The Pyrethroids Permethrin and Cyhalothrin are Potent Inhibitors of the Mitochondrial Complex I

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ABSTRACT

The synthetic pyrethroid derivatives permethrin and cyhalothrin are widely used insecticides that are considered to be relatively nontoxic to higher animals. However, a variety of toxic effects on mammals have been reported. We investigated the effect of these drugs on energy coupling by mitochondria and on the activity of the individual respiratory complexes. Using isolated rat liver mitochondria, a concentration-dependent inhibition of activity of the individual respiratory complexes. The effect of glutamate and succinate sustained state 3 respiration was found for both compounds in the micromolar range. The effect of the complexes I to V were assessed individually in submitochondrial particles (complex I) and in freeze-thawed mitochondria (complexes II–V). Complex I (EC 1.6.5.3) was found to be the most sensitive link within the electron transport chain. Half-maximal inhibition was observed at 0.73 μM permethrin and 0.57 μM cyhalothrin, respectively, and exhibited sigmoidal inhibition kinetics. Complexes II, III, IV and V (EC 1.3.5.1, 1.10.2.2, 1.9.3.1, 3.6.1.34) were not significantly inhibited by up to 50 μM of these drugs. Thus, our results reveal a mode of action of synthetic pyrethroid insecticides not previously reported.

Pyrethroid insecticides are synthetic analogues of the natural pyrethrins contained in flowers of the genus Chrysanthemum. They form, together with chlorinated hydrocarbons (DDT, dieldrin, lindane), organo-phosphorus compounds (parathion, malathion, diazinon) and methycarbamate esters (aldicarb, carbofuran, carbaryl) the four major classes of insecticides. Pyrethroids are neurotoxins. Based on the symptomology after acute intoxication of insects and mammals, they fall into two classes: type I, non-α-cyano-pyrethroids, such as permethrin, show generally peripheral activities and type II pyrethroids, such as cyhalothrin, incline to central action (Leahy, 1985). The latter are characterized chemically by a cyano substituent (fig. 1).

Although synthetic pyrethroids are classified as well known and safe substances (Casida et al., 1983), their widespread use, their high, nonselective potency, and their considerable stability in the environment make them potentially harmful. In fact, intoxications of mammals, including higher animals, by pyrethroids has been observed (Ghosh, 1989). However, effects of pyrethroids on energy conservation by mammalian mitochondria have never been reported. In several toxicological models, movement disorders were linked to a dysfunction of mitochondria. Also, models exist for the development of movement disorders, based on toxins inhibiting complexes I and II of mitochondria (Schulz and Beal, 1994). In this light it appeared important to investigate the effects of pyrethroids on the

ABBREVIATIONS: DCCD, N,N′-dicyclohexylcarbodiimide; MOPS, 3-morpholinopropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid; NADH, nicotinamideadeninedinucleotide; RO*glutamate, respiratory quotient with glutamate; MPTP, 1-methyl-4-phenylpyridinium; RQ, respiratory quotient, defined as the ratio of state 3 to state 4 respiration; DMSO, dimethylsulfoxide.
activity of respiratory chain enzymes of mammalian mitochondria.

Here, we show that submicromolar concentrations of pyrethroids inhibit the respiratory complex I of rat liver mitochondria. This disturbance of the mitochondrial respiratory chain by pyrethroids could provide a new explanation for some of the symptoms of pyrethroid intoxication.

Methods

Materials. Cyhalothrin, (3-(2-chlor-3,3,3,-trifluor-1-propenyl)-2,2-dimethyloctan-3-yl)methyl carboxylic acid [1α,3α(Z)]-(-)-cyano(3-phenoxyphenyl)methyl ester) and permethrin, (m-phenoxymethyl(RS)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) from Riedel-de Haën (Seelze, Germany), were dissolved in dimethylsulfoxide and were made fresh every day. Rotenone, horse heart cytochrome c and antimycin A were purchased from Sigma Chemical Co. (St. Louis, MO), threonyltrifluoroacetan, 2,6-dichlorophenolindophenol and DCCD were purchased from Sigma Chemical Co. (St. Louis, MO) and antimycin A were purchased from Sigma Chemical Co. (St. Louis, Missouri). 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate from Riedel-de Haën (Seelze, Germany). Biuret reagent (Merkotest, Total Protein) was from Merck (Darmstadt, Germany). Duroquinol was synthesized using a Potter-Elvehjem homogenizer with a loose fitting Teflon pestle. Nuclei and cell debris were removed by centrifugation at 7000 g for 10 min. The resulting mitochondrial pellet was washed twice with buffer A by resuspension and centrifugation as before and suspended in buffer B to a concentration of approximately 100 mg mitochondrial protein per ml.

