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Functional neurotoxic effects in rats acutely exposed to insecticide combinations

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Abstract

The aim of the present study was to analyse the alterations in the cortical and peripheral electrophysiological activity of rats acutely treated with combinations of insecticides. Young adult male Wistar rats were treated with 1/5 and 1/25 LD_{50} of the insecticides dimethoate, propoxur, cypermethrin and amitraz, given alone or in triple or quadruple combinations. After 24 h, spontaneous cortical activity, and stimulus-evoked cortical and peripheral responses, was recorded and analysed. All treatments changed the cortical activity spectrum. The effect of the 1/5 LD_{50} combinations indicated non-additive interactions. In the cortical-evoked responses, dimethoate and its combinations gave the strongest change in the latency, while amitraz and its combinations, in the response duration. In the tail nerve, relative refractory period was the most sensitive parameter. The frequency dependence of the cortical responses was the most strongly altered by propoxur, and the least, by amitraz. Our results indicate that simultaneous exposure by various pesticide agents, which happens possibly also in humans, deserves further investigation in, among others, neurotoxicological points of view.

Keywords: Insecticides; Combined exposure; Neurotoxicity; Cortical activity; Peripheral activity; Rat

1. Introduction

Chemical plant protection results in spread of pesticide agents in the environment, potentially causing multiple occupational, or even food-borne, exposure in humans. Relevant toxicological knowledge is, at the same time, insufficient; although it is well known that the most frequently used insecticide agents attack the nervous system of the target, and also of non-target, species.

Organophosphates (OPs) [1] cause permanent inhibition of acetylcholinesterase [2]. In human OP intoxication, a variety of nervous system effects have been found, first of all EEG abnormalities [3,4]. Such alterations were observed also in animal experiments [5,6] as were alterations in cortical-evoked potentials [7,8]. Dimethoate, (DIM) the OP chosen for our study, has moderate human toxicity [9] and widespread use in numerous countries. In our previous experiments, a low dose DIM was applied to rats by different routes of administration and in various timing schemes, and was found to alter neurophysiological parameters in acute [10] and subchronic [11] application.

Carbamates, derivatives of carbaminic acid [12] are another group of insecticide agents with cholinesterase blocking as their main action [13]. This effect of carbamates is, however, reversible. Propoxur (PRP), the carbamate used in the present study, is applied mainly in household pest control and for residual spraying in malaria eradication programs, thanks to its long-lasting activity. In humans, the symptoms of PRP poisoning (diarrhoea, nausea, vomiting, abdominal pain, profuse sweating, salivation, blurred vision, temporary paralysis of the extremities, etc.) are typical for cholinergic overweight [14], although atropine-like effects following PRP exposure are also known [15]. In animal experiments, a single dose of ca. 1/10 LD_{50} caused a 60% drop in cholinesterase activity and marked

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disturbances in higher nervous functions [16]. The similarity of the functional neurotoxicity of PRP and an OP (methyl parathion) was demonstrated by Institóris et al. [17].

Pyrethroids (synthetic derivatives of pyrethrins, toxins contained in the flowers of some *Chrysanthemum* species) are widely used as insecticides because of their high insecticidal potency, low mammalian toxicity, and biodegradability [18]. The agent studied, cypermethrin (CYP), belongs to the type II pyrethroids which have mostly central action [19], leading to poisoning manifested in hypersensitivity, choreoathetosis, tremors, and paralysis [20,21]. Within the nervous system, Na^+ channels are the primary target of pyrethroids [22]. Other known effects include block of Ca^{2+} channels [23], inhibition of ATPases [24], and of acetylcholine [25], GABA [26], serotonin [27] and benzodiazepine [28] receptors.

In patients intoxicated with amitraz (AMI, a formamidine-type insecticide), depression of the CNS was found in all cases [29] together with respiratory depression, bradycardia, hypotension and convulsions. In rats, AMI altered visual evoked potentials [30], reduced motor activity and inhibited monoamine oxidase but not acetylcholinesterase [31].

