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Synergism between insecticides permethrin and propoxur occurs through activation of presynaptic muscarinic negative feedback of acetylcholine release in the insect central nervous system

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Abstract

Although synergism between pesticides has been widely documented, the physiological mechanisms by which an insecticide synergizes another remains unclear. Toxicological and electrophysiological studies were carried out on two susceptible pest species (the mosquito *Culex quinquefasciatus* and the cockroach *Periplaneta americana*) to understand better the physiological process involved in pyrethroid and carbamate interactions. Larval bioassays were conducted with the susceptible reference strain SLAB of *C. quinquefasciatus* to assess the implication of multi-function oxidases and non-specific esterases in insecticide detoxification and synergism. Results showed that the general theory of synergism (competition between pesticides for a common detoxification enzyme) was unlikely to occur in the SLAB strain since the level of synergy recorded between permethrin and propoxur was unchanged in the presence of piperonyl butoxide and tribufos, two inhibitors of oxidases and esterases, respectively (synergism ratios were similar with and without synergists). We also showed that addition of a sub-lethal concentration of nicotine significantly increased the toxicity of permethrin and propoxur at the lower range of the dose–mortality regression lines, suggesting the manifestation of important physiological disruptions at synaptic level. The effects of both permethrin and propoxur were studied on the cercal-afferent giant-interneuron synapses in the terminal abdominal ganglion of the cockroach *P. americana* using the single-fibre oil-gap method. We demonstrated that permethrin and propoxur increased drastically the ACh concentration within the synaptic cleft, which thereby stimulated a negative feedback of ACh release. Atropine, a muscarinic receptor antagonist, reversed the effect of permethrin and propoxur mixtures. This demonstrates the implication of the presynaptic muscarinic receptors in the negative feedback regulation process and in synergism. Based on these findings, we propose a cascade of molecular events explaining the occurrence of synergistic effects between pyrethroid and carbamate on many susceptible insects including *C. quinquefasciatus*, a mosquito of medical importance.

Keywords: Insecticide; Synergism; Pyrethroid-carbamate mixture; Synaptic transmission; Acetylcholine; Feedback mechanism; Mosquitoes; Cockroaches

1. Introduction

Since the 1980s, pyrethroids have been widely used to control insect pests in public health, applications such as house spraying and in mosquito nets (Zaim et al., 2000). Unfortunately, their important use in the last 20 years has led to the development of resistance in many insect species especially those of medical and veterinary importance (Bills, 2001). Mechanisms involved in pyrethroid resistance are: (1) alterations in the sequence of the target protein, the sodium channel (the well known "*Kdr* and *super Kdr* mutations") and (2) an increase of detoxification and/or metabolism (Bloomquist, 1993). Since the arsenal of safe, cost-effective insecticides is severely depleted by the development of resistance, the management of pest populations and

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strategies to slow down the evolution of pesticide resistance is currently based on the optimal use of existing compounds (NRC, 1986). For example, to avoid selecting for any particular type of resistance in pests of medical and agricultural importance, operational programmes may apply alternative classes of insecticides in sequence or rotation. In addition, the use of various mixtures of compounds each acting on different target sites has been adopted (Kurtak et al., 1987; Martin et al., 2000; Penilla et al., 1988).

Among the various strategies proposed, the practical advantage for using insecticide mixtures derives from the finding that synergistic interactions may occur between different components used in combination, thereby reducing the cost and toxicity of a given treatment. Synergism between pyrethroids and organophosphates (OPs) or carbamates has already been demonstrated in the control of agricultural pests (Bynum et al., 1997; Martin et al., 2003; Ozaki et al., 1984). In the sphere of public health, Hemingway (1984), has shown a high degree of synergism between IBP (a fungicide) and malathion (an OP) against susceptible and malathion-resistant strains of *Anopheles stephensi*. Recently, studies have also demonstrated the existence of synergistic interactions between pyrethroids and carbamates (or organophosphates) against susceptible and pyrethroid-resistant strains of *Culex quinquefasciatus*, a major vector of *Bancroftian filariasis* (Corbel et al., 2003a, 2004) and in *Anopheles gambiae*, the main malaria vector in Africa (Bonnet et al., 2004; Darriet et al., 2003).

Despite the literature referring to synergism in insects, the physiological mechanisms by which one insecticide synergizes another remains unclear. According to Corbett (1974), the general theory of synergism results from the ability of one molecule to interfere with the metabolic detoxification of the other. Indeed, some authors have shown that synergism between pyrethroids and OPs was caused by an inhibition by OPs of either esterases (Gunning et al., 1999), or oxidases (Kulkrani and Hodgson, 1980), thereby preventing degradation of the pyrethroid. In such cases, pyrethroid and OP mixtures provide a level of synergism by competitive substrate inhibition.

However, authors have also suggested that synergism may be related to a disruption of different physiological systems in insects (Bodnaryk, 1982; El-Sayed and Knowles, 1984; Plapp, 1979). In our previous works on *C. quinquefasciatus* (Corbel et al., 2003a, 2004), synergistic and antagonistic interactions were detected between permethrin and propoxur against two mosquito strains both having an identical enzymatic background but differing only by an insensitive acetylcholinesterase. Thus, it is possible that the basis of synergism may be complex involving target sites of both pyrethroid (sodium channel) and carbamate insecticides (acetylcholinesterase). Indeed, the toxic effect of carbamates in susceptible insects is due to their inhibition of acetylcholinesterase (AChE, EC3.1.1.7), a specific enzyme involved in the hydrolysis of acetylcholine (ACh) at cholinergic synapses (Corbett et al., 1984). On the other hand, many studies have shown that repetitive firing of nerves induced by pyrethroids stimulated acetylcholine release at cholinergic nerve terminals (Hue and Mony, 1987; Lund and Narahashi, 1983). Then, simultaneous application of pyrethroid and carbamate in insects may contribute to increase ACh concentration at a critical level leading to an early block of cholinergic synaptic transmission.

To test this hypothesis further, toxicological and electrophysiological studies have been undertaken on *C. quinquefasciatus* mosquitoes and the cockroach *Periplaneta americana*, the latter being a suitable model for investigating the mode of action of insecticides on cholinergic synaptic transmission (Hue and Callec, 1990). The effects of two types of insecticides, the pyrethroid, permethrin and the carbamate, propoxur, were studied alone and in combination. We demonstrate for the first time that synergistic interactions between permethrin and propoxur occur at cholinergic synapses, via an unexpected mechanism involving negative feedback of acetylcholine release.

2. Materials and methods

2.1. Biological materials

The standard susceptible strain S-Lab of *C. quinquefasciatus*, originating from California (Georghiou et al., 1966) was used for larval bioassay. This strain has been colonized for many years in laboratory and is free of any detectable insecticide resistance mechanism. Electrophysiological experiments were performed on adult male cockroaches *P. americana* obtained from our laboratory stock colony maintained at 29 °C with a photoperiod of 12-h light–12-h dark.

