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Mitochondrial Phospholipase A_2 Activity and Mitochondrial Aging†

J. Wallace Parce,‡ Carol C. Cunningham,* and Moseley Waite§

ABSTRACT: The changes in mitochondrial phospholipid metabolism and energy-linked functions have been followed as coupled mitochondria are allowed to age in isotonic sucrose at 18 °C. Analysis of the aging process has provided an approach for studying the structure-function relationships within the mitochondrion without adding external agents to perturb the membrane structure. The initial event observed in this process of deterioration is a loss of respiratory control which is paralleled by diminishing levels of ATP. As ATP levels decline, so do the rates of reacylation of monoacylglycerophosphorylethanolamine and fatty acid oxidation. In most cases the previously inactive phospholipase A_2 (EC 3.1.1.4, phosphatide 2-acyl-hydrolase) begins rapid hydrolysis of membrane phosphatidylethanolamine as ATP levels approach zero. The final energy-linked phenomenon observed to decline is the anilinonaphthalenesulfonic acid fluorescence response. Evidence is presented which suggests strongly that the activity of the mitochondrial phospholipase A_2 on endogenous phospholipids is suppressed in tightly coupled mitochondria. This suppression is temporally linked to ATP levels in the mitochondrion. Furthermore, this study demonstrates that mitochondria which are only slightly damaged have the potential to effect membrane repair through reacylation of monoacyl phospholipids.

In the past few years the process of mitochondrial aging has received considerable attention (Waite et al., 1969a; Scarpa & Lindsay, 1972; Cheah et al. 1973; Siliprandi et al., 1973; Jurkowitz et al., 1974; Ozelkok et al., 1974; Yamaguchi & Satomura, 1974). Analysis of this process provides an approach for studying the structure-function relationships within the mitochondrion without adding external agents to disrupt the membrane. A number of the above studies have suggested that the endogenous phospholipase A_2 may be involved in the deterioration of mitochondrial energy-linked functions. Waite et al. (1969a) have shown a direct relationship between endogenous phospholipase A_2 activity and irreversible mitochondrial swelling, a change generally associated with progressive loss in energy-linked functions (Jurkowitz et al., 1974). Moreover, Scarpa & Lindsay (1972) have presented evidence suggesting that the phospholipase is partially responsible for the loss in respiratory control observed during the aging process.

In the present study we have investigated more completely the participation of the endogenous phospholipase A_2 in the mitochondrial aging process. In order to determine the contribution of this enzyme in the loss of energy-linked properties we have measured simultaneously the changes in RCR,[1] ATP levels, the energy-linked Ans fluorescence response, and phospholipase A_2 activity as mitochondria were aged at 18 °C. We have also measured the kinetics of loss of monoacyl phospholipid reacylation during the aging process. Moreover, this

† From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103. *Received October 4, 1977.* This work was supported by North Carolina Heart Association Grants 1973-74A-5, 1974-75-A-29, and 1975-76-A-5, National Science Foundation Grant GB 36345, and National Institutes of Health Grant AM 11799.

‡ Portions of this work were taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Wake Forest University, Winston-Salem, North Carolina. Present address: Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305.

§ Recipient of U.S. Public Health service research career Development Award AM 17392.

[1] Abbreviations used: RCR, respiratory control ratio; Ans, 1-anilino-8-naphthalenesulfonic acid; diacyl-GPE, diacylglycerophosphorylethanolamine; monoacyl-GPE, monoacylglycerophosphorylethanolamine; FFA, free fatty acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; diacyl-GPC, diacylglycerophosphorylcholine.

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approach has allowed us to determine some of the interrelationships between energy-linked functions and lipid metabolism in the mitochondrion.

Preliminary reports of this work have appeared elsewhere (Parce & Cunningham, 1974; Parce et al., 1976).

Experimental Procedures

Materials. Sucrose for the preparation and aging incubation of the mitochondria was purchased from Schwarz/Mann (special enzyme grade). The 8-anilino-1-naphthalenesulfonic acid was obtained from Sigma Chemical Co. and recrystallized twice from water as the magnesium salt. ATP, ADP, and rotenone were also purchased from Sigma. Sodium succinate, NADP, glucose-6-phosphate dehydrogenase, and hexokinase were purchased from Boehringer Mannheim. The [2-^{14}C]ethan-1-ol-2-amine hydrochloride was obtained from Amersham/Searle Corp., and [^{3}H]oleic acid was from New England Nuclear. Pentadecanoic acid was obtained from Analabs, and FCCP was a gift from Dr. P. G. Heytler, E. I. duPont deNemours and Co., Inc.