Although the mentioned insecticide agents are in widespread use so that multiple occupational exposure is possible and simultaneous presence of residues in the environment cannot be excluded, information on their simultaneous effects and interactions is minimal. Therefore, the aim of the present study was to record and analyse the alterations in the cortical and peripheral electrophysiological activity of rats acutely treated with combinations of the above insecticides. (Acute application is not a realistic model of human exposure, previous experiments have, however, shown that the alterations of cortical electrical activity following acute and subchronic administration of OPs in rats can be quite similar [32].) It was also supposed that the results of this work will be applicable as prime data in more realistic, longer-term exposure models.

2. Methods

Young adult male Wistar rats of ca. 250 g body weight, obtained at the Breeding Centre of the University of Szeged, were used. The animals (four rats in a cage) were housed under conventional conditions (22–24 °C, 12 h light/dark cycle with light starting at 6:00 a.m.). Standard rodent chow and drinking water was given *ad libitum*.

The insecticides were given in a single oral dose, administered by gavage, 24 h before electrophysiological recording, according to the doses given in Table 1A. The higher dose chosen was 1/5 LD_{50}, in which the effect of both the single agents and their combinations was tested. The lower dose was 1/25 LD_{50} where only combinations were used. The LD_{50} values (Table 1B) were determined, based on data found in the literature, in previous studies (DIM [33], PRP [34], CYP [35]) or in pre-experiments of the present study (AMI). DIM, PRP and CYP were dissolved in sunflower oil of pharmaceutical quality to 0.5 ml/kg b.w. AMI was suspended in 2.5% methylcellulose mucus (5 ml/kg b.w.) and was, in the combinations, given separately 30 min before the other substances. The control animals received

Table 2
Time schedule of the experiments

Time elapsed from the beginning (h:min)	Action
0:00–0:30	Oral administration of the insecticide(s)
23:10	Urethane injection (ip)
23:20–23:30	Preparation
24:00	Start ECoG recording
24:06	Start somatosensory EP recording
24:09	Start visual EP recording
24:10	Start auditory EP recording
24:12	Start tail nerve recording
24:20	End of recording

Table 1
Doses administered in the single and combined treatments (A), and LD_{50} data of the insecticides used (B)

Groups	Control	D	P	C	A	DPC[a]	DPA	DCA	PCA	DPCA
(*A*)										
High dose (1/5 LD_{50})	oil	92	17	111	105.8	92 17 110.8	92 17 105.8	92 110.8 105.8	17 110.8 105.8	92 17 110.8 105.8
Low dose (1/25 LD_{50})	oil	—	—	—	—	18.4 3.4 22.2	18.4 3.4 21.2	18.4 22.2 21.2	3.4 22.2 21.2	18.4 3.4 22.2 21.2
		Dimethoate		Propoxur		Cypermethrin				Amitraz
		DIM, D		*PRP, P*		*CYP, C*				*AMI, A*
(*B*)										
LD_{50} (mg/kg b.w.)		460		85		554				529

[a] In the combinations, the doses are given in the order signalized by the one-letter symbols (see Table 1B) of the agents used.

0.5 ml/kg b.w. sunflower oil. Each group, treated and control, consisted of ten rats, giving a total number of animals of 150.

For recording, the rats were prepared in urethane anaesthesia (1000 mg/kg ip). The animal's head was fixed in a stereotaxic frame, the skull was opened and the left hemisphere was exposed. Lidocaine (10%) was applied on the wounds and liquid paraffin on the exposed dura. Following ca. 30 min recovery, silver recording electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas, and electrocorticogram (ECoG) was simultaneously recorded from these sites for 6 min. Subsequently, cortical evoked potentials (EPs) were recorded from the same sites by application of series of 50 peripheral sensory stimuli; first SS at 1, 2 and 10 Hz repetition rate, then VIS at 1 Hz, and finally AUD at 1 Hz rate. For somatosensory stimulation, a pair of needles was inserted in the contralateral whisker pad and electric shocks (3–4 V, 0.05 ms) were applied. For visual stimulation, flashes (ca. 60 lux) of a flashbulb were directed to the contralateral eye via an optical conductor. Acoustic stimulation was performed by clicks (40 dB), led through the hollow ear bar into the contralateral ear of the rat. After that, the tail nerve was stimulated at the base of tail by electric shocks (4–6 V, 0.05 ms) delivered via a pair of needles, and the compound action potential was recorded 50 mm

