BINDING OF PIERICIDIN A ROTENONE AND AMYTAL IN BEEF HEART MITOCHONDRIA

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Piericidin A (Hall et al, 1966), rotenone and amytal (Ernster, Dallner and Assone, 1963) specifically inhibit NADH oxidation in beef heart mitochondria and are thought to act in the NADH dehydrogenase region of the respiratory chain. Horgan, Singer and Casida (1968) have shown that incubation of beef heart electron transport particles (ETP) with [14-C]rotenone resulted in binding of the inhibitor to the particles. Pretreatment of the ETP with piericidin A or amytal decreased the binding capacity for [14-C] rotenone, indicating that all three compounds were binding at the same site in the respiratory chain.

We now wish to report that labelled piericidin A is tightly bound to beef heart mitochondria at low concentrations. The amount of piericidin A specifically bound is proportional to the inhibition observed. Preincubation of mitochondria with inhibitory concentrations of rotenone or amytal results in a decrease in the binding of labelled piericidin A. Preincubation with antimycin A, an inhibitor which does not act in the NADH dehydrogenase region of the respiratory chain, did not affect the binding of piericidin A. The concentration of the rotenone / piericidin A-sensitive factor is in the region of 20 mμmole/g. mitochondrial protein.

METHODS

Beef heart mitochondria were prepared by the method of Sanadi and Fluarty (1963) and were suspended in 0.25M-sucrose and 50mM-Tris-HCl, pH 7.6. 18-25 mg. mitochondrial protein (1.0 ml.) were incubated at 30° for 8 min. with 4.0 ml. 0.25M-

sucrose and 50mM-Tris-HCl, pH 7.6, containing 2% (w/v) bovine serum albumin (BSA). Inhibitors were added as their ethanolic solutions. The mitochondria were collected by centrifugation at 30,000 x g and then washed in sucrose-BSA medium. Finally the pellets were suspended in 4.0 ml. sucrose-tris medium. 0.1 ml. aliquots were added to 10.ml. methanol: toluene (30:70) containing 0.8% (w/v) 2,5-diphenyloxazole (PPO) and 0.05% (w/v) 1,4-bis[2-(5-phenyloxazoyl)] benzene and counted in a Packard Tri-Carb scintillation counter. The specific activity of the [3-H]piericidin A was 56.5 mC/mmole.

RESULTS

When [3-H] piericidin A at a concentration of 52 mμmole/g. protein was added to mitochondria in sucrose-tris medium 38.4 mμmole became bound and NADH oxidation was inhibited 88%. When the concentration of [3-H] piericidin A was increased beyond that required for maximal inhibition to 1.04 μmole/g. protein then 0.68 μmole became bound, indicating that piericidin A binding was not restricted to the specific site. Addition of 2% BSA to the incubation medium resulted in a decrease in the non-specific binding so that the biphasic nature of the curve became evident (Fig.1, curve B). The rapidly rising phase of the curve could be abolished by titrating the mitochondria to maximal inhibition with unlabelled piericidin A before addition of [3-H] piericidin A (Fig. 1, curve C).

Figure 1 shows that the initial part of the curve is due to binding of piericidin A at the specific site. Similar results were obtained with [3-H] octahydropiericidin A. A measure of the piericidin A sensitive site was obtained by subtracting curve C (non-specific binding) from curve B (non-specific plus specific binding). The maximum binding at the specific site was found to be in the region of 18-20 mμmole/g. protein. Repeated washing of the mitochondria with the sucrose-tris-BSA medium resulted in the removal of the non-specific binding (Table 1).

A comparison of the amount of piericidin A bound and the degree of inhibition obtained showed that there was a direct correlation. (Table 2).

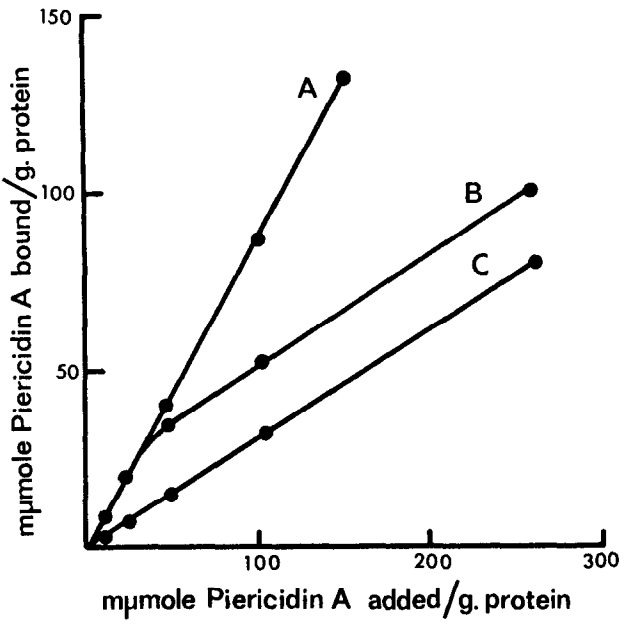

Fig.1. Binding of piericidin A to beef heart mitochondria and inhibition of NADH oxidase. Curve A, binding in sucrose tris medium; Curve B, binding in sucrose-tris-BSA medium; Curve C, binding in sucrose-tris-BSA medium after preincubation with 35.8 mμmole unlabelled piericidin A/g protein.