2.2. Insecticides and synergists

Two technical grade compounds were used, representing carbamate and pyrethroid classes of insecticides, propoxur 99.4% (Bayer AG, Leverkussen, Germany) and permethrin 94.4% *cis*:*trans* 25:75 (Agrevo plc, Berkhamsted, U.K.). In addition, two classical synergists were used for larval bioassays; Piperonyl butoxide (5-((2-(2-butoxyethoxy)ethoxy)methyl)-6-propyl-1,3-benzodioxole) or PBO (Merck, Darmstadt, Germany) an inhibitor of mixed-function oxidases and tribufos (*S*,*S*,*S*-tributyl phosphorotrithioate) or DEF (Interchim, Montluçon, France), an inhibitor of esterase. In addition, nicotine (Sigma, St. Quentin, France), an ACh agonist, was used as ''synergist'' of permethrin and propoxur. Concentrations used for larval bioassays were 1 mg/l PBO, 0.008 mg/l DEF and 40 mg/l nicotine, the maximum sub-lethal concentrations for SLAB strain (Chandre et al., 1997). Stocks solutions were prepared in absolute ethanol and stored at 4 °C for no more than 2 months.

2.3. Larval bioassay procedure

The larval bioassays were performed using a standard protocol described by the World Health Organization (WHO, 1996). Each bioassay was repeated three times using late third- and early fourth-instar larvae of SLAB *C. quinquefasciatus*. For each bioassay, 20 larvae of each strain were transferred to cups

containing 99 ml of distilled water. For each bioassay, we used five cups per concentration (100 larvae) and five to eight concentrations of each insecticide in a range that caused 0–100% mortality. One milliliter of each insecticide, at the desired concentration, was added to the cups. Control treatments of 1 ml ethanol were performed for each test. Each bioassay was maintained at 27 ± 1 °C throughout all tests. Larval mortality was recorded after 24-h exposure, corrected by the formula of Abbott (1925) if necessary, and data were analysed by the log-probit method of Finney (1971) using the Probit software (Praxème) programmed by Raymond et al. (1997). This software uses the iterative method of maximum likelihood to fit a regression between the log of concentration and the probit of mortality. The goodness of fit is estimated by a weighted χ^2. It also estimates the slope of the regression lines and the lethal concentrations (LC_{50} and LC_{95}) with their 95% confidence intervals.

2.4. Synergism study

In previous toxicological studies carried out on *C. quinquefasciatus* (Corbel et al., 2003a,b, 2004), synergism was evaluated by adding a sub-lethal concentration (actually LC_0) of permethrin (4×10^{-4} mg/l) or propoxur (1×10^{-2} mg/l) to increasing doses of propoxur and permethrin, respectively. To compare with previous data, the same procedure was adopted to evaluate the toxicity of pyrethroid and carbamate combinations in the presence/absence of synergists. The different steps of the study were as follows. (1) To assess the implication of esterases and oxidases in pyrethroid and carbamate detoxification, mosquito larvae were exposed to PBO and DEF in combination with increasing levels of propoxur and permethrin, respectively. (2) To determine the role of metabolic-based detoxification in synergism, PBO and DEF were combined to permethrin–propoxur combinations. Finally, (3) to evaluate the role of ACh in synergistic interactions between permethrin and propoxur, a sub-lethal (LC_0) dose of nicotine (40 mg/l) was added to various doses of each insecticide.

Log dosage–probit mortality lines were produced for each insecticide and for the various insecticide mixtures and their slope were compared using a χ^2-parallelism test. Synergism ratio's 50 (SR_{50}) were obtained by calculating the ratio between LC_{50} of each insecticide with and without synergist. The synergism ratios and the slopes of each regression line (as well as their confidence intervals) were given by the Probit software programmed by Raymond et al. (1997). A SR significantly higher than 1 (i.e., confidence interval of SR did not include the value 1) indicated a synergistic effect, whereas a SR equal 1 indicates an additive effect.

2.5. Electrophysiology

Adult male cockroaches *P. americana* were pinned dorsal side up on a dissection dish. The dorsal cuticles were removed to allow access to the ventral nerve cord. The terminal abdominal ganglion (TAG) with the nerve cord were then carefully dissected under a binocular microscope and placed in normal cockroach saline containing (in mM): NaCl 208, KCl 3.1, $CaCl_2$ 10, sucrose 26, HEPES 10; pH was adjusted to 7.2 with NaOH. The synaptic preparation was composed of a cercus, the corresponding cercal nerve XI, the de-sheathed TAG (containing the studied synapse) and the abdominal part of the nerve cord. Electrophysiological recordings of synaptic events were obtained using the single-fiber oil-gap method (Hue and Callec, 1990). With this technique it is possible to record unitary excitatory postsynaptic potentials (uEPSP) resulting from the activity of presynaptic cercal mechanoreceptors. In addition, composite excitatory postsynaptic potentials (cEPSP) were also triggered in response to short electrical presynaptic stimulation applied at a frequency of 0.1 Hz to the ipsilateral cercal nerve XI. Direct activation of cholinergic postsynaptic receptors (named ACh potential) located on dendritic membranes of the isolated giant interneuron (GI) was achieved by means of pneumatic pressure micro-ejection system (Medical System Corp., USA) of acetylcholine (ACh, 10^{-2} M, 25 psig) within the neuropil of the TAG using micropipette (tip diameter around 20 μm). During the experiments, resting potential was continuously monitored on a pen chart recorder. The uEPSPs and cEPSPs were recorded using Hameg oscilloscope and stored on PC computer with Hameg software. Experiments were carried out at room temperature (20 °C). Data were expressed as mean ± S.E.M. when quantified. Electrophysiological data were analysed for statistical significance using Analysis Variance (one-way ANOVA) followed by post hoc Tukey test. Differences between data were significant when $p < 0.05$. Data analysis were performed with STATISTICA (StatSoft, Cracow, Poland).

In all electrophysiological experiments, permethrin and propoxur were prepared in dimethylsulfoxide (DMSO, stock solution 10^{-2} M) and absolute ethanol (stock solution 10^{-2} M), respectively. Final dilution contained at most 0.1% DMSO and absolute ethanol. These concentrations of solvents were found to be without effect on the synaptic transmission. Excepted for permethrin and propoxur, all compounds were purchased from Sigma Chemicals (L'isle d'Abeau Chesnes, France).

3. Results

3.1. Synergism studies of permethrin and propoxur on C. quinquefasciatus

Synergism studies carried out on *C. quinquefasciatus* were summarized in Figs. 1A and B and 2A and B. All log(dose)–probit(mortality) curves for permethrin and propoxur, with and without synergists, were fitted by linear regression and mortality never exceeded 7% in the control batches. When applied alone, all synergistic compounds (PBO, DEF and nicotine) were non-toxic at the doses administered (<5% mortality).