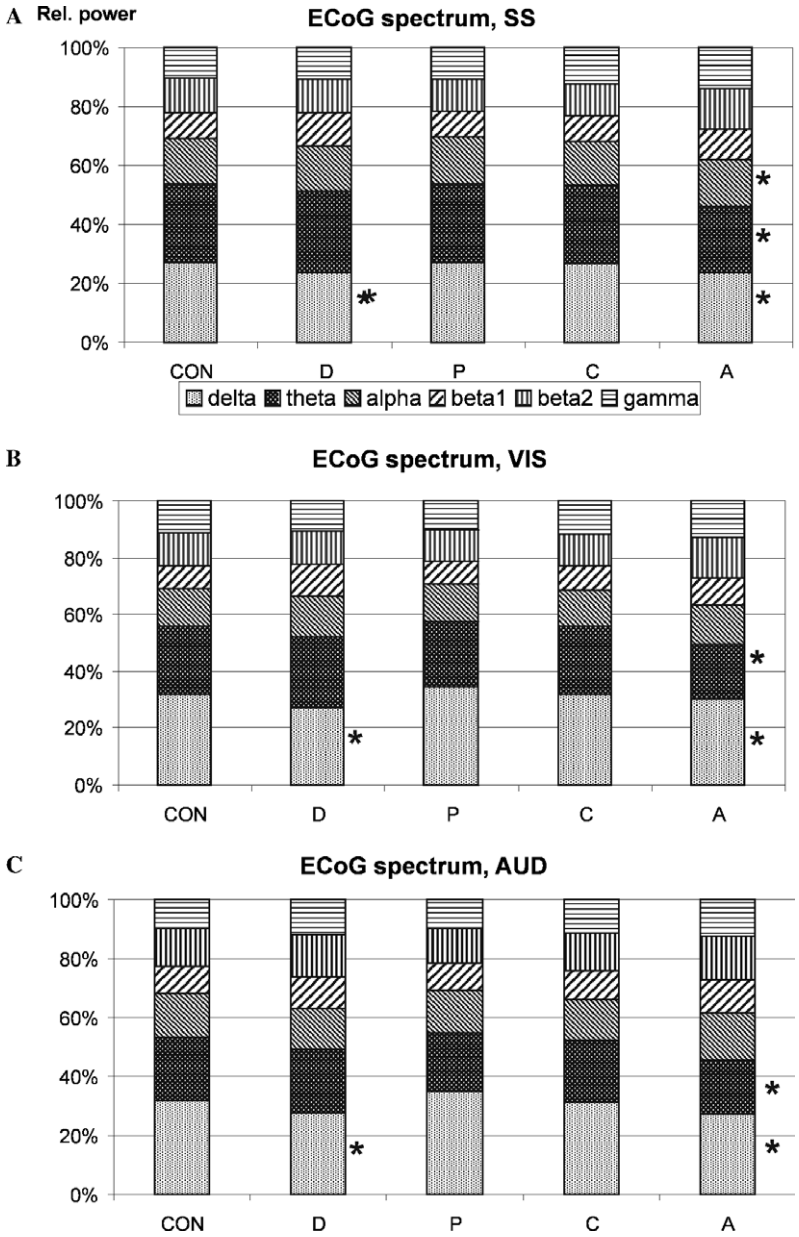

Fig. 1. Spectral distribution of the ECoG in the somatosensory (A), visual (B) and auditory (C) cortical centre after acute application of 1/5 LD_{50} of the insecticides. CON, control; D, dimethoate; P, propoxur; C, cypermethrin; A, amitraz. Ordinate: percents within the total ECoG power. Insert in A: frequency bands. $^{*}p < 0.05$ vs. control in the same band.

distally by another pair of needle electrodes. The time schedule of the experiments is given in Table 2.

The analysis of the ECoG records yielded power spectra by bands (δ to γ [36]).

On the cortical EPs, latency and duration of the main waves were manually measured after averaging. From the tail nerve records, conduction velocity and relative refractory period was determined as described in Dési and Nagymajtényi [8]. Recording and analysis was PC-based, using the NEUROSYS 1.11 software (Experimetria, UK). Finally, the rats were sacrificed by an overdose of urethane.

