Regulation of the Ca\(^{2+}\)-Independent Phospholipase A\(_2\) in Liver Mitochondria by Changes in the Energetic State.

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Abbreviations

The abbreviations used are BEL; bromoeno lactone: BSA; bovine serum albumin: CCCP; carbonyl cyanide m-chlorophenylhydrazone: CSA; cyclosporin A: FAME; fatty acid methyl esters: FFA; free fatty acids: iPLA2; Ca\(^{2+}\)-independent phospholipaseA2: sn-1; position 1 within a glycerophospholipid molecule as defined by the stereo specific numbering system: sn-2; position 2 within a glycerophospholipid molecule as defined by the stereo specific numbering system: TMPD; N,N,N’N’-tetramethylphenylenediamine: TPP\(^+\); tetraphenylphosphonium cation: TTFA; thenoyltrifluoroacetone.
ABSTRACT

The effect of electron transport chain redox status on activity of the mitochondrial calcium-independent phospholipase A2 has been examined. When oxidizing NAD-linked substrates, the enzyme is not active unless deenergization occurs. Uncoupler, rotenone, antimycin A and cyanide are equally effective at up regulating the enzyme while oligomycin is ineffective. TTFA causes deenergization and activates the enzyme but only if succinate is the respiratory substrate. These findings show that the mitochondrial iPLA2 responds to the energetic state overall, rather than to the redox status of individual electron transport chain complexes. With NAD-linked substrates, and using rotenone to deenergize, iPLA2 activation can be reversed by adding succinate to reestablish a membrane potential. For this purpose, ascorbate plus TMPD can be used instead of succinate and is equally effective. With succinate as substrate, the membrane potential can be reduced in a graded and stable fashion by adding increasing concentrations of malonate, which is a competitive inhibitor of succinate utilization. A partial and stable activation of the iPLA2 accompanies partial deenergization. These findings suggest that in addition to the several functions that have been proposed, the mitochondrial iPLA2 may help to coordinate local capillary blood flow with changing energy demands.
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Relationships between phospholipase activity in mitochondria and degradation of the structure and bioenergetic capacities of these organelles were already under investigation during the 1950s and 1960s [e.g. (1-3)]. Towards the end of that period Waite and coworkers described an apparent relationship between mitochondrial swelling and the activity of an endogenous phospholipase A (4). Evidence for such a relationship grew stronger during the subsequent decade (5,6), and during the same period the loss of coupled function which accompanied swelling became known as the mitochondrial permeability transition. There was a tendency to interpret results in that emerging area within the context of energy coupling mechanisms underlying the synthesis of ATP (7-9), as being related to the process of Ca²⁺ release from mitochondria (10,11), or to the control of steroid hormone biogenesis in cell types having that capacity (12,13).

Attempts to purify and characterize the phospholipase of interest led to the description of a low molecular weight phospholipase A₂ that requires Ca²⁺ (14-16) and is subject to inhibition by a product, monolysocardiolipin (17). Its enzymatic properties, together with cloning and sequencing data, led to classification of the enzyme as a type IIA secretory phospholipase (16,18). However, the occurrence of the enzyme in mitochondria from hepatocytes was later called into question and it was thought ultimately that it is present primarily in the Kupfer cells of liver (19,20). This latter view raised again the question of the identity of the apparent phospholipase A₂ activity in hepatocyte mitochondria and how it is related to the occurrence of the mitochondrial permeability transition and related phenomena.

The way in which phospholipase A₂ activity might promote the permeability transition became more apparent as evidence accumulated showing that small amounts of free fatty acids promote opening of the permeability transition pore and related phenomena (21-25). The question of enzyme identity was illuminated, in part, when it was found that rat liver mitochondria contain a phospholipase A₂ that does not require Ca²⁺ while appearing to be much larger than the type IIA secretory enzyme that was of interest before. Based on sensitivity of enzyme activity to bromoenol lactone (BEL) but not to other phospholipase inhibitors, the absence of a Ca²⁺ requirement, and its apparent recognition by a commercial antibody raised against partial sequence from a phospholipase found in a macrophage-like cell line, we concluded that the predominate activity is an iPLA₂ (26,27). Independent work has shown that an analogous enzyme is found in heart mitochondria (28), the mitochondria of kidney (29-31) and brain (32), and in those of lung and spleen (33).

