(4,5) P_2 depletion induced fragmenting of mitochondria is not due to a loss of integrity associated with apoptosis.

2.3. Removal of mitochondrial PtdIns(4,5) P_2 triggers autophagy

The exclusion of apoptosis prompted us to investigate whether autophagic processes, which are characterised by digestion of

mitochondria in acidic lysosomal compartments [20], are involved in the PtdIns(4,5) P_2 dependent mitochondrial fragmentation. To test whether the degradation of mitochondria observed after PtdIns(4,5) P_2 removal through pOMML-Skip or masking through pOMML-PLCδPHd is caused by autophagy, acidic compartments were stained with lysotracker red (LTR), a well established marker for the lysosomal compartment [21–23], and examined for co-localisation with mitochondria in HepG2 cells, a cell line that has been used successfully

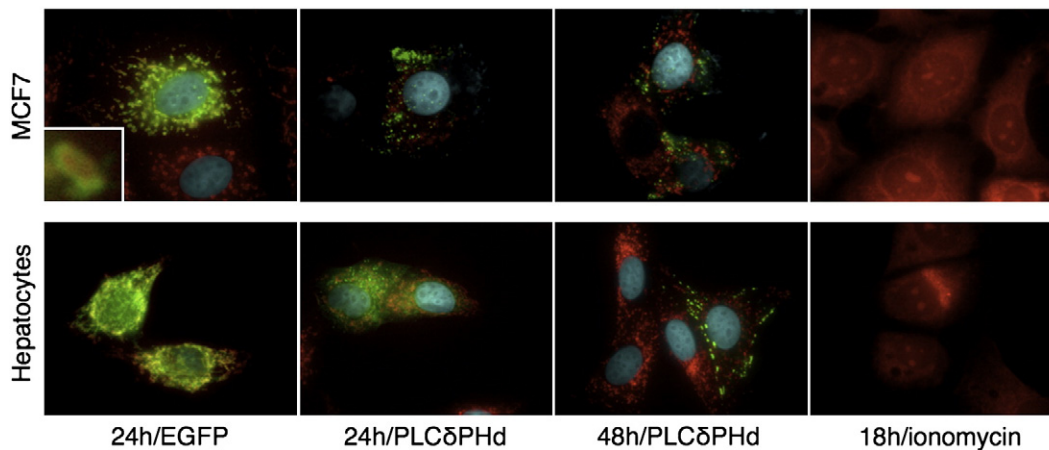


Fig. 3. PtdIns(4,5) P_2 depletion at the OMM is not causing cytochrome c release. MCF-7 cells were fixed and immunostained with Alexa Fluor 594 (red) labelled anti-cytochrome c at the indicated times after transfection of pOMML-EGFP (green) or pOMML-PLCδPHd (green). Counterstained with DAPI (blue) and examined by fluorescence microscopy. Cytochrome c is localised to the mitochondrial interspace, surrounded by mitochondria outer membrane targeted green fluorescence (inset). For comparison, cells were treated overnight with 100 μM Ionomycin before fixation followed by cytochrome c immunostaining (red).

to induce autophagy via glucagon treatment coupled with starvation [24–26]. We found that mitochondrial fragmentation induced by the expression of pOMML-Skip results in co-staining of mitochondria with acidic compartments, which is enhanced upon amino acid starvation and glucagon stimulation (Fig. 4A). The same effect was seen in pOMML-PLC δ PHd transfected cells (data not shown) confirming the notion that removal of targeted mitochondria takes place in acidic lysosomal compartments. The presence of LC3 in autophagosomes (puncta formation) has been used as indicator of autophagy [27]. Immunofluorescence of LC3 with an antibody revealed red puncta formation and co-staining with fragmented mitochondria in pOMML-Skip expressing cells (Fig. 4B; Skip) indicating the formation of autophagosomes induced by removal of PtdIns(4,5)P₂. Puncta did not appear in mock transfected cells (Fig. 4B; EGFP).

3-Methyladenine (3-MA) has been used to inhibit autophagy [28]. It is a class III PI3-kinase inhibitor that has been used to block the formation of the autophagosome, a process that seems to depend on the presence of PtdIns(3)P [29]. Fig. 4C reveals that 3-MA treatment does not inhibit the fragmentation of mitochondria in pOMML-PLC δ PHd transfected cells. Similar effects were observed in pOMML-Skip treated cells, whereas in mock transfected cells 3-MA did not influence the amount of mitochondria (data not shown). As 3-MA inhibits the formation of the autophagosomes, we conclude that fragmentation of mitochondria caused by PtdIns(4,5)P₂ loss precedes autophagy, rather than it being an effect of lysosomal degradation of mitochondria [30].