Larval bioassay first showed that DEF did not significantly modify the toxicity of permethrin [$SR_{50} = 1.2(1.0 - 1.3)$] and propoxur [$SR_{50} = 0.8(0.7 - 0.9)$], indicating that esterases did not play physiological role in pyrethroid and carbamate

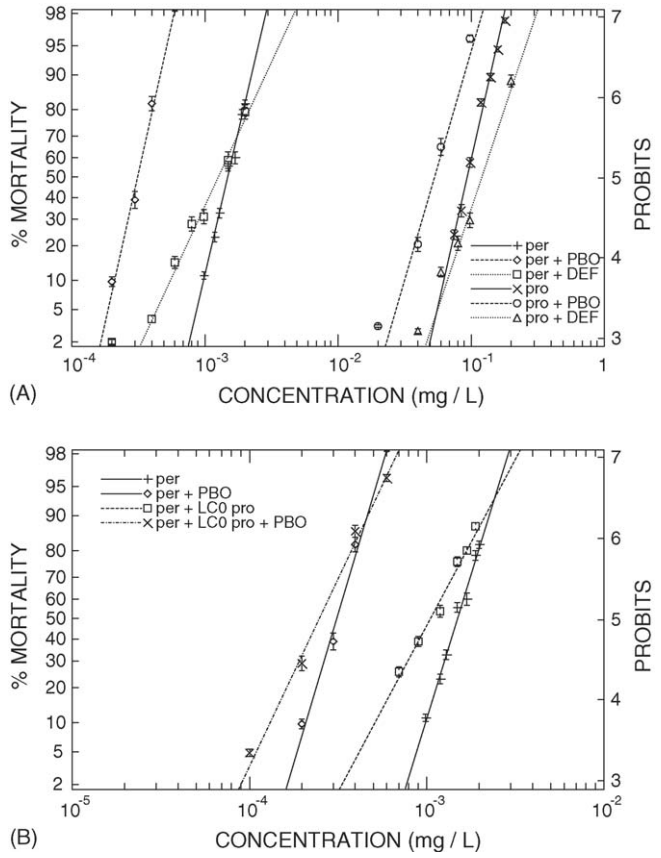

Fig. 1. (A) Synergist modification of concentration–mortality regression lines for permethrin and propoxur with the susceptible reference strain of *C. quinquefasciatus* in the presence of esterase (DEF) and oxidase (PBO) inhibitors. (B) Dose/mortality regression lines for permethrin on *C. quinquefasciatus*, in the presence of PBO and/or propoxur LC_0.

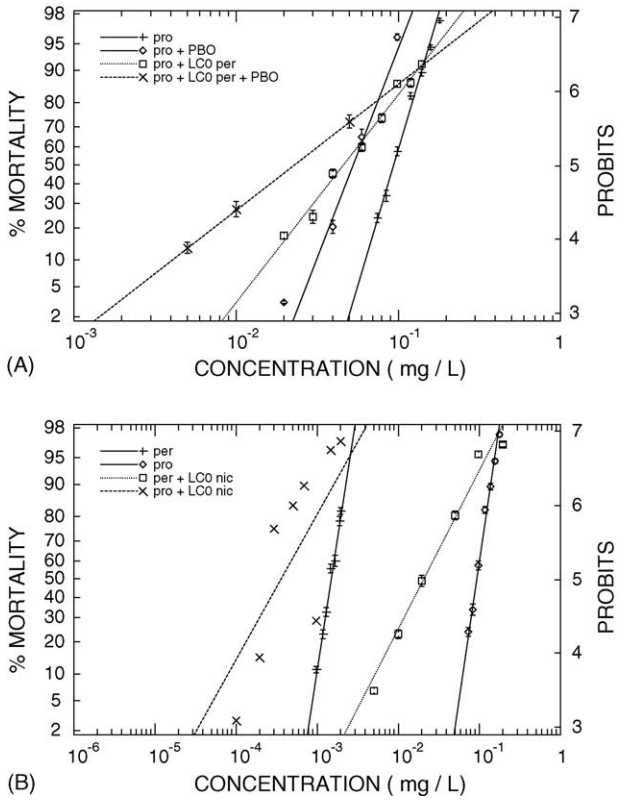

Fig. 2. (A) Dose/mortality regression lines for propoxur on *C. quinquefasciatus* in the presence of PBO and/or permethrin LC_0. (B) Dose/mortality regression lines for permethrin and propoxur on *C. quinquefasciatus* in the presence of nicotine LC_0.

detoxification in the SLAB strain (Fig. 1A). By contrast, PBO significantly decreased the tolerance to permethrin [$SR_{50} = 4.8(4.0 - 5.6)$] and propoxur [$SR_{50} = 1.8(1.5 - 2.2)$]. This indicated that detoxification of these two chemicals was mainly mediated by P450-dependent monooxygenases (Fig. 1A). In the presence of PBO, the slopes of the regression lines for permethrin (7.4 ± 0.6) and propoxur (5.7 ± 0.6) were steep, indicating a homogeneity of detoxifying oxidative system in the SLAB strain.

As previously observed by Corbel et al. (2003a,b), when sublethal dose (LC_0) of permethrin (4×10^{-4} mg/l) and propoxur (1×10^{-2} mg/l) and vice versa, was added, synergism occurred at lower range of the dose–mortality regression lines (Figs. 1B and 2A). Synergism ratios recorded from LC_{10} ($2.0 < SR_{10} < 4$) to LC_{80} ($1.2 < SR_{80} < 1.4$) differed significantly from 1 ($p < 0.05$) and were higher when permethrin synergized the propoxur (confidence interval of SR did not overlap, $p < 0.05$). Interestingly, the level of synergism detected between permethrin + LC_0 propoxur [$SR_{50} = 1.4(1.3 - 1.5)$] or between propoxur + LC_0 permethrin [$SR_{50} = 2.0(1.9 - 2.2)$] was not significantly modified in the presence of PBO [$SR_{50} = 1.3(1.1 - 1.5)$ and $2.0(1.8 - 2.3)$ for permethrin and propoxur, respectively]. This suggested that monooxygenase played little or no role in synergism in the SLAB strain. Since esterases were shown to be not involved in permethrin and propoxur detoxification in these mosquitoes (SR_{50} confidence interval included the value 1, Fig. 1A), no regression lines were established for pyrethroid and carbamate combinations in the presence of DEF.

Finally, a strong synergism was detected when LC_0 nicotine (40 mg/l) was added to increasing concentrations of permethrin [$SR_{50} = 4.5(4.1 - 5.0)$] and propoxur [$SR_{50} = 4.2(2.3 - 7.5)$]. Synergism effect was more pronounced at lower range of concentrations (Fig. 2B), as indicated by the weaker slopes of the regression lines for permethrin + LC_0 nicotine (2.0 ± 0.9 mg/l) and propoxur + LC_0 nicotine (2.1 ± 0.13 mg/l) compared to permethrin (7.1 ± 0.3 mg/l) and propoxur (7.4 ± 0.3 mg/l) alone. The fact that the dose–response relationships for propoxur and permethrin were quite similar whatever the pesticide used as a synergists (i.e., nicotine, permethrin or propoxur) suggested that synergism between pyrethroids and carbamates may result from a general physiological perturbation involving an increase of ACh concentration in the synaptic cleft.

To verify this assumption, the role of ACh in synergism was investigated using electrophysiological experiments performed on the commonly used cockroach, *P. Americana* synaptic transmission. It occurs between sensory fibers, which originate from mechanoreceptors of the cerci and giant interneurons. The use of this biological model for such investigations is encouraged by the fact that considerable literature already

exists dealing with cockroach central nervous system physiology and anatomy, so it should not be necessary to establish parameters for a completely new organism. Furthermore, and most significantly, in neurobiology, electrophysiological techniques like oil-gap techniques are well adapted on the cockroach central nervous system for investigating the effects of compounds at synaptic level. Many functional analogies are established between cockroach and other insect systems.

