BBA 45908

STRUCTURAL AND RESPIRATORY EFFECTS OF *AGKISTRODON PISCIVORUS* PHOSPHOLIPASE A ON RAT LIVER MITOCHONDRIA

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(Received October 20th, 1969)

SUMMARY

1. Oxygen electrode tracings show that isolated *Agkistrodon piscivorus* phospholipase A alters mitochondrial respiration and phosphorylation in a manner identical to whole venom: at low concentrations it increases mitochondrial respiration in the absence of phosphate acceptor; at high concentrations it causes severe inhibition of electron transport; and, at intermediate concentrations it produces a stage of respiratory decline in which ADP acts as an inhibitor (phosphate acceptor inhibition).

2. Electron microscope studies confirm that whole *A. piscivorus* venom and isolated phospholipase A produce identical morphological alterations in mitochondria and that structural disruption accompanies respiratory decline.

3. Serum albumin can reverse the uncoupling caused either by whole *A. piscivorus* venom or its purified phospholipase A.

4. Incubation in the presence of succinate, or succinate and ADP, protects mitochondria against the respiratory damage produced by phospholipase A; but incubation in the presence of glutamate–malate affords no protection.

5. There is a remarkable similarity among the overall effects produced on mitochondria by venom phospholipase A, the classical uncouplers and oleic acid.

INTRODUCTION

In 1954, NYGAARD *et al.*[1] obtained electron micrographs which suggested that morphological changes accompanied the hydrolysis of mitochondria by crotoxin, a partially purified phospholipase A from the venom of *Crotalus terrificus terrificus*. Other workers using different partially purified venom phospholipase A's have also arrived at the same conclusion as a result of studying changes in mitochondrial absorbance due to swelling or performing thin-layer chromatography on venom-treated mitochondria[2], but conclusive evidence using a purified venom phospholipase A has been lacking.

We have isolated an essentially homogeneous low molecular weight protein

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showing phospholipase A activity from the venom of *Agkistrodon piscivorus*[3] and the comparison of the respiratory and structural alterations produced by whole venom with those produced by purified phospholipase A is presented in this communication.

MATERIALS AND METHODS

A. piscivorus phospholipase A was prepared as described by AUGUSTYN AND ELLIOTT[3].

Rat liver mitochondria were prepared in 225 mM mannitol–75 mM sucrose–0.1 mM EDTA (pH 7.4)[4]. The reaction medium contained 45 mM mannitol–15 mM sucrose–40 mM KCl, 20 mM $MgCl_2$–20 mM KH_2PO_4 (pH 7.4)–0.02 mM EDTA (pH 7.4). Measurements of respiratory and phosphorylative activity were determined polarographically[5] in a sealed vessel[6].

In studies in which mitochondria were incubated with phospholipase A for long periods of time, incubations were carried out outside of the oxygen electrode vessel in a shaking water bath held at 25° and aliquots withdrawn for assay in the oxygen electrode vessel.

In other studies 3-min incubations were made in the oxygen electrode vessel.

After substrate and ADP were added and the rate of respiration recorded, the entire contents of the oxygen electrode vessel were removed and aliquots promptly used for osmium tetroxide fixation and negative staining.

Osmium tetroxide fixation

Osmium tetroxide fixation was done using Palade's fixative solution. Samples were fixed for 30 min and dehydrated first with increasing concentrations of ethyl alcohol (50, 70, 95 and 100% (v/v)) and then with propylene oxide. Final embedding was done in epon 812. The resulting blocks were sectioned with an ultramicrotome (LKB), stained with lead citrate and examined with an electron microscope (Jem 7).

Negative staining

Negative staining was done according to PARSONS' method of surface spreading using phosphotungstic acid[7].

RESULTS

The respiratory effects produced by increasing concentrations of either whole *A. piscivorus* venom or its purified phospholipase A (which hydrolyzes the β-acyl ester linkage of phosphatidyl choline or ethanolamine) into three categories as illustrated by their characteristic oxygen electrode tracings in Fig. 1. Curve A is a control which shows a normal respiratory control ratio. Curve B is obtained when either 10.4 μg of whole, fresh venom or 1.2 μg of phospholipase A per mg mitochondrial protein are preincubated for 3 min and shows that uncoupling results. Curve C shows that when 16.7 μg of whole, fresh venom or 2.4 μg of phospholipase A per mg mitochondrial protein are added, ADP acts to inhibit respiration, that is, respiration on substrate alone proceeds more rapidly than respiration in the presence of substrate and ADP. This condition was called phosphate acceptor inhibition. When 41.9 μg of whole, fresh venom (or 3.6 μg of phospholipase A) per mg mitochondrial protein

are added, respiration is severely inhibited, both in the presence of substrate alone or of substrate and ADP (Curve D).