Methods. Male Sprague-Dawley rats (8–12 weeks old) were injected intraperitoneally with 20 μCi of an aqueous solution of [2-^{14}C]ethan-1-ol-2-amine hydrochloride (20 Ci/mol) 40 min prior to sacrifice. Liver mitochondria were then isolated by the method of Schneider (1948). With this procedure freshly prepared mitochondria generally have 92% of the total mitochondrial radioactivity in diacyl-GPE, 6% in diacyl-GPC, and 2% in monoacyl-GPE. Mitochondria were then incubated in 0.25 M sucrose at 30 mg/mL either in a test tube in an 18 °C water bath or in a water-jacketed vial held at 18 °C and attached to a magnetic stirrer. These incubation procedures are referred to as the aging incubation. At various times during the aging incubation aliquots of mitochondria were removed and assayed for respiratory control, energy-linked Ans fluorescence response, ATP levels, diacyl-GPE hydrolysis, calcium-stimulated phospholipase A_2 activity, oleic acid incorporation into acyl-GPE and FFA levels.

Respiratory control ratios were measured by the method of Estabrook (1967) using a Clark electrode. During a single measurement, ADP was added three times. Mitochondrial ATP levels were measured using the hexokinase, glucose-6-phosphate dehydrogenase couple and following NADP reduction fluorometrically (Williamson & Corkey, 1969). The energy-linked Ans fluorescence response (Azzi 1969; Azzi et al., 1970; Datta & Penefsky, 1970; Layton et al., 1974) was measured as follows. Three-tenths of a milliliter of mitochondria, 30 mg/mL protein, was added to a stoppered 3-mL cuvette containing 2.7 mL of a fluorescence assay mixture which gave final concentrations of 0.3 M sucrose, 20 mM Tris-HCl, pH 7.4, 10 μM rotenone, and 100 μM Ans. The fluorescence was measured in an Aminco Bowman fluorometer with front face optics (excitation 335 nm, emission 465 nm). Succinate was added with mixing to give a final concentration of 10 mM, and the resulting decrease in fluorescence was recorded. After the relative emission reached a minimum, FCCP was added to give a final concentration of 0.67 μM, and the relative emission was recorded until it reached a maximum. The percent change in relative emission was calculated as the highest relative emission minus the lowest relative emission divided by the average of the highest and the lowest relative emission.

The above procedure was modified to determine the energy state of mitochondria that were being maintained at 18 °C. A flow through device was attached to a Farrand fluorometer such that the mitochondrial suspension could be drawn up into the cuvette for immediate fluorescence analysis without being exposed to air. The cuvette was a 1.0-mm quartz capillary which allowed fluorescence measurements with very turbid samples. A small volume of a concentrated Ans solution was added to mitochondria (30 mg of protein/mL) to give a final concentration of 100 μM. These mitochondria were then incubated at 0 or 18 °C, and the fluorescence assays were performed as follows. A 1.0-mL syringe was attached to the free end of the tubing which connected to the top of the capillary cuvette, and 1.0 mL of anaerobic deionized water was flushed through the system. The free end of the tubing attached to the bottom end of the capillary cuvette was then immersed in the mitochondrial preparation and 0.1 mL of the suspension was drawn up into the fluorometer. The fluorescence of this sample was recorded immediately. The sample was then ejected from the cuvette into a small test tube which contained 1.0 nmol of FCCP. The sample was then mixed rapidly and drawn back into the fluorometer. The fluorescence response was then recorded until the signal was stable. Respiratory control was constant in mitochondria preparations throughout the time required for these measurements.

