EFFECTS OF β-BUNGAROTOXIN ON MITOCHONDRIAL RESPIRATION ARE CAUSED BY ASSOCIATED PHOSPHOLIPASE A ACTIVITY

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SUMMARY Mitochondria prepared from tissue that had been incubated with β-bungarotoxin exhibited abnormal respiration. The respiratory rate in the presence of substrate only was apparently normal, but it did not increase upon the addition of ADP. This effect could also be obtained by treatment with V. russelli phospholipase A or oleate. Treatment with lesser amounts of these agents caused the mitochondria to become uncoupled.

β-Bungarotoxin, a snake venom neurotoxin, causes neuromuscular blockade by a presynaptic mode of action occurring in two stages (1,2). An initial phase of increased rate of spontaneous acetylcholine release (increased miniature end-plate potential frequency) is followed by complete inhibition of nerve impulse-induced release of acetylcholine.

Studies in this laboratory have shown that β-bungarotoxin also affects the storage of several putative neurotransmitters and non-transmitter compounds in mammalian brain synapses in vitro (3,4). We have proposed that the effects of β-bungarotoxin on brain and neuromuscular synapses result from an interference with energy metabolism caused by a phospholipase A activity that was discovered to be associated with the toxin (4). We found that toxin-treated brain synapses had decreased levels of ATP and increased production of CO_2. Treatment of various membrane fractions with the toxin produced free fatty acids that partially uncoupled oxidative phosphorylation when added to mitochondria. In addition, a second effect on mitochondria was detected: Mitochondria prepared from toxin-treated brain tissue exhibited a limitation

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Abbreviations used: BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-aminoethylether) N,N'-tetraacetic acid.

in the maximal rate of respiration. In this paper we give evidence that this second effect on mitochondria is also due to the phospholipase A activity associated with the toxin.

MATERIALS AND METHODS

β-Bungarotoxin was purified as described (3). Vipera russelli venom phospholipase A, BSA and ADP were obtained from Sigma (St. Louis, MO); oleic acid was from Matheson, Coleman and Bell (Norwood, OH).

Mitochondria were prepared from the cerebral cortex or liver of adult Sprague Dawley rats as described (4). Mitochondrial respiration was measured at 37° in a Rank oxygen electrode. A 0.2-0.5 ml sample of mitochondria was added to 2.5 ml of respiration buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA) and 0.1 ml of 0.4 M potassium succinate, pH 7.4. When added, ADP and BSA were at 1.7 mM and 1.7 mg/ml respectively.

Protein was determined by the method of Lowry et al. (5).

RESULTS

As shown in Fig. 1 (tracing a), mitochondria prepared from brain minces that had been incubated with 7 μg of β-bungarotoxin per ml were abnormal in that the respiratory rate of the mitochondria failed to increase upon addition of ADP. The respiratory rate of control mitochondria increased by a factor of 1.7 after addition of ADP (Fig. 1, tracing c).

This effect of β-bungarotoxin on mitochondria depended on the amount of toxin present during incubation of the brain minces. When β-bungarotoxin was at 0.35 μg/ml, the mitochondria, rather than exhibiting limited respiration, respired at a near maximal rate even before addition of ADP (Fig. 1, tracing b). Thus, with a lower concentration of β-bungarotoxin the mitochondria were almost completely uncoupled.

The phospholipase A activity of our β-bungarotoxin preparation is heat stable; approximately 60% of the phospholipase activity remains after heating at 100° for 5 min (unpublished results). We therefore examined the heat stability of the toxin's ability to limit mitochondrial respiration. Minced cerebral cortex was incubated as described for Fig. 1 with β-bungarotoxin that had been heated at 100° for 5 min. The toxin was at 14 μg/ml. The respiration of mitochondria prepared from that tissue was not distinguishable from that shown in Fig. 1, tracing a, for mitochondria from tissue treated with