The primary data were compared by one-way ANOVA, separately for each cortical area, after the Kolmogorov–Smirnov normality test. Then, ECoG index, EP amplitude and EP duration were normalized, on the basis of the control mean value in each area, and compared by two-way ANOVA (treatment × area) to see if the alterations in the three cortical areas (if any was indicated by the one-way ANOVA) were significantly different. For post hoc analysis, LSD was used with $p < 0.05$ as criterion of significance throughout.

During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University of Szeged (based on the EU-harmonized animal welfare act of Hungary) were strictly followed.

3. Results

The 1/5 LD_{50} doses of the insecticides had some clear-cut effects on the ECoG. When given alone, the relative power

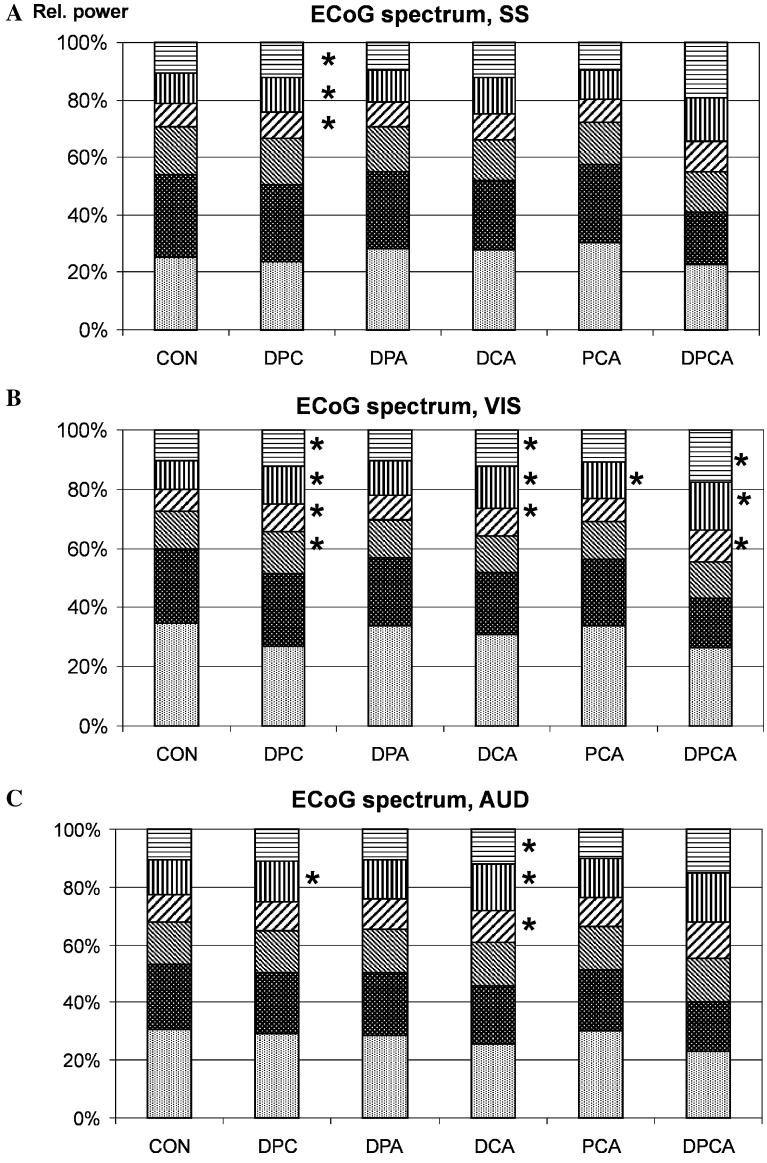

Fig. 2. Spectral distribution of the ECoG in the somatosensory (A), visual (B) and auditory (C) cortical centre after acute application of 1/5 LD_{50} of the insecticides in combinations. Displayed as in Fig. 1. CON, control; DPC, dimethoate-propoxur-cypermethrin; DPA, dimethoate-propoxur-amitraz; DCA, dimethoate-cypermethrin-amitraz; PCA, propoxur-cypermethrin-amitraz; DPCA, dimethoate-propoxur-cypermethrin-amitraz. *$p < 0.05$ vs. control in the same band.