3.2. Effect of permethrin and propoxur on uEPSPs, cEPSPs and ACh potential

Previous findings have reported that in cockroach, cEPSPs result from the interaction of ACh with postsynaptic nicotinic ACh receptors of giant interneurons (Hue and Callec, 1990). In this study, the superfusion of the experimental chamber with saline containing permethrin induced a biphasic effect according to the different concentrations tested (Fig. 3A). Permethrin used at relatively low concentration (10^{-8} M) produced a slight increase in cEPSP amplitude form 4.1 ± 0.2 to 4.7 ± 0.3 mV (Fig. 3Ac, $n = 3$), which was not statistically significant. By contrast, higher concentrations of permethrin induced a concentration-dependent decrease in cEPSP amplitude from 4.3 ± 0.1 to 3.4 ± 0.2 mV ($n = 3$, $F_{1.5} = 30.9$, $p < 0.005$) for 10^{-7} M permethrin and from 4.3 ± 0.2 to 3.1 ± 0.4 mV ($n = 3$, $F_{1.5} = 9.0$, $p < 0.05$) for 3×10^{-7} M permethrin (Fig. 3Aa–c). It is interesting to note that the same concentration (i.e., 3×10^{-7} M) of permethrin also decreased the uEPSPs (from 1.3 ± 0.1 to 0.9 ± 0.1 mV, $F_{1.58} = 43.8$, $p < 0.001$; Fig. 3Ba–c) which are known to result from the spontaneous activity of presynaptic cercal mechanoreceptors. According to these first results, it is tempting to suggest that the biphasic effect of permethrin, a pyrethroid insecticide known to affect voltage-dependent sodium channels (Zlotkin, 1999), seemed to reflect changes in presynaptic activity. In other words, low concentration of permethrin, via alteration of sodium channels, produce an elevation of ACh released into synaptic cleft whereas cEPSP depression observed with higher concentrations strongly suggests that the amount of ACh released was sufficiently high for activating the negative feedback acting through presynaptic muscarinic receptors which thereby decrease subsequent release of ACh (Hue et al., 1989). To substantiate this hypothesis, additional experiments were conducted using

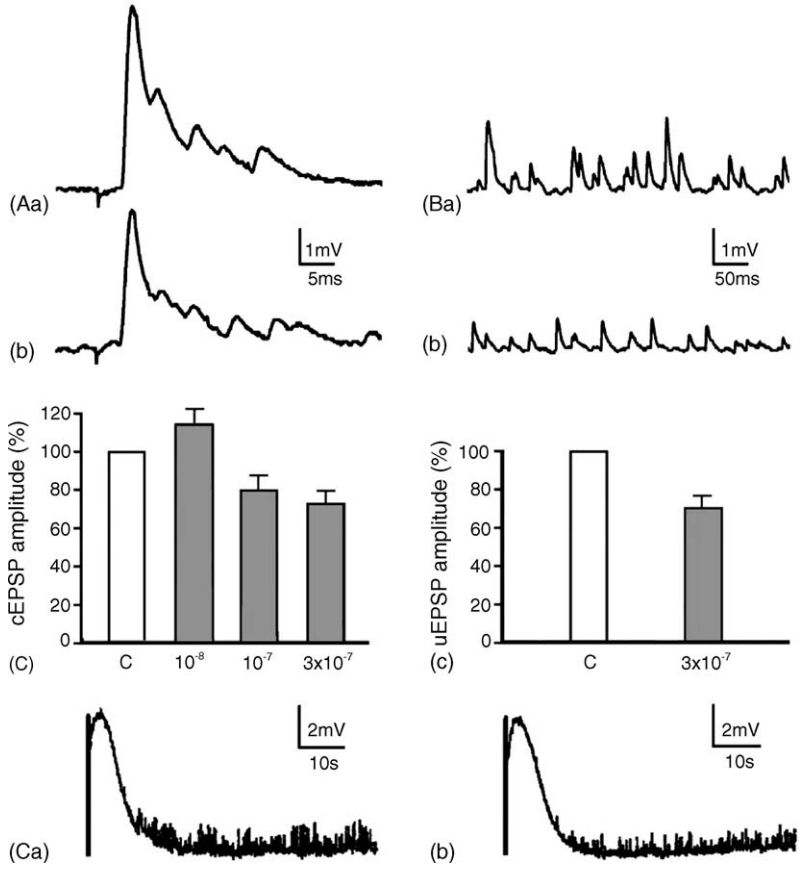

Fig. 3. Effects of permethrin on cEPSPs, uEPSPs and PACh amplitudes. (A) Control cEPSP (Aa) was reduced in amplitude 20 min after 3×10^{-7} M permethrin treatment (Ab). As expected, uEPSPs (Ba control) reflecting spontaneous activity of presynaptic cercal mechanoreceptors were also reduced by 3×10^{-7} M permethrin (Bb). Quantification of these effects were illustrated in a comparative histogram summarizing the concentration-dependent effect of permethrin on cEPSP amplitude (Ac, $n = 3$) and the reduction of uEPSP amplitude observed after 20 min of 3×10^{-7} M permethrin treatment. Data are means ± S.E.M. and expressed as % of control (i.e., for cEPSP 4.1 ± 0.2 mV, $n = 3$; 4.3 ± 0.1 mV, $n = 3$; 4.3 ± 0.2 mV, $n = 3$ for 10^{-8}, 10^{-7} and 3×10^{-7} M permethrin, respectively and for uEPSP 1.3 ± 0.1 mV from 30 measured uEPSP from three independent experiments). (C) Lack of effect of 3×10^{-7} M permethrin on PACh evoked by pressure ejection of ACh within the neuropil of the TAG (a) was control and (b) represented PACh recorded after 30 min in the presence of permethrin. It is interesting to note that the thick baseline in the PACh represented uEPSPs which were reduced in amplitude in the presence of permethrin.

pneumatic-pressure ejection of ACh within the neuritic tree of giant interneuron in the TAG. With this method, the ACh potential (PACh) is only a reflection of postsynaptic nicotinic ACh receptor activation which is independent of presynaptic stimulation. As illustrated in Fig. 3Ca,b, in the presence of 3×10^{-7} M permethrin, ACh, even after 30 min, could evoke postsynaptic potential with an amplitude very similar (6.5 ± 1.5 mV, $n = 3$) to that recorded in control (6.6 ± 1.3 mV, $n = 3$). It should be noted that the thick baseline of the ACh potential, which represents uEPSPs, was reduced in the presence of permethrin. This apparent lack of effect of permethrin on PACh amplitude confirmed that the cEPSP depression might be explained by changes in the concentration of endogenous ACh which was assumed to be increased by high concentration of permethrin, thus reducing subsequent ACh release.

To check whether cEPSP and PACh were also affected by an anticholinesterase compound, similar experiments were carried out in the presence of the carbamate, propoxur used at different