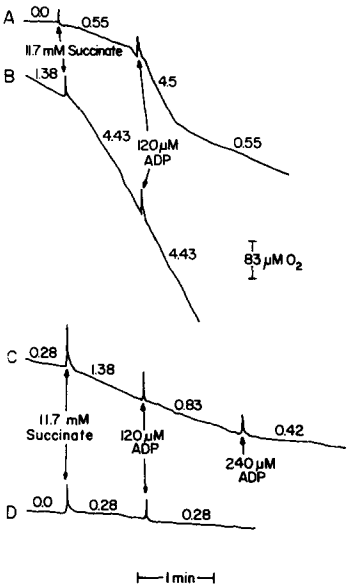

Fig. 1. The effects of increasing concentrations of whole *A. piscivorus* venom (or of purified phospholipase A) on rat liver mitochondria. The numbers above the curves indicate respiratory activity represented as μM O_2/sec. Concentration of reagents added indicated at point of addition. Letters identifying each curve are explained in the text. The respiratory vessel was maintained at 25° and contained 3.0 ml. The end of each tracing indicates the point at which aliquots were withdrawn for osmium tetroxide fixation and negative staining.

Changes in mitochondrial structure are induced by whole venom or purified phospholipase A. Fig. 2 shows osmium tetroxide fixed control mitochondria which appear intact with outer and inner membranes clearly visible, tubular cristae and minimal swelling. In negative staining experiments with control mitochondria, cristae appeared as long, thread-like structures with inner membrane subunits (diameter approx. 100 Å) clearly visible.

An osmium tetroxide fixed sample of uncoupled mitochondria showed some swelling (Fig. 3), but no other change in overall architecture of the mitochondria.

Mitochondria which showed phosphate acceptor inhibition exhibited moderate to severe degrees of swelling on fixation with osmium tetroxide (Fig. 4). There are areas in which the outer membranes were completely disrupted. The degrees of swelling of the cristae were much greater than that seen in uncoupled mitochondria.

The osmium tetroxide fixed sample of severely inhibited mitochondria showed complete disruption with formation of small vesicles (about 1 μ in diameter) of unknown nature (Fig. 5). They may represent a fragment of disrupted outer membrane which have folded to form an apparent discrete structure. Larger particles with swollen cristae representing fragments of mitochondria are also detectable. In the negatively stained section (bottom) some portions of the cristae are detectable with inner membrane subunits present on their surface. Larger fragments of mitochondria are also visible.