The iPLA₂ activity of rat liver mitochondria acting upon endogenous phospholipids is regulated in some manner by the energetic status of the organelle. That is to say, little or no activity is seen when
Regulation of mitochondrial iPLA$_2$ activity mitochondria are intact and fully energized. The enzyme becomes active upon the addition of a protonophoric uncoupler (CCCP), when the permeability transition pore is opened, or when they are permeabilized by the pore forming compound alamethicin (26,27). Here we investigate the origin of this energy dependence in greater detail, including the relationship between activity and the redox status of individual respiratory chain complexes, the effect of partial deenergization on activity, and the reversibility of enzyme activation brought about by restoring the energetic state.

EXPERIMENTAL PROCEDURES

Preparation and incubation of mitochondria. Rats were maintained by University Laboratory Animal Resources, and all procedures were approved by the Institutional Animal Care and Use Committee of the Ohio State University. Briefly, the rats were sacrificed by an overdose of Nembutal that was administered IP. When they were no longer responsive, the abdominal and chest cavities were opened with scissors; the liver was removed, and was then transferred to ice cold isolation medium.

Rat liver mitochondria were prepared by a standard procedure in which bovine serum albumin (BSA) and EGTA were present in the homogenization medium but were absent from the medium used for washing (34). They were incubated at 25°C in media containing 0.23 M mannitol, 0.07 M sucrose, and 3 mM Hepes (Na$^+$), pH 7.4, before the addition of respiratory substrates. 10 mM each of glutamate and malate, both Na$^+$ salts, were normally used as the substrate but in some cases respiration was supported by 10 mM of Na$^+$ succinate, or by 6 mM ascorbic acid in the presence of 100 µM N,N,N',N'-tetramethyl-phenylenediamine (TMPD). When succinate alone was the exogenous substrate, rotenone was present at 1 nmol/mg protein. For all substrates, as they were added to the media, the concentrations of mannitol and sucrose were reduced in proportion to maintain a total osmotic pressure of 300 mOsM.

Incubations were conducted in vessels that were open to the atmosphere, with stirring maintained to provide an availability of O$_2$ throughout the time course (35). Because the experiments were conducted over relatively long time frames (up to 2 hours) all incubations pertaining to a given experiment were conducted simultaneously to prevent variations arising from aging of the preparation. Cyclosporin A was always present to prevent occurrence of the permeability transition and the effectiveness of this reagent was verified by monitoring swelling using a Brinkman probe colorimeter that was interfaced to a computer. Alternatively, swelling was sometimes monitored discontinuously using the same instrument, by moving the probe from vessel to vessel and manually recording the reading.

Changes in membrane potential were also of interest and were monitored in parallel with other
Regulation of mitochondrial iPLA₂ activity parameters using a TPP⁺ electrode as previously described (35). For calculation of the potential in units of mV, the matrix volume was taken to be 1 µl/mg protein (36) and the fraction of TPP⁺ bound nonspecifically to mitochondrial components was accounted for (37). Expected redox states of complex 4 (cytochromes aa₃) were verified by dual wavelength spectroscopy (38). Protein concentrations were determined by the Biuret reaction in the presence of 1% deoxycholate, while oxygen consumption was monitored with a Clark-type electrode.

**Determination of iPLA₂ activity:** Recently distilled solvents were used throughout. Phospholipase activity was determined by monitoring the accumulation of free fatty acids (FFA) derived from the endogenous phospholipids (34). To extract these products, a 3 mL aliquot of the incubation was added to 4 mL of cold methanol to which 5.00 µg of heptadecanoic acid (17:0) had previously been added as an internal standard. The aqueous methanol solution was mixed before addition of 8 mL CHCl₃, and the resulting mixture was centrifuged to separate the organic and the aqueous phases. The aqueous phase (upper phase) was removed by aspiration, after which the lower organic phase was transferred to a 10 mL conical screw cap tube. This phase was brought to dryness under N₂ and the free fatty acids were converted to methyl esters (FAME) by reaction with diazomethane (39). During this procedure, the dried lipid phase was taken up in 1.1 mL of ether: methanol (10:1) to which 0.2 mL of the diazomethane solution was added. Reaction was allowed to continue for 15 minutes at room temperature. The samples were then brought to dryness and taken up in 1 mL hexane. Typically they were thereafter stored overnight, at -80°C and under argon, prior to analysis by GLC.

To begin the analysis the stored samples were dried and the lipids were taken up in 0.2 mL hexane. These solutions were applied to silica gel mini columns for the separation of FAME from other mitochondrial lipids (34). The columns were washed successively with 1.0 mL hexane, 1.8 mL CHCl₃ and finally 2.0 mL hexane:ether (1:1). FAME are obtained in the hexane:ether wash. The hexane:ether solvent mixture was removed under N₂ and the FAME were taken up in 12 µL of hexane. They were separated and quantitated using a GLC equipped with a capillary column and a computing integrator. Peak areas representing the original level of individual FFA were converted to units of nmol/mg of mitochondrial protein. To convert the areas to units of mass they were compared to the area of the internal standard peak, which represented the 5.00 µg of heptadecanoic acid that was added to the original extract.

