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The iPLA$_2\gamma$ is identified as the membrane potential sensitive phospholipase in liver mitochondria

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ABSTRACT

Previous reports from our lab identified a mitochondrial calcium-independent phospholipase A$_2$ activity that is activated when the mitochondrial membrane potential is decreased. This activity was demonstrated to influence occurrence of the permeability transition. Originally, this activity was ascribed to the iPLA$_2\beta$ protein. Recently, both iPLA$_2\beta$ and iPLA$_2\gamma$ knock out mice have been generated. It has been shown by others that the iPLA$_2\gamma$ plays a significant role in progression of the permeability transition. In this paper, using the iPLA$_2\beta$ and iPLA$_2\gamma$ knock out mice we show that the membrane potential sensitive activity is the iPLA$_2\gamma$.

1. Introduction

In a previous publication, we identified a calcium-independent phospholipase A$_2$ (iPLA$_2$) activity in rat liver mitochondria that is activated under conditions that produce loss of membrane potential ($\Delta\psi$), including addition of a protonophore, addition of alamethicin, or opening of the permeability transition pore [1]. This activity was subsequently shown to play a facilitating role in occurrence of the mitochondrial permeability transition and to modulate release of cytochrome *c* from isolated mitochondria [2]. More recently, through use of select combinations of electron transport inhibitors and substrates, we demonstrated that increased activity is not determined by the redox status of any particular electron transport complex, that activation occurs in a graded fashion, and that activation by de-energization is reversed when an energized state is restored [3]. These regulatory features suggest that this activity is involved in sensing and coordinating cellular bioenergetics and cellular fate.

Members of the phospholipase A$_2$ superfamily are organized into 16 groups (Group I–Group XVI) that are further divided into many subgroups (For review see [4]). Group VI iPLA$_2$s include the calcium-independent phospholipase A$_2$ that have activity in the absence of calcium and have in common a catalytic serine at the active site. Group VI is further divided into six subgroups based on genetic code and physical structure. Group VIA and VIB iPLA$_2$s have been named the iPLA$_2\beta$ and iPLA$_2\gamma$, respectively. It has been reported that each of these isozymes have a population that is localized to the mitochondria [1,5–8].

The iPLA$_2\beta$, encoded by the PNPLA9 gene, was first purified and characterized from macrophage-like cells by Dennis and colleagues [9]. Immunoblot analysis of Percoll purified mitochondria, using a commercial antibody raised against the activity obtained by Dennis, showed the presence of the iPLA$_2\beta$ and we initially attributed the calcium-independent phospholipase A$_2$ activity to this protein [1]. A similar activity was found independently in mitochondria from rabbit heart [5]. Additional Ca^{2+}-independent iPLA$_2$ activities were identified in mitochondria from kidney [7,10], brain [6], lung and spleen [8]. Based on immunoblot analysis, using the commercially available antibody, the activities in heart, and brain were also attributed to iPLA$_2\beta$. An iPLA$_2\beta$ KO

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mouse has been generated by Turk and colleagues through homologous recombination [11,12], and these mice have been employed to identify roles for the iPLA$_2\beta$ in processes dependent upon arachidonic acid derived signaling molecules [13] and in generation of platelet activating factor [13,14].

Gross and colleagues cloned the iPLA$_2\gamma$ encoded by the PNPLA8 gene in 2000 [15]. Examination of the gene showed the presence of both peroxisomal and mitochondrial targeting sequences [16]. This dual localization was verified at the protein level in a myocardial over expression model [17]. Additional evidence for a mitochondrial location of iPLA$_2\gamma$, came from the group of Schnellmann, who showed inhibition of iPLA$_2$ activity in isolated kidney mitochondria by the stereo specific inhibitor (R) bromoenol lactone (R-BEL), which is relatively selective as an inhibitor of iPLA$_2\gamma$ [7]. Gross and colleagues have generated an iPLA$_2\gamma$ global knockout mouse model by removing exon 5 from the PNPLA8 gene [18]. Using mitochondria isolated from the iPLA$_2\gamma$ global knockout, Moon et al. [19], showed a reduction in iPLA$_2$ activity and that progression of the permeability transition, as evidenced by the rate of swelling, was reduced when compared to wild type counter parts. Rapid swelling was recovered when palmitic acid was added supporting a hypothesis that iPLA$_2\gamma$ activity plays a role in opening the permeability transition pore through release of FFA.

