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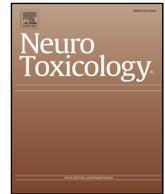
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Full Length Article

Permethrin enhances the agonist activity of dinotefuran on insect cholinergic synaptic transmission and isolated neurons



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ABSTRACT

Insect resistance mechanisms against pesticides lead to the development and the search of new pesticide combinations in order to delay the resistance. The combination of neonicotinoids with pyrethroids was currently proposed but the mode of action of these compounds at synaptic and extrasynaptic levels needs to be further explored. In the present study, we evaluated the effect of the combination of two insecticides, permethrin and dinotefuran, on cockroach cholinergic synaptic transmission and on isolated cell bodies. We first found that combination of 5 μ M permethrin and dinotefuran enhances depolarization of the sixth abdominal ganglion compared to dinotefuran alone, without an inhibition of the spontaneous activity. However, a pretreatment with 1 μ M dinotefuran or permethrin before bath application of the mixture inhibits the ganglionic depolarization. Compared to permethrin, 1 μ M dinotefuran induces a persistent enhancement of spontaneous activity. Interestingly, at extrasynaptic level, using dorsal unpaired median neurons and Kenyon cells, we found that combination of both 1 μ M dinotefuran and permethrin resulted in an increase of the mixture-induced current amplitudes. Pretreatment with 1 μ M dinotefuran strongly decreases the currents whereas permethrin induces a time-dependent inhibition. These data demonstrate that the combination of dinotefuran and permethrin enhances the effect of dinotefuran.

1. Introduction

The resistance of insects to insecticides represents a serious threat for programs aimed at controlling and preventing their effects. Considering this problem, there is a renewed interest in improving the efficacy of the existing insecticides by combining those with different modes of action to enhance insecticidal activity and to delay the development of resistance (Darriet and Chandre, 2012; Guillen and Bielza, 2012; Taillebois and Thany, 2016). In this framework, several combinations used pyrethroid and neonicotinoid insecticides to achieve effective control of resistant insect populations. Indeed, they remain the most important and widely used classes of synthetic insecticides for both agricultural applications and vector controls of animal and human diseases (Jeschke et al., 2011). Neonicotinoids act as agonists of insect neuronal nicotinic acetylcholine receptors (nAChRs) (Jeschke et al., 2013; Thany, 2009; Tomizawa and Casida, 2003, 2005), they stimulate synaptic transmission increasing excitatory activity (Buckingham et al., 1997). Pyrethroids exert their effects by interacting with the voltage-

gated sodium channel, increasing its sensitivity to depolarization and prolonging its opening by inhibiting the inactivation and deactivation processes, thereby stabilizing the open state of the channel (Soderlund et al., 2002). Thus, it was proposed that the combination of spinosad with thiamethoxam and clothianidin, increased toxicity in spinosad-resistant thrips strain. The effect of the combination was associated to a possible interaction at the nAChRs (Guillen and Bielza, 2012). Moreover, it was demonstrated that the mixture containing permethrin, dinotefuran and pyriproxyfen had anti-feeding and insecticidal efficacy against *Triatoma infestans*, a vector of *Trypanosoma cruzi* (Tahir et al., 2017), *Ctenocephalides felis* (Varloud and Fourie, 2015; Varloud et al., 2015) and *canis* (Lienard et al., 2013) fleas that may transmit tapeworm and bartonellosis. Similar efficacy of pyrethroid-neonicotinoid mixture was found with the *Anopheles gambiae*. The mixture containing clothianidin and deltamethrin induced high overall hut mortality and exiting rates, providing prolonged control of malaria transmitted by pyrethroid resistant *Anopheles gambiae* populations (Ngufoer et al., 2017).

The physiological effect of pesticides was studied using the

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cholinergic synapse between the cockroach afferent giant interneuron and the cercal nerve XI, localized in the sixth abdominal ganglion (A6), well recognized and used as a model to study the pharmacology of several compounds (Blagburn and Sattelle, 1987; Harrow et al., 1980; Hill and Blagburn, 2001; Hue et al., 2007; Hue and Callec, 1990; Sattelle et al., 1983). It was demonstrated that neonicotinoid insecticides dinotefuran, clothianidin and thiamethoxam increased ganglionic depolarization and blocked the excitatory postsynaptic potentials (EPSPs) evoked by electrical stimulation of the nerve XI (Thany, 2009, 2011) whereas co-exposure of permethrin and propoxur, a carbamate insecticide which blocked acetylcholinesterase activity, decreased EPSP amplitudes (Corbel et al., 2006). The mechanism leading to the toxicity was explained by a general physiological perturbation involving an increase in acetylcholine concentration at the synaptic level (Corbel et al., 2006). However, despite that most of the afferent excitatory fibers are carried in cercal nerve XI and the cholinergic nature of the excitatory transmitter (Flattum and Shankland, 1971; Pitman, 1985; Sattelle et al., 1983), few studies investigated the physiological mechanisms leading to the toxic effect of the combination of pyrethroids and neonicotinoids at synaptic and extrasynaptic levels.

In the present study, we investigated the mechanism of action of the mixture composed of a pyrethroid, permethrin and a neonicotinoid, dinotefuran on cockroach cholinergic synaptic transmission and isolated neurons expressing nAChR subtypes. More precisely, we used dorsal unpaired median (DUM) neurons which are neurosecretory cells generating endogenous spontaneous action potentials (Grolleau and Lapiéd, 2000) and mushroom body Kenyon cells involved in learning and memory processes (Farris et al., 2004). We found that the mixture increases ganglionic depolarization and ionic currents, suggesting that permethrin enhances the excitatory activity and the agonist effect of dinotefuran.

2. Materials and methods

2.1. Isolation of the sixth abdominal ganglion and the nerve XI

Adult male cockroaches *Periplaneta americana* were dissected and opened along the longitudinal dorsal-median line as illustrated in Callec et al. (Callec, 1974; Callec and Boistel, 1966; Callec and Sattelle, 1973). A fine pair of forceps was used carefully to remove the alimentary canal and overlying muscle and tracheae. The abdominal nerve cord, one circus and the corresponding cercal nerve XI were isolated and immediately flooded with saline solution (in mM): NaCl, 208; KCl, 3.1; CaCl₂, 5.4; NaHCO₃, 2; Sucrose, 26; pH adjusted to 7.4 with NaOH (Buckingham et al., 1997; Thany, 2009). The preparation was then removed and transferred to the recording chamber and continuously superfused with a saline plus mannitol (87 g/l) solution. The disposition of the preparation within the chamber facilitated electrical stimulation of the cercal nerve and allowed to preserve the EPSPs, the action potentials and the postsynaptic polarization (Callec and Sattelle, 1973). EPSPs were evoked using electrical stimulation of cercal nerve XI (Buckingham et al., 1997; Thany, 2009).