To measure calcium stimulated phospholipase A_2 activity, 4.5 mg of mitochondria (aliquot B) was added to an assay mixture comprised of 50 mM Tris-HCl, pH 7.5, 0.20 M sucrose, and 2 mM $CaCl_2$. This mixture was incubated for 30 min at 37 °C. The reaction was quenched by addition of 3 mL of chloroform:methanol (1:2, v/v). The lipids were extracted by the method of Bligh & Dyer (1959), brought to dryness under a stream of N_2, and redissolved in chloroform:methanol (9:1, v/v). For measurement of diacyl-GPE hydrolysis occurring during the aging incubation, an aliquot of mitochondria (aliquot A) from the incubation suspension was added to the assay mixture and the lipids were immediately extracted by the method of Bligh & Dyer and concentrated as described above. The extracts from both aliquots A and B were fractionated by thin-layer chromatography on silica gel G plates and developed in chloroform–methanol–H_2O–NH_4OH system (65:35:2:3, v/v). The plates were exposed to iodine vapors and the spots corresponding to monoacyl-GPE, diacyl-GPC, and diacyl-GPE were scraped into scintillation vials and counted in 10 mL of a scintillation fluid composed of toluene, Triton X-100, and water in a ratio of 2:1:0.2 (by volume) containing 2.9 g of Omnifluor/L. The percent diacyl-GPE hydrolysis was calculated as [cpm in monoacyl-GPE/cpm in monoacyl-GPE + cpm in diacyl-GPE] × 100. For aliquot A this was the percent diacyl-GPE hydrolyzed at 18 °C. The percent diacyl-GPE hydrolyzed during incubation for 30 min (Ca^{2+} stimulated activity) was determined as the percent diacyl-GPE hydrolyzed in B minus the percent diacyl-GPE hydrolyzed in A.

To measure monoacyl phospholipid reacylation, 4.5 mg of mitochondria from the aging incubation was added to an assay mixture comprised of 50 mM Tris-HCl, pH 7.5, 0.2 M sucrose, 2 mM $CaCl_2$, and 18.4 pmol of [^{3}H]oleic acid (90 nCi). The resulting mixture was incubated at 37 °C for 30 min, and the lipids were extracted and concentrated as described above. The lipids were fractionated on silica gel G plates developed first in chloroform–methanol–acetic acid (90:12:2, v/v) and then in the same direction with chloroform–methanol–H_2O–NH_4OH (65:35:2:3, v/v). The lipids were exposed to iodine vapors, and radioactivity in the individual phospholipids and free fatty acids was determined as described above. The β oxidation of [^{3}H]oleate was estimated by the decrease in recovery of [^{3}H]oleate in the organic phase of the lipid extraction mixture obtained at the end of the reacylation assay. This calculation assumes that loss of fatty acid occurs primarily by β oxidation in isolated mitochondria.

FFA levels were measured by the following procedure. At

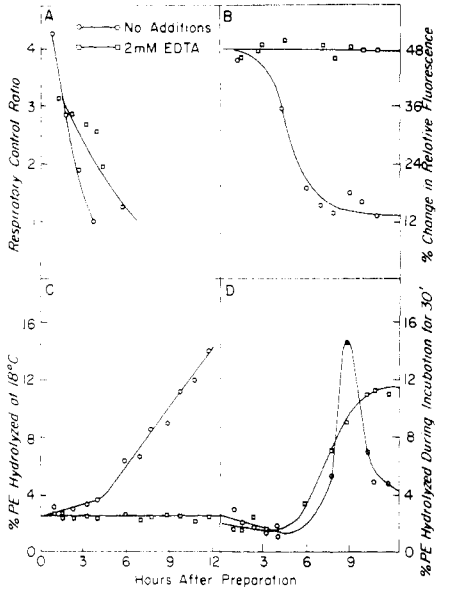

FIGURE 1: Protection of mitochondrial functional and structural integrity by addition of EDTA. The mitochondrial preparation was divided in half, and both tubes were placed in an 18 °C water bath. One tube was made 2 mM with respect to EDTA by the addition of a small volume of concentrated EDTA solution (□—□). The other tube served as a control without EDTA (○—○). The assays are described in the Methods section. In this and all following figures the abbreviation PE is used for diacylglycerophosphorylethanolamine.