Our earlier work also identified a facilitating role for iPLA$_2$ activity in the permeability transition [2]. The iPLA$_2$ activity described there has the specific regulatory feature of being membrane potential dependent [2] and it is of interest to determine whether that potential sensitive activity can also be attributed to the iPLA$_2\gamma$. The identity of the potential sensitive activity has not been addressed by those examining isozymes present in mitochondria. Assays employed by those groups do not utilize intact mitochondria. Their assay systems commonly employ sonicated mitochondria and exogenous radiolabeled phospholipid. That system is not useful in determining the identity of the membrane potential sensitive iPLA$_2$, as sonication would disturb the membrane barrier, resulting in mitochondria unable to maintain a membrane potential. This precludes observation of iPLA$_2$ activity differences between conditions that generate energized or de-energized mitochondria and thus cannot specifically address the identity of an enzyme activity modulated by membrane potential.

With this in mind, we isolated liver mitochondria from both iPLA$_2\beta$ and iPLA$_2\gamma$ knockout animals and performed an *in organello* product accumulation assay to determine iPLA$_2$ activity after mitochondrial depolarization. We observed a significant reduction in release of polyunsaturated fatty acids (PUFA) in uncoupled mitochondria from iPLA$_2\gamma$ knockout animals, although a residual activity remained. There was no decrease in membrane sensitive release of fatty acids upon iPLA$_2\beta$ knockout. Thus, the present study demonstrates that iPLA$_2\gamma$ is the predominant membrane potential sensitive activity, but leaves room for the interpretation that additional mitochondrial phospholipases become active in de-energized mitochondria. Additionally, it suggests that while iPLA$_2\beta$ may be present in mitochondria, it is not the membrane potential sensitive phospholipase and has distinct regulatory properties and physiological functions.

2. Materials and methods

2.1. Animals and reagents

Male iPLA$_2\beta$ null mice were a gift from Dr. John Turk, University of Washington, St. Louis, MO, USA; male iPLA$_2\gamma$ null mice were a gift from Dr. Richard W. Gross, University of Washington, St. Louis, MO, USA. Male Sprague–Dawley rats (~250 g) and male C57/B6 mice, the background strain for the iPLA$_2\beta$ null and iPLA$_2\gamma$ null mice, were obtained from Harlan Laboratories, Indianapolis, IN, USA. Animals were maintained by University Laboratory Animal Resources. All procedures were approved by the Institutional Animal Care and Use Committee of the Ohio State University.

Specific enantiomers of bromoenol lactone (R- and S-BEL) were purchased from Cayman Chemical, Ann Arbor, MI, USA. The iPLA$_2\beta$ specific antibody was a gift from Dr. Sasanka Ramanadham, University of Alabama, Birmingham, AL, USA. iPLA$_2\gamma$ specific antibody was purchased from Abcam, Cambridge, ENG. Anti-VDAC1 antibody was purchased from Cell Signaling Technology, Beverly, MA, USA. Anti-mouse/anti-rabbit-HRP were purchased from Cayman Chemicals, Ann Arbor, MI, USA. HRP chemiluminescent substrate was purchased from Thermo Fisher Scientific, Waltham, MA, USA. All other reagents were purchased from Sigma Aldrich, St. Louis, MO, USA.

2.2. Mitochondrial isolation

Mouse or rat liver mitochondria were isolated by methods previously described [20]. Two mouse livers or one rat liver were homogenized in ice cold media containing 230 mM mannitol, 70 mM sucrose, 3 mM HEPES, 0.2 mM EGTA and 2 mg/mL fatty acid free BSA, pH 7.4. Centrifugation was performed as previously described [20]. The final mitochondrial pellet was suspended in media containing 230 mM mannitol, 70 mM sucrose, and 3 mM HEPES, pH 7.4 (MSH media) at a concentration of 50–60 mg/mL. Protein concentration was determined by the Biuret method.

2.3. Determination of iPLA$_2$ activity

Phospholipase activity was determined by monitoring accumulation of polyunsaturated free fatty acids (PUFA). Incubations were conducted simultaneously in vessels open to the air with constant stirring. Mitochondria were diluted to 1.0 mg/mL in MSH solution containing 10 mM succinate and 0.5 nmol/mg rotenone. Cyclosporine A (0.5 nmol/mg) was present. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (1.0 nmol/mg) was added 2 min after addition of mitochondria to dissipate the proton gradient. When utilized, BEL (5 μM) was added 45 s after addition of mitochondria.