Preparation of SMP. SMP were obtained form mitochondria according to Ragan et al. (1987). Briefly, male Sprague-Dawley rats, 500 g, were killed by decapitation and the liver was quickly removed. It was placed in ice-cold buffer A (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4). The liver was minced and washed with buffer A. After the addition of 2 mM EDTA, a 10% suspension (w/v) of the minced liver was homogenized using a Potter-Elvehjem homogenizer with a loose fitting Teflon pestle. Nuclei and cell debris were removed by centrifugation at 7000 × g for 10 min. Mitochondria were separated by centrifugation of the supernatant at 7000 × g for 10 min. The resulting mitochondrial pellet was washed twice with buffer A by resuspension and centrifugation as before and suspended in buffer A to a concentration of 50 mg mitochondrial protein per ml.

Oxygen electrode studies. Oxygen consumption of coupled mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) at 30°C. The incubation medium [250 mM sucrose, 10 mM KCl, 1 mM P1 (Tris-salt), 25 mM Tris-Cl, pH 7.2] contained 0.87 ± 0.05 mg/ml mitochondrial protein. Permefrin and cyhalothrin were added to the medium 2 min before starting the reaction with 3 mM glutamate or succinate. After the recording of stable basal respiration, state 3 respiration was transiently initiated by the addition of 200 nmol ADP. In all the experiments described in this report, the concentration of DMSO never exceeded 1.5% (v/v) and controls with the same amount DMSO were always conducted. To uncouple mitochondria, 1.5 mM carbonyl cyanide p-(tri-fluoromethoxy)phenylhydrazone were added to the assay from a 100-fold concentrated stock solution in ethanol.

Spectrophotometric assay of enzyme activities. Mitochondrial enzyme activities were determined using freeze-thawed SMP (complex I) or freeze-thawed mitochondria (complexes II–IV). The experiments were performed at 30°C. Mitochondrial preparations and pyrethroids or specific inhibitors were incubated in 3 ml medium. After an equilibration period of 5 min, the reaction was initiated by the addition of the substrate. Changes in absorbance were registered using a Perkin Elmer Corp. (Norwalk, CT) Lambda 5 UV/VIS spectrophotometer.

NADH-duroquinone oxidoreductase (EC 1.6.5.3, complex I) activity was determined using 0.06 mg/ml of mitochondrial protein in 20 mM KP, 2 mM KCN, 0.1 mM EDTA, 0.3 mM duroquinone, 0.13 mM NADH, pH 7.4 (Pecchi et al., 1994; Singer, 1974). The decrease in absorbance at 340 nm was recorded. Rotenone at 30 μM was used as a specific inhibitor of complex I. The rotenone-sensitive activity was 70 to 80% of the total activity and was taken as 100% activity of complex I.

Succinate:2,6-dichloroindophenol oxidoreductase (EC 1.3.5.1, complex II) activity was determined in 50 mM KP, 3 mM KCN, 0.1 mM EDTA, 0.5 mM duroquinone, 0.1 mM 2,6-dichloroindophenol, 20 mM succinate, pH 7.4 and 0.3 mg/ml mitochondrial protein (Hatefi and Stiggall, 1978). The decrease in absorbance at 600 nm was monitored. One mM threonyltrifluoroacetan was used as a specific inhibitor of complex II.

Ubiquinol:ferricytochrome c oxidoreductase (EC 1.10.2.2, complex III) activity was determined in 50 mM KP, 0.1 mM EDTA, 3 mM KCN, 0.4 mM duroquinol, 0.1 mM cytochrome c, pH 7.4 and 0.3 mg/ml mitochondrial protein. Duroquinol was added to the medium after the preincubation period and the reaction was started by the addition of an aqueous solution of cytochrome c. The decrease in absorbance at 520 nm was monitored. Background activity from the spontaneous reaction of duroquinol with cytochrome c was subtracted. Antimycin A at 10 μg/ml was used as a specific inhibitor of complex III (Krähenbühl et al., 1991).

Ferrocyanochrome c:oxygen oxidoreductase (EC 1.9.3.1, complex IV) activity was determined in 50 mM KP, 0.1 mM EDTA, 1 mM reduced cytochrome c, pH 7.4 and 0.3 mg/ml of mitochondrial protein. The reaction was started by the addition of cytochrome c, which was reduced before the experiment with sodium dithionite. The increase in absorbance at 520 nm was monitored. Three mM KCN were used as specific inhibitor of complex IV.

