Pesticide Biochemistry and Physiology 99 (2011) 131–139

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Binary mixtures of pyrethroids produce differential effects on Ca^{2+} influx and glutamate release at isolated presynaptic nerve terminals from rat brain ☆

Steven B. Symington [a,1], Hilliary E. Hodgdon [b], Richard K. Frisbie [c], J. Marshall Clark [b,c,*]

[a] Department of Biology and Biomedical Science, Salve Regina University, Newport, RI 02840, USA
[b] Department of Veterinary & Animal Sciences, University of Massachusetts, Amherst, MA 01003, USA
[c] Molecular & Cellular Biology Program, University of Massachusetts, Amherst,MA 01003, USA

ARTICLE INFO

Article history:
Received 26 April 2010
Accepted 9 November 2010
Available online 26 November 2010

Keywords:
Pyrethroids
Binary mixtures
Voltage-sensitive calcium channels
Voltage-sensitive sodium channels
Voltage-sensitive chloride channels
Glutamate release
Calcium influx
Presynaptic nerve terminals
Rat brain
Synaptosomes

ABSTRACT

Isolated rat brain synaptosomes were used to evaluate the action of pyrethroid mixtures on Ca^{2+} influx and subsequent glutamate release under depolarizing conditions. In equipotent binary mixtures at their respective and/or estimated EC_{50}s with deltamethrin always as one of the two components, cismethrin, λ-cyhalothrin, cypermethrin, esfenvalerate and permethrin were additive and *S*-bioallethrin, fenpropathrin and tefluthrin were less-than-additive on Ca^{2+} influx. In binary mixtures with deltamethrin always as one of the two components, esfenvalerate, permethrin and tefluthrin were additive and λ-cyhalothrin was less-than-additive on glutamate release. Binary mixture of *S*-bioallethrin and cismethrin was additive for both Ca^{2+} influx and glutamate release. Only a subset of pyrethroids (*S*-bioallethrin, cismethrin, cypermethrin, and fenpropathrin) in binary mixtures with deltamethrin caused a more-than-additive effect on glutamate release. These binary mixtures were, however, only additive (cismethrin and cypermethrin) or less-than-additive (*S*-bioallethrin and fenpropathrin) on Ca^{2+} influx. Therefore, increased glutamate release evoked by this subset of pyrethroids in binary mixture with deltamethrin is not entirely occurring by Ca^{2+}-dependent mechanisms via their action at voltage-sensitive calcium channels. These results suggest that pyrethroids do not share a common mode of toxicity at presynaptic nerve terminals from rat brain and appear to affect multiple target sites, including voltage-sensitive calcium, chloride and sodium channels.

1. Introduction

Since its enactment, the Food Quality Protection Act (FQPA)[2] has significantly altered the Federal Insecticide Fungicide and Rodenticide Act and the Federal Food Drug and Cosmetic Act, leading to the restricted use and/or elimination of several pesticides, with more restrictions likely to follow [1]. To maintain adequate agricultural productivity and to control insect vectors of human and animal diseases, the use patterns for currently registered pest control agents, such as the pyrethroids, are likely to increase in order to compensate for the discontinued or restricted use of certain pesticides set forth by the FQPA.

Pyrethroid insecticides have been used in agricultural and urban/home environments for over 30 years and account for ~25% of the worldwide insecticide market [2]. They are synthetic insecticides derived from the botanical insecticide mixture, pyrethrum, and are remarkably effective and relatively safe insecticides as they disrupt the insect nervous system at concentrations that result in little to no mammalian toxicity [1,3–5]. Given the widespread use of pyrethroids in agriculture, home environments and vector control, they are likely to be subject to increased regulation by the Environmental Protection Agency (EPA) under the amendments set forth by the FQPA [6,7].

Pyrethroids are neurotoxic and produce hyperexcitation, convulsions, seizures, and eventual paralysis and death in mammals. There are least two distinct classes of pyrethroids based on the syndromes of intoxication produced in mammals: the Tremor syndrome (T-syndrome) [4,8] and the Choreoathetosis with Salivation

☆ This work was supported by the Pyrethroid Working Group; a global conglomerate of the major pyrethroid manufactures and is comprised Aventis CropScience (Dublin, NJ); Bayer Corporation (Kansas City, MO); DuPont Agricultural Products (Newark, NJ); FMC Corporation (Philadelphia, PA); Pytech, GmbH (Horgen, CH); Cheminova (Denmark); Dow AgroSciences (Indianapolis, IN); Syngenta (Cheshire, UK) and Valent USA Corporation (Montvale, CA).