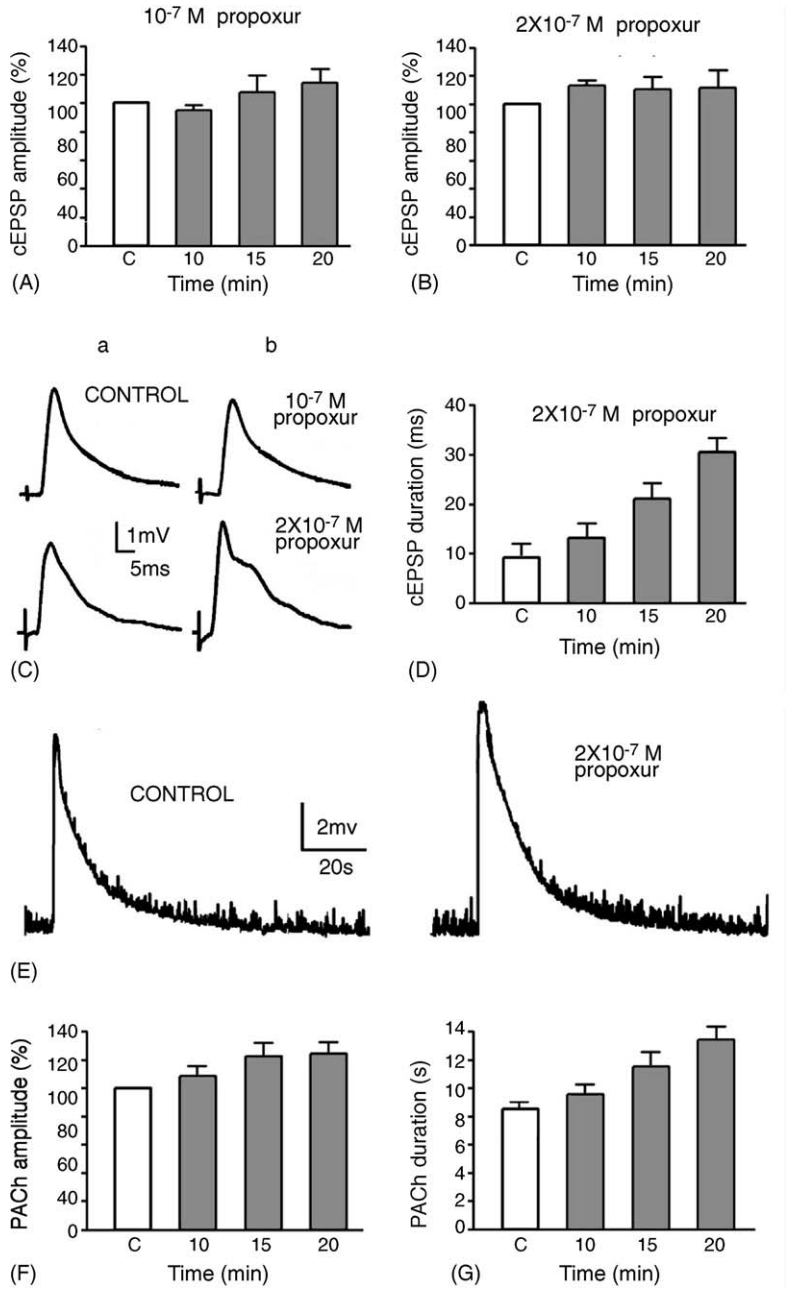

Fig. 4. Effects of propoxur on cEPSP amplitude and PACh. (A and B) Comparative histogram summarizing the effect of 10^{-7} M (A) and 2×10^{-7} M (B) propoxur vs. time of exposure, on cEPSP amplitudes ($n = 4$). Data are expressed as % of control (i.e., 4.2 ± 0.4 mV for 10^{-7} M propoxur and 4.4 ± 0.6 mV for 2×10^{-7} M propoxur, $n = 4$). (C) Effect of propoxur on cEPSP duration, observed after 10 min treatment, depending on the concentration tested (10^{-7} M and 2×10^{-7} M for (a) and (b), respectively, $n = 4$). (D) Histogram illustrating that the effect of propoxur (2×10^{-7} M) on cEPSP duration was more important according to time of exposure ($n = 4$). Data are means $\pm$ S.E.M. (E) Typical example of the effect of propoxur (2×10^{-7} M) on both amplitude and duration of ACh potential (PACh) 20 min after exposure. (F and G) Comparative histogram illustrating the effects of 2×10^{-7} M propoxur on PACh amplitude. Data are expressed as % of control, 7.5 ± 0.9 mV (F) and duration (G) during time of exposure. Data are means $\pm$ S.E.M. ($n = 4$).

concentrations. As illustrated in Fig. 4A and B cEPSP amplitudes slightly increased after 20 min of exposure, with propoxur used at 10^{-7} and 2×10^{-7} M (114 ± 8% and 111 ± 6% relative to control, respectively, $n = 4$). Interestingly, the stabilization of the cEPSP amplitude increase observed with higher concentration of propoxur led us to suspect the participation of the negative feedback mechanism which counteracted further enhancement of cEPSP amplitude as predicted with higher concentration of propoxur. Nevertheless, as shown in Fig. 4B, the raising effect of 2×10^{-7} M propoxur appeared more rapidly compared to lower concentration (Fig. 4A). In addition, cEPSP durations were also increased (up to 30.5 ± 0.5 ms, $n = 4$, after 20 min of 2×10^{-7} M propoxur treatment compared to control 9.2 ± 2.1 ms, $n = 4$, $F_{1.4} = 29.3$, $p < 0.01$) (Fig. 4C and D). Finally, PACh was increased in amplitude, which was not statistically significant and prolonged in duration (from 8.5 ± 0.6 in control to 13.6 ± 0.9 s, $n = 4$, $F_{1.4} = 26$, $p < 0.01$, after 20 min of treatment Fig. 4E–G). This indicated that propoxur exerted its inhibitory effect on acetylcholinesterase localized at postsynaptic level.

3.3. Presynaptic muscarinic receptors modulate the effects of permethrin and propoxur

As indicated above, it was previously demonstrated that presynaptic muscarinic receptors were involved in the modulation of ACh release. In other words, cEPSPs were potentiated in the presence of muscarinic antagonists (e.g., atropine) and reduced with muscarinic agonists (Hue et al., 1989; Le Corronc et al., 1991). In order to estimate the participation of presynaptic muscarinic receptors in the effect of permethrin and propoxur, new set of experiments was designed in the presence of atropine, known to block muscarinic receptors in the cockroach synaptic transmission (Hue et al., 1989; Le Corronc et al., 1991). As shown in Fig. 5A, 3×10^{-7} M permethrin produced an important reduction of cEPSP amplitude (73 ± 7% relative to control, $n = 3$) after 20 min of treatment. If this effect reflected activation of presynaptic muscarinic receptors, application of atropine should reverse this effect. Pretreatment with atropine (10^{-6} M) for 10 min counteracted the cEPSP depression induced by 3×10^{-7} M permethrin and cEPSP amplitudes were enhanced sometimes even more than in control (Fig. 5B). For 10, 15 and 20 min, differences were always statistically significant ($F_{1.8} = 25$, $p < 0.05$; $F_{1.7} = 6.7$, $p < 0.05$; $F_{1.6} = 16$, $p < 0.05$, respectively). It should be noted that during the first 10 min, atropine applied alone induced an increase of cEPSP amplitude (7 ± 1%, $n = 8$, relative to control). In addition, bath application of atropine (10^{-6} M) also modulated the effect of 2×10^{-7} M propoxur as indicated in Fig. 5C (superimposed histogram) where cEPSP amplitudes were potentiated compared to those recorded in the presence of propoxur applied alone ($F_{1.4} = 14.1$, $p < 0.05$; $F_{1.4} = 12.1$, $p < 0.05$; $F_{1.4} = 9.1$, $p < 0.05$ after 10, 15 and 20 min, respectively; Fig. 5C, black bars). This reinforced the assumptions made just