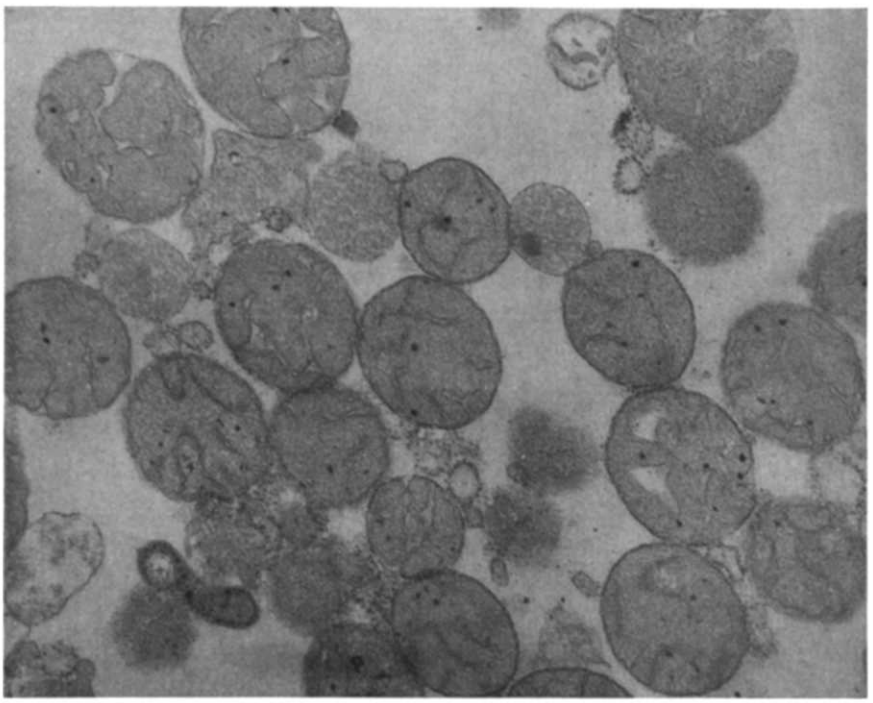

Fig. 2. Control mitochondria, magnification 34 500 ×. Conditions of the experiment as described for Fig. 1.

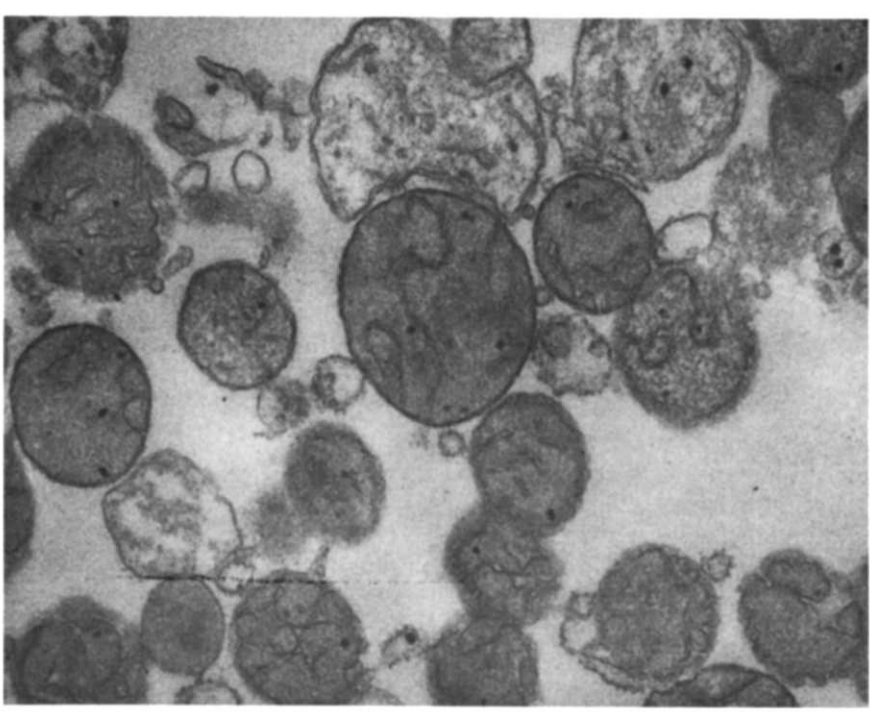

Fig. 3. Uncoupled mitochondria, magnification 34 500 ×. Conditions of the experiment as described for Fig. 1.