* Corresponding author. Address: Department of Veterinary & Animal Sciences, Morrill 1 N311B, 639 North Pleasant St., University of Massachusetts, Amherst, MA 01003, USA. Fax: +1 413 577 4267.
E-mail address: jclark@vasci.umass.edu (J.M. Clark).

[1] Partial support for S.B. Symington from RI-INBRE Grant # P20RR016457, National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

[2] *Abbreviations used:* ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-bis-(β-aminoethylether) *N,N,N,N′*-tetraacetic acid; EPA, Environmental Protection Agency; FQPA, Food Quality Protection Act; Fura-2 AM, (5-oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-acetyloxymethyl ester; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*(2-ethanesulfonic acid); PWG, Pyrethroid Working Group; TTX, tetrodotoxin; VSCC, voltage-sensitive calcium channels; VSClC, voltage-sensitive chloride channels; VSSC, voltage-sensitive sodium channels.

0048-3575/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2010.11.009

syndrome (CS-syndrome) [5,9–11]. Not all pyrethroids, however, fit neatly into the classic T- or CS-syndrome response, and some produce mixed signs of intoxication [1,12].

Voltage-sensitive sodium channels (VSSCs) are modified by pyrethroids [4,13–16]. In whole cell voltage-clamp experiments, non-cyano pyrethroids (Type I) elicit depolarizing after potentials and repetitive discharges. Pyrethroids that have an α-cyano (nitrile) group in their alcohol moieties (Type II) generally result in substantial membrane depolarisations, leading to conduction block [17]. Recently, it has been shown that not all pyrethroids modify VSSC in the same manner [15,18].

Other target sites, including voltage-sensitive calcium channels (VSCCs) and voltage-sensitive chloride channels (VSClCs), are also implicated with pyrethroid neurotoxicity [1,19–28]. Using isolated presynaptic nerve terminals, both cismethrin and deltamethrin stimulated Ca^{2+} influx, but only deltamethrin enhanced Ca^{2+}-dependent neurotransmitter release [21]. The release of neurotransmitter was unaltered by tetrodotoxin (TTX), but was blocked by ω-conotoxin GVIA, implicating $Ca_v2.2$, the N-type VSCCs in presynaptic nerve terminals, as an additional target site for deltamethrin [21]. In subsequent experiments, λ-cyhalothrin, β-cyfluthrin, cypermethrin, esfenvalerate and permethrin also increased Ca^{2+} influx and subsequent endogenous glutamate release assays [22,23], supporting the contention that other CS-syndrome pyrethroids, in addition to deltamethrin, also act as VSCC agonists [24,25]. Three additional pyrethroids (*S*-bioallethrin, tefluthrin and fenpropathrin), which produce the T-syndrome, failed to elicit concentration-dependent responses and/or responses that saturated in the Ca^{2+} influx and/or glutamate release assays.

A subset of pyrethroids (*S*-bioallethrin, β-cyfluthrin, cypermethrin, deltamethrin and fenpropathrin) block maxi VSClCs [24]. Similarly, a volume-activated chloride channel ($I_{Cl,vol}$) in ventricular myocytes was inhibited by fenpropathrin but not cypermethrin or tetramethrin [26]. These collective results suggest a direct action of certain pyrethroids on selective chloride channels.

In the current study, the actions of α-cyano and non-cyano pyrethroids individually and in binary mixtures are investigated using isolated presynaptic nerve terminals from rat brain. An expected result of applying pyrethroids in binary mixtures would be simple additivity if a single common mechanism of toxicity is assumed. However, data exist that shows different VSSC isoforms have variable sensitivity to pyrethroids [14], only a subset of pyrethroids act as VSCC agonists [22,23], and only a subset of pyrethroids act as maxi VSClC antagonists [24]. Therefore, we hypothesize that selected binary mixtures should produce less-than-additive, additive, and more-than-additive results when applied to a tissue preparation that possesses all three target sites.