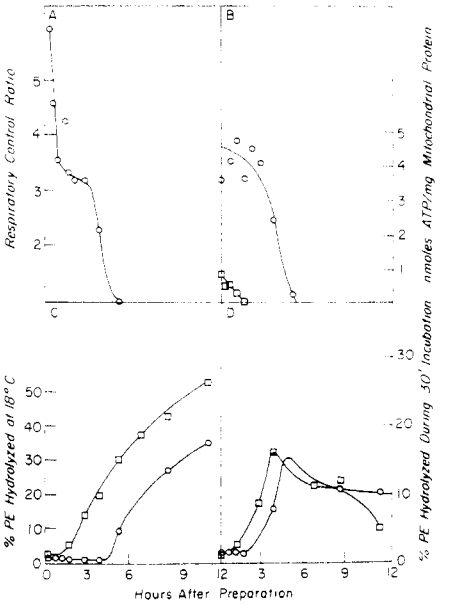

FIGURE 2: Effect of FCCP on monoacyl-GPE production. The mitochondrial preparation was divided in half. Both halves were placed in an 18 °C water bath immediately after preparation, and one-half (□—□) was titrated with enough FCCP to cause complete loss in respiratory control. The other half (○—○) served as the control. The various assays are described in the Methods section.

the times indicated 0.3-mL aliquots were removed from the aging incubation suspension and the lipids were extracted by the Bligh & Dyer technique. Ten microliters of a 5.0 mM solution of pentadecanoic acid in methanol was added to each sample during the extraction procedure prior to the point at which two phases were formed. The extracted lipids were then separated by thin-layer chromatography on silica gel H plates developed in a petroleum ether–diethyl ether–formic acid system (70:30:1.5, v/v), and the thin-layer plates were dried under N_2. The lipids were detected under ultraviolet light after the plates were sprayed with 0.1% 2,7-dichlorofluorescein in 95% ethanol. The silica gel containing the FFA was scraped from the plate. The FFA were then extracted from the silica gel by the Bligh & Dyer procedure, blown dry under N_2, and redissolved in 5.0 mL of dry methanol in preparation for esterification. Methylation was carried out using the procedure of Morrisett et al. (1975). The fatty acid methyl esters were analyzed on a Barber-Colman gas chromatograph with a column packing of 10% Silar 10 C on 100–120 mesh Gas Chrom Q. Micrograms of FFA in the mitochondria were calculated based on the internal standard pentadecanoic acid.

Results

The relationship between the changes in RCR, percent change in relative fluorescence, diacyl-GPE hydrolysis, and phospholipase A_2 activity as the mitochondria are incubated at 18 °C is illustrated in Figure 1 (curves designated by the circles). (See paragraph at the end of this paper concerning supplementary material.) At the beginning of the aging incubation, the respiratory control ratio exhibits the typical decay (Baldwin et al., 1975) reaching a value of 1 in 3 to 6 h. In general, the time at which respiratory control approaches 1.0 (Figure 1A) is the same as the time at which the rapid phase of diacyl-GPE hydrolysis begins (Figure 1C). Prior to complete loss in respiratory control, there is no apparent hydrolysis of diacyl-GPE. In contrast to the kinetics for loss of RCR, the percent change in relative fluorescence exhibits a lag before it begins to decrease (Figure 1B). In calcium-stimulated phospholipase assays (Figure 1D), the level of monoacyl-GPE was observed to stay constant or decrease slightly until the mitochondria lose respiratory control even though Ca^{2+} levels are in excess (2 mM). At this point the Ca^{2+}-stimulated phospholipase A activity increases rapidly. The decrease in activity toward the end of the experiment is probably due to decreased level of substrate, diacyl-GPE, or to product inhibition by the high amount of monoacyl phospholipid (Waite et al., 1969a). In this study we did observe some variability in the rates at which these mitochondrial properties changed when comparing preparations. The same temporal sequence for alterations in properties was always observed, however, with respiratory control being lost before either phospholipase A activity was expressed or the energy-linked fluorescence response declined.

Figure 1 also shows the results obtained when the 18 °C aging incubation suspension is made 2 mM with respect to EDTA (squares). In Figure 1C, mitochondria from the same preparation incubated in the presence of EDTA show no diacyl-GPE hydrolysis during the 12-h incubation period. Phospholipase A_2 activity can be restored by adding back Ca^{2+} ions as shown in Figure 1D. EDTA does extend the period of respiratory control as expected (Scarpa & Lindsay, 1972); however, even in the complete absence of diacyl GPE hydrolysis, respiratory control is still lost. The percent change in relative fluorescence as shown in Figure 1B is maintained in the presence of EDTA. Mitochondrial swelling, measured as turbidity decrease at 520 nm, was lower at all times in samples incubated in the presence of EDTA. Furthermore, very little swelling was observed in either control or EDTA-containing samples until after respiratory control is lost.