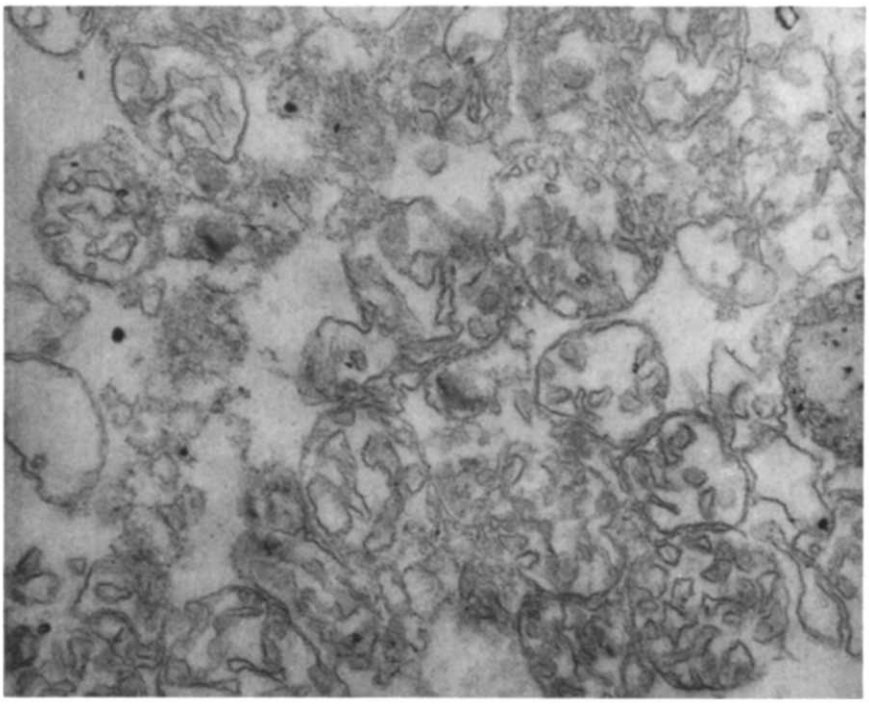

Fig. 4. Mitochondria showing phosphate acceptor inhibition, magnification 34 500 ×. Conditions of the experiment as described for Fig. 1.

Time studies in which mitochondria were incubated with a minimal amount of purified phospholipase A (0.72 μg per mg mitochondrial protein) for periods of time ranging from 1 to 10 min were monitored by oxygen electrode (Table I) and electron microscope studies. In the first and second minutes of incubation mitochondria showed a decrease in the respiratory control ratio from 5.0 in the control (succinate as substrate) to 1.8 and 1.2, respectively. Osmium tetroxide fixed thin sections of these mitochondria showed a structural integrity identical to that seen in Fig. 2. After 3 min of incubation mitochondria were uncoupled and after the fourth and fifth minutes showed phosphate acceptor inhibition and the degree of structural deterioration at each of these times corresponded to the respiratory condition of the mitochondria. After 7- and 10-min incubation periods, the mitochondria were fixed with osmium tetroxide and appeared identical to those seen in Fig. 5 confirming the loss of structural integrity accompanying respiratory inhibition. Mitochondria incubated for 10 min in the absence of phospholipase A, were structurally intact and showed good respiratory control.

In 1965, ZIEGLER *et al.*[8] showed that bovine serum albumin (0.1 % final concn.) could reverse the uncoupling activity produced by *Bungarus fasciatus* venom and restore respiratory control. Serum albumin also reversed the uncoupling caused either by whole *A. piscivorus* venom or purified phospholipase A (Fig. 6). Osmium tetroxide fixation of aliquots of uncoupled mitochondria treated with albumin showed morphology identical with that shown in Fig. 2. The mitochondria had undergone some swelling but cristae as well as inner and outer membranes appeared intact. In contrast,