2.4. Mitochondrial PtdIns(4,5)P₂ loss in glucagon induced autophagy

The data suggest that PtdIns(4,5)P₂ loss is causing fragmentation, which is then followed by a process consistent with autophagy. However, it remained to be determined whether the induction of autophagy under physiologic conditions would also exhibit a loss of PtdIns(4,5)P₂ on the mitochondrial membrane. It is well established that autophagy can be induced in HepG2 cells by amino acid starvation and glucagon induction [24–26,31]. Thus, it would be possible to exploit these conditions for HepG2 cells to correlate PtdIns(4,5)P₂ on the OMM with autophagy. In order to obtain the lipid levels on the OMM, isolated mitochondria were probed with a recombinant GST-tagged PLC δ PHd, a lipid recognition domain that is frequently employed to detect specifically PtdIns(4,5)P₂ in overlay assays and microscopy [8,32–35]. Here, we utilize the recombinant GST-tagged PLC δ PHd in a pull-down with isolated mitochondria from hepatocytes, where the amount of captured recombinant PLC δ PHd should be a direct indicator of the PtdIns(4,5)P₂ levels on the OMM. Importantly, our data (Fig. 5C and D) validate the usefulness of this approach, since stripping off the phosphates of the inositol lipids by the specific inositol phosphatase Synaptojanin [36,37] abolished the pull-down of the recombinant PLC δ PHd domain. This method could therefore specifically monitor organelle PtdIns(4,5)P₂ levels and was subsequently employed to evaluate the PtdIns(4,5)P₂ levels on the OMM of isolated mitochondria from HepG2 cells that had been amino acid starved and glucagon treated in order to induce autophagy. For comparison, isolated mitochondria from cells that were kept in

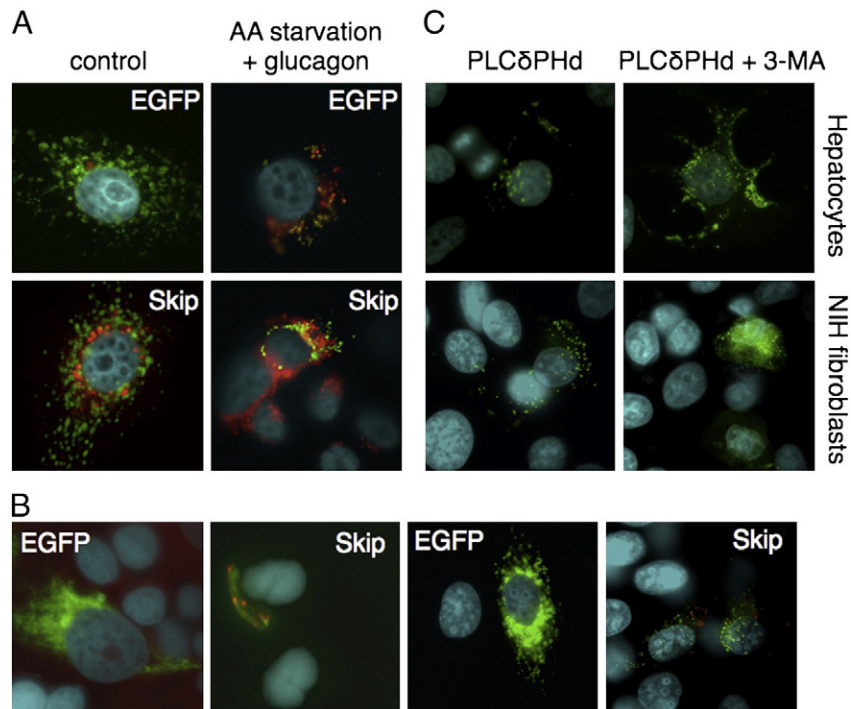


Fig. 4. Removal of mitochondrial PtdIns(4,5)P₂ leads to fragmentation and mitophagy. **A:** 24 h after transfection, HepG2 cells were amino acid starved and glucagon induced for 1 h (right panel), while control cells (left panel) were kept in normal growth medium. HepG2 cells were then treated with lysotracker red (LTR), washed, fixed and counterstained with DAPI (cyan). Acidic compartments (red) that co-localise with mitochondria (green) appear yellow. Mock transfected cells (upper panel) have only a few acidic compartments, but mitochondria do not localise with them. Upon induction of autophagy through amino acid starvation (right panel) mitochondria become fragmented and co-localise with lysosomes. pOMML-Skip transfected cells (lower panel) have fragmented mitochondria with and without starvation and co-localise with acidic compartments. The phenotype is enhanced upon amino acid starvation and glucagon stimulation. **B:** Immunofluorescence of LC3. 16 h after transfection HepG2 cells were fixed and immunostained for the autophagy marker LC3 (red) and DAPI counterstained (cyan). Punctuate staining of LC3 indicates sites of autophagosome formation which partly co-localise with fragmented mitochondria in pOMML-Skip transfected cells (Skip). Puncta formation is absent in pOMML-EGFP transfected cells (EGFP). Images from two independent experiments are shown (left and right, resp.). **C:** HepG2 and NIH cells were transfected with pOMML-PLC δ PHd (green). After 12 h growth medium was supplemented with the autophagy inhibitor 3-MA or solvent control. After another 12 h cells were fixed, DAPI (cyan) counterstained and observed under the microscope. Fragmented mitochondria accumulate in 3-MA treated pOMML-PLC δ PHd (right) transfected cells as compared to untreated pOMML-PLC δ PHd transfected cells (left).