Aliquots were taken at times indicated and a modified Folch extraction performed [20]. 5.0 μg heptadecanoic acid was employed as internal standard. The Folch emulsion was centrifuged at 1000 rpm for 5 min. The lower organic phase was collected and brought to dryness under nitrogen. Extracted lipids were dissolved in 1.0 mL freshly distilled ether containing 100 μL methanol. Diazomethane, 200 μL, was added to convert FFA to fatty acid methyl esters (FAME). After 15 min at RT, samples were brought to dryness under nitrogen. Samples were re-suspended in 1.0 mL hexane and stored overnight at −80 °C under argon.

Prior to analysis, stored samples were brought to dryness under nitrogen and suspended in 200 μL hexane. Samples were applied to silica gel mini-columns and washed successively with 1.8 mL hexane and 2.0 mL chloroform. FAME were eluted in 2.0 mL hexane:ether (1:1). FAME were brought to dryness under nitrogen and suspended in 12 μL hexane. They were separated and quantified using a GLC equipped with a capillary column and a computing integrator. Peak areas representing the amount of individual FFA were converted to units of nmol/mg of mitochondrial protein by comparison to the area of the heptadecanoic acid peak retention times of individual FFA were determined by comparison to those of known components in a commercially available standard mixture (Nu-Chek Prep, Elysian, MN, USA). For the present study, the sum of the released linoleic acid (18:2), arachidonic acid (20:4), and docosahexenoic acid (22:6) were used as the indicator of

phospholipase A_2 activity [21]. In wild type animals, the percent distribution was 59%, 36%, and 5% respectively. In the iPLA$_2\gamma$ KO animals, there was variation in that percent distribution, as follows: 41%, 43%, 17%.

2.4. Immunoblot analysis

Isolated mitochondria were diluted and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% Triton x-100, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, and 5 mM EGTA) supplemented with a protease inhibitor cocktail (Roche, Nutley, NJ, USA). Equal amounts of mitochondrial protein were loaded into the lanes of a polyacrylamide gel. Gels were run at a constant 100 mV until separation was achieved, as determined by the separation of a pre-stained protein standard (Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins were transferred to a PVDF membrane by the wet method at a constant 100 mV for 1.5 h. Membranes were incubated in blocking solution (5% defatted dry milk in TBST) for 1 h at room temperature before being washed three times in TBST to remove excess blocking solution. Incubation with primary antibodies took place overnight at 4 °C. The primary antibody solution was removed and membranes washed three times with TBST before secondary HRP-linked antibody was added in blocking solution. Membranes were visualized using ECL and exposed on X-ray film.

3. Results and discussion

3.1. Effect of R- and S-BEL

The halide compound, BEL, selectively inhibits group VI iPLA$_2$s [22,23]. BEL contains one stereogenic center and exists as two enantiomers. Purified enantiomers have been used to show that S-BEL is 10-fold more reactive towards iPLA$_2\beta$, whereas R-BEL is selective for iPLA$_2\gamma$ [24]. We employed S- and R-BEL to pharmacologically identify the iPLA$_2$ species in rat liver mitochondria that is activated upon mitochondrial depolarization by the chemical uncoupler, CCCP. When this protonophore was used to activate iPLA$_2$ there was no significant difference in release of PUFA when S-BEL was present compared to vehicle alone (Fig. 1). However, when R-BEL was present there was an approximately 55% reduction in release of PUFA (Fig. 1). This data supports a hypothesis that the iPLA$_2\gamma$ and not the iPLA$_2\beta$ accounts for a significant portion of the membrane potential regulated release of FFA in isolated mitochondria. It is important to note that, in the presence of R-BEL, some PLA$_2$ activity remains. Experiments by Schnellmann and colleagues [7,10], utilizing BEL enantiomers to inhibit mitochondrial iPLA$_2$s, show similar relative inhibitory activities.