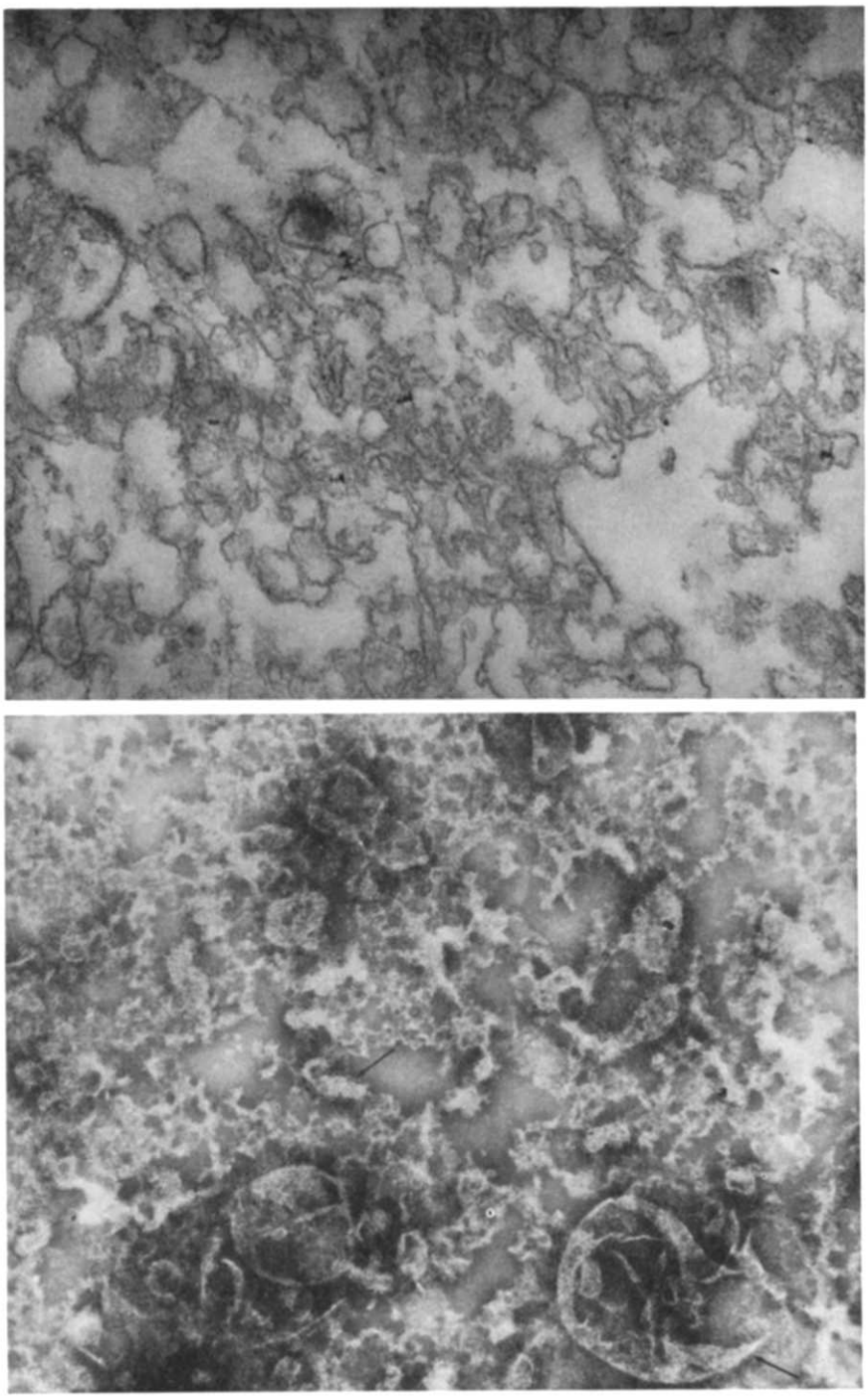

Fig. 5. Mitochondria showing complete inhibition: osmium tetroxide fixed sample (top) magnification 34 500 ×; negatively stained sample (bottom) magnification 70 000 ×. Conditions of the experiment as described for Fig. 1.

TABLE I

EFFECT OF INCUBATING MITOCHONDRIA IN THE PRESENCE OF PHOSPHOLIPASE A

Phospholipase A, 0.3 μg/mg mitochondrial protein.

Incubation time (min)	*μM O_2/min per mg mitochondrial protein*			*Control ratio**
	Mitochondria	*+ Succinate (11.6 mM)*	*+ ADP (120 μM)*	
1	4.1	16.4	73.8 (16.4)	4.5
2	12.3	24.6	69.7 (20.5)	3.4
3	24.6	45.1	57.4	—
4	4.1	16.4	12.3	—
5	4.1	12.3	12.3	—
7	0.0	8.2	8.2	—
10	0.0	8.2	4.1	—
Control	0.0	12.3	73.8 (12.3)	6.0

* Control ratio, ratio of respiratory rate in the presence of added ADP to the rate obtained following its (ADP) expenditure. Respiratory rate following expenditure of ADP given in parenthesis in column titled: + ADP.

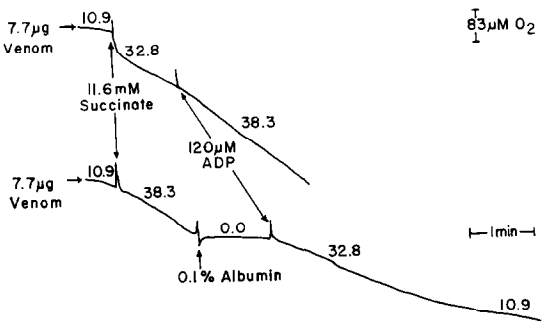

Fig. 6. Reversal by albumin of the uncoupling caused by whole *A. piscivorus* venom. The upper tracing shows mitochondria incubated with 7.7 μg whole venom per mg mitochondrial protein for 3 min in the oxygen electrode vessel; mitochondria are uncoupled. The lower tracing shows reversal of uncoupling by albumin.

it was observed that when use of higher venom or phospholipase A concentrations resulted in phosphate acceptor inhibition or complete inhibition, serum albumin could not protect respiration nor restore respiratory control.