2.2. Electrophysiological recordings

2.2.1. Mannitol-gap recordings

Synaptic experiments were performed on the cercal nerve giant interneuron synapses located within the cockroach A6, using the mannitol-gap method pioneered by Callec (Callec and Sattelle, 1973; Callec et al., 1980). Electrical events were recorded using extracellular electrodes. A non-electrolyte medium (mannitol) was interposed between the recording sites (Callec et al., 1980). The main advantages of this method were to preserve the recordings of the unitary or evoked EPSPs and the postsynaptic polarization. Consequently, monitoring the variations of excitatory postsynaptic potential amplitudes and/or polarization induced by drug application enables dose-response curves to

be recorded. Moreover, this set-up allows long-term experiments to be performed and test solutions can be readily applied without any of the technical problems associated with intracellular recording (Callec et al., 1980).

The A6 was carefully desheathed to facilitate penetration of bath-applied drugs. The recording electrodes were connected to the input of high-impedance amplifier, whose outputs were passed to a numeric oscilloscope (PCSGU250, Velleman instruments, France) and a computer connected with Minidigidata 1B (Axon instruments, USA). Variation of postsynaptic polarization was monitored on a chart recorder and the EPSPs were evoked by electrical stimulations of the ipsilateral cercal nerve XI using a dual pulse stimulator (Campden 915, USA). The mixture was applied during 3 min under the same conditions as previously published (Buckingham et al., 1997; Thany, 2009), with a micropump fast perfusion (Harvard Apparatus) that produced a constant solution exchange (500 μL/min). For pretreatment, all compounds were bath-applied for at least 10 min before a single application of the mixture. Recordings were made at room temperature.

2.2.2. Patch clamp recordings

Permethrin and dinotefuran-induced currents were recorded using the patch-clamp technique in the whole-cell recording configuration under voltage-clamp mode. Membrane potential and input membrane resistance were recorded under current-clamp mode. Ejection pipettes and patch-clamp electrodes were pulled from borosilicate glass capillary tubes (GC150T-10; Clark Electromedical Instruments Harvard Apparatus, UK) using a P-97 model puller (Sutter Instruments, USA). Patch pipettes had resistances ranging from 1 to 1.2 MΩ when filled with internal pipette solution (see composition below). The liquid junction potential between extracellular and intracellular solutions was always corrected before the formation of a gigaohm seal (> 1 GΩ). Signals were recorded with an Axopatch 200 A (Axon instruments, USA). Currents induced were displayed on a computer with software control pClamp (version 10.0, Axon Instruments, USA) connected to a digitizer (DIGIDATA 1322, Axon Instruments, USA). DUM neuron somata were voltage-clamp at a steady-state holding potential of −50 mV. Experiments were carried out at room temperature (20–22 °C).

2.3. Compounds

All compounds were provided by Ceva Santé Animale (France).

2.4. Statistical analysis

For statistical analysis, a nonparametric Mann-Whitney test was employed, using Prism program (GraphPAD Software, San Diego, CA). Dose-response curve was fitted to the following equation :

$$y = \max / (1 + 10^{(\log EC_{50})H})$$

where max is the maximum response obtained, EC₅₀ is the concentration giving half the maximum response and H is the Hill slope. Data are presented as the mean ± S.E.M using different concentrations of the mixture on the same ganglion. Statistical analysis was expressed as nonsignificant (NS) for $p > 0.05$ and significant for $p < 0.05$.

3. Results

3.1. Effects of the permethrin and dinotefuran mixture on the sixth abdominal ganglion

In a previous study, we demonstrated that 1 mM dinotefuran applied in the sixth abdominal ganglion of the cercal afferent/giant interneuron synapses induced a strong depolarization of postsynaptic interneurons (Le Questel et al., 2011). Using the same experimental