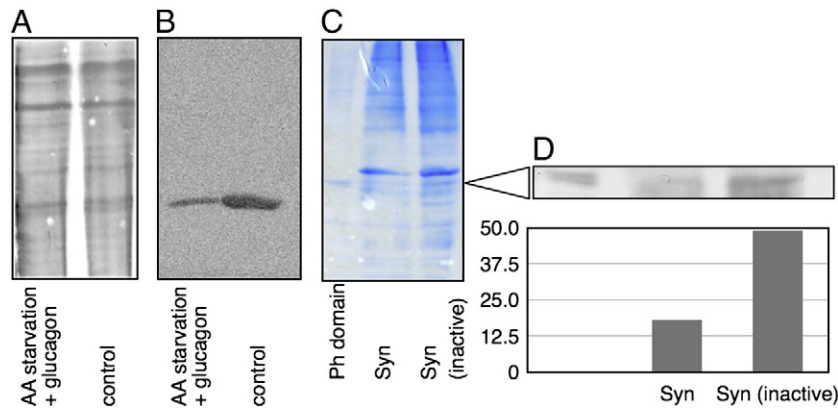


Fig. 5. Mitochondrial PtdIns(4,5)P₂ loss in amino acid starved/glucagon treated HepG2 cells. Mitochondria from +/- autophagy (AA starvation + glucagon) induced HepG2 cells were probed with recombinant GST-PLCδPHd that specifically binds PtdIns(4,5)P₂. A: After stringency washes, lysates were subjected to SDS-PAGE and the gel was Coomassie stained. B: Western blot analysis of the same lysates with anti-GST antibody confirms that mitochondria isolated from cells in full medium (control) were recovered with more GST-PLCδPHd bound, indicating a loss of PtdIns(4,5)P₂ during physiologic autophagy (42.45 +/- 2.85% of control PIP₂ levels; mean and standard error for 2 independent experiments). C: To validate that PtdIns(4,5)P₂ can be detected with a recombinant GST-tagged PLCδPHd isolated mitochondria were stripped (30 min) of their PtdIns(4,5)P₂ with a recombinant catalytic subunit of Synaptojanin 1 (a PtdIns(4,5)P₂ 5-phosphatase). For comparison the corresponding inactive enzyme, was employed. GST-tagged recombinant PLCδPHd was then added to both washed samples and the mitochondria were washed again and sedimented. The recovered mitochondrial pellet was lysed and subjected to SDS-PAGE (Coomassie stain). The triangle indicates the position of the PLCδPHd. The first lane reveals the purity of the PLCδPHd preparation. D: The same lysates were also subjected to Western blotting. Blots were probed with anti-GST antibodies for the amount of mitochondrial-bound PLCδPHd, which in turn correlates with the amount of PtdIns(4,5)P₂ present on the OMM. Indeed, less PLCδPHd was detected in the sample treated with active Synaptojanin, indicating that PtdIns(4,5)P₂ is present on the mitochondrial membrane, readily accessible for PtdIns(4,5)P₂ metabolising enzymes. Importantly, these data indicate that the PLCδPHd can be used as a tool to specifically detect PI(4,5)P₂ on the mitochondrial membrane.

normal growth medium were used. The pull-down experiments reveal a substantial loss of PtdIns(4,5)P₂ levels in mitochondria undergoing amino acid starvation (Fig. 5A and B), which implies that PtdIns(4,5)P₂ levels in the OMM are an important element in the signalling controlling autophagy.