The observation that monoacyl-GPE levels do not increase until respiratory control is lost prompted us to examine the possibility of an energy-linked inhibition of the phospholipase. The effect of depletion of mitochondria with respect to ATP is shown in Figure 2. A mitochondrial suspension was divided in half immediately after preparation, and one-half was titrated

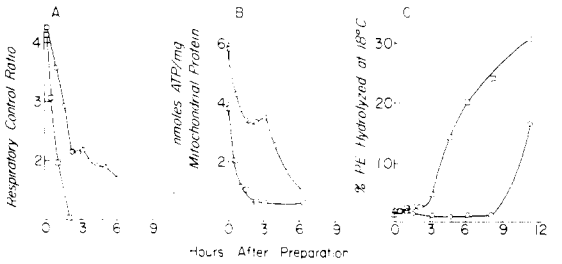

FIGURE 3: Effect of anaerobiosis on mitochondrial properties and activities. In the last wash step of the mitochondrial preparation, equal volumes of suspended mitochondria were placed in two separate tubes and centrifuged. One pellet was resuspended in a volume of 0.25 M sucrose to give a 30 to 40 mg/mL mitochondrial suspension (O—O). The other pellet was resuspended in the same volume of sucrose which had been previously made anaerobic by bubbling with N_2 (□—□). This sample was held anaerobic by passing a stream of N_2 over the surface of the suspension for the entire incubation period.

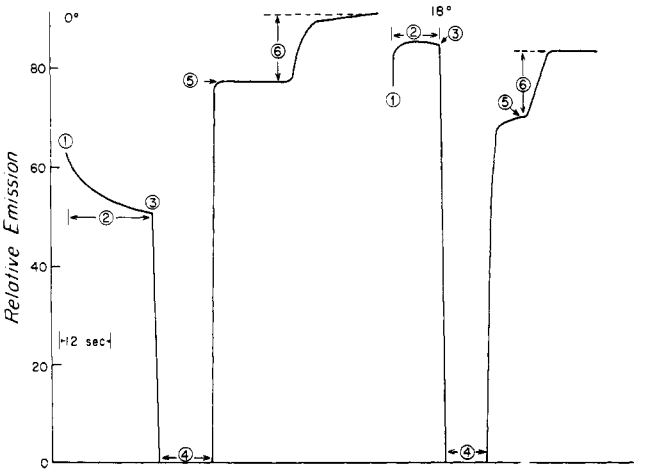

FIGURE 4: Energy state of the inner membrane in the aging incubation at 0 and 18 °C. The detailed procedure is outlined in the Methods section. The numbers on the figure designate the following steps and/or events: (1) the mitochondria–Ans mixture was drawn into the capillary cuvette; (2) period of thermal equilibration; (3) the mitochondria–Ans mixture was forced from cuvette into tube containing FCCP and mixed (equilibration with atmosphere occurred); (4) time for step 3; (5) Ans fluorescence level in presence of FCCP; (6) change in Ans fluorescence as mitochondrial sample becomes anaerobic.

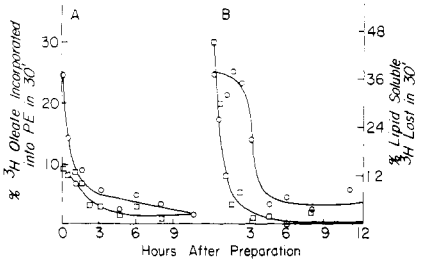

FIGURE 5: Effect of anaerobiosis on mitochondrial monoacyl phospholipid reacylation and β-oxidation activities. This experiment was carried out with the same mitochondrial preparation used in Figure 3 and the conditions are described in the legend for Figure 3.

with enough FCCP to cause complete uncoupling (RCR = 1). The rate of decline of respiratory control ratios and of ATP levels for the control mitochondria is similar (Figures 2A and 2B). Again, the rapid phase of diacyl-GPE hydrolysis began at about the time respiratory control reached one and ATP levels approached zero (Figures 2C and 2D). ATP levels in the mitochondria treated with FCCP decreased very rapidly, and consequently the lag period for net diacyl-GPE hydrolysis was very short (Figures 2C and 2D).