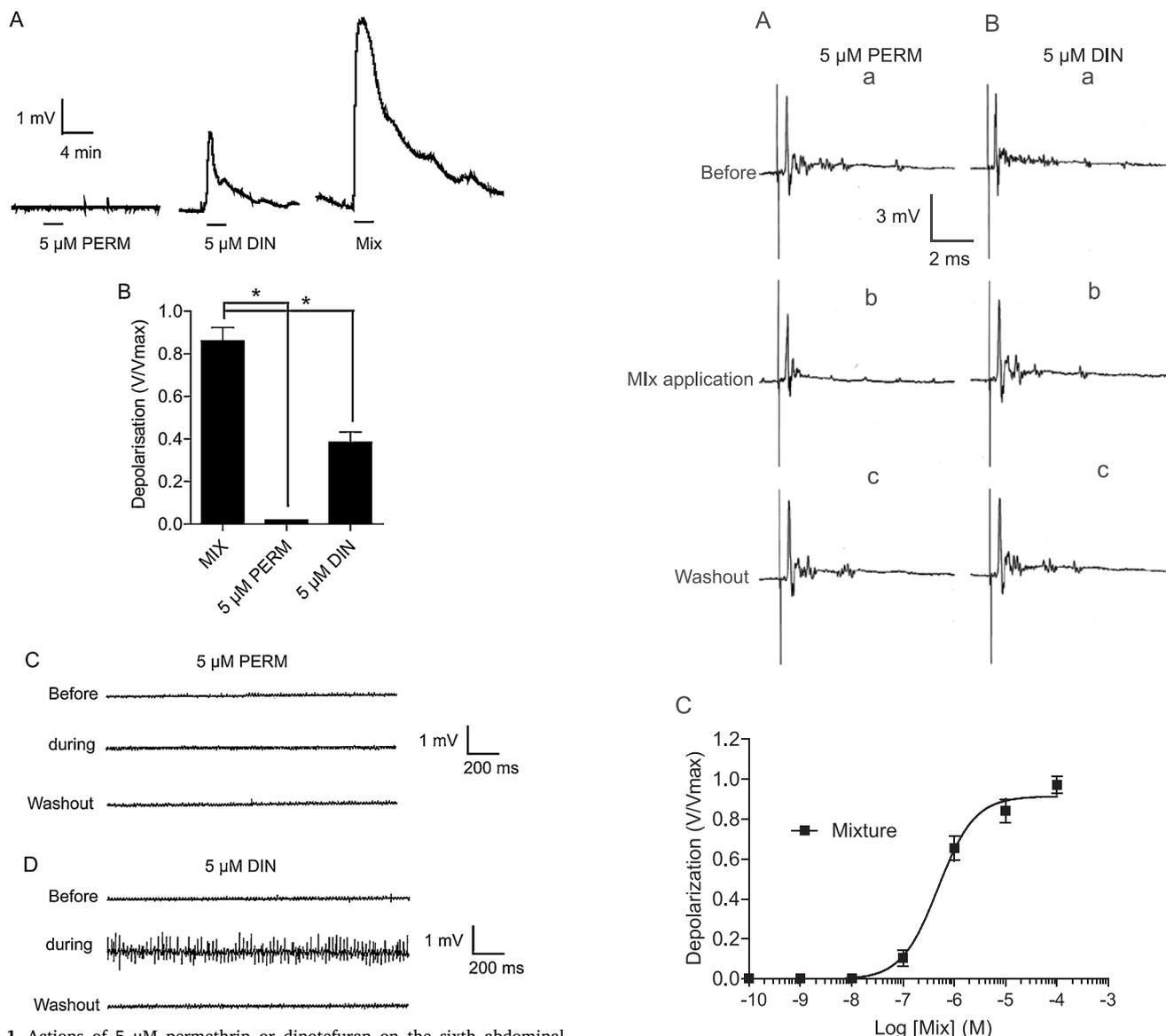
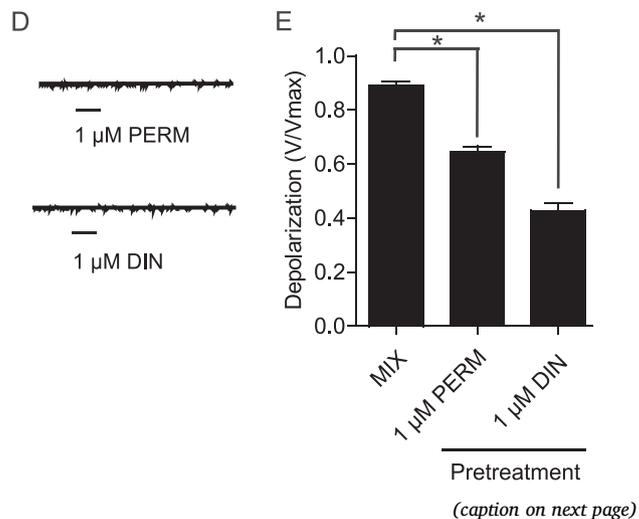


Fig. 1. Actions of 5 μM permethrin or dinotefuran on the sixth abdominal ganglion. (A) Example of 5 μM permethrin (PERM), 5 μM dinotefuran (DIN) and the mixture effects on the cercal afferent giant interneurons. The bars indicate 3 min bath application of the compound. Note that, 5 μM dinotefuran induces a ganglionic depolarization compared to permethrin. (B) Histogram showing the effect of 5 μM permethrin or dinotefuran and the mixture on ganglionic depolarization. Data are mean ± S.E.M. Each bar represents n = 12 experiments. * p < 0.05. (C and D) Spontaneous background activity (Unitary excitatory postsynaptic potential) recorded during the rising phase of depolarization under 5 μM permethrin (C) or 5 μM dinotefuran (D). In each case, upper trace represents before application of the compound, middle the effect during 3 min bath application and the lower trace during washout. Note that no background activity was observed during bath application of 5 μM permethrin.

conditions, we were interested to study the effects of the mixture of dinotefuran and permethrin at low concentration. First, we compared dinotefuran and permethrin effects at low concentration and found that permethrin at 5 μM had no effect on ganglionic depolarization compared to dinotefuran. The mixture containing both 5 μM permethrin and dinotefuran increased significantly the ganglionic depolarization compared to the effect of 5 μM dinotefuran alone. The amplitude of the mixture evoked depolarization was 6.1 ± 0.5 mV whereas we found 2.6 ± 0.2 mV for dinotefuran alone ($p < 0.05$, $n = 12$, Fig. 1A and B). This result suggested that permethrin could enhance the agonist effect of dinotefuran in the cockroach sixth abdominal ganglion. However, 5 μM permethrin completely blocked spontaneous background activity



(caption on next page)

Fig. 2. Effect of 5 μM permethrin or dinotefuran on excitatory postsynaptic potential recorded after electrical stimulation of the nerve XI. (A) Effect of 5 μM permethrin (PERM). (Aa) Before bath application of 5 μM permethrin. (Ab) During bath application of 5 μM permethrin, stimulation of the nerve XI evoked excitatory postsynaptic potential. The decrease was not significantly different compared to control condition (See Table 1). (Ac) Recording of excitatory postsynaptic potential during washout. (B) Effect of 5 μM dinotefuran on excitatory postsynaptic potential. (Ba) recording before bath application of 5 μM dinotefuran. (Bb) Recording under 3 min application of the compound. (Bc) The effect was reversed after 20 min washout. (C) Dose-response curve recorded using successive applications of the mixture using the same ganglion. Responses are normalized to the maximum response and fitted to the Hill equation describes in materials and methods section. (D) Effect of 1 μM permethrin (PERM) or 1 μM dinotefuran (DIN) on ganglionic depolarization. Note that at this concentration permethrin and dinotefuran did not induce ganglionic depolarization. (E) Pretreatment of the sixth abdominal ganglion with 1 μM permethrin (PERM) or 1 μM dinotefuran (DIN). In each case, data are mean \pm S.E.M of 12 experiments. * $p < 0.05$.

Table 1

Effect of 5 μM permethrin or dinotefuran on excitatory postsynaptic potential recorded after electrical stimulation of the nerve XI. In each experimental condition (Permethrin or dinotefuran), identical letters indicate no significant difference ($p < 0.05$). Data are expressed as mean \pm S.E.M.