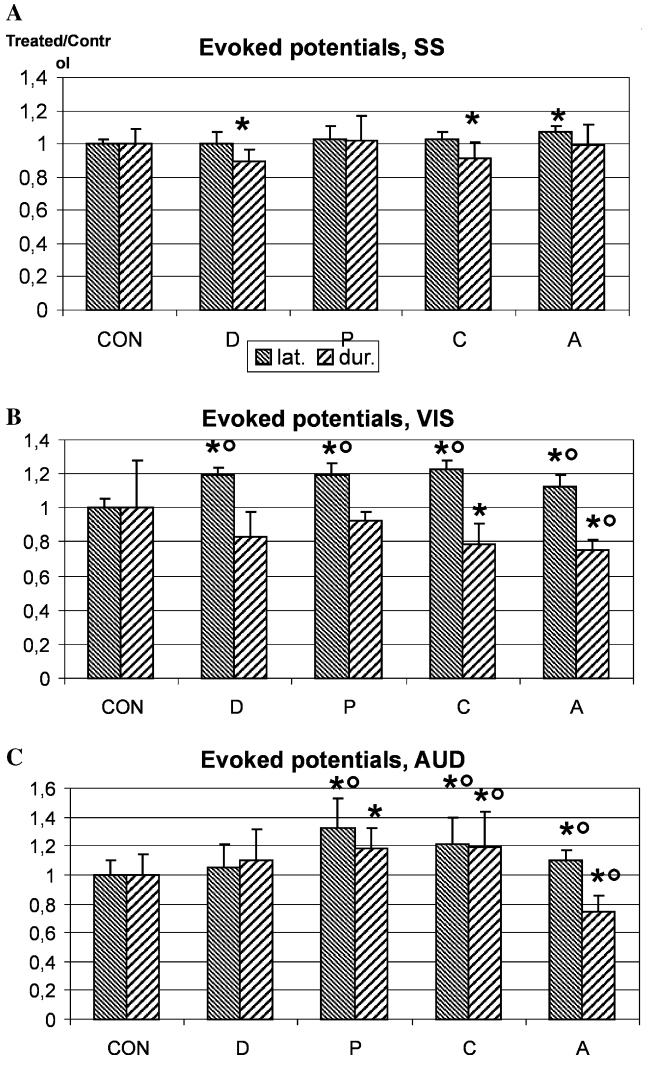

Fig. 3. Change of latency (lat.) and duration (dur.) of the cortical sensory evoked potentials in the rats treated with 1/5 LD_{50} of the insecticides. The values displayed, both mean and SD, are normalized to the mean values of the control, and constitute thus relative changes. (A), somatosensory; (B), visual; (C), auditory areas. Bar patterns as in Fig. 1. Ordinate: relative change (mean + SD, $n = 10$). $^{*}p < 0.05$ vs. the data of the same cortical area in the control group. $^{\circ}p < 0.05$ vs. data of the other cortical areas from the same treatment group.

of the δ band was reduced by DIM and AMI in all three cortical areas (Fig. 1A–C). In the rats treated with the triple and quadruple 1/5 LD_{50} combinations, changes in the fast bands were the more pronounced. The DPC combination increased β1, β2 and γ bands in the SS, α to γ in the VIS, and β2 in the AUD area (Fig. 2A–C). β1, β2 and γ were increased vs. control in the DCA group in the VIS and AUD area, and in the DPCA group, only in the VIS area (Fig. 2B and C). Among the 1/25 LD_{50} combinations, only the effect of DPCA was comparable to that of 1/5 LD_{50}.

In the cortical EPs, high dose of DIM (Table 1A) caused latency increase in the VIS area only ($p < 0.05$ vs. control, and vs. SS and AUD in the DIM-treated group; Fig. 3A–C), while duration decreased only in the SS area ($p < 0.05$ vs. control; n.s. vs. the other areas). PRP and CYP increased the latency of the VIS and AUD EP (both significant vs. control and vs. SS EP in the treated group; n.s. among themselves). In case of AMI, latency increase and duration decrease was significant in the VIS and AUD area.