Changes in FFA levels can usually be related to activity of a PLA₂ by considering the composition of products because positional analysis data have shown which fatty acids are normally located at the sn-1 and sn-2 positions of mitochondrial phospholipids (6,26). For the present study we used the sum of
Regulation of mitochondrial iPLA2 activity accumulating linoleic acid (18:2), arachidonic acid (20:4), and docosahexenoic acid (22:6) as the indicator of phospholipase A2 activity. In sum, these fatty acids are present at a level of ~170 nmol/mg protein and constitute 83% of the acyl groups esterified at the sn-2 position (6). The results of FFA analysis are reported as totals of the three specified above and are in units of nmol/mg mitochondrial protein. Individual peaks were identified by their retention time, obtained by comparison to those of known components in a commercial standard mixture (Nu-Chek Prep, Elysian MN). Repetitive analysis of single samples has shown that the standard deviation for individual peak area values is approximately ± 4% (34). During this study we further determined that the standard deviation on maximal activity of the mitochondrial iPLA2, from rat to rat (animal variability) is ±16%.

All experiments were repeated twice, and in most cases three times, with the data shown being representative.

RESULTS

Effects of electron transport inhibitors;

In principle, activation of the iPLA2 upon deenergizing mitochondria could reflect a direct effect of proton motive force on the conformational state of the enzyme, or an interaction of enzyme with some other mitochondrial component that is affected by the energetic state. Electron transport chain complexes are prominent possibilities of components that might interact with the iPLA2, with the interaction affecting activity, and with the nature of the interaction affected by a change in the redox status of the complex. To examine the effect of individual electron transport chain complexes on iPLA2 activity we used well established inhibitors that leave chain components in particular states of reduction, and compared these states to enzyme activity. Results are shown in figure 1. The inhibitor rotenone blocks electron transport through complex 1, leaving that complex reduced when NADH linked substrates are present, while the other components become oxidized due to the presence of excess O2 (see scheme 1 and the associated table). In addition, rotenone leads to deenergization of mitochondria under these conditions, similar to the effect of the uncoupler CCCP (figure 2, Rotenone and CCCP). As seen in figure 1A, in mitochondria oxidizing glutamate plus malate the iPLA2 is active when rotenone is present, and the activity level is the same as when CCCP is present. These findings are consistent with the notion that the iPLA2 is activated by deenergization per se, but as already noted, mitochondrial components change their redox state upon deenergization, and it might be that it is the state of these that is actually affecting the enzyme. Among the various possibilities figure 1A tends to rule out an important role for the redox state of complex 1 because it will be reduced when rotenone is present but
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(substantially) oxidized in the presence of CCCP.

To further probe for possible effects of redox state on iPLA₂ activity we examined the effects of antimycin A, which inhibits electron transport at complex 3 (scheme 1). As seen in figure 1B, this agent also activates the enzyme. It is equally effective compared to rotenone in this regard and in promoting loss of membrane potential (figure 2, Antimycin A). Antimycin A, like rotenone, will cause a persistent reduction of complex 1 in the presence of an NAD⁺ linked substrate and will also produce a persistent reduced state of complex 3. Thus it would seem that as with complex 1, there is also no special role for the redox state of complex 3 in regulating the iPLA₂ since equal activity is seen when complex 3 is oxidized (rotenone present, antimycin absent) and when it is reduced (rotenone absent, antimycin A present).

Cyanide blocks electron transport at complex 4, resulting in reduction of the entire electron transport chain while also maintaining a state of deenergization (figure 2, KCN). Like the other inhibitors employed to this point, the iPLA₂ is active when cyanide is present (figure 1C).

The enzyme is also active when succinate plus rotenone is used as substrate and TTFA is present (figure 1D). The latter agent inhibits complex 2 (40,41) and deenergizes mitochondria that are oxidizing succinate [figure 3, TTFA (Succinate)]. It does not deenergize mitochondria when malate-glutamate are present together with succinate [figure 3, TTFA (Glutamate/Malate/Succinate)] or when malate-glutamate is used alone [figure 3, TTFA (Glutamate/Malate)]. Likewise, TTFA does not activate the iPLA₂ when NAD-linked substrates are present (figure 1D).