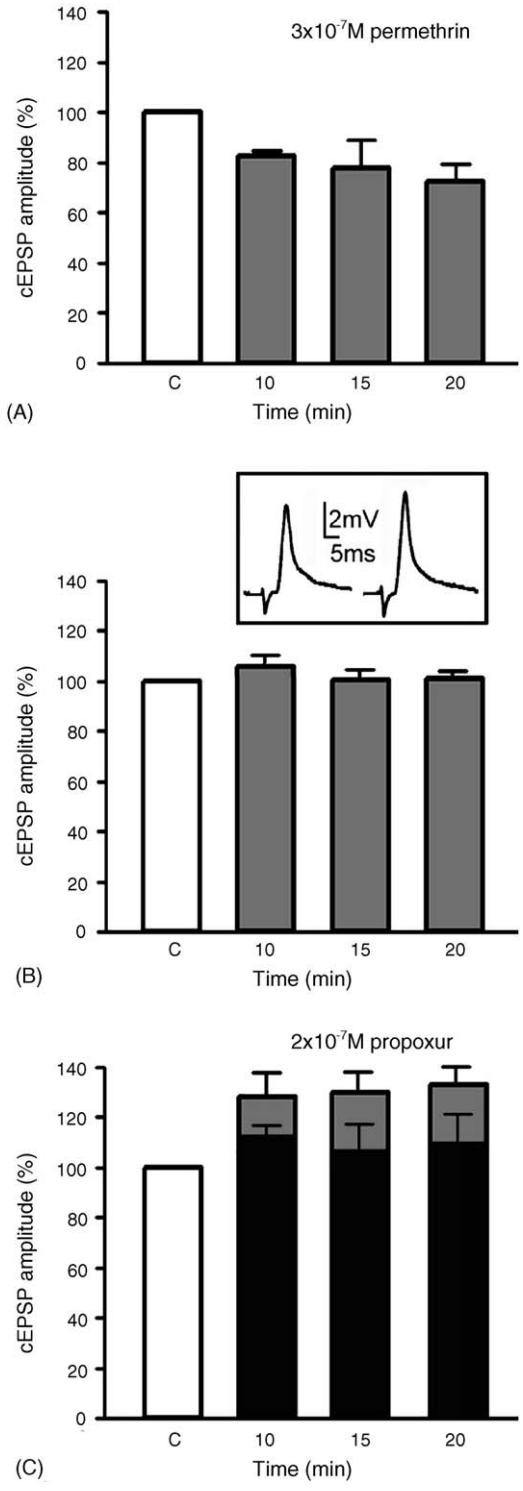

Fig. 5. Effects of the muscarinic antagonist, atropine on permethrin- and propoxur-induced cEPSP amplitude modification. (A) Histogram illustrating the time-dependent decrease in cEPSP amplitude produced by 3×10^{-7} M permethrin ($n = 10$). Data are expressed as % of control 4.3 ± 0.2 mV ($n = 4$). (B) Pretreatment with atropine (10^{-6} M) for 10 min clearly reversed the cEPSP depression observed in the presence of 3×10^{-7} M permethrin as illustrated in both inset and histogram ($n = 8$, control amplitude, i.e., after atropine treatment 4.6 ± 0.6 mV). (C) Pretreatment with atropine (10^{-6} M) for 10 min also counteracted the effect of 2×10^{-7} M propoxur on cEPSP amplitudes. The superimposed histogram represented the effect of 10^{-6} M atropine on cEPSP amplitude measured in the presence of 2×10^{-7} M propoxur (grey bars, $n = 4$). Black bars were taken from Fig. 4B ($n = 4$). Data are expressed as mean ± S.E.M.

above for Fig. 4B. These results confirmed the involvement of the muscarinic negative feedback in the modulation of the effects of permethrin and propoxur in the synaptic transmission.

3.4. Effects of permethrin and propoxur mixture on synaptic transmission

Based on our previous findings, the neurotoxic activity of permethrin and propoxur applied alone occurred through elevation of ACh within the synaptic cleft, which thereby activated the negative feedback mechanism. To understand further the physiological mechanism underlying the synergism observed between these two insecticides on *C. quinquefasciatus*, the effects of permethrin and propoxur applied in combination were studied on synaptic transmission, under the same experimental conditions. As illustrated in Fig. 6, the results revealed synergism depending on the concentration of permethrin used. The combined effect of 10^{-7} M permethrin and 2×10^{-7} M propoxur co-exposure (at 10 min) produced a synergistic $64 \pm 4\%$ depression (Fig. 6A and Ca) relative to cEPSP amplitude measured with 2×10^{-7} M propoxur applied alone. For comparison, the calculated additive effect of these two insecticides applied separately only revealed a slight decrease of cEPSP amplitude ($96 \pm 8\%$; Fig. 6Ca). Difference between these two effects was statistically significant ($F_{1.7} = 6.9$, $p < 0.05$). The synergism between permethrin and propoxur was improved after pretreatment for 10 min with higher concentration of permethrin (i.e., 3×10^{-7} M). In this case, 3×10^{-7} M permethrin/2×10^{-7} M propoxur co-exposure produced an important decrease of cEPSP amplitude ($34 \pm 3\%$; Fig. 6B and Cb, measured at 10 min). The calculated additive effect of these two insecticides applied separately only revealed a slight decrease of cEPSP amplitude ($92 \pm 9\%$; Fig. 6Cb). The calculated values obtained under these two experimental conditions was statistically significant ($F_{1.7} = 17.6$, $p < 0.005$; Fig. 6Cb).

We then examined if this synergism occurred through the activation of presynaptic muscarinic receptors involved in the negative modulation of ACh release. The experimental procedure was designed in order to obtain the most important synergism between permethrin and propoxur (i.e., pretreatment with 3×10^{-7} M permethrin for 10 min and 3×10^{-7} M permethrin/2×10^{-7} M propoxur co-exposure). As shown in Fig. 7A and B, addition of 10^{-6} M atropine clearly reversed the combined effects of permethrin and propoxur on cEPSP amplitude. Quantification of such effect of atropine was illustrated in Fig. 7C. The superimposed histogram indicated that atropine counteracted the insecticide mixture-induced potentiation of cEPSP depression compared to the effect of combined insecticides alone (black bars of Fig. 7C). These results confirmed, for the first time, the idea that permethrin and propoxur applied in combination produced important cEPSP depression via the activation of the presynaptic muscarinic negative feedback.