3.2. iPLA$_2$ activation in knockout animals

A membrane potential sensitive iPLA$_2$ was also identified in mice liver mitochondria that is sensitive to inhibition by racemic BEL (Fig. 2). CCCP stimulated accumulation of PUFA was reduced by ~60% when racemic BEL was present in the incubation. This was observable after 45 and 180 min of incubation. Since it is known that the specificities of individual BEL enantiomers are not absolute [24], we moved directly to examining iPLA$_2$ activity in the transgenic mice strains described above to determine the isozyme responsible for the membrane potential sensitive activity. Utilizing the iPLA$_2\beta$ and iPLA$_2\gamma$ knockout mice, we monitored PUFA accumulation in isolated liver mitochondria and demonstrate that iPLA$_2\gamma$, is the enzyme responsible for the membrane potential sensitive activity.

In Fig. 3A, it is shown that mitochondria isolated from wild type mice, like those from rats, exhibited increased phospholipolysis and accumulation of PUFA after uncoupling when compared to those left untreated (Fig. 3A). The relative increase in iPLA$_2$ activity seen in the presence of uncoupler is similar to that seen in rat liver mitochondria, although total free fatty acid accumulation was greater in mouse liver mitochondria (Fig. 3A). Mitochondria from the two knockout animals showed different magnitudes of PUFA release in response to treatment by uncoupler. Mitochondria isolated from iPLA$_2\gamma$ knockout mice displayed a substantial decrease in membrane potential sensitive iPLA$_2$ activity when compared to mitochondria from wild type mice similarly treated (Fig. 3A and B). In contrast, mitochondria from iPLA$_2\beta$ knockout mice exhibited uncoupler induced iPLA$_2$ activity similar to that observed in wild type animals (Fig. 3A and C). The reduction in accumulation of PUFA after uncoupling in mitochondria from iPLA$_2\gamma$ knock out mice corresponds to 80% of that observed in mitochondria from

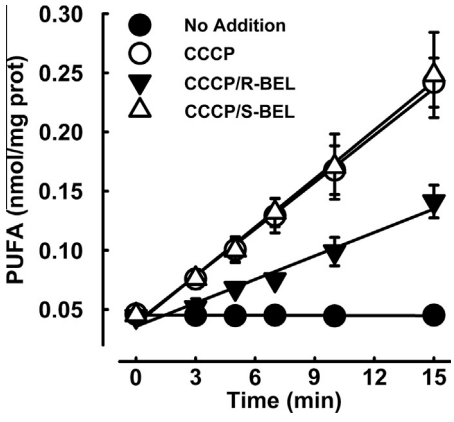

Fig. 1. Effect of R- and S-BEL on iPLA$_2$ activity: rat liver mitochondria were incubated at 1.0 mg/mL in the MSH medium containing succinate (10 mM), rotenone (0.5 nmol/mg), and cyclosporine A (0.5 nmol/mg). The temperature was 25 °C. BEL (5 μM) was added 45 s after the addition of mitochondria. CCCP (1.0 nmol/mg) was added 2 min after the addition of mitochondria. Samples were taken at the indicated time points and processed for free fatty acid determination as described in the Section 2. ● No addition of CCCP, ○ CCCP, but no addition of BEL, △ S-BEL added 45 s after addition of mitochondria, CCCP added, ▼ R-BEL added 45 s after addition of mitochondria, CCCP added. Values are the average of 3 independent preparations ± S.E. (error bars).

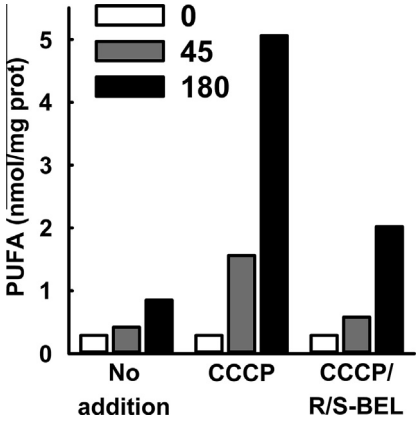

Fig. 2. Mouse liver mitochondria contain a BEL sensitive iPLA$_2$: incubations were conducted as in Fig. 1, however mitochondria were isolated from mouse liver. The first group of three bars was obtained from an incubation in the absence of CCCP and BEL. The second group of three bars was obtained from an incubation containing CCCP (1.0 nmol/mg). The third set of three bars was obtained from an incubation containing CCCP (1.0 nm/mg) and racemic BEL (5 μM). In each set, the clear bar is the value obtained at $t = 0$, the lighter gray bar is the value determined at 45 min. and the darkest bar is the value determined at 180 min. The data is the average from two separate preparations with individual values within 15% of each other at each time point.