The effect of an anaerobic mitochondrial environment on the aging phenomenon is shown in Figure 3. One-half of the mitochondrial preparation was resuspended in an anaerobic sucrose solution and maintained anaerobic by passing a stream of nitrogen over the surface of the mitochondrial suspension. Both the respiratory control ratio and ATP levels of mitochondria in an anaerobic environment declined more rapidly than they did in the control mitochondria (Figures 3A and 3B). Likewise the lag period in net diacyl-GPE hydrolysis was much shorter for the anaerobic mitochondria (Figure 3C).

The above observations suggest that the depression of phospholipase A activity during the early stages of in vitro aging is the result of an energy-linked inhibition of the enzyme. Possibly the energy state of the mitochondrial inner membrane controls the expression of the phospholipase. In the following experiment the energy state of the inner membrane was measured in mitochondria that were being incubated at 18 °C. This measurement was carried out by determining the energy-linked Ans fluorescence response in mitochondria in the *absence* of added substrate. In this experiment Ans was added to the aging incubation mixture and aliquots of the Ans–mitochondria complex were monitored for Ans fluorescence as described in the Methods section. The results of this experiment are shown in Figure 4 for mitochondria stored at 0 and 18 °C. For mitochondria stored at 0 °C there is a short period of thermal equilibration in the fluorometer during which time the fluorescence decreased. During this period of thermal equilibration the oxygen content of the sample is not exhausted. Addition of FCCP to the sample resulted in an increase in fluorescence which was followed by a further increase as the cuvette became anaerobic. When the same procedure was applied to mitochondria incubated at 18 °C, a stable initial fluorescence value was obtained immediately. Addition of FCCP caused a decrease in fluorescence which was then followed by an increase as the cuvette became anaerobic. The initial fluorescence and the final anaerobic fluorescence were essentially the same. These data indicate that fresh mitochondria stored at 30 mg/mL at 0 °C demonstrate an energy-linked Ans fluorescence response that can be dissipated by FCCP. When these mitochondria are incubated at 18 °C, however, no energy-linked fluorescence response is noted. The addition of FCCP requires mixing, at which time some oxygen is introduced into the sample. The decrease in fluorescence observed after addition of FCCP indicates that the mitochondria are essentially anaerobic when stored at 18 °C. Direct measurements with a Clark electrode demonstrate less than 2% oxygen saturation at 18 °C in these suspensions. Higher levels of oxygen saturation in mitochondrial suspensions at 0 °C are a result of decreased rates of electron transport and the increased solubility of oxygen in water at the lower temperature. Phospholipase A activity was suppressed completely during the time required for these measurements; therefore, it was not responsible for the lack of an energy-linked Ans fluorescence response at 18 °C. Moreover, this experiment (Figure 4) demonstrates that the expression of phospholipase A activity is not controlled by the energy state of the mitochondrial membrane.

The depression of phospholipase A activity during the period when ATP levels were measurable suggested the possibility that the lack of monoacyl-GPE accumulation could be a competition between the phospholipase and the reacylase system. Figure 5A demonstrates monoacyl phospholipid reacylation in mitochondria and the effect of anaerobiosis on such activity. A maximum of 25% of the lipid extractable tritium

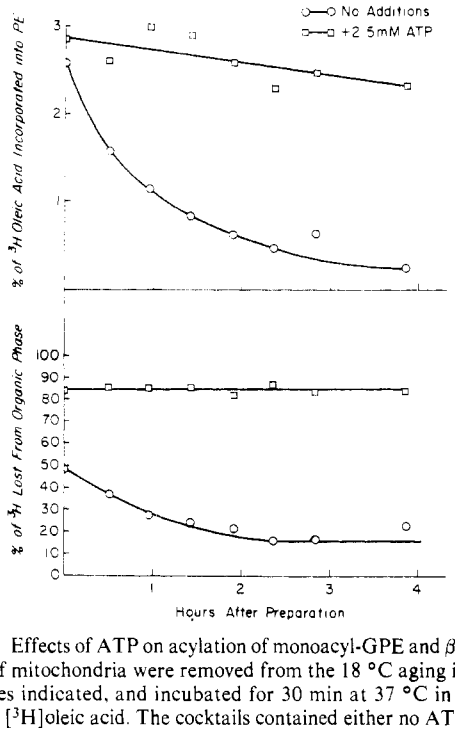

FIGURE 6: Effects of ATP on acylation of monoacyl-GPE and β oxidation. Aliquots of mitochondria were removed from the 18 °C aging incubation at the times indicated, and incubated for 30 min at 37 °C in a cocktail containing [^{3}H]oleic acid. The cocktails contained either no ATP (O—O) or 2.5 mM ATP (□—□).