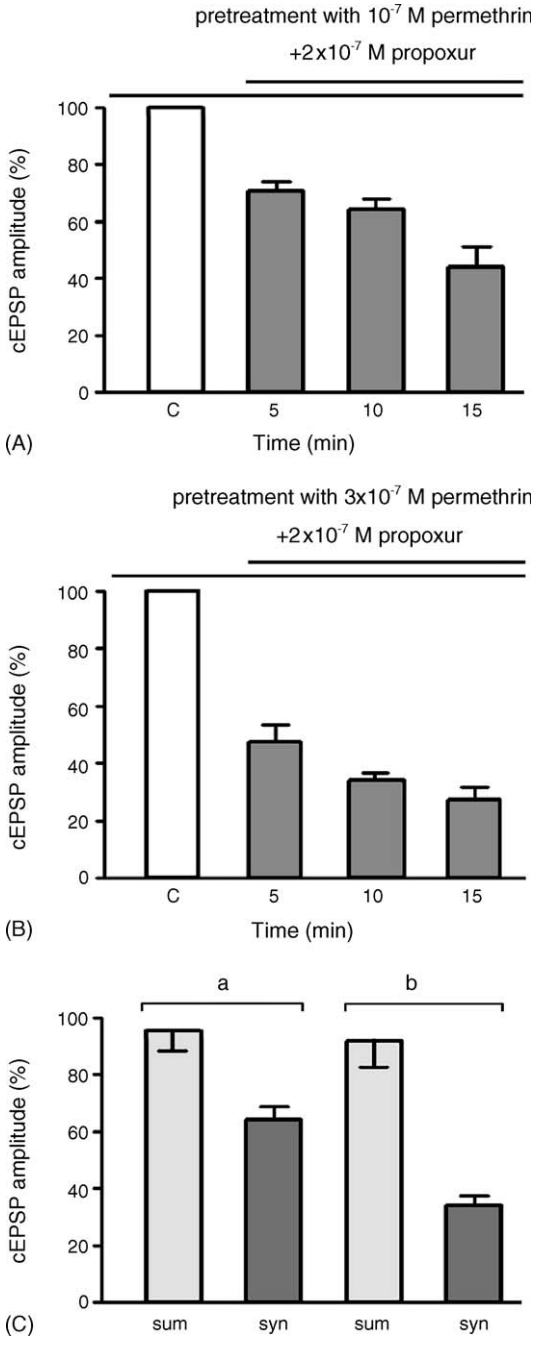

Fig. 6. Effects of permethrin and propoxur mixture on cEPSP amplitudes. Two different experimental conditions were used for studying the concentration-dependent synergism observed between permethrin and propoxur. The effect of 2×10^{-7} M propoxur on cEPSP amplitude was measured after 10 min-pretreatment with 10^{-7} M permethrin (A, control values 4.1 ± 0.6 mV, $n = 4$) and after 10 min-pretreatment with 3×10^{-7} M permethrin (B, control values 4.3 ± 0.7 mV, $n = 4$). The results clearly indicated that pretreatment with 3×10^{-7} M permethrin, 2×10^{-7} M propoxur/3×10^{-7} M permethrin co-exposure produced a greater cEPSP depression compared to propoxur applied alone at the same concentration (see Fig. 4B). Control experiments are expressed as normalized percentage (four independent experiments in all cases). Summation (sum) of effects of permethrin and propoxur applied separately (measured at 20 and 10 min, respectively) was much smaller than that of obtained with permethrin and propoxur co-exposure (syn, measured at 10 min). (Ca) Pretreatment for 10 min with 10^{-7} M permethrin and (Cb) pretreatment for 10 min with 3×10^{-7} M permethrin. Data are expressed as mean ± S.E.M.

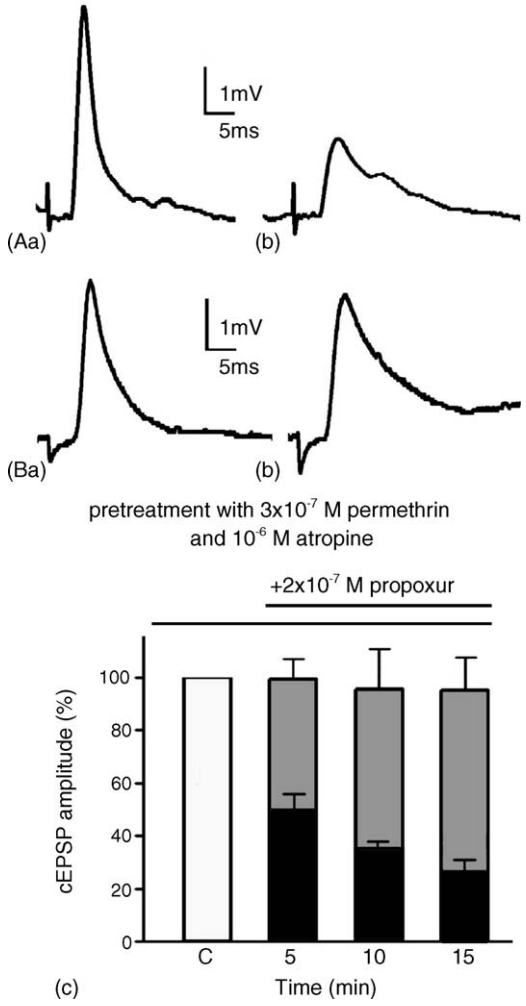

Fig. 7. Effects of atropine on synergism between permethrin and propoxur. (A) Typical examples of cEPSP recorded in control (a) and 15 min after 3×10^{-7} M permethrin/2×10^{-7} M propoxur co-exposure (b). (B) Atropine (10^{-6} M) strongly limited cEPSP depression observed with 3×10^{-7} M permethrin/2×10^{-7} M propoxur. In this case, cEPSP amplitude (b) was very similar to that recorded in control (a). (C) Superimposed histogram (grey bars) illustrated how pretreatment with 10^{-6} M atropine also abolished the synergism between 3×10^{-7} M permethrin and 2×10^{-7} M propoxur. Data are expressed as % of control 4.9 ± 0.7 mV, after atropine, $n = 3$. For comparison black bars were taken from Fig. 6B. Control experiments are expressed as normalized percentage (three independent experiments in all cases). Data are expressed as mean ± S.E.M.

4. Discussion

In this study, larval bioassays and electrophysiological experiments were undertaken to understand better the physiological mechanisms involved in insecticide synergism in insects. Toxicological studies carried out on the susceptible reference strain (SLAB) of *C. quinquefasciatus* first showed that DEF (an esterase inhibitor) did neither synergize permethrin nor propoxur whereas PBO (an oxidase inhibitor) greatly increased the toxicity of permethrin and, at a lesser extent, propoxur. However, the degree of synergy observed between permethrin and propoxur was not significantly modified by the presence of PBO (SR_{50} with and without synergist were not significantly different from each other), indicating that inhibition of monooxygenase activity was not the physiological mechanism of synergism in this strain. Since DEF did not modify the toxicity of permethrin and propoxur, it is also unlikely that esterase inhibition played a significant role in the synergistic interactions observed in these mosquitoes. Such findings differ from data previously reported in the literature which showed that synergism, in most cases, results from a competition of two compounds for a common detoxification enzyme. For example, Miyata et al. (1981) demonstrated synergism between malathion and IPB on the green rice leafhopper and the authors suggested that, since both compounds contain a carboxylester bond, the potentiation action was due to a competition of the two compounds for the same carboxylesterase. This was supported in their study by the synergistic action of IBP and malathion observed in the resistant but not in the susceptible strain, and the fact that IPB appeared to have an inhibitory effect on ^{14}C-malathion degradation in vitro.

However, many authors emphasized the fact that inhibition of enzymatic activity by one of the two component of the association could not only explain the synergistic or antagonistic interactions observed in their studies (Bodnaryk, 1982; Hemingway, 1984; Horowitz et al., 1987). For example, Hemingway (1984) demonstrated synergism by topical applications between IPB and malathion against both susceptible and carboxylesterase-resistant strains of *A. stephensi*. The authors underlined the fact that the high degree of synergism with the mixture against the susceptible strain would not be expected if IPB were simply competing with malathion for the same carboxylesterase. To date, the reason for such a synergism in susceptible mosquitoes remains unknown.

In our study, the fact that a sub-lethal dose of nicotine strongly increased the toxicity of both permethrin and propoxur against susceptible mosquitoes, suggests the manifestation of an important physiological disruption at the synaptic level. The degree of synergy was similar when nicotine synergized either permethrin or propoxur and the slopes of the regression lines with the mixtures were weak as previously observed by Corbel et al. (2003a) with insecticide combinations. This finding suggests that permethrin and propoxur may synergize each other by a similar mechanism, most probably by increasing the concentration of ACh in the synaptic cleft.

