The Pyrethroids Permethrin and Cyhalothrin are Potent Inhibitors of the Mitochondrial Complex I 1

BEAT GASSNER, ANDREAS WÜTHRICH, GÜNTER SCHOLTYSIK and MARC SOLIOZ
Institute of Veterinary Pharmacology (B.G., A.W., G.S.) and Department of Clinical Pharmacology (M.S.), University of Berne, 3012 Berne, Switzerland
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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. Complexes I, III, IV and V (EC 1.6.5.3, 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 methylcarbamate esters (aldrin, carbofuran, carbarly) the four major classes of insecticides. Pyrethroids are neurotoxins. Based on the symptoms of 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 (Leahey, 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 humans, by pyrethroids has been observed (Chen et al., 1991). The underlying mechanisms of these toxic effects are not known.

A number of biochemical interactions of pyrethroids have been described in the literature. Alteration of the sodium channel kinetics is supposed to be the principal molecular mode of action of synthetic pyrethroids (Vijverberg et al., 1982; Vijverberg and van den Bercken, 1990; Tatebayashi and Narahashi, 1994). But inhibitory effects have also been described for Ca++-channels (Kadous et al., 1994), ATPases (Reddy et al., 1991; Michelangeli et al., 1990), and the receptors for acetylcholine (Abbassy et al., 1983), GABA (Lawrence and Casida, 1983), serotonin (Oortgiesen et al., 1989) and benzodiazepine (Devaud and Murray, 1988). However, whether any of these interactions are responsible for the toxicological effects of pyrethroids in higher animals remains unclear. Recently, an effect of pyrethroids on mitochondria was observed in fish exposed to these drugs in vivo. It was found that pyrethroids affected O2-consumption and led to a decrease in the activity of mitochondrial enzymes (Reddy and Philip, 1992; 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 mitochondrial energy conservation of mammals, including humans.

The activities 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.

ABBREVIATIONS: DCCD, N,N‘-dicyclohexylcarbodiimide; MOPS, 3-morpholinopropanesulfonic acid; EDTA, ethylenediamintetraacetic acid; EGTA, ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid, NADH, nicotinamideadeninedinucleotide; RQ Glutamate, respiratory quotient with glutamate; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+ , 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. Cytochalasin B (for isolation of rat liver mitochondria), 3-(2-chloro-3,3,3-trifluor-1-propenyl)-2,2-dimethylcyclopropanecarboxylic acid [1α,3α(Z)]-(-)-cyano(3-phenoxophenyl)methyl ester) and permethrin, (m-phenoxbenzyl(RS)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) from Riedel-de Hae¨n (Seelze, Germany), were dissolved in dimethylsulfoxide (DMSO). Duroquinol (Fluka), 2,6-dichloroindophenol, 0.5 mM EDTA, a 10% suspension (w/v) of the minced liver was homogenized with a Potter-Elvehjem homogenizer with a loose fitting Teflon pestle. Nuclei and cell debris were removed by centrifugation at 700 x g for 10 min. Mitochondria were separated by centrifugation of the supernatant at 7000 x g for 30 min. The resulting pellet was washed twice by resuspension and centrifugation as before and suspended in buffer B to a final concentration of 5 mg mitochondrial protein per ml.

Oxygen electrode studies. Oxygen consumption of coupled mitochondrial activity 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 Pi (Tris-salt), 25 mM Tris-Cl, pH 7.2) contained 0.87 ± 0.05 mg/ml mitochondrial protein. Duroquinol was 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 mitochondrial, 1.5 μM carbonyl cyanide p-(tri-fluoromethoxy)phenoxyhydradrazine were added to the assay from a 1000-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 (Hatfield and Stiggall, 1978). The decrease in absorbance at 600 nm was monitored. One mM threonyltrifluoroaceton 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).

Ferrocytochrome 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-
Inhibition of Complex I by Pyrethroids

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 \( RQ_{\text{glutamate}} \) = 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$^{-1}$. Half maximal inhibition occurred at \([\text{permethrin}]_{50} = 22 \mu$M (max. inhibition 107%); \([\text{cyhalothrin}]_{50} = 7 \mu$M (max. inhibition 72%). Uncoupled mitochondrial respiration was similarly inhibited by cyhalothrin (not shown).

Using succinate as the substrate (fig. 3) assay duplicates from four animals (respiratory quotient with glutamate \( RQ_{\text{glutamate}} \) = 4.1 ± 0.5) and \([\text{cyhalothrin}]_{50} = 2.4 \mu$M (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 \([\text{permethrin}]_{50} = 0.73 \mu$M (max. inhibition 76%, \( n = 2.4 \)) and \([\text{cyhalothrin}]_{50} = 0.57 \mu$M (max. inhibition 75%, \( n = 3.5 \)).

\[ y_{[p]} = \frac{y_{\text{max}}[p]^n}{K + [p]} \] (1)

The value of \( n \) increases with the degree of cooperativity and indicates the number of binding sites in the system. Half maximal inhibition \( (y_{0.5}) \) is reached at

\[ [p]_{0.5} = \frac{K}{n} \] (2)

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

\[ y_{[p]} = \frac{y_{\text{max}}[p]^n}{K + [p]} \]
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 $76 \pm 11 \text{ nmol min}^{-1} \text{ mg}^{-1}$ and was $97\%$ inhibited by $1 \text{ mM}$ of the complex II inhibitor threo-nitratfluoroacetone. 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 $(\text{permethrin})_{0.5} = 0.5 \mu \text{M} (\text{max. inhibition } 26\%)$ and $(\text{cyhalothrin})_{0.5} = 3 \mu \text{M} (\text{max. inhibition } 36\%)$.

**Assay of duroquinol:ferricytochrome c oxidoreductase.** In the assay of complex III ($bc_1$-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 $66 \pm 7 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for controls (three experiments) and was not affected by up to $50 \mu \text{M}$ pyrethroids (fig. 6). The measured activity of complex III was $98\%$ inhibited by $10 \mu \text{g/ml}$ 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 $238 \pm 14 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (triplicates, three animals) and $1.5 \text{ mM}$ 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 $205 \pm 14 \text{ nmol min}^{-1} \text{ mg}^{-1}$ and was $80\%$ inhibited by $1 \text{ mM}$ DCCD (triplicates, three animals). The mitochondrial F-type ATPase consists of the polar, detachable $F_1$ head portion and the membrane-embedded $F_o$ part. DCCD blocks the proton channel in the $F_o$-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-
Inhibition of Complex I by Pyrethroids 859

Artificial analysis, a causative link between the exposure to synthetic pyrethroid insecticides and the resulting observed neurotoxic symptoms after intoxication with synthetic pyrethroid insecticides. Clearly, further studies of the inhibitory effects of pyrethroids on mitochondria from different tissues, particularly brain, are required to prove this point.

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Schweizer, R., Becker, K., Class, T., Eckrich, W., Hoffmann, G., Hoppe, H. W.,


Send reprint requests to: Dr. Marc Solioz, Department of Clinical Pharmacology, Murtenstrasse 35, CH-3010 Berne, Switzerland.