Among the 1/5 LD_{50} combinations (Fig. 4) DPCA induced the greatest latency increase in all three areas ($p < 0.05$ vs. control in each area; n.s. in area-to-area comparison). In the SS and VIS area, DPC and DPA had a similar effect. Duration of the VIS EP was altered by DPC and DPA; and of the AUD EP, by DPCA. The effects of the low dose (1/25 LD_{50}) combinations was negligible, except for a slight latency increase of the VIS EP (not shown).

Somatosensory stimulation was applied also at repetition rates of 2 and 10 Hz, beside 1 Hz, to reveal any frequency dependence of the measured parameters of the EPs (Fig. 5). The most conspicuous effect here was that, in contrast to the controls, a latency increase was seen in the treated groups also at 2 Hz. At 10 Hz stimulation frequency, the difference in latency increase between the control and treated groups was visible but no more significant.

In the tail nerve, relative refractory period was altered in nearly all treated groups (Fig. 6). This effect was significant vs. control with DIM, CYP and AMI, and with the high dose combinations DPA and + DPCA. The low dose combinations had no effect. The alterations of the nerve conduction velocity were below significance.

4. Discussion

On the spontaneous cortical activity, DIM and AMI had the most pronounced effect. The decrease of slow and increase of fast activity seen in the DIM-treated rats was in line with our previous observations where DIM and other OPs were given in similar doses in various timing from acute to subchronic [37]. A likely explanation of that lies in the cholinergic mechanism of the ascending cortical activation system [38]. Cholinesterase inhibitors would be expected to increase the effect of the activation and, this way, to shift the ECoG spectrum to higher frequencies. Given the known common mechanism [2,13] one would also expect a synergism between DIM and the carbamate PRP. This, however, was not seen and PRP even seemed to antagonize the effects of DIM; similarly to what was found earlier in a different dosing scheme [17]. The reason for that is not clear but it is possible that the doses, equitoxic in general outcome (i.e., fractions of LD_{50}) are not equitoxic to a specific endpoint like cortical activity. CYP had no noteworthy effect on the spontaneous cortical activity either alone or in combinations (high similarity of the DPA and DCA columns in Fig. 2A–C). Although some of the actions of CYP at receptor level can take place also in the central nervous system [25–27], functional effects in the CNS have not been described [39,18]. The lack of interaction of CYP with OPs is likewise known [40], and our results showed that CYP had also no influence on the effects of AMI. The CNS effects of AMI seem to be based on its effect on monoaminergic control. AMI inhibits monoamine oxidase