ATPase (EC 3.6.1.34, complex V) activity was determined in a coupled enzyme assay containing in a reaction volume of 1 ml 100 mM Tris- SO4 pH 7.4, 20 mM MgSO4, 0.2 mM NADH, 0.53 mM phosphoenolpyruvate, 0.013 mg mitochondrial protein and 0.5 U each of lactate dehydrogenase and pyruvate kinase. After preincu-
In a first set of experiments, we looked at effects of permethrin and cyhalothrin on the respiratory control of mitochondria from four animals (respiratory quotient with glutamate (RQ\textsubscript{glutamate}) = 4.1 ± 0.5), we measured glutamate and succinate sustained oxygen consumption (state 4 respiration), ADP-stimulated oxygen consumption (state 3 respiration) and the P/O ratio. Although state 4 respiration and the P/O ratio were not affected by permethrin or cyhalothrin, ADP-stimulated respiration was similarly inhibited by cyhalothrin (not shown).

Using succinate as the substrate (fig. 3) assay duplicates (two animals) gave a 100\% activity of 61 nmol min\(^{-1}\) mg\(^{-1}\) and half maximal inhibition occurred at [permethrin]\(_5\) = 22 μM, (max. inhibition 107\%); [cyhalothrin]\(_5\) = 7 μM (max. inhibition 72\%). Uncoupled mitochondrial respiration was similarly inhibited by cyhalothrin (not shown).

## Results

### Effect of pyrethroids on respiratory control of mitochondria

In a first set of experiments, we looked at effects of permethrin and cyhalothrin on the respiratory control of mitochondria by measuring the \(O_2\)-consumption. In quadruplicate assays with isolated, coupled rat liver mitochondria from four animals (respiratory quotient with glutamate (RQ\textsubscript{glutamate}) = 4.1 ± 0.5), we measured glutamate and succinate sustained oxygen consumption (state 4 respiration), ADP-stimulated oxygen consumption (state 3 respiration) and the P/O ratio. Although state 4 respiration and the P/O ratio were not affected by permethrin or cyhalothrin (data not shown), state 3 respiration was inhibited by micromolar concentrations of these drugs (fig. 2). Oxygen consumption of controls using glutamate as the substrate was 47.8 ± 9.9 nmol min\(^{-1}\) mg\(^{-1}\). Maximal inhibition occurred at [permethrin]\(_5\) = 22 μM, (max. inhibition 107\%); [cyhalothrin]\(_5\) = 7 μM (max. inhibition 72\%). Uncoupled mitochondrial respiration was similarly inhibited by cyhalothrin (not shown).

Using succinate as the substrate (fig. 3) assay duplicates (two animals) gave a 100\% activity of 61 nmol min\(^{-1}\) mg\(^{-1}\) and half maximal inhibition occurred at [permethrin]\(_5\) = 7.6 μM, (max. inhibition 100\%) and [cyhalothrin]\(_5\) = 2.4 μM (max. inhibition 54\%). From the observation that state 4 respiration was not affected by permethrin and cyhalothrin, we could exclude uncoupling of oxidation and phosphorylation by the pyrethroids. However, our findings indicated that a component of mitochondrial respiration was inhibited. This inhibition was only apparent at the increased rates of ADP-stimulated state 3 respiration or in uncoupled mitochondria. Direct inhibition of a respiratory complex and/or the inhibition of an accessory function such as the transport system for glutamate could be responsible for the observed effects. To identify possible sites of inhibition in the respiratory chain, we individually assayed the activities of the respiratory complexes.

### Assay of NADH:duroquinone oxidoreductase

Complex I activity was determined in extensively washed SMP from two animals. In these preparations, the activity was 151 ± 18 nmol min\(^{-1}\) mg\(^{-1}\) and was 72 ± 8\% rotenone sensitive (19 experiments, SMP from two animals). The rotenone sensitive activity of complex I was inhibited in a concentration dependent manner by permethrin and cyhalothrin (fig. 4). According to equation 1, half maximal inhibition occurred at [permethrin]\(_5\) = 0.73 μM (max. inhibition 76\%, n = 2.4) and [cyhalothrin]\(_5\) = 0.57 μM (max. inhibition 75\%, n = 3.5).

\[
y(p) = \frac{y_{max}[p]^n}{K'[p]^n} \\
\]

The value of \(n\) increases with the degree of cooperativity and indicates the number of binding sites in the system. Half maximal inhibition \((y_{50})\) is reached at

\[
[p]_{50} = \frac{K'}{n} \\
\]

\(K'\) comprises the dissociation constant of pyrethroids in the system (Segel, 1975).