Previous electrophysiological findings clearly showed that mAChRs sharing pharmacological properties very similar to vertebrate M2 mAChR-subtypes (Le Corronc et al., 1991) were involved in the modulation of ACh release at cercal-afferent giant-interneuron synapses of the cockroach. In this case, application of muscarinic agonists decreased the cEPSP amplitude without any interaction with postsynaptic cholinergic receptors. Any means that increased ACh concentration within the synaptic cleft can mimic direct activation of presynaptic mAChRs. This was already shown particularly with organophosphate insecticides (e.g., dichlorvos) known to exhibit dual effects on the synaptic transmission (Lapied et al., 1989). Based on simultaneous analysis of cEPSP and PACh the well known anticholinesterasic effect was described at postsynaptic level. However, during prolonged application of

dichlorvos a decrease in cEPSP amplitude was observed whereas the amplitude and duration of PACh progressively increased. This secondary effect of dichlorvos was attributed to a direct action of the non-hydrolyzed ACh remaining in the synaptic cleft on presynaptic mAChRs, which thereby reduced the ACh release by a negative feedback mechanism (Hue et al., 1989; Lapied et al., 1989). According to these findings, and based on our results which demonstrated for the first time the implication of functional presynaptic mAChRs in the synergism, we suspect the following cascade of molecular events that underlies the synergistic effect observed with permethrin and propoxur mixture (Fig. 8). Modification of electrophysiological properties of the axonal voltage-dependent sodium channels by permethrin results in an increase of the ACh release in the synaptic cleft. In parallel, application of low concentration of propoxur, known to block acetylcholinesterase activity at postsynaptic level, also produces an elevation of non-hydrolyzed ACh concentration (Fig. 8, step 1). The cumulative effects of both permethrin and propoxur on ACh concentration in the synaptic cleft result in direct activation of presynaptic mAChRs involved in the negative feedback mechanism (Fig. 8, step 2). The latter decrease subsequent release of ACh which is confirmed by the cEPSP depression observed.

The physiological mechanism by which permethrin and propoxur synergized each other in the American cockroach is likely to occur in the susceptible strain of *C. quinquefasciatus*, which is free of any detectable resistance mechanism (Georghiou et al., 1966). In the S-LAB strain, mortality levels are consistent with AChE inhibition by propoxur and repetitive firing of nerves by permethrin, which lead to an ACh accumulation in the synapses and then to the death of the insect. Interestingly, the negative feedback inhibition of ACh release observed in *P. americana* may also explain unusual toxicological responses previously observed in a carbamate-resistant strain (MSE) of *C. quinquefasciatus*. Indeed, these authors have shown that in the MSE strain (300 000-fold less

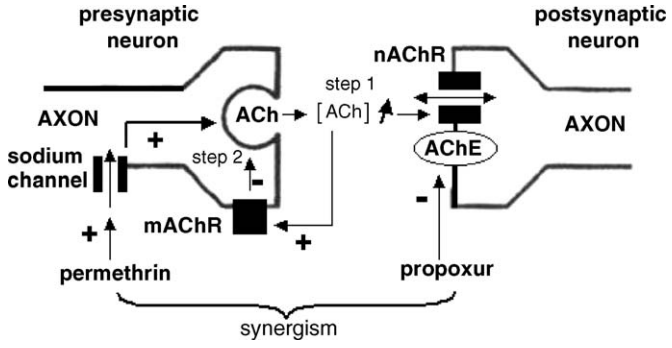

Fig. 8. Hypothetical pattern of events underlying the synergistic interaction between permethrin and propoxur that occurred through activation of the presynaptic muscarinic negative feedback mechanism. Permethrin, via alteration of voltage-dependent sodium channels, increased the release of ACh within the synaptic cleft. In the same way, the inhibition of acetylcholinesterase (AChE) induced by propoxur applied in combination with permethrin further increased ACh concentration (step 1). The large excess of non-hydrolyzed ACh released activated presynaptic muscarinic receptors involved in the negative feedback mechanism (step 2). This decreased subsequent release of ACh resulting in cEPSP depression.

sensitive than the S-LAB strain), AChE activity was unaffected by the treatment of propoxur, even at doses of insecticides giving 100% mortality. The authors suspected that mortality of MSE larvae was not due to AChE inhibition but to the interaction with another target site, known as choline acetyltransferase or ChAT (EC2.3.1.6) involved in ACh synthesis (Pitman, 1971). This was supported by the behavioural abnormality of moribund larvae (which stayed on the water surface without moving and shrivelled up and died) arising through a lack of ACh in the synapse (''ACh-mortality'') and the fact that in vitro inhibition of ChAT activity (about 30% inhibition at 2 g/l propoxur) was caused by doses of propoxur inducing mortality of MSE strain.

However, since there is no direct binding between CHAT and OP or carbamate insecticides (Scaps et al., 1997), one can assume that ACh-mortality induced by high doses of propoxur might occur through a different process involving a regulation of ACh release in the synapses. Indeed, some authors demonstrated that cholinesterase inhibitors (chlorpyrifos, paraxon, eserine, etc.) were shown to interfere with agonist binding to M2 and M4 muscarinic acetylcholine receptors in rat and human brains (Huff et al., 1994; Jett et al., 1991; Van Den Beukel et al., 1997). The authors showed that direct interactions between OPs or carbamates and muscarinic receptors occur and might play a role in the neurotoxicity of these compounds, usually known for their AChE inhibition.

Consequently, at high doses, propoxur which is also an ACh substrate analogue may interact with the presynaptic M2 receptors in the MSE strain, thereby disturbing neurological function in insects. Such ligand–receptor complex could then block the synaptic transmission through an activation of negative feedback process. This phenomenon would also explain the unusual decrease of mortality observed in heterozygotes (SLAB × MSE) with increasing concentrations of propoxur. In this case, the regulation of ACh release via a negative feedback manner would balance the previous ACh accumulation due to inhibition of sensitive fraction of AChE and then decrease the mortality. Further investigations would be now required to understand better the interaction pathway between carbamates/OPs and muscarinic receptors in the cockroach *P. americana* and the mosquito *C. quinquefasciatus* sharing highly insensitive acetylcholinesterase.

Toxicological and electrophysiological investigations have proved promising at identifying the physiological mechanisms involved in synergism in insects. Such approach allows effectively a better understanding of the mode of actions of pesticides and may then contribute to improving the control of pests in the field. Synergism between insecticides has been widely reported in the literature and is generally considered as of great importance for controlling resistant insects. In mosquitoes of medical importance, the use of pyrethroid and carbamate or OP ''two in one'' treated nets were effective at killing and reducing blood feeding of both susceptible and pyrethroid-resistant mosquitoes (Asidi et al., 2005; Guillet et al., 2001; Hougard et al., 2003). Unlike mono-treatments, the absence of selection for an insensitive acetylcholinesterase by mosaics and mixtures (Corbel et al., 2003b) was another good

indication for a better management of insecticide resistance in *A. gambiae*.

In conclusion, our recent findings on the toxicity of insecticide mixtures in insects revealed that synergism between pesticides may not only occur by enzymatic competition between components but also through complex physiological process involving target sites (sodium channel, acetylcholinesterase, muscarinic receptors, etc.) also present in mammals and humans. Subsequently, one should be very careful when using such mixtures for treated materials (bednets, fabrics, etc.) since synergistic effects might occur in humans. Risk assessment should be now carefully investigated with combined pyrethroids and cholinesterase inhibitors to ensure that such strategies present effectively no risk for human safety.

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