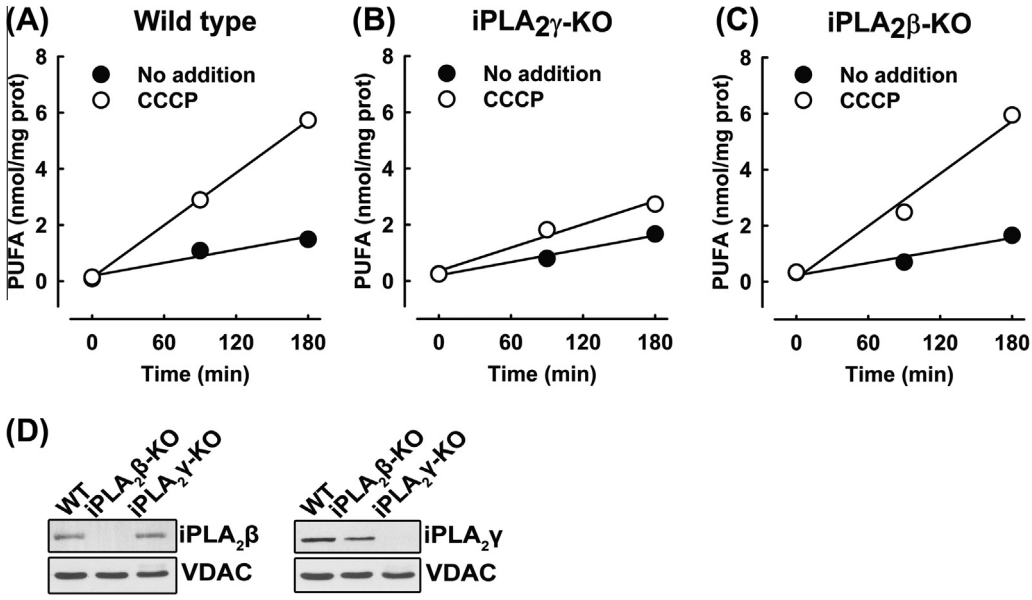

Fig. 3. iPLA$_2\gamma$ is the enzyme responsible for most of the iPLA$_2$ activity seen in depolarized liver mitochondria: Incubations were conducted as in Fig. 1, however mitochondria were isolated from mouse liver. (A) Wild type mitochondria were incubated in the absence ● or presence ○ of CCCP (1.0 nmol/mg). (B) iPLA$_2\gamma$-KO mitochondria were incubated as in (A). (C) iPLA$_2\beta$-KO mitochondria were incubated as in (A). (D) Western blot analysis of mitochondria isolated from wild type, iPLA$_2\beta$-KO, and iPLA$_2\gamma$-KO mice probed with antibodies against iPLA$_2\beta$, iPLA$_2\gamma$, and the mitochondrial marker VDAC as loading control. Values in Panel (A) are the average of 5 independent preparations and have a standard error of 10% or less. Values in Panels, (B), and (C) are the average of two independent preparations with values differing by 15% or less at all time points.

either wild type or iPLA$_2\beta$ knock out mice (open circles, Fig. 3A–C). In the absence of uncoupling, a low level of PUFA release was observed for all animal genotypes (closed circles, Fig. 3A–C). Western blot analyses were conducted in order to verify animal genotype (Fig. 3D). No iPLA$_2\beta$ or iPLA$_2\gamma$ protein was detected in mitochondria isolated from iPLA$_2\beta$ and iPLA$_2\gamma$ knockout mice, respectively. Both proteins were found in wild type animals. This data indicates that iPLA$_2\gamma$ is the phospholipase species primarily responsible for the depolarization dependent iPLA$_2$ activity observed in isolated rat and mouse liver mitochondria and is consistent with results of the experiments carried out in rat liver mitochondria utilizing BEL enantiomers (Fig. 1).