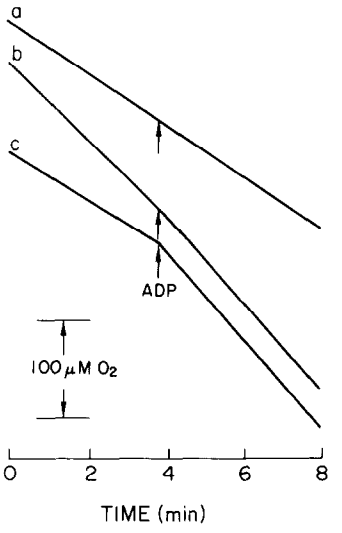

Fig. 1. Oxygen uptake by a mitochondrial fraction prepared from toxin-treated and control cerebral cortex. One-half of a rat cerebral cortex was minced in 2.0 ml of 20 mM Tris-HCl, pH 7.4, 83 mM NaCl, 74 mM sucrose, 3 mM KCl, 3 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose. The mince preparations were incubated with or without β-bungarotoxin for 60 min with shaking at 37° under an atmosphere of 95% O_2, 5% CO_2. After incubation the tissue was washed by centrifugation, homogenized, and a mitochondrial fraction prepared as described (4). The respiration of this fraction was measured as described under Methods. The concentration of β-bungarotoxin during incubation of the brain minces was 7 μg/ml, 0.35 μg/ml and 0 μg/ml for tracings a, b and c respectively. The control sample (tracing c) contained 3.6 mg of protein, and each toxin-treated sample for tracings a and b contained 3.5 mg of protein. The arrow marks the addition of ADP.

unheated toxin. Thus, the toxin's activity on mitochondria is very heat stable as is its associated phospholipase activity.

Table 1 describes experiments similar to those in Fig. 1. As shown in Table 1, a 1:1 mixture of mitochondria from control and toxin-treated tissues also fails to respond to ADP, indicating that the toxin causes this effect by the production of a diffusible factor that was able to alter the control mitochondria. When BSA was present in the respiration buffer during measurement of oxygen uptake, the mixture of mitochondria responded normally to ADP. This finding suggests that the active diffusible factor produced by β-bungarotoxin could be a fatty acid; BSA protects mitochondria against other effects of fatty acids (6) presumably because of its ability to bind fatty acids tightly

Table 1. Effect of β-bungarotoxin on oxygen uptake by brain mitochondria

Mitochondria	BSA added	Respiratory rate* Substrate	+ADP
Control (3.8)	-	0.61	1.05
Toxin-treated (3.9)	-	0.65	0.67
Control (1.9) + toxin-treated (2.0)	-	0.62	0.65
Control (1.9) + toxin-treated (2.0)	+	0.52	0.93
Control (3.1) + toxin-treated (0.8)	-	0.83	1.08
Toxin-treated (3.9)	+	0.50	0.78

Mitochondria were prepared from brain minces that had been incubated without or with 7 μg of β-bungarotoxin per ml, and oxygen uptake was measured as described in Methods and the legend to Fig. 1. The number in parenthesis is the mg of protein in each sample of mitochondria added to the oxygen electrode. When used, BSA was added before the mitochondria.

*μmol O_2 per min per 100 mg of protein.

Table 2. Effect of V. russelli phospholipase A on oxygen uptake by brain mitochondria

Phospholipase* (μg/ml)	Mitochondria	Respiratory rate† Substrate	+ADP
0	Control (3.2)	0.62	1.07
0.5	Phospholipase-treated (2.8)	1.07	1.07
0.5	Control (1.6) + phospholipase-treated (1.4)	0.97	1.10
10	Phospholipase-treated (3.1)	0.61	0.37
10	Control (1.6) + phospholipase-treated (1.5)	0.59	0.59

Mitochondria were prepared from brain minces that had been incubated with or without V. russelli phospholipase A, and oxygen uptake was measured as described in Methods and the legend to Fig. 1. The number in parenthesis is the mg of protein in each sample added to the oxygen electrode. V. russelli phospholipase had three times the specific phospholipase A activity of the β-bungarotoxin-associated enzyme.

*Phospholipase concentration during 60 min incubation of minces.

†μmol O_2 per min per 100 mg of protein.

(7). When the mixture of mitochondria contained a lower proportion (1:4) of mitochondria from toxin-treated tissue, the mitochondria were uncoupled, an effect that can be caused by fatty acids (6). The limitation in maximal respiratory rate of toxin-treated mitochondria alone was also less evident when BSA was present.

As shown in Table 2, the effects of β-bungarotoxin can be reproduced by incubating minced brain tissue with phospholipase A from V. russelli venom. Mitochondria from brain tissue that had been treated with 0.5 μg of V. russelli phospholipase A per ml were uncoupled. Mitochondria from brain tissue that had been treated with 10 μg of the phospholipase A per ml exhibited the limitation in maximal rate of respiration. In fact the respiratory rate was lowered by the addition of ADP, a reverse acceptor control effect (8,9) also occasionally seen with β-bungarotoxin treated mitochondria.

Table 3 shows that the respiration of liver mitochondria is altered by treatment with oleate or with brain mitochondrial fractions prepared from tissue that had been incubated with β-bungarotoxin or V. russelli phospholipase A. Addition of β-bungarotoxin-treated brain mitochondria caused the liver mitochondria to become uncoupled. This was the same preparation of toxin-treated brain mitochondria that produced the second effect (limitation in maximal respiratory rate) when added to freshly prepared mitochondria from untreated brain. Addition to liver mitochondria of brain mitochondria from tissue treated with V. russelli phospholipase A did limit the maximal respiratory rate of the liver mitochondria.