2.5. PKCα inhibition mimics the phenotype caused by mitochondrial PtdIns(4,5)P₂ removal

We have shown that mitochondrial PtdIns(4,5)P₂ levels are reduced during amino acid starvation induced autophagy and thus have established its presence in the signalling leading to autophagy. It is clear that the loss of PtdIns(4,5)P₂ caused by the phosphatases could impair other PtdIns(4,5)P₂ metabolising enzymes such as the PI-PLC or the PtdIns(4,5)P₂ dependent PLD. In order to elucidate this, we focused on chemical inhibitors that are known to interfere with PtdIns(4,5)P₂ dependent pathways as well as being able to cause mitochondrial phenotypes. Cells were treated with a series of compounds acting as Ca²⁺ ionophore, Ca²⁺ chelator, PC-PLD inhibitor, PC-PLC and cPLA(2) inhibitor, PI-PLC inhibitor, PI3-kinase inhibitor or PKC inhibitors (data not shown) and examined for mitochondrial size and shape changes using fluorescence microscopy imaging. It became apparent that calcium and/or PKC may play a role, which prompted a further investigation of this matter. Fig. 6A shows that long-term incubation of MCF-7 cells with the phorbol ester PMA (12-O-tetradecanoylphorbol 13-acetate), which is known to cause downregulation of PKCα, δ and ε [38,39], leads to fragmentation of mitochondria. Mitochondria in control cells showed an average fragment size of 2.3 μm², whereas in PMA treated or pOMML-PLCδPHd expressing cells showed an average fragment size of 0.4 μm². Similar or synergistic effects were seen with Bapta AM, a Ca²⁺ chelator (data not shown). As PKCε is not Ca²⁺ dependent and Rottlerin, a PKCδ-inhibitor, did not cause such a phenotype (data not shown), we hypothesised that the sensing of PtdIns(4,5)P₂ deprivation on the mitochondrial membrane is PKCα mediated.

In order to test this hypothesis, we examined mitochondrial lysates by Western blotting with an antibody that recognises specifically the motif that conventional PKCs (cPKC) phosphorylate [40]. This phospho-(serine) PKC substrate antibody has been shown to detect endogenous levels of known and unknown proteins, when

phosphorylated at serine residues surrounded by arginine or lysine at the -2 and +2 positions and a hydrophobic residue at the +1 position [41]. We used mitochondrial lysates from MCF-7 cells that had been transfected with pOMML-PLCδPHd (70% of cells were GFP positive as judged by fluorescence microscopy), pOMML-EGFP control cells, chelerythrine chloride (a selective inhibitor of group A and B PKC isoforms) [42] treated cells and amino acid starved cells. Fig. 6B shows that PKC serine substrates are less phosphorylated in mitochondria from autophagy induced cells (pOMML-PLCδPHd transfectants and amino acid starved cells) and chelerythrine chloride-treated cells. The most striking difference is a phosphorylated 35 kDa substrate, which can only be detected in control mitochondrial lysates and is completely absent from autophagy induced cells. Notably, despite loading of equal amounts of total protein, mitochondria from chelerythrine chloride-treated cells seem to be driven towards apoptosis, as less cytochrome c, is present in mitochondrial lysates.

Taken together, PtdIns(4,5)P₂ removal reduces PKC activity on mitochondria. It is evident that loss in cPKC substrate phosphorylation is a common hallmark of PtdIns(4,5)P₂ removal and starvation induced autophagy. The sensitivity of this process to calcium and PKC modulating agents as well as the PKC isozyme specific inhibition by chelerythrine chloride points to PKCα as the main downstream target.

2.6. Co-expression of mitochondrial targeted PKCα and PtdIns(4,5)P₂ phosphatase Skip rescues from mitophagy

If PKCα is the downstream signal of reduced PtdIns(4,5)P₂ levels in cells undergoing autophagy, the overexpression of PKCα at the site of PtdIns(4,5)P₂ deprivation may rescue such a phenotype. We therefore constructed a DsRed tagged full-length PKCα with the same localisation signal as for the PtdIns(4,5)P₂ modulation tools used earlier and transfected it at the same time as the EGFP tagged pOMML-Skip. In cells that were co-expressing both constructs we found that pOMML-DsRed-PKCα inhibits fragmentation of mitochondria and the cells survive (Fig. 6C). pOMML-DsRed mock vector does not change the phenotype caused by pOMML-EGFP-Skip. These data confirm that PKCα is an effector of PtdIns(4,5)P₂ dependent mitophagy and that its mitochondrial overexpression rescues mitochondria from fragmentation and consequently from elimination by autophagy.