	5 μM Permethrin	5 μM dinotefuran
Control	4.6 \pm 0.8 mV ^a	4.7 \pm 0.5 mV ^b
During application	4.2 \pm 0.5 mV ^a	5.0 \pm 0.6 mV ^b
Washout	5.1 \pm 0.3 mV ^a	4.6 \pm 0.4 mV ^b

(unitary excitatory post synaptic potentials) whereas dinotefuran increased spontaneous activity (Fig. 1C and D). Furthermore, at this concentration, permethrin and dinotefuran had no effect on the EPSPs evoked by electrical stimulation of the nerve XI recorded at the maximum ganglionic depolarization (Fig. 2A and B). The corresponding data are reported in Table 1. Successive applications of the mixture using the same ganglion resulted in dose-dependent depolarization of postsynaptic interneurons (Fig. 2C). Because these compounds acted on different receptors which differently affected cholinergic synaptic transmission, we suggested that pretreatment with low dose permethrin could modulate the agonist effect of the mixture consistent with an inhibitory action on presynaptic sodium channels resulting to a decrease of the ganglionic depolarization. Thus, because cockroach synapses expressed a high affinity binding site for dinotefuran (Miyagi et al., 2006), we suggested that the mixture could interact competitively with dinotefuran binding sites, resulting to a reduction of the ganglionic depolarization. When synapses were pretreated with 1 μM permethrin or dinotefuran, the mixture-induced depolarization was 30% less with 1 μM permethrin ($p < 0.05$, $n = 12$, Fig. 2D and E) and 58% less with 1 μM dinotefuran, compared to the effect of the mixture alone ($p < 0.05$, $n = 12$, Fig. 2D and E). Note that 1 μM permethrin or dinotefuran did not induce a ganglionic depolarization (Fig. 2D). These data confirmed that pretreatment with permethrin or dinotefuran reduced the mixture-induced ganglionic depolarization. Moreover, we also found that bath application of the mixture induced an increase of the spontaneous background activity (Fig. 3A). But, pretreatment with 1 μM permethrin had no significant difference. The frequency of action potentials was not significantly different compared to the effect with the mixture alone (Fig. 3B). Interestingly, the frequency of action potentials increased strongly when the synapse was pretreated with 1 μM dinotefuran (Comparing before and during mixture application, the percent of spike was increased to 47% under pretreatment with 1 μM dinotefuran) and the effect was persistent during the time-scale before and after mixture application (Fig. 3C). The increase represented $31.6 \pm 1.8\%$ of the total background activity under pretreatment with 1 μM dinotefuran (Fig. 3D).

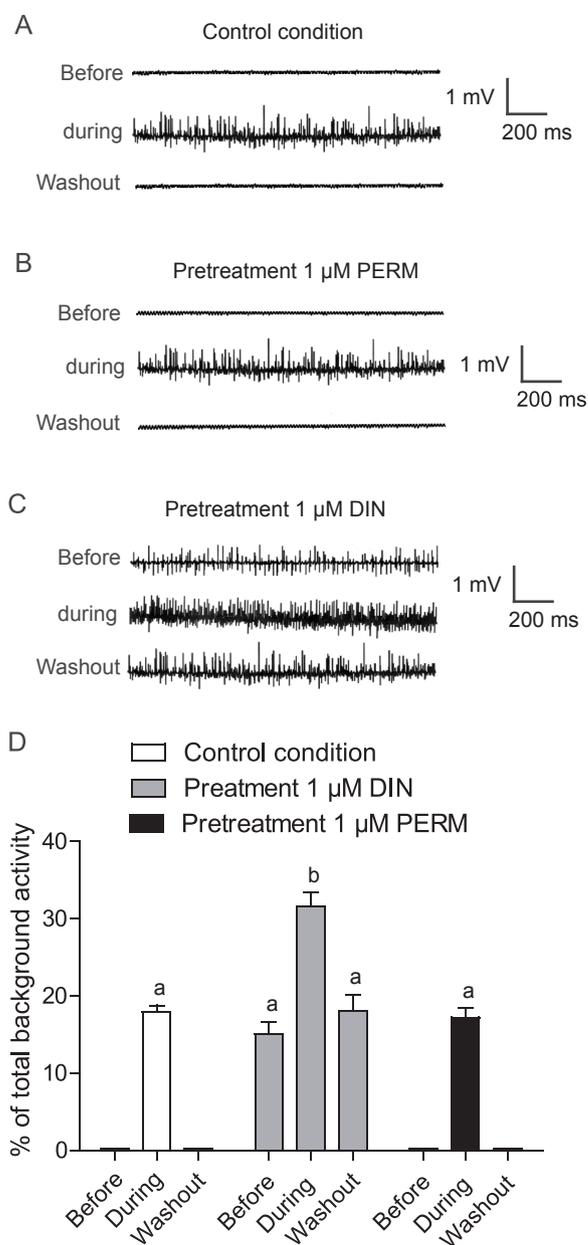


Fig. 3. Effect of the mixture containing 5 μM dinotefuran and 5 μM permethrin on background spontaneous activity after pretreatment with 1 μM dinotefuran or permethrin. (A) Effect of the mixture on excitatory background activity recorded at the maximum ganglionic depolarization. (Aa) Before the application of the mixture (upper trace) no spontaneous background activity was observed. Middle trace: spontaneous background activity during bath application of the mixture. Lower trace: the background activity stopped when the depolarization ceased. (B) Pretreatment with 1 μM permethrin. Upper trace: before application of the mixture, no background activity was found. Middle trace: Under pretreatment of 1 μM permethrin (PERM). Application of the mixture induced a background activity which ceased during washout (lower trace). (C) The terminal abdominal ganglion was pretreated with 1 μM dinotefuran. Upper trace: background activity was observed before application of the mixture, during pretreatment. Middle trace: during application of the mixture, we found a strong increase of background activity. Lower trace: background activity was persistent during washout. (D) Histograms of the percentage of total excitatory background activity. A different letter in the histogram indicates a significant difference ($p < 0.05$). Data are mean \pm S.E.M of at least $n = 12$ experiments in each condition.

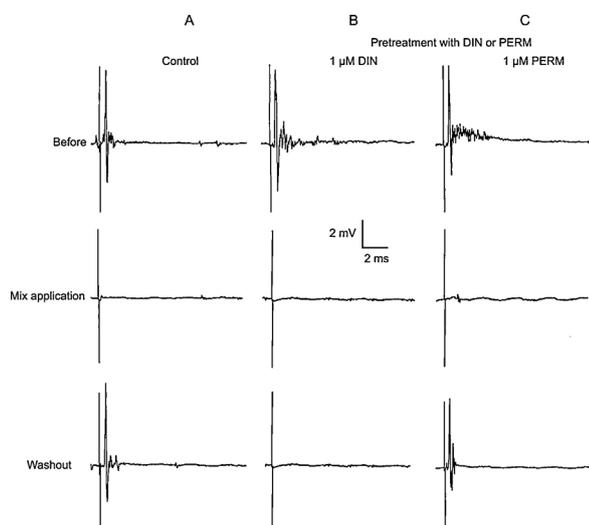


Fig. 4. Effects of the mixture on excitatory postsynaptic potentials obtained after electrical stimulation of the nerve XI after pretreatment of the sixth abdominal ganglion with 1 μM dinotefuran or permethrin. (A) The effect of the mixture was first recorded using saline solution (Control condition). Application of the mixture completely blocks the EPSP recorded after electrical stimulation of the nerve XI. No effect was found before application of the mixture and during washout. (B) Before pretreatment with 1 μM dinotefuran, we obtained excitatory postsynaptic potentials after electrical stimulation of the nerve XI (upper trace), during application of the mixture excitatory postsynaptic potential are blocked (middle trace). The effect is not reversible 10 min after washout (lower trace). (C) Similar method is used with 1 μM permethrin pretreatment. We found the same blocking of the excitatory postsynaptic potential during application of the mixture (middle trace) but compared with pretreatment with 1 μM dinotefuran, the effect is reversible (lower trace).