Incubation of mitochondria with succinate or succinate and ADP, showed a protective effect against phospholipase A (Tables I–III). In these experiments mitochondria were incubated in a shaking water bath at 25° for varying time periods with (1) phospholipase A alone or (2) in the presence of succinate (11.6 mM) or (3) in the presence of succinate and ADP (120 μM). At the end of each incubation period, the effect produced on respiration of the mitochondria by incubation in each respiratory state was followed in an oxygen electrode.

The effect of adding 0.3 μg of phospholipase A per mg mitochondrial protein on mitochondria incubated in the absence of succinate and ADP, *i.e.* uncoupling

followed by phosphate acceptor inhibition and complete inhibition described earlier was apparent (Table I). An immediate fall in the respiratory control ratio coincided with the uncoupling of respiration and preceded the decline in rate of respiration. Doubling the phospholipase A present during the incubation accelerated the rate of respiratory decline.

Succinate protects mitochondria against phospholipase A (Table II), since respiratory control, though decreased, was maintained for 3 times the time shown in Table I. This protective action of substrate was seen to a lesser extent when the concentration of phospholipase A was doubled. Mitochondria incubated in the presence

TABLE II

EFFECT OF INCUBATING MITOCHONDRIA *plus* SUCCINATE IN THE PRESENCE OF PHOSPHOLIPASE A

Phospholipase A, 0.3 μg/mg mitochondrial protein.

Incubation time (min)	*μM O_2/min per mg mitochondrial protein*		*Control ratio**
	Mitochondria + succinate	*+ ADP (120 μM)*	
1	16.4	65.6 (16.4)	4.0
2	16.4	61.5 (16.4)	3.75
3	20.5	57.4 (16.4)	3.5
4	20.5	49.2 (20.5)	2.4
5	24.6	49.2 (20.5)	2.4
7	16.4	32.8 (20.5)	1.6
10	16.4	16.4	—
Control	16.4	73.8 (16.4)	4.5

* Control ratio as in Table I.

TABLE III

EFFECT OF INCUBATING MITOCHONDRIA *plus* SUCCINATE *plus* ADP IN THE PRESENCE OF PHOSPHOLIPASE A

Phospholipase A, 0.3 μg/mg mitochondrial protein.

Incubation time (min)	*μM O_2/min per mg mitochondrial protein*		*Control ratio**
	Mitochondria + succinate + ADP	*+ ADP (120 μM)*	
1	12.3	65.6 (12.3)	5.3
2	12.3	57.4 (16.4)	3.5
3	16.4	57.4 (16.4)	3.5
4	12.3	45.1 (16.4)	2.75
5	12.3	49.2 (16.4)	3.0
7	12.3	28.7 (20.5)	1.4
10	12.3	16.4	—
Control	8.2	24.6 (4.1)	6.0

* Control ratio as in Table I. The ADP added before incubation is converted to ATP when the mitochondria remain coupled and does not prevent the measurement of respiratory control ratios.

of succinate and ADP showed a slight increase in protection against phospholipase A over mitochondria incubated with succinate alone (Table III). At double the phospholipase A concentration mitochondria retained weak control after 5 min of incubation.

The results of incubating mitochondria with phospholipase A when glutamate (10 mM) and malate (5 mM) replace succinate as the substrate showed that incubation in the absence of substrate and ADP leads to a larger initial decrease in respiration and respiratory control than with succinate or no substrate. Weak control was retained for about 3 min and then mitochondria were completely inhibited. Incubation of mitochondria with phospholipase A in the presence of glutamate–malate or glutamate–malate and ADP indicated that no protection of mitochondria such as seen with succinate occurred (Table IV).

TABLE IV

EFFECT OF INCUBATING MITOCHONDRIA + GLUTAMATE–MALATE + ADP IN THE PRESENCE OF PHOSPHOLIPASE A

Phospholipase A, 0.3 μg/mg mitochondrial protein.