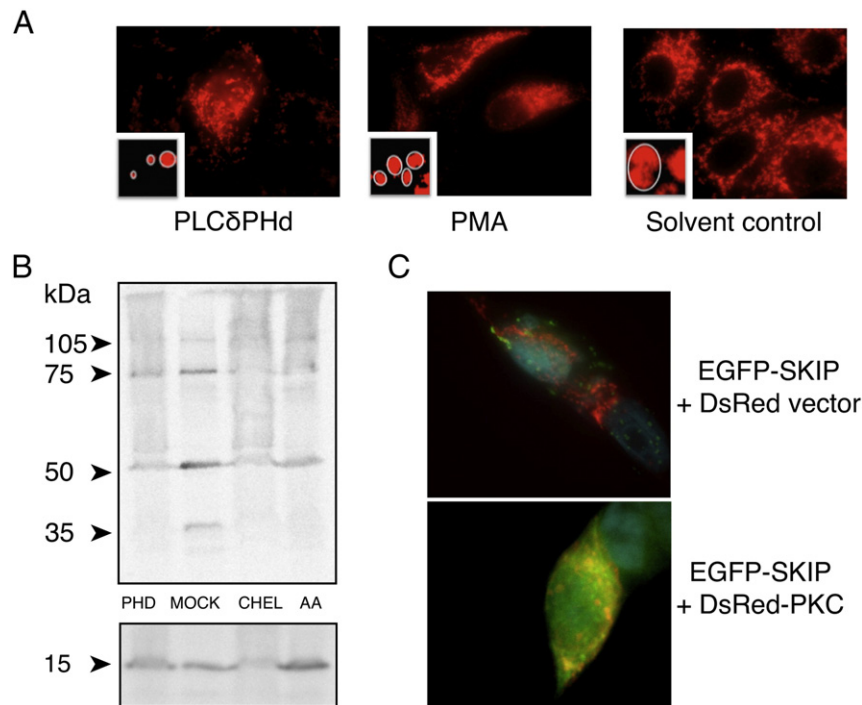


Fig. 6. Mitophagy induced by masking PtdIns(4,5)P₂ can be rescued by PKC. **A:** Overexpression of the PtdIns(4,5)P₂ binding domain PLCδPHd on the outer mitochondrial membrane results in fragmentation of mitochondria soon after expression and leads to mitophagy as outlined above (left). PMA long-term treatment also results in mitochondrial fragmentation (middle), whereas treatment with solvent control has no effect on the mitochondrial network and mitochondria keep a tubular shape (right). Insets: automatic area recognition (IPLab Software) shows single fragments encircled. Mitochondria were stained with an Alexa Fluor 594 labelled cytochrome c antibody. **B:** Detection of phospho-serine PKC substrates in mitochondrial lysates. MCF-7 cells were transfected with pOMML-PLCδPHd (lane PHD) or pOMML-EGFP (lane MOCK) and mitochondria were isolated 24 h after transfection. Mitochondria were also isolated from MCF-7 cells that had been treated with PKC inhibitor chelerythrine chloride (lane CHEL) or amino acid starved (lane AA). Mitochondria were lysed and subjected to Western blotting. The membrane was probed with anti phospho-serine PKC substrate antibody (top). Anti cytochrome c was used as a loading control on the stripped membrane (bottom). PHD and AA have significantly decreased phospho PKC substrate levels comparable to negative control CHEL. Compared to MOCK control mitochondria from autophagy induced cells (PHD and AA) are lacking phosphorylated 35 kDa substrate and phosphorylation of 50 kDa, 75 kDa and 105 kDa substrates is reduced. Chelerythrine chloride treatment inhibits phosphorylation, but mitochondria are leaking cytochrome c, as can be seen from the faint band, despite loading equal amounts of protein. **C:** Co-expression of mitochondrial targeted PKCα and PtdIns(4,5)P₂ phosphatase Skip. 24 h of pOMML-Skip overexpression leads to fragmentation of mitochondria (green dots) and consequently to mitophagy. Co-expression of the pOMML-DsRed empty Vector does not change this phenotype (left). Co-expression of pOMML-PKCα (red) inhibits fragmentation of 5-phosphatase “attacked” mitochondria and cells display a mitochondrial network with co-localised PKCα (co-localisation appears yellow) (right). Nuclei were counterstained with DAPI.

3. Discussion

Before this study, little was known of the role played by the mitochondrial membranes and their phospholipid constituents. It is proven that PtdIns is present in the OMM [43] and immunogold labelling has also implied the presence of PtdIns(4,5)P₂ [5]. However, regulation of mitochondrial phosphoinositides and their specific function has been neglected, partly because of difficulties in measuring these lipids. Here we present evidence that PtdIns(4,5)P₂ is present on the outer mitochondrial membrane. Firstly, recombinant PLCδPHd domain, which binds specifically to the headgroup of PtdIns(4,5)P₂, could be recovered from isolated mitochondria that had been probed with the recombinant PH domain (Fig. 5). Secondly, the expression of the phosphatase Skip, which specifically dephosphorylates PtdIns(4,5)P₂ [36], on the OMM leads to a severe phenotype change of mitochondria, indicating an important function for this lipid in mitochondria. Since PtdIns(4,5)P₂ binding and metabolising proteins have been found on mitochondria [6,44,45], it is not surprising that mitochondria have PtdIns(4,5)P₂ on the outer membrane. But the question is whether PtdIns(4,5)P₂ is only a structural part of the mitochondrial membrane, or whether it also has an important signalling function, similar to PtdIns(4,5)P₂ on the plasma membrane.