Table 2

Effect of the mixture on excitatory postsynaptic potential recorded under pretreatment with 1 μM permethrin (PERM) or dinotefuran (DIN). Data are mean ± S.E.M. of n = 12 experiments in each condition. Identical letters indicate no significant difference.

	Control	Pretreatment 1 μM PERM	Pretreatment 1 μM DIN
Before	4.8 ± 0.6 mV ^a	5.1 ± 0.5 mV ^a	5.2 ± 0.4 mV ^a
Mixture application	0	0 mV	0
Washout	5.3 ± 0.3 mV ^a	0 mV	4.9 ± 0.8 mV ^a

We then evaluated the effect of the mixture on EPSPs evoked after electrical stimulation of cercal nerve XI at the maximum ganglionic depolarization. When the ganglion was superfused with standard saline solution (Control condition) for 3 min, an evoked EPSP was recorded after electrical stimulation of the cercal nerve XI. The mean amplitude was 4.8 ± 0.6 mV. When the ganglion was superfused with the mixture, the EPSP was completely blocked. If the ganglion was washed with standard saline solution for 10 min, the EPSP returned to normal values of 5.3 ± 0.3 mV. No significant difference was found compared to the EPSP recorded before application of the mixture ($p > 0.05$, $n = 12$, Fig. 4A). Fig. 4B and C show recordings under pretreatment with 1 μM dinotefuran or permethrin. Pretreatment with 1 μM dinotefuran induced a blocking of EPSP which was not reversible 10 min after washout whereas the effect with 1 μM permethrin was reversible under the same conditions (Fig. 4B and C). The quantitative study of this blocking effect of dinotefuran and permethrin is reported in Table 2.

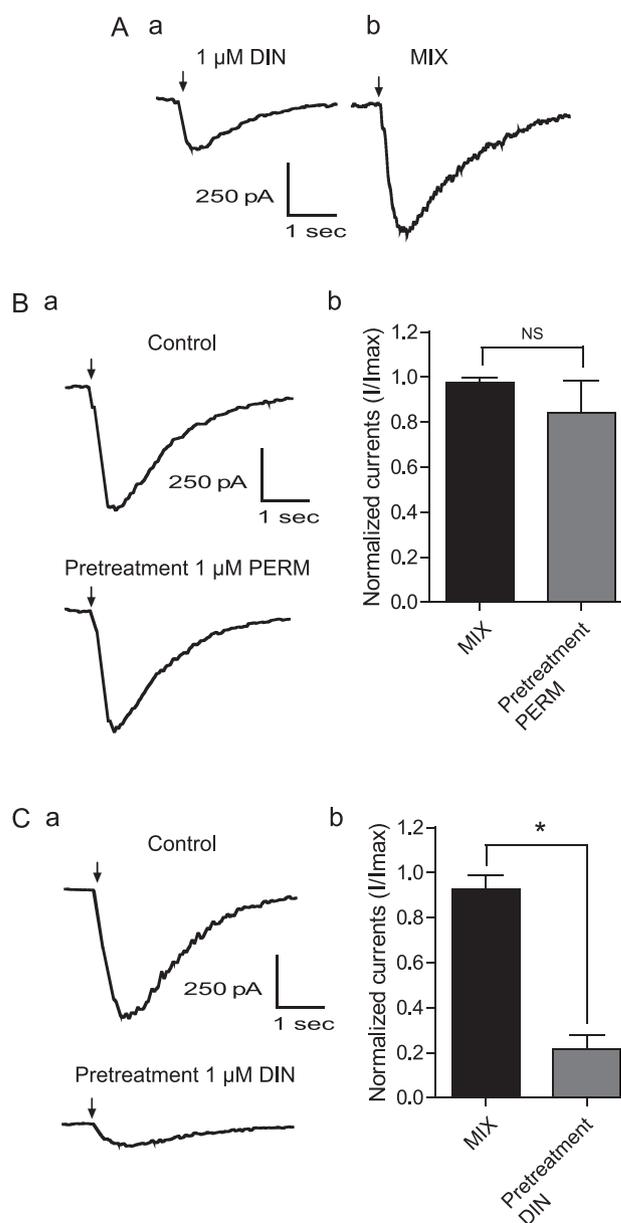


Fig. 5. Currents recorded on nicotinic acetylcholine receptors expressed on cockroach DUM neurons. (A) Example of currents induced by 1 μM dinotefuran (Aa) or the mixture (Ab) of both 1 μM permethrin and dinotefuran. (B) Effects of 5 min pretreatment with 1 μM permethrin (PERM). (Ba) Typical example of the mixture-induced currents under control condition and after 5 min pretreatment with 1 μM permethrin. (Bb) Histograms summarize the effect of the pretreatment on the mixture-induced current amplitudes. No significant difference was found. In each case, $n = 12$ cells. NS: no significant ($p > 0.05$). (C) Pretreatment of the cells with 1 μM dinotefuran (DIN). (Ca) Currents recorded after application of the mixture. Note that pretreatment with 1 μM permethrin induces a strong decrease of the mixture-induced current amplitudes. (Cb) Histograms represent the decrease of current amplitudes after pretreatment with 1 μM dinotefuran. In each case, histograms are represented as mean ± S.E.M. of $n = 12$ cells. * $p < 0.05$. MIX: mixture; PERM: permethrin and DIN: dinotefuran. Arrows indicate 300 ms pulse duration of the mixture. Currents are recorded at -50 mV holding potential.