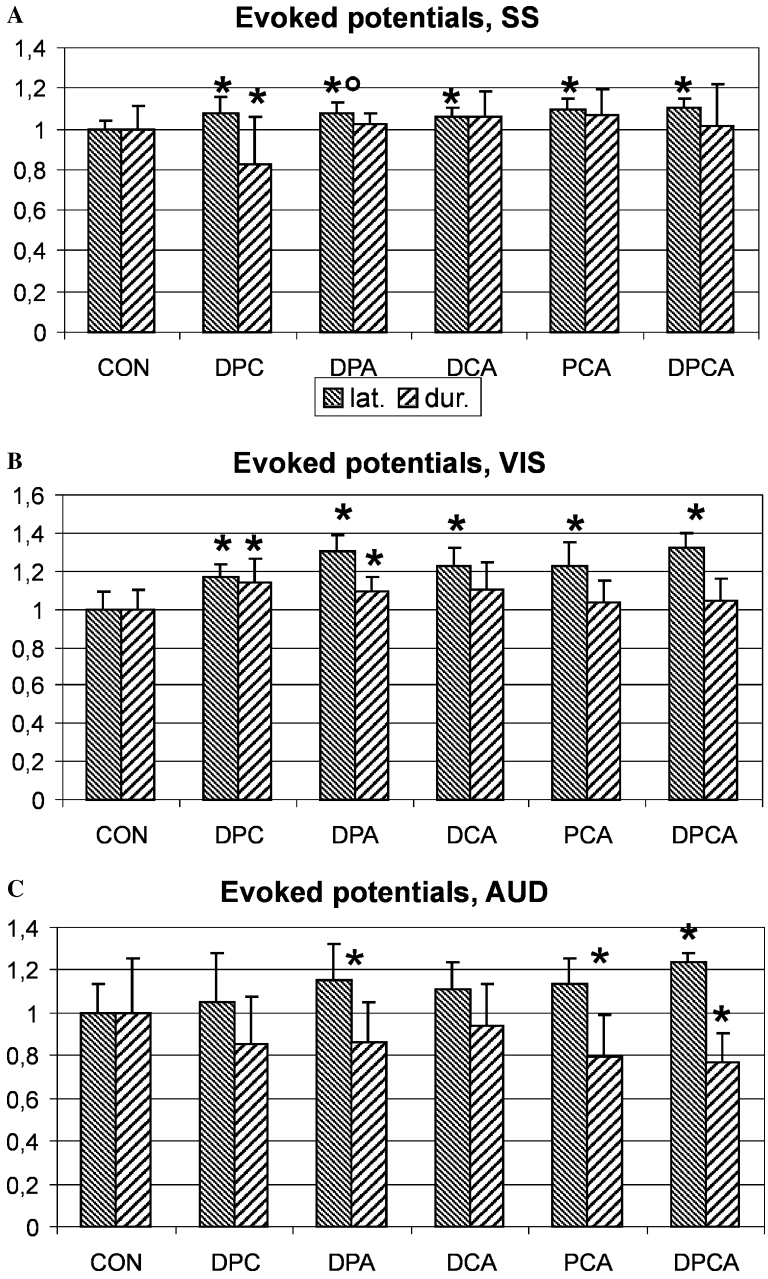

Fig. 4. Change of latency (lat.) and duration (dur.) of the cortical sensory evoked potentials in the rats treated with 1/5 LD_{50} of the insecticides in combinations (A), somatosensory; (B), visual; (C), auditory areas. Values normalized and displayed as in Fig. 3. Bar patterns as in Fig. 2.

[41] and acts as an α-2-adrenergic agonist [42]. With a dose close to ours (100 mg/kg b.w.) increased excitation and reduced inhibition in the hippocampus was observed [43]. In our results, AMI, given alone, significantly decreased low frequency (δ, θ and α) ECoG. In the combinations, increase of the fast ($\beta 2$ and γ) bands was more pronounced, first of all if DIM was also present in the combination. As AMI was shown not to be a cholinesterase inhibitor [31], this positive interaction shows more a common final outcome than a common mechanism.

The effect of the insecticides on the EPs was, generally, depression. Lengthened latency and duration of EPs was seen in earlier experiments of us after several weeks of treatment with various OPs [8] and with PRP [17]. This time, a single acute dose caused partly similar changes (which was not unexpected, see [32]) albeit the strength of effect was quite different in the three cortical areas recorded. Considering the lack of noteworthy effect on the peripheral conduction velocity, it was likely that the latency increase was due to central effects like the well known relationship of spontaneous and stimulus-evoked cortical activity [44,45]. Under conditions of increased spontaneous activity, evoked responses tend to be depressed. Analysis of the frequency dependence of the somatosensory EP showed that in case of 500 ms inter-stimulus interval (corresponding to 2 Hz stimulation frequency) the responses have a significant