We conclude that mitochondrial PtdIns(4,5)P₂ is an important signalling molecule, rather than a structural component.

We show that [36] masking of PtdIns(4,5)P₂ by overexpression of OMM targeted PLCδPHd creates fragmented mitochondria (Fig. 1).

The same effect is seen when the lipid is removed by dephosphorylation through the PtdIns(4,5)P₂ specific 5-phosphatase Skip. While employing the PH domain renders the headgroup of PtdIns(4,5)P₂ invisible for signalling molecules on the OMM, such as Synaptojanin 2A [45], without changing the membrane composition, the 5-phosphatase would effectively alter membrane composition by converting PtdIns(4,5)P₂ into PtdIns(4)P. Yet both methods create the same phenotypes leading to the conclusion that mitochondrial PtdIns(4,5)P₂ is an important signalling molecule, rather than a structural component of the membrane. On the contrary, overexpression of the PH domain on the inner mitochondrial membrane has no detectable mitochondrial phenotype, hinting that either PtdIns(4,5)P₂ has no signalling function there or the inner membrane is devoid of this lipid. The latter would not be surprising, considering that it is generally believed that the inner membrane is a relic of endosymbiotic origin [46,47] and thus resembling a prokaryotic membrane in composition.

Investigating the role of mitochondrial PtdIns(4,5)P₂ demonstrated its importance for organisation of mitochondrial network in the cell. The loss or masking of this lipid resulted in the fragmentation of mitochondria, which was then followed by autophagy, as judged from co-localisation with lysosomes and LC3 staining (Fig. 4) [26]. This prompted the question whether “naturally” (nutrient deprivation) triggered autophagy would correlate with changes in mitochondrial PtdIns(4,5)P₂ levels. Indeed, we discovered that PtdIns(4,5)P₂ levels are greatly reduced during autophagy that has been triggered through nutrient deprivation (Fig. 5), indicating that PtdIns(4,5)P₂ acts as an

important component of the machinery controlling the events leading to autophagy in general. In essence one could perceive mitochondrial PtdIns(4,5)P₂ levels as a maintenance indicator for mitochondria, but it is also possible that this inositol lipid is a precursor to other lipid mediators that may be participating in this process.

For that reason, we probed known PtdIns(4,5)P₂ dependent pathways by pharmacological profiling of our phenotype. Our results indicated an involvement of calcium and PKCs prompting us to investigate the role of the classical (calcium dependent) PKCs in PtdIns(4,5)P₂ dependent induction of mitochondrial fragmentation. Employing a specific antibody recognising the phospho-serine motifs phosphorylated by classical PKCs revealed that mitochondrial lysates from cells that had been PtdIns(4,5)P₂ deprived or amino acid starved contained less PKC-phosphorylated proteins (Fig. 6).

It is clear that PtdIns(4,5)P₂ removal on the mitochondria could result in the reduction of PI-PLC second messenger products, DAG and Ins(1,4,5)P₃, which are needed for the activation of classical PKC isoenzymes. Indeed, PLC δ -1 has been found to be present on the mitochondria to regulate mitochondrial calcium uptake in liver cells [44]. Furthermore, we could induce mitochondrial fragmentation in MCF-7 cells with the selective PLC inhibitor U-73122, but not with the inactive analogue U-73343 (data not shown). Recently, it was demonstrated that the isoenzyme PKC α translocates to the mitochondria in myocytes upon PMA treatment [48], and the C2 domain of PKC α , which binds directly to PtdIns(4,5)P₂, has been shown to be important for the localisation and activity of this enzyme [49]. We reasoned that in both cases, overexpression of mitochondrial targeted PKC α should rescue the PtdIns(4,5)P₂ deprived mitochondria, if a cPKC substrate was the signal that protects the mitochondrion from the autophagic machinery when phosphorylated. We found that overexpression of mitochondrial targeted full-length PKC α did indeed rescue PtdIns(4,5)P₂ deprived mitochondria from mitophagy, indicating that the interaction between PtdIns(4,5)P₂ and PKC α suffices for its activation on the mitochondria, because we did not use a constitutively active enzyme.

In summary, this result indicates that PKC α a well established mitochondrial PKC isoform that controls apoptosis [50], is also an important mediator in PtdIns(4,5)P₂ deprivation induced fragmentation and mitophagy.