3.2. Effects of the mixture on isolated cell bodies expressing nicotinic receptor

We have demonstrated that the permethrin and dinotefuran mixture induced an increase of ganglionic depolarization and inhibited EPSPs evoked by electrical stimulation of the nerve XI at the maximum

ganglionic depolarization. The effect of the mixture was significantly higher to the one of dinotefuran alone, suggesting that permethrin could enhance dinotefuran effect. We hypothesized that this effect was not specific to the synapses between cercal afferent and giant interneurons and could be observed on isolated neurons such as DUM neurons, isolated from the same sixth abdominal ganglion. Thus, we used a mixture of both 1 μ M dinotefuran and permethrin. Under the same conditions, as expected, permethrin enhanced the agonist effect of dinotefuran. We found that application of the mixture strongly induced an inward current of -0.58 ± 0.03 nA compared to currents induced by 1 μ M dinotefuran (-0.21 ± 0.01 nA, $p < 0.05$, $n = 18$ cells, Fig. 5A). We then evaluated the effect of cells pretreatment during 5 min with 1 μ M permethrin or dinotefuran. No significant change of the mixture-induced current amplitudes was observed when cockroach DUM neurons were pretreated during a short term delay with 1 μ M permethrin ($p > 0.05$, $n = 12$, Fig. 5B). Nevertheless, we found a strong decrease of the mixture-induced currents after 5 min pretreatment with 1 μ M dinotefuran. Indeed, 1 μ M dinotefuran significantly reduced the currents induced by the mixture to -0.12 ± 0.02 nA ($p < 0.05$, $n = 12$, Fig. 5C). These data seemed to suggest that dinotefuran may interact with the mixture on cockroach nAChRs expressed on DUM neurons. Thus, in both cases, the effect of the mixture and dinotefuran were sensitive to nicotinic acetylcholine receptor antagonist mecamylamine (Data not shown). Moreover, when cells were treated for a long period with 1 μ M permethrin, the current amplitudes decreased strongly and no effect on current amplitudes was observed if 1 μ M dinotefuran was applied during the decrease (Fig. 6A). Using the same method, we found that the decrease of the mixture-induced currents could be reversed after bath application with 1 μ M permethrin (Fig. 6B). These data suggested that dinotefuran had a direct and rapid effect on current amplitudes. Interestingly, similar effect was not found with cockroach Kenyon cells. Indeed, despite the fact that the mixture induced an increase of current amplitudes (The mean current amplitude was -208.7 ± 0.3 pA for the mixture and -82 ± 0.07 pA for dinotefuran at -50 mV holding potential, Fig. 7A and B), pretreatment with 1 μ M permethrin or dinotefuran revealed that 1 μ M permethrin had no effect on current amplitudes (Fig. 8A and B). 1 μ M dinotefuran reduced to 82% the mixture-induced current amplitudes ($p < 0.05$, $n = 12$, Fig. 8C and D). In all cases, we found that the kinetics of the mixture evoked currents were affected after pretreatment with both 1 μ M permethrin or dinotefuran.

4. Discussion

4.1. Effects of the mixture containing pyrethroids and neonicotinoids

In the present study, the cockroach cholinergic synaptic transmission was used to evaluate the effect of the permethrin and dinotefuran combination at low concentration. First, we found that the combination of low concentration permethrin and dinotefuran increased ganglionic depolarization, inhibited the spontaneous background activity and the EPSPs. The effect of the mixture at synaptic level could be explained by an action of permethrin on sodium channels. At the presynaptic level, permethrin bound to sodium channels and counteracted the excitatory effect of dinotefuran resulting to an inhibition of the background activity. Indeed, the effect of permethrin on background activity was consistent with previous data demonstrating that low permethrin concentration inhibited spontaneous activity of cockroach presynaptic cercal mechanoreceptors (Corbel et al., 2006) and cholinergic minisynaptic current in projection neurons of the drosophila dorsal antennal lobe glomeruli (Yan et al., 2011). This hypothesis could explain the finding that pretreatment with dinotefuran resulted to an enhancement of the spontaneous activity whereas permethrin inhibited the activity. Our point of view is consistent with the previous observation that permethrin blocked cholinergic transmission at the cercal-afferent giant-interneuron synapses (Hue and Mony, 1987). Moreover, we

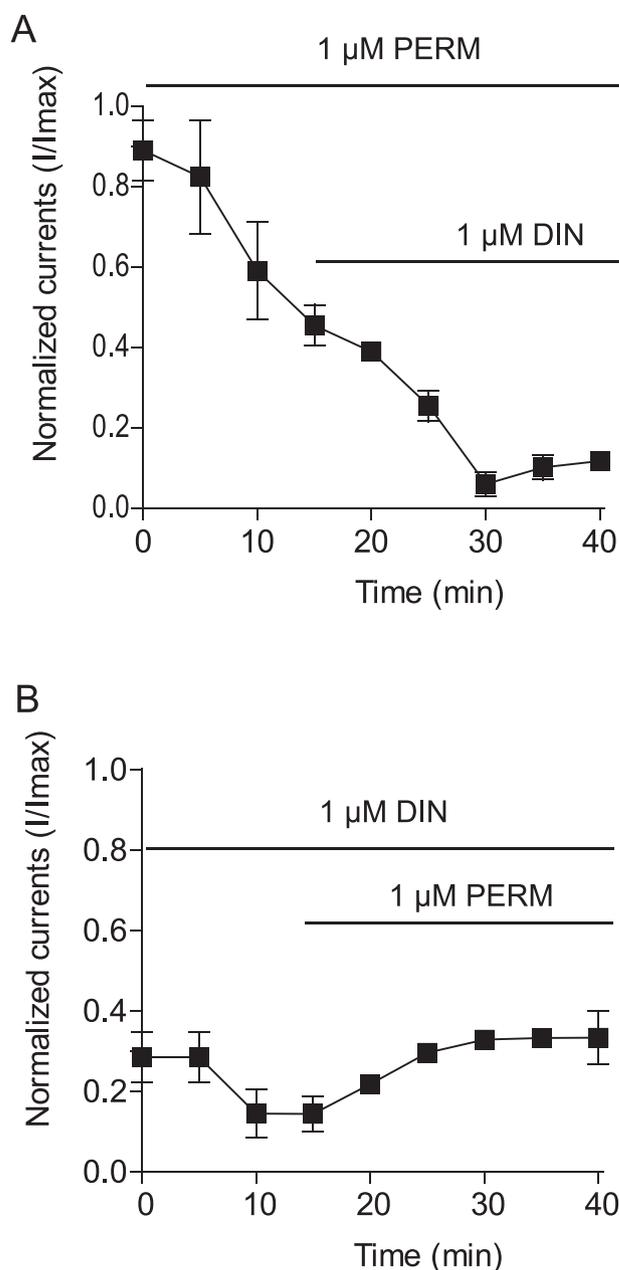


Fig. 6. Time-dependent effect of the mixture during bath application of 1 μ M permethrin or dinotefuran. (A) Mixture was continuously bathed with 1 μ M permethrin, resulting to a decrease of mixture-induced currents. The current amplitude continuously decreases despite application of 1 μ M dinotefuran (DIN). (B) The effect of the mixture under application of 1 μ M dinotefuran (DIN) was reversed if we apply 1 μ M permethrin. Data are mean \pm S.E.M. Each point represents $n = 12$ cells.