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**Fig. 2.** Dose-response curves for the effects of permethrin (●) and cyhalothrin (○) on glutamate sustained \(O_2\)-consumption during state 3 respiration of freshly isolated rat liver mitochondria. \(O_2\)-consumption was monitored with a Clark-type electrode as described in “Methods.” The data are the means ± S.E.M. of assay quadruplicates.

**Fig. 3.** Dose-response curves for the effects of permethrin (●) and cyhalothrin (○) on succinate sustained \(O_2\)-consumption of rat liver mitochondria during state 3 respiration. Data points are the means of assay duplicates. Details of the procedure are as described for figure 2.

**Fig. 4.** Dose-response curves for the effects of permethrin (●) and cyhalothrin (○) on rotenone sensitive NADH:duroquinone oxidoreductase of SMP. The oxidation of NADH was monitored spectrophotometrically as described in “Methods.” The data are the means ± S.E.M. of assay triplicates.
Thus, complex I was substantially inhibited by micromolar concentrations of pyrethroids.

**Assay of succinate:2,6-dichloroindophenol oxidoreductase.** Complex II activity was assessed in triplicates from three animals. The activity of controls was 76 ± 11 nmol min⁻¹ mg⁻¹ and was 97% inhibited by 1 mM of the complex II inhibitor threo-nyltrifluoroacetone. The observed inhibition of complex II by pyrethroids leveled off at 26% indicating it may not be a specific effect (fig. 5). Using the Hill model, half maximal inhibitions were calculated to be at (permethrin)₀.₅ = 0.5 μM (max. inhibition 26%) and (cyhalothrin)₀.₅ = 3 μM (max. inhibition 36%).

**Assay of duroquinol:ferricytochrome c oxidoreductase.** In the assay of complex III (bc₁-complex, triplicates, three animals), the background activity from the spontaneous oxidoreduction of duroquinol:cytochrome c was 38% of the total activity and was subtracted. The enzymatic activity of complex III was 66 ± 7 nmol min⁻¹ mg⁻¹ for controls (three experiments) and was not affected by up to 50 μM pyrethroids (fig. 6). The measured activity of complex III was 98% inhibited by 10 μg/ml of antimycin A, a specific inhibitor of complex III.

**Assay of ferrocytochrome c:oxygen oxidoreductase.** Complex IV (cytochrome c oxidase) activity was not inhibited by permethrin, but appeared somewhat inhibited by cyhalothrin in the concentration range tested (fig. 7). However, the difference between the inhibitory action of the two drugs was not statistically significant. The measured control activity was 238 ± 14 nmol min⁻¹ mg⁻¹ (triplicates, three animals) and 1.5 mM KCN, the classical inhibitor of complex IV, blocked the activity completely.

**Assay of ATPase.** Complex V activity was not influenced by permethrin or cyhalothrin in the concentration range tested (fig. 8). The control activity was determined to be 205 ± 14 nmol min⁻¹ mg⁻¹ and was 80% inhibited by 1 mM DCCD (triplicates, three animals). The mitochondrial F-type ATPase consists of the polar, detachable F₁ head portion and the membrane-embedded F₀ part. DCCD blocks the proton channel in the F₀-part of the ATPase. Only in the complete structure, ATP hydrolysis is coupled to proton flux and thus inhibited by DCCD. The high degree of inhibition we observed with this reagent indicates that our preparation consisted of 80% complete complex V molecules.

**Discussion**

Our study was undertaken to answer the question of whether permethrin, a type I pyrethroid, and/or cyhalothrin, a type II pyrethroid, interfered with the mitochondrial respiratory chain. We found that micromolar concentration of both compounds inhibited glutamate and succinate sustained state 3 respiration in a concentration-dependent manner. This inhibition could in principle be due to an effect of pyre-
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...toxins has been postulated to play a key role in the pathogenesis of movement disorders (Schulz and Beal, 1994). Inhibition of the mitochondrial complexes I and II (Pecci et al., 1994; Veitch and Hue, 1994) has drawn particular attention in this context, as some neurotoxins are known to interact with these sites. Model systems that use this mode of action for the induction of neurodegenerative diseases have recently been described. Even a slight inhibition of complex I induced concentration dependent, cell specific apoptosis in PC12 cells (Hartley et al., 1994). Also, suppression of complex I activity was found to be the ultimate step in the mode of action of MPTP. MPTP was discovered as a contaminant of an illicit synthetic pyrethroid insecticides (Kadous, A., Matsumura, F. and Enan, E.: High affinity binding of 3-verapamil to rat brain synaptic membrane is antagonized by pyrethroid insecticides. J. Pharmacol. Exp. Ther. 247: 14-22, 1988.)...


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