4. Material and methods

4.1. Tissue culture

NIH 3T3 mouse fibroblasts and MCF-7 human breast cancer cells were grown in DMEM supplemented with 10% foetal calf serum (FCS) (Gibco-Invitrogen), and HepG2 cells were grown in Minimal Eagle's Medium supplemented with 10% FCS, glutamate (Sigma, G7513) and non-essential amino acids at 37 °C in an atmosphere of 5% CO₂. For amino acid starvation, cells were cultured in serum-free Krebs-Ringer-HEPES buffer (KRH: 25 mM HEPES, 115 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, and 2 mM CaCl₂, pH 7.4 at 37 °C) containing 1 μ M glucagon (Sigma, G3157). In some experiments, 10 mM 3-MA (Sigma, M9281) was added 30 min before and again during autophagic induction. Media and supplements for cell culture were purchased from Sigma-Aldrich unless otherwise stated. Cell lines were obtained from ATCC.

4.1.1. Other inhibitors and reagents

The following concentrations were used: 10 μ M U73122 (Calbiochem, 662035), 40 μ M Bapta AM (Calbiochem, 196419), 100 μ M Ionomycin (Calbiochem, 407950), 100 nM PMA (Calbiochem, 524400), 50 μ M LY294002 (Calbiochem, 440202), 10 μ M Rottlerin (Calbiochem, 557370), 200 μ M D609 (Calbiochem, 251400), and 0.4% n-Butanol (Sigma, B7906). Solvent controls: DMSO (Sigma, D2650) and 10 μ M U73343 (Calbiochem, 662041).

4.2. Transfection

Cells were cultured in 24-well plates (containing 500 μ l medium per well) on glass coverslips and transfected at 70% confluence. 0.5 μ g plasmid DNA was diluted in 50 μ l serum-free medium and 1 μ l Turbofect (Fermentas, R0531) was added. The transfection mixture was allowed to stand for 20 min before it was added to the cells. The transfectants were analysed 24 h after transfection unless specified differently.

4.3. Plasmids

Inner mitochondrial membrane localisation Vector: A Kozak sequence followed by the mitochondrial targeting sequence derived from the precursor of subunit VIII of human cytochrome c oxidase targeting sequence from the commercially available pAcGFP1-Mito was cloned directionally in frame between the NheI and AgeI restriction sites at the N-terminus of the fluorescent protein coding sequence of commercially available pEGFP-C1 (pMito, mock control). The original in frame restriction sites of the multiple cloning sites were used to insert Skip full-length phosphatase.

Outer mitochondrial membrane localisation Vector: A Kozak sequence followed by the minimal targeting sequence from the mitochondrial import receptor TOM20 (CAGCGTTGTAGCTAGCATGGTGGGTCGGAAACAGCGCCATCGCCGCGGTGTATGCGGGGCCCTTTTCATTGGGTACTGCATCTACTTCGACCGCAAAAAGACGAAGTGACCCCAACTTCAAGAAACCGGTTTAGAG) was cloned directionally in frame between the NheI and AgeI restriction sites at the N-terminus of the fluorescent protein coding sequence of commercially available pEGFP-C1 (pOMML for outer mitochondrial membrane localisation) or pDsRed-Express (pOMML-DsRed) (mock controls), respectively. The original in frame restriction sites of the multiple cloning sites were used to insert the proteins of interest: PH domain of PLC δ -1 (PLC δ PHd), PLC δ PHd R37D, Skip, and PKC α , respectively.

PLC δ PHd was mutated to PLC δ PHdR37D by site directed mutagenesis using the sense primer 5'-gaagtcagctcgtgggataggaacgcttc-tac-3' and antisense primer 5'-gtagaagcgtccctatcccagagctggactc-3'. pAcGFP1-Mito (PT3730-5), pEGFP-C1 (PT3028-5), and pDsRed (PT3678-5) were purchased from BD Biosciences Clontech. Human cDNA clone TOMM20 (accession no. NM_014765.1; order number SC114860) was purchased from OriGene Technologies. NheI and AgeI restriction sites, flanking the targeting sequence, were introduced by PCR using the following primers 5'-cagcgttgtagctagcatggt-3' and 5'-ctctaaaccggtttctgaagt-3'.

All constructs were propagated in *E. coli* XL1-Blue (Stratagene, 200268), isolated with Qiagen Maxiprep and verified by sequencing (Yorkshire Biosciences).

4.4. Microscopy and image analysis

Cell preparations were observed under a Nikon TE 2000 fluorescence microscope using a 100 \times Fluor oil lens. Filters used in the fluorescence experiments were band pass for DAPI, FITC and TRITC with excitation wavelength of (nm) 340–380, 465–495 and 540–580, respectively, and with emission wavelength of (nm) 435–485, 515–555 and 572–605, respectively. Images were digitally acquired with a CCD camera (Hamamatsu) for each fluorophore separately and processed using IPLab software v 3.65a and ImageJ (National Institutes of Health, Bethesda, MD).