As shown in Table 3, oleate at 50 μM caused the liver mitochondria to become uncoupled while oleate at 250 μM limited the maximal rate of respiration. In the presence of 1 mM oleate oxygen uptake was eliminated.

BSA protected the liver mitochondria against the effects of the added oleate and treated brain mitochondria. There was severe limitation of respiration with 350 μM oleate, but uncoupling and almost maximal respiration was observed with 350 μM oleate in the presence of BSA.

Table 3. Respiratory rate of liver mitochondria after addition of treated brain mitochondria or oleate

Additions to respiration buffer			Respiration rate of liver mitochondria*	
Brain mitochondria	Oleate	BSA	Substrate	+ADP
-	-	-	1.12	4.03
Control (1.9)	-	-	1.19	4.12
Toxin-treated (2.0)	-	-	2.89	4.32
Toxin-treated (2.0)	-	+	1.03	4.15
Phospholipase-treated (1.5)	-	-	1.73	2.05
Phospholipase-treated (1.5)	-	+	1.31	4.05
Phospholipase-treated (0.6)	-	-	3.20	4.34
-	50 μM	-	3.18	4.26
-	250 μM	-	1.05	1.15
-	250 μM	+	1.10	3.54
-	350 μM	-	0.55	0.41
-	350 μM	+	2.81	3.75
-	1000 μM	-	0	0

Mitochondria were prepared from brain minces that had been incubated without or with 7 μg of β-bungarotoxin per ml or 10 μg of V. russelli phospholipase A per ml as described in the legend to Fig. 1. For this experiment the brain mitochondria had been kept frozen for 7-10 days before use. The number in parenthesis is the mg protein in each sample of brain mitochondria used. Liver mitochondria were prepared the day of the experiment; each sample of liver mitochondria contained 1.1 mg of protein. When used, BSA was added before the mitochondria. Oxygen uptake by the brain mitochondria was negligible relative to that by the liver mitochondria used.

*μmol O_2 per min per 100 mg of protein.

DISCUSSION

The phospholipase A activity associated with β-bungarotoxin accounts for all known effects of the toxin on mitochondrial respiration. The same effects are also produced by treatment with V. russelli phospholipase A or free fatty acid. At certain concentrations, each of these 3 agents causes mito-

chondria to become uncoupled. Higher concentrations produce a second effect on mitochondria, a limitation in the maximal rate of respiration.

BSA can protect the mitochondria against both effects. Under some conditions of treatment with these agents, the second effect was observed when BSA was absent during measurement of respiration, but the mitochondria were uncoupled and respired at a new maximal rate in the presence of BSA. These studies indicate the second effect predominates over and obscures the uncoupling effect. This conclusion is consistent with the finding that the uncoupling agents, 2,4-dinitrophenol and 5-chloro-3-t-butyl-2'-chloro-4-nitrosalicylanilide (S-13) did not increase the substrate-dependent respiratory rate of toxin-treated mitochondria that were exhibiting the limitation in maximal respiratory rate (4). We have also observed the effect when respiration was measured with pyruvate and glutamate, which are NADH-linked substrates, and with ascorbate using N,N,N',N',-trimethylphenylenediamine (TMPD) as an electron transfer agent between ascorbate and the cytochrome c region of the respiratory chain (4).

The main conclusion to be reached from these studies is that the phospholipase A activity associated with β-bungarotoxin can cause all described effects on mitochondrial respiration. These studies do not answer the crucial question of whether this phospholipase A activity is just a contaminant in our β-bungarotoxin preparation. Elsewhere we will give evidence that the phospholipase A and neurotoxin activities of our preparation reside on the same protein.

REFERENCES

1. Lee, C.Y. and Chang, C.C. (1966) Mem. Inst. Butantan Simp. Internac. 33, 555-572.
2. Chang, C.C., Chen, T.F. and Lee, C.Y. (1973) J. Pharmacol. Exp. Ther. 184, 339-345.
3. Wernicke, J.F., Oberjat, T. and Howard, B.D. (1974) J. Neurochem. 22, 781-788.
4. Wernicke, J.F., Vanker, A.D. and Howard, B.D. (1975) J. Neurochem. 25, 483-496.

5. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
6. Wojtzcak, L. and Lehninger, A.L. (1961) Biochim. Biophys. Acta 51, 442-456.
7. Boyer, P.D., Ballou, C.A. and Luck, J.M. (1947) J. Biol. Chem. 167, 407-424.
8. Lehninger, A.L. and Gregg, C.T. (1963) Biochim. Biophys. Acta 78, 12-26.
9. Gregg, C.T. and Lehninger, C.T. (1963) Biochim. Biophys. Acta 78, 27-44.