In previous publications, we discussed the relationships between membrane potential, iPLA$_2$ activity, and opening of the permeability transition pore [1,2]. The data here, coupled with that presented by Gross [19] strengthens those proposed relationships and supports the hypothesis that changes in membrane potential can affect occurrence of the permeability transition though release of PUFA via a membrane potential sensitive phospholipase. This could occur in numerous physiological and pathological conditions including, reperfusion injury [25], identification of poorly functioning mitochondria [26], and apoptosis [27]. Since the magnitude of the mitochondrial membrane potential can be an indicator of the functional status of mitochondria, modulations in iPLA$_2\gamma$ activity and released fatty acids are proposed to play a role in coordinating cellular bioenergetics.

While identifying iPLA$_2\gamma$ as the predominant membrane potential sensitive activity, these results question interpretations of earlier data ascribing the membrane potential sensitive activity to the iPLA$_2\beta$ [1,5,6]. This early hypothesis was developed based on immunological studies of fractionated mitochondria showing the presence of this protein in the membrane portion of Percoll purified liver mitochondria. Although that data provides evidence of a mitochondrial localization for the iPLA$_2\beta$, the fact that mice devoid of this isozyme retain iPLA$_2$ activity after uncoupling (Fig. 3C), argues against this protein being responsible for release of FFA after depolarization. It has been shown by others, that the iPLA$_2\beta$ interacts with numerous proteins, some of which increase phospholipase activity [28] while others decrease phospholipase activity [29,30], we propose that iPLA$_2\beta$ activity is not observed in our experimental system because this enzyme is not regulated by membrane potential and that an additional extra-mitochondrial factor required for activity is not present. The iPLA$_2\beta$ likely functions in a distinct or secondary role within the mitochondria rather than as a primary sensor of mitochondrial bioenergetics as originally proposed [1]. Additional work is necessary to reevaluate the role of the iPLA$_2\beta$ in mitochondria.

A final aspect of the data presented here is that when iPLA$_2\gamma$ is ablated, a small level of membrane potential dependent activity remains (Fig. 3B). We have also observed a low level of iPLA$_2$ activity in the presence of racemic BEL in rat liver mitochondria when activated by a number of different de-energizing compounds [1,2] and in mouse liver when activated by addition of CCCP (Fig. 2). These data imply the presence of an additional lipolytic activity that may be partially regulated by membrane potential and may rely on μM levels of calcium for activity. The presence of other PLA$_2$ activities is consistent with the data of Gross and colleagues [19]. In their recent paper, while immunoblot analysis confirmed the absence of iPLA$_2\gamma$ in their knock out mice, significant release of arachidonic acid still observed (~60% compared to wild type) in mitochondria isolated from those mice. Additionally, although progression of the permeability transition was significantly decreased in iPLA$_2\gamma$ knock out mice, further inhibition of the transition was observed when either R- or S-BEL was added, indicating additional BEL sensitive activity.

Others have observed iPLA$_2$ activities with unique regulatory properties in mitochondria isolated from different tissues. In isolated brain mitochondria an iPLA$_2$ activity is present under basal conditions that increases in activity upon slight depolarization as induced by BAX and truncated BID. This activity is reduced below basal activity in the presence of BEL [6,31]. Schnellmann and colleagues have identified an iPLA$_2$ activity in rabbit renal cortex mitochondria that is inhibited by CCCP rather than activated [10]. The relationship of this activity to that identified in our studies is unclear due to the differing assay systems as mentioned above. It is clear that further work is required to determine the

regulation and roles of $iPLA_2$ activity in mitochondria from a variety of tissues.

3.3. Conclusion

Herein, we have demonstrated that the predominant protein responsible for membrane potential sensitive $iPLA_2$ activity is the $iPLA_2\gamma$ encoded by the PNPLA8 gene. Because activity of this enzyme is closely linked to the bioenergetic status of mitochondria, it is an excellent candidate for coordination of cellular processes with the functional status of mitochondria.

Additionally, by utilizing $iPLA_2\beta$ knockout mice we have demonstrated that the $iPLA_2\beta$ does not contribute to depolarization induced $iPLA_2$ even though its physical presence in mitochondria has been documented [1,5,6].

Our results are consistent with the presence of additional phospholipase activities that operate in conjunction with those already identified or have distinct roles in mitochondrial function. Activation of additional lipolytic activities may be linked to or dependent upon activation of the membrane potential dependent activity or may require additional factors.

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