Analysis of fragmentation of mitochondria by microscopy was carried out with the automatic particle size recognition tool of ImageJ software (public domain, NIH Bethesda, Maryland, USA). Fluorescence microscopy images of mitochondria were masked and converted into a binary image. Particle size and count were measured using an exclusion area (area occupied by a single mitochondrial fragment) of >0.6 μ m².

4.5. Immunofluorescence

Cells were fixed in 4% (w/v) PFA for 10 min, quenched with 50 mM NH₄Cl/PBS, permeabilized with 0.1% (v/v) Triton X-100 for 7 min and blocked with 1% (w/v) BSA/PBS (fatty acid free; Sigma, A6003) for 1 h at room temperature. Cells were then incubated with the fluorescently labelled antibody (1:200 diluted in blocking solution) for 1 h followed by three extensive PBS washes before treatment with 300 nM 4',6-diamidino-2-phenylindole (DAPI; Calbiochem, 268298) for nuclear counter staining. Thoroughly washed coverslips were mounted on glass slides using Mowiol supplemented with 0.6% (w/v) 1,4-diazabicyclo-[2.2.2]octane (DABCO). Cytochrome c antibody (BD Pharmingen, 556432) and LC3 antibody (Cell Signaling, 2775) were labelled with an appropriate Alexa fluor dye using the Zenon Antibody labelling kit (Invitrogen, Z-25005).

4.5.1. Loading of lysotracker red (LTR)

500 nM LTR (Invitrogen, L-7528) was added to the living cells for 20 min. Then cells were washed twice with PBS and fixed with 4% paraformaldehyde. Coverslips were mounted as described earlier. JC-1 staining (Sigma, T4069) was carried out according to the manufacturer's instructions and mitochondria were checked under the microscope for bright red fluorescence. Control samples were pretreated with valinomycin.

4.6. Mitochondria extraction with a Parr Cell Bomb (45 ml)

Adherent cells were washed twice, harvested in ice cold PBS and pelleted at 500×g for 5 min. The cell pellet was resuspended in an equal volume of cold cavitation buffer (250 mM sucrose, 20 mM Hepes pH = 7.5, 2 mM MgCl₂, 1 mM NaEDTA, 1 mM PMSF, 1 mM DTT), which contained 5 μM pV(phenbig) [33] and a Protease Inhibitor Cocktail III (Calbiochem, 539134), and transferred to the pre-chilled cavitation bomb [51]. The chamber was pressurised at 300 psi and incubated on ice for 5 min before the pressure was slowly released. The recovered suspension was checked in the microscope for efficient cell disruption. All following steps were carried out in the cold or on ice. The lysate was cleared of nuclei and debris at 500×g. The pellet was discarded and the supernatant was centrifuged 3 times (each time transferred to a fresh tube) at 1300×g for 5 min. Then the supernatant was split into 300 μl aliquots and centrifuged at 14000×g for 20 min. The pellet was resuspended in a desired volume of cavitation buffer and analysis of mitochondria was carried out. Alternatively the suspension was supplemented with 50% glycerol for storage at −80 °C. The quality of the mitochondria was checked by microscopy and JC-staining [52] revealing a greater than 95% purity and integrity (data not shown).

4.7. Protein expression

pGEX-4T1 containing the PLCδPHd or the catalytic subunit of Synaptojanin, respectively [36], was transformed into *E. coli* XL1-Blue. A single colony was picked transferred into 5 ml of LB-medium and shaken at 37 °C. The culture was subsequently scaled up to 4 l. Protein expression was induced by 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, 59740) addition and incubation at 18 °C overnight. Glutathione S-transferase (GST)-fusion protein was purified according to the manufacturer's manual using glutathione-Sepharose 4B (GE Healthcare, 17-07-56-01). Protein integrity was confirmed by Western blot using a GST antibody (Novagen, 71097).

4.7.1. Western blotting

Mitochondria were lysed in SDS sample buffer, boiled and separated by SDS-PAGE and blotted on PVDF membrane. After blocking in 5% (w/v) milk, membranes were probed with a primary antibody, followed by a secondary HRP conjugated antibody. The GST antibody was used at a 1:1000 dilution whereas the PKC phospho-

serine substrate antibody (Cell Signaling, 2261) was used at 1:200. HRP secondary antibody conjugates (Bio-Rad, 172–1019) were employed at 1:5000. Quantification of band densities was carried out with ImageJ software.

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