Thus, the iPLA₂ in liver mitochondria becomes active upon deenergization, regardless of what redox states in the electron transport chain are maintained by the agents used to produce deenergization. Oligomycin, which inhibits complex 5 (the ATP synthase complex) and so leads to depletion of ATP in the matrix space, has no effect on iPLA₂ activity (figure 1E) or on mitochondrial membrane potential under these conditions (figure 2, Oligomycin).

Reversibility and partial activation;

As further discussed below, one can imagine several physiological or pathophysiological processes wherein the energy dependence of mitochondrial iPLA₂ activity might be of significance. These possibilities are perhaps best considered within the context of whether or not activation of the enzyme is reversible, and in light of a more detailed understanding of the relationship between the extent of deenergization and the magnitude of enzyme activity. To examine the potential for reversibility, mitochondria oxidizing glutamate plus malate were deenergized with rotenone and maintained in that state for a period of 60 min. Succinate was then added to reestablish electron flow below complex 1 and
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the incubation was continued. As seen in figure 4A, succinate addition reestablished a large membrane potential and prevented further accumulation of free fatty acids during a subsequent period (figure 5A). There was furthermore a reduction in the preexisting level of free fatty acids which occurred during further incubation. These findings were confirmed by using antimycin A to produce deenergization and subsequently adding the artificial electron carrier TMPD in the presence of ascorbic acid. Those conditions reestablish electron flow to complex 4 and reestablished a significant membrane potential as expected (figure 4B). This membrane potential was also sufficient to thereafter limit activity of the iPLA$_2$ and a reduction in the preexisting level of free fatty acids was again observed (figure 5B)\textsuperscript{4}.

To investigate how a partial loss of membrane potential affects iPLA$_2$ activity methods were needed to generate various degrees of deenergization and to sustain these over an extended period. The activity of malonate as an inhibitor of succinate oxidation can be used in both regards, as illustrated in figures 6A and 6B. Increasing the concentration of malonate progressively decreases the succinate oxidase activity of respiring mitochondria with a half maximal effect seen at about 3 mM, and a maximal effect at about 15 mM, when the succinate concentration is 10 mM (figure 6A). As the succinate oxidase activity falls, the membrane potential also falls following a lag that is apparent at the lowest malonate concentrations employed. This lag in the concentration dependence can be attributed to the need for less than the available succinate oxidase activity to maintain a full potential when mitochondria are not under a significant load, but more importantly it is seen that the partially reduced membrane potentials produced at intermediate levels of malonate are stable for periods of 2 hrs or longer when an open incubation vessel is employed (figure 6B). This time frame allowed us to compare activity to membrane potential when the latter parameter was suppressed to different degrees. As seen in figure 6C, there is a clear relationship between these parameters wherein a partially reduced membrane potential produces a partial activation of the iPLA$_2$ that is maintained over time. In more detail, decreasing the membrane potential from about 170 mV to about 130 mV was sufficient to activate the iPLA$_2$ to $\frac{1}{2}$ of the maximal activity. Presuming that the same situations pertain \textit{in vivo} one can imagine that relatively modest energy loads would bring forth some activity of the iPLA$_2$ which would return to the resting, no activity level, when the energy load was dissipated.

\textit{Inhibition of induced activities by BEL:}

The effectiveness of BEL as an inhibitor of activity induced by the various inhibitors used here was also examined. As seen in figure 7, BEL was equally effective with all of them, while the fraction of activity inhibited was limited to ~50%. The inhibited fraction was not increased by using higher levels of BEL, by making a second addition at 1hr after the first, by using the (R) and (S) BEL enantiomers
Regulation of mitochondrial iPLA₂ activity individually, or by including an excess of EGTA in the medium (data not shown). The failure of EGTA to alter the fraction of BEL insensitive activity shows that the insensitive activity is not Ca²⁺-dependent.

DISCUSSION

As documented in the Introduction, there has been considerable effort directed at determining which proteins are responsible for releasing fatty acids from mitochondrial phospholipids. It is also important to understand how the process is regulated biochemically as investigate here. In our preceding papers describing iPLA₂ activity in liver mitochondria, we emphasized that deenergization is required to activate the enzyme (26,27). Chemical uncoupling, pore formation, and ion transport loads were emphasized as the approaches to deenergization. We can now add to this group a number of electron transport inhibitors, all of which are equally effective, and as effective as the approaches used earlier. This is significant because each of the agents used produce well-understood states of the electron transport chain in which the individual complexes are highly reduced or highly oxidized during continued incubation. Thus, in light of this data, it seems unlikely that enzyme activity is controlled by association with electron transport chain components, with the interaction changing as the redox state of the interactive partner changes, as once seemed probable.