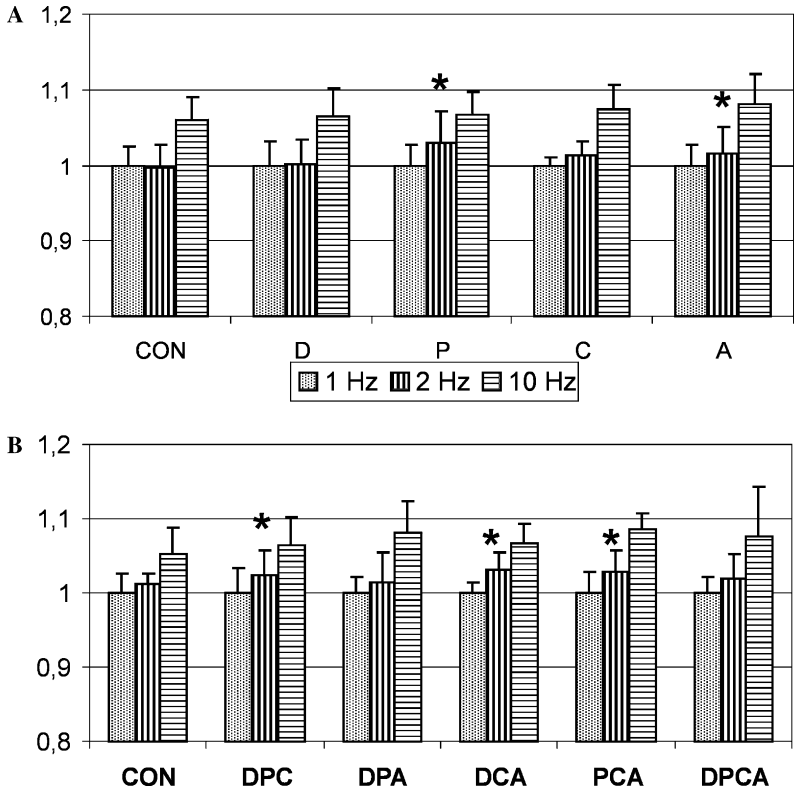

Fig. 5. Dependence of the latency of the somatosensory evoked potential on the frequency of stimulation. (A), single 1/5 LD_{50} doses; (B), 1/5 LD_{50} combinations, the bars represent relative values (ratio of [latency at the given frequency]/[latency at 1 Hz frequency]). Insert: stimulation frequencies. $^{*}p < 0.05$ vs. the value at the same frequency in the control group.

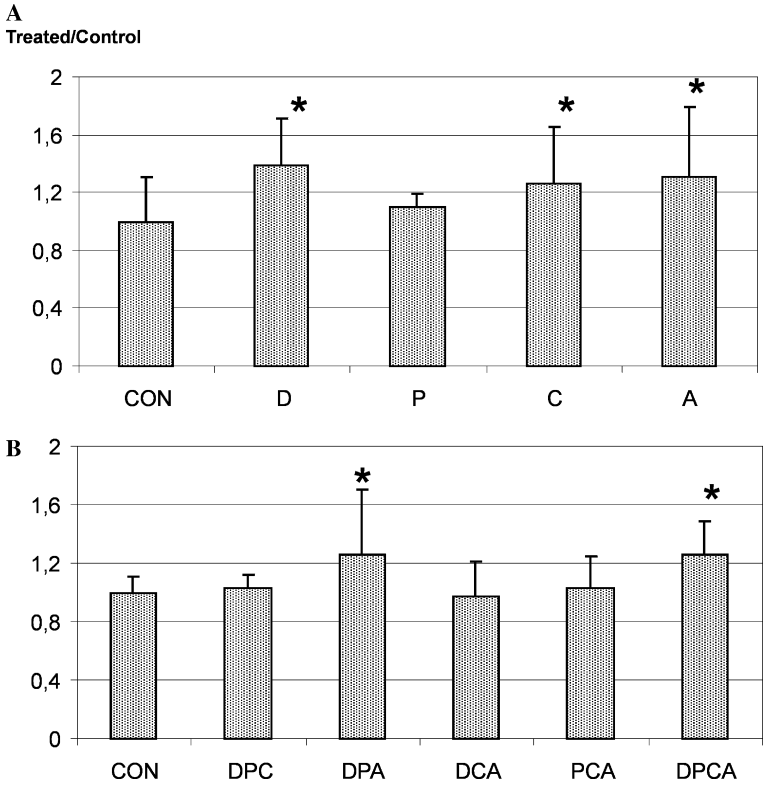

Fig. 6. Change of the relative refractory period of the tail nerve. Relative values calculated and displayed as in Fig. 3. (A), single 1/5 LD_{50} doses; (B), 1/5 LD_{50} combinations. $^{*}p < 0.05$ vs. control in the same band.

delay compared to those obtained with 1000 ms interval in most of the treated groups, but not in the controls. This time range is far from the relative refractory period of the peripheral nerves (as observed in the present study and in [8]), also indicating that the changes, brought about by the insecticide agents, were generated within the brain.

In the results presented, there were several cases where the effect of insecticide combinations on certain parameters of nervous activity did not correspond to what was expected on the basis of known mechanisms of action. This indicates that safety limits, based on single substance effects, may be inadequate in case of combined exposure, and emphasizes the need for further studies in combination toxicology.

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