hypothesized that the mixture effect resulted to both presynaptic activity of permethrin and postsynaptic activity of dinotefuran. For instance, pretreatment with permethrin could increase the depolarizing effect of permethrin, reducing the sensitivity of sodium channels and resulting to a decrease or an inhibition of background activity whereas pretreatment with dinotefuran will strongly increase the effect of the mixture on postsynaptic nAChRs.

The mode of action of the mixture on isolated cell bodies appears more complex. Combination of both compounds enhanced the mixture-induced current amplitudes compared to dinotefuran alone. The agonist effect induced by the mixture was reduced after pretreatment with dinotefuran whereas permethrin had a time-dependent effect. We

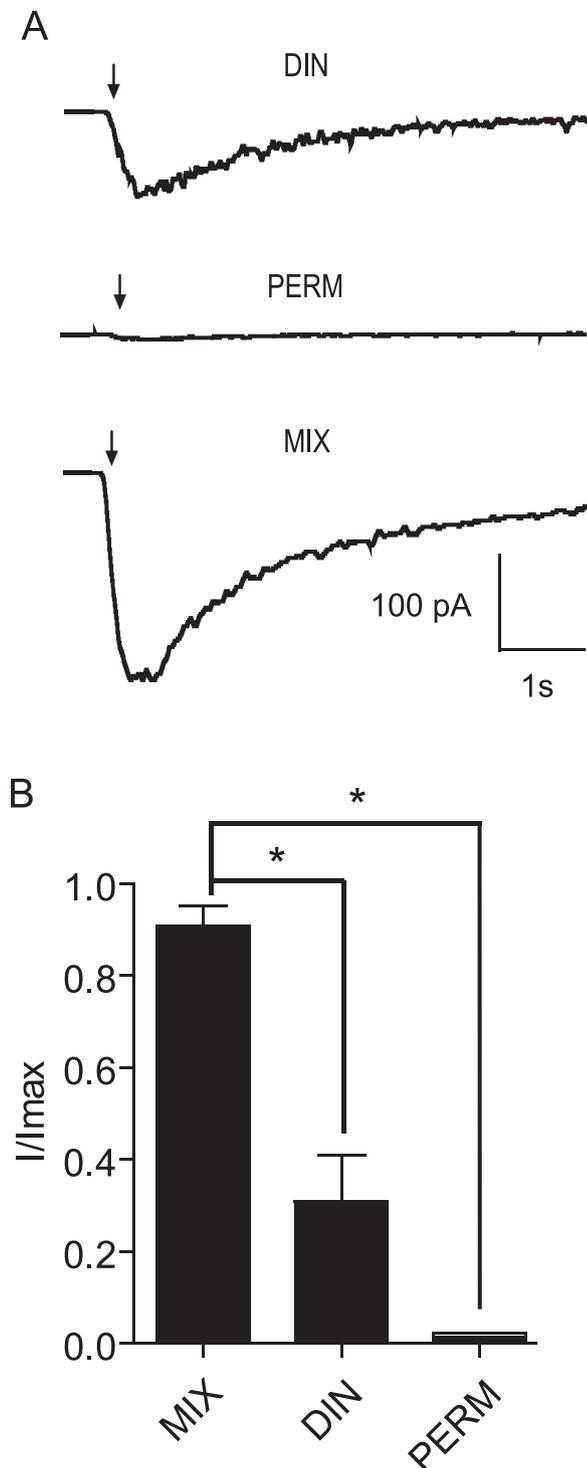


Fig. 7. Effect of dinotefuran, permethrin and the mixture on cockroach nAChRs expressed on Kenyon cells. (A) Currents induced by 1 μ M dinotefuran (DIN), 1 μ M permethrin (PERM) and the mixture (MIX) of 1 μ M permethrin and 1 μ M dinotefuran. Arrows indicate 300 ms pulse (15 psig) duration. (B) Histograms illustrating the effects of the mixture and of each compound on cockroach Kenyon cells. In each case, data are mean \pm S.E.M. of 12 cells. * $p < 0.05$.

suggested that the effect of the mixture could be associated to a decrease of sodium channel activity. In particular, permethrin may activate sodium channels leading to a membrane depolarization and an increase of sodium ions in the cell. This increase is counterbalanced by a $\text{Na}^+/\text{Ca}^{2+}$ exchanger and Ca^{2+} entry through nAChRs activated by dinotefuran. The combined action of permethrin and dinotefuran and

intracellular Ca^{2+} flux increases the mixture-induced current amplitudes. Indeed, we recently demonstrated that neonicotinoid insecticide effect, such as clothianidin is associated with an increase of intracellular calcium (List et al., 2014). Low Ca^{2+} increase leads to a low clothianidin-induced current amplitude whereas increasing intracellular Ca^{2+} induces higher currents (List et al., 2014). We suspect that joint activity of $\text{Na}^+/\text{Ca}^{2+}$ exchanger and nAChRs could enhance intracellular Ca^{2+} concentration. Secondly, pretreatment with permethrin induces a failure in depolarizing the membrane, leading to a decrease of Na^+ influx, consequently no Ca^{2+} entry (or strong Ca^{2+} entry) which results to a decrease of the mixture-induced current amplitudes. This mechanism could explain the finding that permethrin effect is time-dependent compared to dinotefuran which had a rapid effect.