The question now becomes what other consequences of deenergization might lead to activation of the iPLA₂, and do so in a graded and reversible fashion? The possibilities include a direct sensing of membrane potential by the enzyme whereby changes in potential alter conformation with attendant changes in activity, changes in the state of covalent modification (phosphorylation for example) affecting activity, changes in the level of small molecule effectors which are normally bound by the enzyme controlling its activity, or possibly an altered physical state of phospholipids which renders the enzyme more or less able to associate and act upon individual molecules. No firm choice between these possibilities can be made at present although some further points can be added.

It is first of all clear that small molecule effectors involved in activation would have to be inhibitors rather than activators. This is because activation by opening the permeability transition pore, or by adding alamethicin, will be accompanied by the release of all matrix space components up to a molecular weight of approximately 1,500 (42). Thus potential activators will be diluted by about $10^3$ when activation results from pore formation, rather than rising in concentration as one would expect if an activator was controlling. Likewise, control by phosphorylation/dephosphorylation would have to be
Regulation of mitochondrial iPLA₂ activity such that the phosphorylated form was poorly active while activation occurred by dephosphorylation. This is because ATP (and other nucleotides) is released when the enzyme is activated by pore formation and would not be available to support a phosphorylated state. Also arguing against a poorly activated phosphorylated form, which becomes active upon dephosphorylation, is the failure of oligomycin to produce activation. This is because this agent, by inhibiting ATP synthesis, should antagonize the phosphorylation. If dephosphorylation was proposed to activate the enzyme following loss of membrane potential, because ATP synthesis is interrupted, then one is left to wonder why oligomycin alone would not have the same effect. Thus, while several potential activating mechanisms can be ruled out, several others remain. At present we favor a direct effect of membrane potential on the enzyme because it is straightforward and supported by the failure of oligomycin to produce an activation, and because it does not conflict with any of other available data on regulation of activity.

While deenergization is clearly required to observe activity in rat liver mitochondria, this may not be the case in mitochondria from all sources. Schnellmann and coworkers, examining kidney mitochondria from rabbit, found that uncoupling was not required (31). Brustovetsky and coworkers showed that similar to kidney mitochondria, there is a background level of iPLA₂ activity observed in brain mitochondria without uncoupling. This level was increased by the presence of tBid plus full length Bax, possibly relating to ROS formation, and without requiring the permeability transition (32). Williams and Gottlieb, who identified an iPLA₂ in rabbit heart mitochondria (28), used an activity assay based upon an artificial and exogenous substrate, which itself may affect bioenergetics status. They also obtained their mitochondria from perfused hearts which were subject to ischemia/reperfusion and/or ischemic preconditioning, which does indeed affect their biochemical properties [e.g.(43)]. Thus, it is not clear from that study if the heart mitochondrial enzyme is regulated similarly to the enzyme in liver mitochondria.

More recently, Gross and coworkers have described how multiple products can arise from the gene encoding iPLA₂γ (44,45). These studies stop short of revealing how activity is controlled at the protein level, but have identified new possibilities for investigation. Included are the possible significance of a nucleotide binding site located near to the active site (46), activating effects of divalent cations, and inhibitory effects of long chain acylcoenzyme A. The latter two of these possibilities were seen when the enzyme was overexpressed (47). Within the context of activation by divalent cations (Ca²⁺ and Mg²⁺) it can be recalled that maintaining liver mitochondria in a high ionic strength medium (KCl) also yields a relatively high activity of the iPLA₂ (26). Thus, activating effects of electrolytes added to the medium should be evaluated within the context of how they affect ionic strength and the mitochondrial
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While it is clear that regulation of the mitochondrial iPLA$_2$ is not completely understood at the protein level, it can also be said that the same is true regarding the enzymes physiological and pathophysiological functions. We originally demonstrated that activity of the mitochondrial iPLA$_2$ promotes the permeability transition (26,27) and this has been verified and extended by other reports (31,48). That consequence of activity was expected, given the well established effects of FFA on the phenomenon (see Introduction). Thus to some degree, the question of mitochondrial iPLA$_2$ functions reduces to functions of the permeability transition. Apoptotic and necrotic cell death are prominent among the known functions of the transition and so the iPLA$_2$ is likely involved in those processes as well. In addition, it has long been maintained that the permeability transition, can be seen, in its most basic function, as a way to identify poorly functioning mitochondria within an intracellular population, and thereby to initiate their removal/replacement (35,49,50). The present findings remain consistent with that role since even a modest depolarization produces an acceleration of iPLA$_2$ activity which would raise the probability that that the transition occurs in the affected mitochondrion (figure 6).