2. Materials and methods

2.1. Chemicals

Percoll™ was purchased from Amersham Biosciences Corp. (Piscataway, NJ). Amplex® UltraRed reagent (10-acetyl-3,7-dihydroxyphenoxazine), fura-2 AM (5-oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy) ethoxy)-2-benzofuranyl)-acetyloxymethyl ester and Pluronic® F-127 (polyethylene–polypropylene glycol polymer with oxirane) were purchased from Molecular Probes, Inc., (Eugene, OR). All other chemicals were purchased from Sigma–Aldrich, Inc. (St. Louis, MO) at the highest purity available.

Technical grade pyrethroids used in this study were provided by the Pyrethroid Working Group (PWG): a global conglomerate of the major pyrethroid manufacturers that is comprised of Aventis CropScience (Dublin, NJ); Bayer Corporation (Kansas City, MO); DuPont Agricultural Products (Newark, NJ); FMC Corporation (Philadelphia, PA); Pytech, GmbH (Horgen, CH), Cheminova (Denmark); Dow AgroSciences (Indianapolis, IN), Syngenta (Cheshire, UK) and Valent USA Corporation (Montvale, CA). Crude cismethrin was a gift from Dr. D.M. Soderlund (Cornell University, Geneva, NY) and was purified (99.8%) using a high performance liquid chromatography (HPLC) as described previously [29]. Pyrethroid stock solutions were prepared in dimethylsulfoxide (DMSO) and serial diluted as required. Pyrethroid mixtures were prepared in desired ratios just before treatment.

2.2. Synaptosome preparation

Retired (ex-breeder) female CD® IGS Sprague–Dawley rats (~500 g, ⩾8 months of age) were purchased from Charles River Laboratories (Wilmington, MA) and maintained at the Central Animal Care Facility, Morrill Science Center I, University of Massachusetts (Amherst, MA). Rats were housed (two per cage) in standard plastic animal cages (45 × 24 × 20 cm) on a 12:12 h photoperiod with full access to food and water *ad libitum*. All animal procedures were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines (Protocol No. 23-09-09R).

Isolated presynaptic nerve terminals (synaptosomes) were prepared from whole rat brain except for brain stem as previously described [26,30]. Briefly, animals were decapitated, whole brain removed, and homogenized. Following centrifugation, the resulting crude mitochondrial pellet (P2) was resuspended and transferred to the top of a discontinuous Percoll™ gradient and centrifuged. Bands 3 and 4, containing purified synaptosomes, were collected, placed into saline A (125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 20 mM HEPES, pH 7.4), and centrifuged. Purified synaptosomal pellet was resuspended into saline A containing 10 mM glucose and the protein concentration determined [31].

2.3. Ca^{2+} influx assay

Ca^{2+} influx was measured using the fura-2 AM assay [26,32,33]. Briefly, synaptosomes were loaded with fura-2 AM solubilized in 2% Pluronic® F-127 [33]. A separate aliquot of synaptosomes received only DMSO to determine autofluorescence. Following loading, synaptosomes were diluted 10-fold using saline A with glucose, centrifuged and the resulting pellets resuspended into ice-cold saline A with glucose.

For the microtiter plate assay, fura-2 loaded synaptosomes were treated with the test pyrethroids in DMSO or DMSO alone (0.2%, final concentration) and incubated for 20 min at 37 °C prior to the start of each assay. Pretreated synaptosomes were diluted into 37 °C saline B (2 mM $CaCl_2$, 125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 20 mM HEPES, 10 mM glucose, pH 7.4), mixed for 5 s and basal fluorescence monitored (0–2 min) (Ex λ = 340 and 380 nm, Em λ = 510 nm). Synaptosomes were subsequently depolarized by the addition of KCl (60 mM final concentration) and incubated for an additional 2 min. Fluorescence was again recorded to determine the change (Δ) in relative fluorescence units. Values reported are change (Δ in $[Ca^{2+}]_i$/μg protein as previously described [21,26].

2.4. Neurotransmitter release assay

Endogenous neurotransmitter release from synaptosomes was determined using an enzyme-linked assay with Amplex® UltraRed Reagent for the detection of L-glutamate as previously described [26,34]. Briefly, purified synaptosomes were resuspended into saline A with glucose and incubated with the appropriate pretreatments as above. Pretreated synaptosomes were added to 37 °C saline B with glucose containing Amplex®, glutamate oxidase,