4.2. Synergism between pyrethroids and neonicotinoids insecticides as a strategy against insect pest

The development of resistance to pyrethroids and neonicotinoids lead to the use of pesticide combinations for the control of arthropod pests and vector-borne diseases. In particular, it is expected that the combination will shift the evolution of resistance, reducing the concentration used and increasing the potential of each compound. This strategy may limit environmental effect of pesticides. Moreover, it could help to increase the specificity of some mixtures against insect pests avoiding or limiting negative effects against useful insects. This is an important area for future studies and successful management of pest populations. In this context, combinations between pyrethroids and neonicotinoids were investigated with the challenge to identify the best combination (Taillebois and Thany, 2016; Yu et al., 2016). For example, the neonicotinoid, dinotefuran and pyrethroid, permethrin used in this study, were previously applied to commercialize the VECTRA 3D, an ectoparasiticide combining three active ingredients (dinotefuran, permethrin and pyriproxyfen) against *Stegomyia albopicta* mosquitoes (Tahir et al., 2017). VECTRA 3D revealed a significant anti-feeding and insecticidal efficacy against *S. albopicta*. According to the data reported in the present study, we propose that permethrin concentration in the mixture helps to increase the agonist effect of dinotefuran. The efficacy of the mixture is associated with the finding that in addition to their effect on sodium channels and nAChRs, permethrin and dinotefuran have a shift in their mode of action. Our future investigations will be to test other combinations of pyrethroids. We previously demonstrated that the mixture of deltamethrin and acetamiprid increased the toxicity of each compound against the pea Aphid *Acyrtosiphon pisum* (Taillebois and Thany, 2016). We hypothesize that type I and type II pyrethroids could have different effects because it was proposed that they have different toxicity (Breckenridge et al., 2009).

In conclusion, we designed this study to evaluate the mode of action of pyrethroids and neonicotinoids at synaptic and extrasynaptic levels. We demonstrated that low permethrin concentration was able to increase dinotefuran effect. Our results and previous data give us first insight into the molecular mechanisms of these compounds at low doses which is of great interest for safe applications of pesticides.

Conflict of interest

The authors declare no conflicts of interest. This paper adheres to the principles for scientific rigor and transparency.

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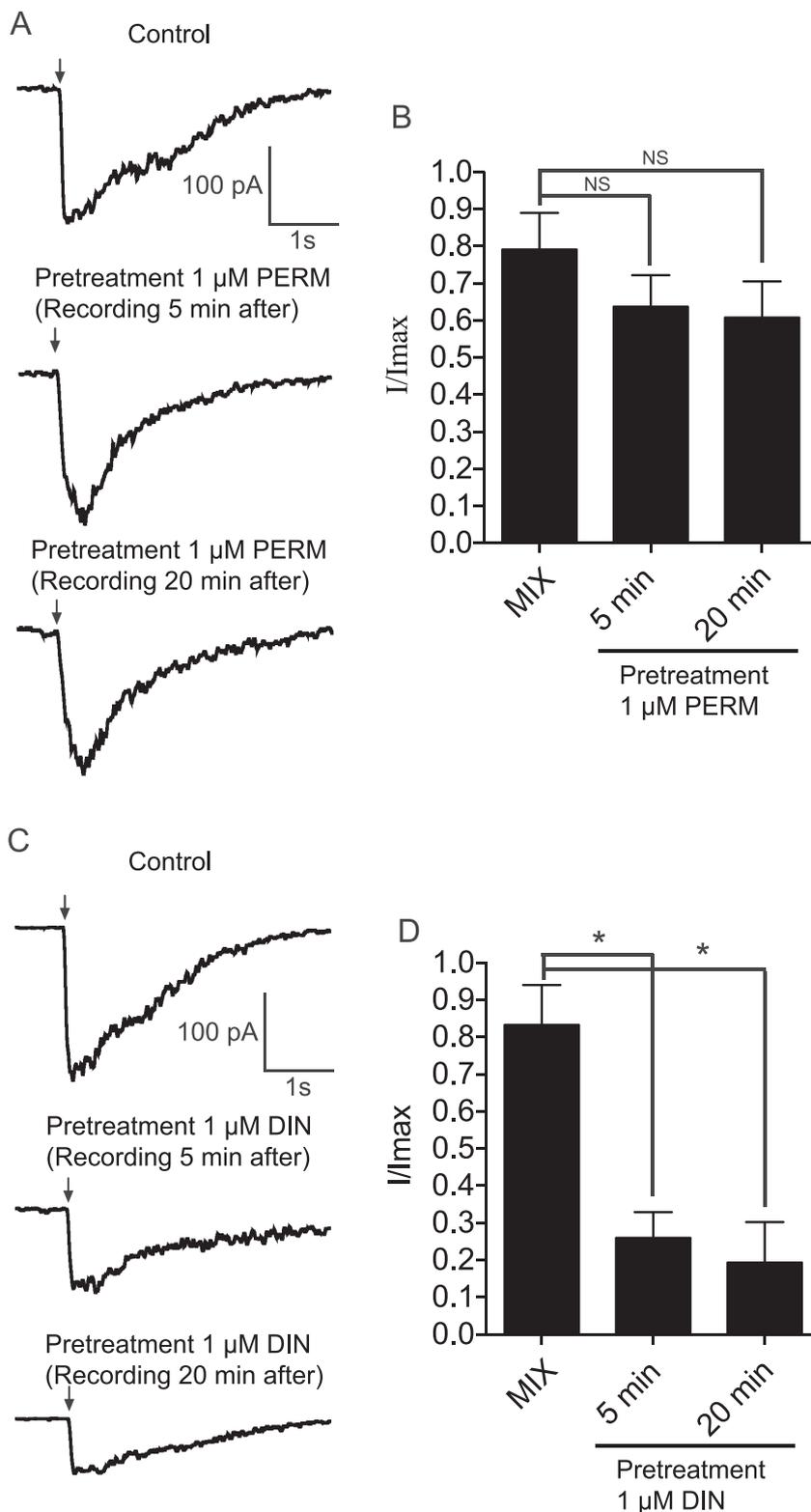


Fig. 8. Pretreatment of Kenyon cells with 1 μ M permethrin (PERM) or 1 μ M dinotefuran (DIN). (A) The mixture-induced currents recorded at -50 mV holding potential in control condition (Control) and 5 min and 20 min during pretreatment with 1 μ M permethrin (PERM). (B) Histograms summarize the data obtained under bath application of 1 μ M permethrin. Each histogram represents mean \pm S.E.M. of $n = 12$ cells. No significant difference (NS) was found at 5 min and 20 min. (C) The mixture-induced currents are also recorded under pretreatment with 1 μ M dinotefuran (DIN). After 5 min pretreatment with 1 μ M dinotefuran we found a reduction of the current amplitudes. The current amplitudes are also reduced 20 min later during pretreatment. (D) Histograms summarize the data obtained in each condition. Data are mean \pm S.E.M of 12 cells. * $p < 0.05$. Arrows indicate 300 ms pulse duration of the mixture.

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