Numerous other functions of iPLA$_2$ have been suggested as summarized by others (33,47,51). Some of these reflect the generation of free arachidonic acid from phospholipids and its expected conversion to lipid signaling molecules, including prostaglandins, HETES, thromboxanes, and leukotrienes. Local circulation through capillaries is influenced by some of these and in particular it is increased by prostaglandins of the E series [e.g.(52,53)]. Given the graded response of iPLA$_2$ activity to decreasing membrane potential (figure 6C), and the reversibility of activation, once a full potential is restored (figure 5), one can imagine that this enzyme provides for a signaling mechanism between mitochondria and the local circulation as regards the adequacy of blood flow. If flow were not adequate to meet the needs for O$_2$ and substrates, the membrane potential would decrease proportionately, iPLA$_2$ activity would rise increasing the availability of arachidonate, and prostaglandins produced from some of this would cause dilation of nearby capillaries. Once the blood supply became sufficient, the membrane potential would rise and enzyme activity would return to a near zero value. With the availability of iPLA$_2$ knockout mice (54-56), this possibility, and others can be further investigated.

A final point to consider here is the partial inhibition of apparent iPLA$_2$ activity produced by the mechanism-based inhibitor BEL. This was seen when BEL is used as a racemic mixture and regardless of which electron transport inhibitor was used to bring about deenergization. More specifically we found that BEL reduced activity by about 50% when it was assessed at 1 hr after deenergization and the BEL had been added at the beginning of the incubation (figure 7). This partial sensitivity to BEL,
Regulation of mitochondrial iPLA₂ activity together with the absence of an external Ca²⁺ requirement, provides evidence that an iPLA₂ participates in the accumulation of free fatty acids that we observed. However, it also suggests that multiple enzymes are participating, or that the duration of BEL effects on mitochondrial iPLA₂ is limited. It is now thought that iPLA₂ activities are derived as splice variants from either the PNPLA8 gene (iPLA₂γ) or from the PLA2G6 gene (iPLA₂β) (57). Gross and coworkers have knocked out the gene producing iPLA₂γ and have shown that this reduces activity against an exogenous substrate, but does not eliminate it in liver mitochondria (45,48). Those findings also allow for the participation of multiple enzymes in hydrolyzing mitochondrial phospholipids at the sn-2 position. In our first paper describing the presence of an iPLA₂ in liver mitochondria, we found that BEL eliminated about 85% of the activity (26). To explain that in light of the present finding we note that in the earlier paper the effectiveness of BEL was evaluated in media having a high ionic strength (KCl-based media). Total iPLA₂ activity is higher in those media than in the mannitol-sucrose media used here, but the BEL insensitive activity is about the same. Hence a larger fraction of total activity is inhibited by BEL at high ionic strength. We did not use high ionic strength in this study because mitochondria swell and lose cytochrome c during extended exposure to KCl-based media (27,58). Those changes are incompatible with goals which require that constant membrane potential be maintained on a time scale of hours. Further work will be required to establish the protein identity of the BEL insensitive activity.

In conclusion, while regulation of the mitochondrial iPLA₂ is not yet completely understood, data presented in this paper contribute to our understanding of how mitochondria likely respond to and modulate iPLA₂, activity under physiological and pathophysiological conditions that produce changes in energization. The reversibility of enzyme activity upon re-energization as well as the graded nature of the activation with partial deenergization fit well with models of signaling and regulatory mechanisms.
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FOOTNOTES

2Adam J. Rauckhorst and Kimberly M. Broekemeier made equal contributions to this investigation.

3Animal to animal variation was determined from six repeat experiments using different mitochondrial preparations, which examined iPLA$_2$ after uncoupling with CCCP. The variation is similar when other approaches to deenergization are employed, as can be seen in Figure 7.

4We did not determine the destination of the free fatty acids that were lost upon reestablishing a membrane potential, which amounted to about 0.3 nmol/mg protein. We did notice, however, that the use of Percoll gradient purified mitochondria or the presence of BEL did not alter that behavior.