TABLE 1

Effect of repeated washing on binding of [3-H] piericidin A to mitochondria.

mμmole piericidin A added / g. protein	mμmole piericidin A bound /g. protein			
	1 wash	3 washes	5 washes	7 washes
10.4	8.16	7.44	6.92	6.80
26.0	17.72	15.76	14.80	13.44
52.0	27.76	22.76	21.24	19.16
104.0	31.36	24.12	20.80	19.12

TABLE 2.

Comparison of inhibition of NADH oxidase with binding of [3-H] piericidin A

mμmole PA[a] added/g.	mμmole PA bound/g (B-C)[b]	% binding (B - C)	mμmole PA bound/g. (7 washes)[c]	% binding (7 washes)	% inhibition
10.4	4.12	22.8	6.80	35	42.5
26.0	11.60	64.4	13.44	71	68.5
52.0	18.00	100	19.16	100	88.0
104.0	17.50	100	10.12	100	96.0

[a]piericidin A: [b]Curve B - curve C (see Fig.1) : [c]piericidin A remaining tightly bound after 7 washes with sucrose-tris-BSA medium: 100% binding it taken as [3-H]piericidin A bound on addition of 104 mμmole [3-H] piericidin A.

[3-H] piericidin A was not removed from the specific site by the washing process since addition of a twenty-fold excess of unlabelled piericidin A to mitochondria previously treated with [3-H] piericidin A did not result in displacement of the [3-H] piericidin A. When mitochondria were treated with unlabelled piericidin A, followed by [3-H] piericidin A, then the labelled piericidin A could be removed by repeated washings, thus no exchange or equilibration was taking place.

The effect of other mitochondrial electron transport inhibitors on the binding of [3-H] piericidin A is shown in Table 3. Mitochondria were incubated with a range of concentrations of unlabelled inhibitors followed by the addition of a fixed amount of [3-H] piericidin A. Rotenone, piericidin A and amytal decreased the binding of [3-H] piericidin A, whereas antimycin A had no effect.

TABLE 3

The effect of rotenone, amytal, piericidin A and antimycin A on binding of [3-H] piericidin A.

Piericidin A		Rotenone		Amytal		Antimycin A	
mμmole added/g.	[3-H]PA bound[a]	mμmole added/g	[3-H]PA bound	[mM]	[3-H]PA bound	mg./g.	[3-H]PA bound
0	16.17	0	16.23	0	16.28	0	16.23
7.15	14.58	5.5	14.14	1.0	14.96	2.75	16.77
14.30	13.20	13.75	13.48	3.0	10.62	5.5	16.12
35.80	10.07	27.50	11.50	5.0	8.53		
71.50	8.20	55.00	8.36				

Unlabelled inhibitors were added at the concentrations shown. After incubation for 8 min. at 30° 26.0 mμmole/g. [3-H] piericidin A was added and incubated for a further 8 min. [a][3-H] piericidin A binding is expressed as mμmole/g. protein.

Extraction of [3-H] piericidin A treated mitochondria with acetone resulted in the removal of 90-95% of the tightly bound radioactivity. Chromatography of the extract showed that piericidin A was not bound to mitochondrial phospholipids. The radioactive compound extracted had the same chromatographic behaviour on various systems as piericidin A standards, indicating that the [3-H] piericidin A was removed unchanged.

DISCUSSION

[3-H] piericidin A became bound to mitochondria at low concentrations but binding was also evident at concentrations well above those required for maximal inhibition of NADH oxidase. It is concluded that piericidin A is not a specific inhibitor of NADH

oxidase. This finding is supported by Palmer et al (1968) who have suggested that piericidin A has a site of action in the cytochrome b - ubiquinone region of the respiratory chain. The addition of BSA decreased the non-specific binding of [3-H] piericidin A and preincubation with unlabelled piericidin A prevented any binding of [3-H] piericidin A at the specific site. The difference between binding in the presence and absence of unlabelled piericidin A permits calculation of the piericidin A-sensitive factor (18-20 mμmole/g. protein). Non-specifically bound piericidin A was removed from mitochondria by repeated washing with BSA. The specifically bound piericidin A remaining was in the region of 19-20 mμmole/g. protein. Cremona and Kearney (1964) have found the NADH dehydrogenase concentration of beef heart mitochondria to be 18.7 mμmole/g. protein. The amount of piericidin A specifically bound to mitochondria is therefore of the same order as the NADH dehydrogenase content. We have also shown that the amount of piericidin A bound is proportional to the inhibition of NADH oxidase observed. The above findings show that piericidin A is reacting stoichiometrically with a component in the NADH dehydrogenase region of the respiratory chain.

Preincubation of mitochondria with unlabelled rotenone, before the addition of [3-H] piericidin A, demonstrates that rotenone and piericidin A bind at the same concentrations (see Table 3). It is concluded that piericidin A and rotenone are acting at or near the same component of the respiratory chain. Similar results were obtained with amytal but antimycin A had no effect on piericidin A binding, showing that reversal of binding is specific to inhibitors that act in the same region of the respiratory chain as piericidin A.

Extraction of mitochondria with acetone results in removal of lipids with loss of NADH oxidase activity (Lester & Fleischer, 1961). The lipophilic nature of piericidin A and rotenone suggests that they may be interacting with a lipid component of the NADH dehydrogenase system. Although piericidin A was removed in the acetone fraction with the mitochondrial lipids, it did not appear to be associated with any of the lipid components.

Octahydropiericidin A has similar binding characteristics to piericidin A indicating that the lipophilic side chain need not be unsaturated in order to achieve full inhibitory capacity

(Jeng. et al, 1968 have shown that the two compounds have similar effects on NADH dehydrogenase).

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