Incubation time (min)	*μM O_2/min per mg mitochondrial protein*			*Control ratio**
	Mitochondria	*10 mM glutamate–5 mM malate*	*+ ADP (120 μM)*	
A. Mitochondria + phospholipase A				
1	5.5	10.9	65.6 (10.9)	6.0
2	10.9	10.9	53.7 (10.9)	5.0
3	16.4	21.9	53.7 (10.9)	3.3
4	—	—	—	—
5	0.0	10.9	10.9	—
7	0.0	10.9	10.9	—
10	—	—	—	—
Control	0.0	10.9	71.1 (5.5)	13.0
B. Mitochondria + 10 mM glutamate–5 mM malate				
	Mitochondria + glutamate–malate			
1	10.9		32.8 (10.9)	3.0
2	10.9		21.9 (10.9)	2.0
3	5.5		10.9	—
4	—		—	—
5	5.5		5.5	—
7	10.9		10.9	—
10	—		—	—
Control	10.9		71.1 (5.5)	13.0
C. Mitochondria + 10 mM glutamate–5 mM malate + ADP				
	Mitochondria + glutamate–malate + ADP			
1	10.9		38.3 (10.9)	3.5
2	10.9		27.3 (10.9)	2.5
3	10.9		21.9 (10.9)	2.0
4	—		—	—
5	10.9		10.9	—
7	10.9		5.5	—
10	—		—	—
Control	10.9		71.1 (5.5)	13.0

* Control ratio as in Table I.

DISCUSSION

A. piscivorus venom induces in rat liver mitochondria the following classical respiratory effects: in low concentrations an increase in mitochondrial respiration in the absence of phosphate acceptor, in high concentrations a complete inhibition of electron transport and at intermediate concentrations a stage of respiratory decline in which ADP acts as an inhibitor (phosphate acceptor inhibition). This inhibitory effect of ADP was found to be nonspecific. It could be produced by AMP, UDP, CDP and GDP. However, the nucleotides which function as phosphate acceptors in mitochondria, namely AMP and ADP, produce about a 50% decrease in respiration while those nucleotides which do not function as phosphate acceptors produce only a 20–25% inhibition of respiration when added at the same level.

MYERS AND SLATER[9,10] and HEMKER[11–13] working with 2,4-dinitrophenol observed that it also had a biphasic action on mitochondria. The biphasic action was identical to that seen with *A. piscivorus* venom, that is, 2,4-dinitrophenol stimulated respiration at low concentrations and inhibited it at higher concentrations. A large variety of uncoupling agents such as dicumarol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and tetrachlorotrifluoromethylbenzimidazole *etc.*, were subsequently also shown to produce similar results[14,15].

VÁZQUEZ-COLÓN *et al.*[16] confirmed the biphasic nature of dinitrophenol action and further noted that oleic acid exhibited the same biphasic behavior and that a condition occurred in the respiratory decline caused by both dinitrophenol and oleic acid in which ADP inhibited respiration. VAN DAM[14] studying the inhibitory effect of several uncouplers of oxidative phosphorylation found that, in intact mitochondria, the presence of ADP and P_i enhanced the inhibitory effect produced by higher concentrations of uncouplers. Thus the respiratory effects observed with snake venom appeared to fit into a general pattern of effects produced in mitochondria by uncouplers.

The purified phospholipase A component of *A. piscivorus* venom induced the same pattern of respiratory effects on mitochondria that are produced by whole venom by either increasing the phospholipase A concentration while keeping the incubation time constant or by increasing the length of incubation periods while keeping the phospholipase A concentration constant.

Electron microscope studies confirmed that alteration in mitochondrial morphology paralleled the progressive respiratory decline. The initial swelling which accompanies uncoupling is seen to progress from minor membrane disruption to disruption severe enough to cause extrusion of cristae (phosphate acceptor inhibition) and finally to the total loss of structural integrity which characterizes severe inhibition.

These electron microscope studies are in agreement with the earlier studies on the effects of *C. terrificus terrificus* venom on mitochondria by NYGAARD *et al.*[1]. The improved detail of fixed samples and increased resolution seen in the present study reflect the recent advances made in ultrastructure studies.