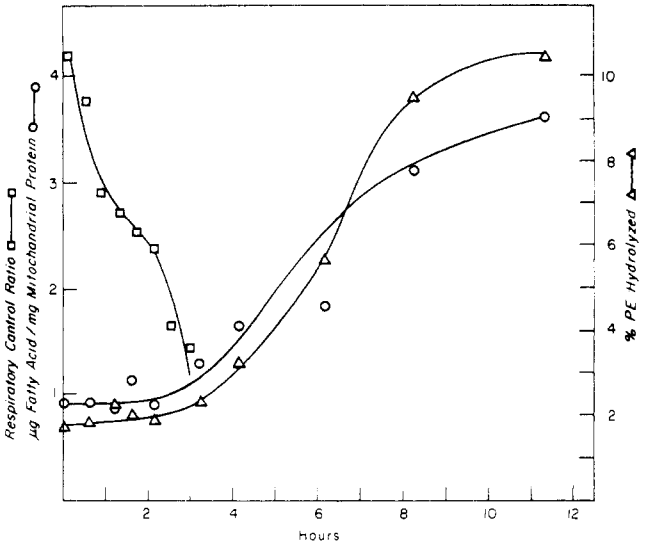

FIGURE 7: Relationship between FFA and monoacyl-GPE levels during mitochondrial aging. Aliquots were removed from the aging incubation at the times indicated and assayed for respiratory control (□—□), monoacyl-GPE levels (△—△), and FFA levels (O—O).

remaining at the end of the assay period was incorporated into diacyl-GPE. Like respiratory control ratios and ATP levels (Figures 3A and 3B), the reacylase activity diminished more rapidly in anaerobic mitochondria than in control mitochondria. In both cases the reacylase activity was very low before phospholipase A_2 activity (Figure 3C) was expressed. Figure 5B demonstrates that the decrease in β-oxidative activity is also much more rapid in the anaerobic mitochondria than in the control.

The decrease in the β-oxidative and reacylase activities is the result of diminishing levels of ATP in aging mitochondria, as is seen in Figure 6. Control mitochondria with no ATP added demonstrate a rapid decay of both reacylase and β-oxidative activities. Maintenance of both of these activities throughout the period of respiratory control is accomplished by the addition of ATP, but not coenzyme A, to the incubation medium. Furthermore, ATP, but not coenzyme A, maintained the capability of the mitochondrial system to reacylate monoacylphosphatidylcholine (data not shown).

Mitochondrial FFA levels also were measured during the aging process (Figure 7). The initial concentration of FFA (3 nmol/mg of mitochondrial protein) remains constant until the levels of monoacyl-GPE begin to increase. The level of FFA then increases in parallel with the level of monoacyl-GPE, as would be expected since there is no mechanism (β oxidation or reacylation) at this point for removal of FFA from the system. Moreover, this low and constant level of FFA observed during the period while respiratory control is being lost suggests that FFA from other contaminating organelles (Boime et al., 1970) are not contributing to loss in energy-linked functions in the mitochondria.

Discussion

In this study we have defined more clearly the temporal sequence of events which occur when mitochondria are allowed to deteriorate under controlled in vitro conditions. Among the first properties to decline during the 18 °C aging process is respiratory control. This is closely paralleled by a decrease in endogenous levels of ATP. The rates of reacylation of monoacyl phospholipids and of fatty acid oxidation also decrease at this time. After the loss of those initial parameters the magnitude of the Ans fluorescence response begins to diminish. Phospholipase A activity is not expressed until respiratory control is lost. Moreover, the increase in the amount of monoacyl phospholipids due to phospholipase A activity can be temporally correlated with the decline in the energy-linked fluorescence response.

Our observation that there is no measurable phospholipase A activity during respiratory control is in contrast to earlier experiments (Waite et al., 1969a; Scarpa & Lindsay, 1972) where no lag in phospholipase A activity on endogenous phospholipids was noted. Experimental conditions such as the medium used for isolation and incubation of mitochondria, the temperature of incubation, and times at which samples were taken for phospholipase A activity measurements (Waite et al., 1969a; Scarpa & Lindsay, 1972) provide possible reasons for the apparent discrepancy in results. Moreover, our observations, when combined with those of Scarpa & Lindsay (1972), suggest that while the phospholipase A can facilitate loss in energy-linked functions such as respiratory control and the Ans fluorescence response, the expression of its activity is not required for loss of energy-linked functions. By poising our incubation conditions as described we have been able to separate loss of respiratory control from apparent hydrolysis of phosphatidylethanolamine. This does not eliminate the possibility that boundary lipids around a protein involved in energy conservation are not being altered during loss in respiratory control, however.