5In addition to chelating any external Ca$^{2+}$, in media containing external Na$^+$, EGTA depletes mitochondria of endogenous Ca$^{2+}$ through action of the Na$^+$/Ca$^{2+}$ antiporter [see (10,11)] for review.
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Independent Phospholipase A2γ Leads to Alterations of Mitochondrial Lipid Metabolism and Function Resulting in a Deficient Mitochondrial Phenotype" J Biol. Chem. 282, 34611-34622


FIGURE LEGENDS

Fig 1. *Effect of Electron Transport Inhibitors on Mitochondrial iPLA₂ Activity*: Rat liver mitochondria were incubated at 1.0 mg protein/ml in the mannitol/sucrose based medium containing 10 mM each of glutamate and malate as respiratory substrates. The temperature was 25°C and cyclosporin A was added at the beginning of the incubation (0.5 nmol/mg protein). Other agents were added 2 min after mitochondria as follows. Panel A: ●, no addition. ○, rotenone (0.5 nmol/mg protein). ▼, the uncoupler CCCP (1 nmol/mg protein). Panel B: ●, no addition. ○, antimycin A (1 nmol/mg protein). Panel C: ●, no addition. ○, KCN (0.50 mM). Panel D: ●, 10 mM succinate was the respiratory substrate in absence of both glutamate and malate and rotenone was present from the beginning (0.5 nmol/mg protein). There was no further addition. ○, glutamate and malate were the respiratory substrates and TTFA was added (50 µM). ▼, same as ● except that TTFA was added in addition to rotenone. △, same as ● except that the CCCP was added. Panel E: Glutamate and malate were the respiratory substrates. ●, no addition. ○, oligomycin was added (1 nmol/mg protein). ▼, the uncoupler CCCP was added. △, same as ● except that oligomycin and CCCP were added.

Fig 2. *Effect of Electron Transport Inhibitors on Mitochondrial Membrane Potential*: Incubation conditions were those described in the legend to figure 1 except that the media contained 2 µM of TPP⁺ to allow monitoring of mitochondrial membrane potential as described in Experimental Procedures. A downward deflection indicates a reduced concentration of TPP⁺ in the medium due to an increase in the fraction accumulated. When used, inhibitors were added at 2 min. Trace labeled No addition: Glutamate and malate were the respiratory substrates and there was no further addition. Trace labeled Rotenone: same as trace labeled No addition, but rotenone was added at 0.5 nmol/mg protein. Trace labeled Antimycin A: same as No addition except that antimycin A was added at 1 nmol/mg protein. Trace labeled KCN: same as no addition except KCN was added at 0.5 mM. Trace labeled CCCP: same as no addition except CCCP was added at 1 nmol/mg protein. Trace labeled Oligomycin: same as no addition except oligomycin added at 1 nmol/mg protein.

Fig 3. *Effect of TTFA on Mitochondrial Membrane Potential*: Experiments are analogous to those shown in Fig. 2, but they examine the effects of TTFA as an inhibitor of complex 2. Trace labeled No addition: Succinate was the respiratory substrate in the presence of rotenone (0.5 nmol/mg protein) and there was no further addition. Trace labeled TTFA (Glut/Mal): 10 mM glutamate and 10 mM malate
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were the respiratory substrates. TTFA (50 µM) was added where indicated. Trace labeled TTFA (Glut/Mal/Succ): glutamate, malate and succinate were the substrates. TTFA was added at 50 µM. Trace labeled TTFA (Succ): Same as the trace labeled No addition (Succ) except that TTFA was added at 50 µM.

Fig 4. Restoration of Membrane Potential in Previously Deenergized Mitochondria:  Panel A: Mitochondria oxidizing glutamate and malate were deenergized with rotenone as shown, and were maintained in that condition for 1 hr. At that point, 10 mM succinate was added, or not added, as indicated in the figure and the incubations were continued for an additional hr. Panel B: Mitochondria oxidizing glutamate and malate were deenergized with antimycin A as shown, with 6 mM ascorbic acid having been present from the beginning. Deenergization was maintained for 1 hr. At that point, 100 µM TMPD was added, or not added, as indicated in the figure and the incubations were continued for an additional 1 hr. In both panels the media contained 2 µM TPP$^+$ in order to monitor changes in membrane potential.

Fig 5. Effect of Reenergization on iPLA$_2$ Activity:  Panel A: Conditions were the same as those described for figure 4A except that TPP$^+$ was not present. ○, 10 mM succinate was added after 1 hr of deenergization. ●, succinate was not added at 1 hr. In both cases the incubations were continued for an addition hr. Panel B: Conditions were the same as described for figure 4B except for the exclusion of TPP$^+$. ○, 100 µM TMPD was added after 1 hr of deenergization. ●, TMPD was not added. In both cases the incubations were continued for an additional 1 hr.