If the negatively stained sections of mitochondria treated with whole *A. piscivorus* venom or its phospholipase A are compared with similar sections of mitochondria which have been extracted with diethyl ether or acetone[17] marked structural differences can be observed although both types of mitochondria show similar changes in enzymatic activity. Mitochondria which have been uncoupled and/or inhibited by

whole venom or its phospholipase A show no alteration or loss of inner membrane structures whereas solvent extracted mitochondria are characterized either by a complete loss of inner membrane structures or by an alteration in their size and appearance. This suggests that the alteration in structural integrity caused by hydrolysis of mitochondrial phospholipid is not equivalent to that caused by the partial or complete removal of mitochondrial phospholipid.

The ability of bovine serum albumin to restore respiratory control and morphology in mitochondria uncoupled by whole venom or its phospholipase A agrees with the finding of CONDREA *et al.*[2] that albumin can reduce phospholipase A induced swelling (monitored by absorbance change at 550 nm). Since thin-layer chromatography showed the same degree of hydrolysis occurred in the presence of albumin as in its absence, these workers concluded that albumin did not act by interfering with phospholipid hydrolysis. Support for this conclusion comes from the present study in that albumin had no protective or restorative ability at the higher venom or phospholipase A concentrations. WEINBACH AND GARBUS[18,19] reported that albumin could restore respiratory control to mitochondria uncoupled with nitro- and halo-substituted phenols, dicumarol, carbonyl cyanide *m*-chlorophenylhydrazone and various other uncoupling agents and VÁZQUEZ-COLÓN *et al.*[16] found that the uncoupling effect produced by dinitrophenol or oleic acid could be reversed by albumin. VAN DEN BERGH[20] reported that the presence of serum albumin in the reaction medium could not only abolish the uncoupling effect of fatty acids but also prevent the inhibition of fatty acid oxidation at higher fatty acid concentrations (see also ref. 21).

These respiratory effects of phospholipase A are most prominent when mitochondria are incubated with phospholipase A in the absence of substrate and ADP. The respiratory decline is accompanied by an immediate and marked decrease in the respiratory control ratio. This fall in the control ratio precedes the decline in respiration and may serve as a sensitive index of the functional integrity of isolated mitochondria[22].

Incubation in the presence of succinate or in the presence of succinate and ADP protects mitochondria against the respiratory damage produced by phospholipase A. Earlier BRAGANCA AND QUASTEL[23] had reported that presence of succinate during the incubation period, protected against the inhibition of succinate dehydrogenase by heated cobra (*Naja naja*) venom. In contrast, mitochondria incubated with phospholipase A in the presence of glutamate and malate *plus* or *minus* ADP showed no protection. The initial periods of weak respiratory control are followed by a rapid decline in respiration as when incubated alone. These results suggest that the sensitivities of NADH oxidase and succinate oxidase to phospholipase A are different, especially in the presence of substrate and ADP, and may reflect a difference in the phospholipid composition of the NADH and succinate portions of the respiratory chain. HEMKER[12,13], comparing the action of dinitrophenol on the succinate and NADH oxidizing portions of the respiratory chain, also observed difference in the sensitivity of these portions of the chain, as did VÁZQUEZ-COLÓN *et al.*[16] when studying the succinate and β-hydroxybutyrate branches of the chain.

It is apparent that (1) all the respiratory effects of whole *A. piscivorus* venom can be reproduced with its purified phospholipase A, (2) the respiratory effects produced by phospholipase A are accompanied by a loss of morphological integrity, and (3) there is a remarkable similarity among the overall effects produced on mito-

chondria by phospholipase A, the classical uncouplers and oleic acid. These observations do not support completely the view which attributes all the effects of phospholipase A on mitochondria to phospholipid splitting or that which attributes these effects solely to the action of free fatty acids. Instead, a more integrated interpretation emerges in which the early uncoupling and swelling effects of phospholipase A are attributable to the action of free fatty acids acting in their capacity as classical uncouplers, while the inhibition, membrane disruption and loss of structural integrity are attributed to an appreciable hydrolysis of membrane phospholipids as well as to the effects of the surface active lysophosphatides. However, ultimate correlations of the observations with events on a molecular level must wait until the relationship of mitochondrial structural integrity to substrate oxidation and phosphorylation is resolved.

ACKNOWLEDGEMENTS

This work was supported by a U.S. Public Health Service grant (GM 06241), a U.S. Public Health Service Research Career Program Award (5-K3-GM15,514) to W.B.E. and grants from the Heart Association of Erie County, Inc. to B.P. and W.B.E. Miss Ann Wadzinski provided valuable technical assistance.

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