There are several possibilities which might explain the delayed expression of phospholipase A activity. The possibility that the activity has been masked during the lag period by reacylation of monoacyl phospholipids has been considered. This investigation indicates, however, that the rate of reacylation is not sufficient to maintain the low levels of monoacyl phospholipids observed if the phospholipase were fully active (see paragraph at end of this paper concerning supplementary material). Furthermore, reacylation activity has ceased before diacyl-GPE hydrolysis begins. If there were a competition between reacylation and diacyl-GPE hydrolysis, one would expect to see an increase in levels of monoacyl-GPE corre-

sponding to a decrease in reacylase activity; this, also, is not the case.

An alternative to explain the initial lag is an inhibition of the phospholipase. Since the enzyme activity is not expressed while there is measurable respiratory control, and therefore relatively high ATP levels, we have considered that there is an energy-linked inhibition of the phospholipase. Inhibition could result from a particular metabolite associated with a high energy state in the mitochondrion (ATP) or an energized state of the membrane. Measurements of the state of energization of the membrane, as measured by the energy-linked Ans fluorescence response (Figure 4), indicate that at 18 °C the inner membrane is not energized. This is probably due to the low levels of oxygen observed at 18 °C in these suspensions. We therefore feel that the observed inhibition of the phospholipase is related more to the levels of ATP than to the energy state of the mitochondrial membrane. The ATP and divalent cation levels in rat liver mitochondria (Munn, 1974), as well as the observed inhibition of the phospholipase by low levels of ATP in in vitro assays (Waite et al., 1969b), suggest that ATP could inhibit by chelating calcium, an essential cofactor of the phospholipase. The observation that added calcium does not stimulate phospholipase A activity at early times during the aging incubation would appear to contradict the above suggestions on the role of ATP in controlling the activity of the phospholipase. However, in coupled mitochondria the phospholipase may be inaccessible to exogenous calcium due to the organization of the mitochondrial membranes and the location of the phospholipase within the mitochondrion (Waite, 1969; Marinetti et al., 1976).

Several studies have been reported on techniques for maintaining energy-linked functions in isolated mitochondria, and some attention has been given to restoration of these functions after significant decay has ensued. Both Ozelkok & Romani (1974) and Siliprandi et al. (1973) have established conditions for increasing the RCR of aged mitochondria from one back to near original levels. Under the conditions used in either of these studies (EDTA present; incubation for 6 h at 0–4 °C) little or no phospholipase A activity would have been expressed. It is likely that this reversibility of damage can occur only in the absence of measurable phospholipase A activity. It is likely that the phospholipase, once expressed, contributes to the irreversible loss in mitochondrial metabolic functions by hydrolyzing membrane phospholipids. Figure 1 suggests that the phospholipase may contribute to the loss in the energy-linked Ans response, a property which is purported to monitor the energization of the mitochondrial inner membrane (Azzi & Montecucco, 1976; Gains & Dawson, 1976; Williams et al., 1977). Indeed, in carrying out this study we have noticed by comparing mitochondrial preparations that the rate of loss of the Ans fluorescence response is proportional to the rate of emergence of the phospholipase A activity.

In conclusion we propose that there are two main stages in the aging process, the first being a reversible loss in energy-linked functions including the decline of RCR and ATP levels. In this stage the loss in reacylase activity and β oxidation are shown to be due to the loss of ATP. The loss in the above mentioned parameters is followed by the irreversible loss in energy-linked functions such as the ability of the membrane to produce an energized state as measured by the Ans fluorescence response. This irreversible phase is facilitated by the action of the phospholipase on membrane phospholipids. We are now focusing our attention on the reversible stage of the aging process in order to determine the reason(s) for the loss of respiratory control.

Supplementary Material Available

A supplementary figure showing energy-linked 1-anilino-8-naphthalenesulfonic acid fluorescence in mitochondria and computations concerning fatty acid turnover in mitochondria (3 pages). Ordering information is available on any current masthead page.

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