Fig. 6. Effect of Partial Deenergization on iPLA$_2$ Activity:  The medium contained 10 mM succinate plus rotenone (0.5 nmol/mg protein) as the respiratory substrate, with increasing concentrations of malonate present as indicated. Malonate was present from the beginning of incubations. Panel A: inhibition of basal succinate oxidation produced by increasing malonate. Panel B: decreasing membrane potential (decreasing TPP$^+$ accumulation) produced by increasing malonate. 2 µM TPP$^+$ was present in these incubations. Panel C: partial and stable activation of iPLA$_2$ produced by selected concentrations of malonate. ●, 1.25 mM malonate. △, 2.5 mM malonate. ○, 5.0 mM malonate. ▼, 10 mM malonate. Panel C inset: ●, iPLA$_2$ activity as a function of malonate concentration and determined as the total PUFA levels found after 60 minutes of incubation. ○, membrane potential determined at 60 minutes, as calculated from the data shown in panel B.
Fig. 7. Effect of Bromoenol Lactone (BEL) on the iPLA₂ Activity of Deenergized Mitochondria: Conditions were as described in the legend to figure 1. Glutamate plus malate were the respiratory substrates when deenergization was produced by CCCP, rotenone, antimycin A, or KCN. When TTFA was employed, glutamate and malate were replaced by succinate plus rotenone as also described in the legend to figure 1. The racemic mixture of BEL was used at 7 µM and when present was added 45 seconds after the mitochondria. Samples were taken at 1 hr and each bar shown represents the activity seen in response to the indicated inhibitor of respiration. The total height of the bar (open plus hatched portions) represents the activity seen when BEL was absent. The hatched area shows the activity that remained when BEL was present. For each inhibitor examined, data were obtained from four separate preparations of mitochondria. The error bars shown are standard deviations of the mean values. When comparing activities seen in the absence and presence of BEL by the paired Student’s-test, BEL present values were lower than BEL absent values at a confidence value of 0.005. When one way Anova was used to compare activities seen when the different deenergizers were employed (BEL absent or present), there were no significant differences at a level of 0.05.
Table 1. The oxidation/reduction status of the major electron transport chain components in the presence of selected inhibitors, when glutamate/malate is the respiratory substrate; * when succinate plus rotenone is the respiratory substrate; **when ascorbate plus TMPD is the respiratory substrate. The status of cytochrome a was checked by dual wavelength spectroscopy (605-630 nm) to verify that the inhibitors were behaving as expected. As used in the table, nd means not determined.
Regulation of mitochondrial iPLA₂ activity

Figure 1

A. No addition
- Rolename
- CCCP

B. No addition
- Antimycin A

C. No addition
- KCN

D. No addition
- TTFA (Glutamate/Malate)
- TTFA (Succinate)
- CCCP (Succinate)

E. No addition
- Oligomycin
- CCCP
- Oligomycin + CCCP
Regulation of mitochondrial iPLA₂ activity

Figure 2

 TPP⁺, μM
0.33 0.66 1.00 1.33 1.66 2.00

Time (min)
0 2 4 6 8 10 12 14 16

Inhibitor
Mito

No addition
Oligomycin

KCN
Rotenone
Antimycin A

Oligomycin
Regulation of mitochondrial iPLA$_2$ activity

Figure 3

 TPP$^-$, μM

Mito

Inhibitor

TTFA (Succ)

TTFA (Glut/Mal/Succ)

TTFA (Glut/Mal)

No addition

min
Figure 4

A. 

TPP⁺, µM

0 20 40 60 80 100 120 min

Mito

Succinate

±Succinate

Rotenone

No addition

± Succinate

B. 

TPP⁺, µM

0 20 40 60 80 100 120 min

Mito

TMPD

±TMPD

Antimycin A

No addition

± TMPD
Figure 5

A. 
- Succinate

- + Succinate

B. 
- TMPD

- + TMPD

PUFA, nmol/mg protein

min

0 20 40 60 80 100 120
Regulation of mitochondrial iPLA₂ activity

Figure 6

A.

O consumption, ng atom/min/mg

0 2 4 6 8 10 12 14 16

0 5 10 15 20

Malonate, mM

B.

TPP⁺, µM

0.33 0.66 1.0 1.33 1.66 2.00

0 20 40 60 80 100 120

Malonate, mM

0 0.4 0.6 0.8 1.0 1.2 1.4 1.6

PUFA, nmol/mg prot

0.0 0.5 1.0 1.5 2.0 2.5

0 0.2 0.4 0.6 0.8 1.0

Malonate, mM

0 2 4 6 8 10 12 14 16

0 5 10 15 20

Malonate, mM

0 20 40 60 80 100 120

Membrane potential, mV

0 100 200 300 400 500 600

Membrane potential, mV

0 100 200 300 400 500 600

Membrane potential, mV

0 100 200 300 400 500 600

Membrane potential, mV
Figure 7

[Diagram showing the regulation of mitochondrial iPLA$_2$ activity with various inhibitors.]