CENTRAL CHOLINERGIC ACTIVATION BY CHLORFENVINPHOS, AN ORGANOPHOSPHATE, IN THE RAT

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Abstract—Effects of 2-chloro-1-(2, 4-dichlorophenyl) vinyl diethyl phosphate, chlorfenvinphos, on spontaneous EEG, EMG and ChE activity in the brain and red blood cells were investigated in male Wistar rats. Chlorfenvinphos up to 1 mg/kg p.o. did not affect the ChE activity and the awake-sleep cycle. In doses over 2 mg/kg, the ChE activity in the brain and red blood cells significantly decreased. The spontaneous EEG showed a prominent arousal pattern and appearance of slow wave sleep and parasleep was markedly depressed. Maximum inhibition of brain ChE activity was obtained 3 hr after the treatment and lasted for more than 72 hr. The duration of arousal pattern was proportional to the doses, however, the awake-sleep cycle returned to control on the 2nd day and a rebound increase in parasleep occurred on the 3rd day. Atropine depressed the EEG arousal pattern induced by chlorfenvinphos, without affecting ChE activity in the brain. The brain noradrenaline level was not altered with chlorfenvinphos. These results indicate that the appearance of EEG arousal pattern after chlorfenvinphos may be derived from central cholinergic activation.

2-Chloro-1-(2, 4-dichlorophenyl) vinyl diethyl phosphate (chlorfenvinphos), an organophosphate, has been used extensively throughout the world as an agricultural insecticide. It is well known that fat-soluble organophosphates such as chlorfenvinphos can easily pass through the blood brain barrier and enter the brain. The central action of organophosphates has been reported by several investigators: EEG changes with DFP (1, 2) and parathion (3), and effects of several kinds of organophosphates on the spontaneous and evoked activities of the cerebral cortex (4). Although acute and chronic toxicities and anti-cholinesterase properties of chlorfenvinphos in experimental animals have been described (5–7), there is still a paucity in information concerning the central action of the compound. The present experiments using chronically electrode-implanted rats were designed to study the effects of chlorfenvinphos on electroencephalogram (EEG), electromyogram (EMG) and cholinesterase (ChE) activity in the brain and red blood cells (RBC).

MATERIALS AND METHODS

1) Assay of ChE and noradrenaline

Male Wistar rats, weighing 150-170 g were housed in separate cages at 22-24°C under

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a constant day-night rhythm. The animals were sacrificed by decapitation and arterial blood was collected into a heparinized centrifuge tube. The whole brain was rapidly removed and homogenized with 25 ml of ice-cold saline. Blood was centrifuged at 1,000 rpm for 5 min. The precipitated RBC was washed with ice-cold saline and centrifuged. After this procedure had been repeated twice, RBC was hemolyzed with 5 volumes of ice-cold distilled water. ChE activity was determined by a decrease in the amount of acetylcholine (ACh) which had been added in an enzymatic reaction mixture as substrate. The enzymatic reaction was performed by addition of 0.1 ml of the brain and RBC samples into a test tube containing 1 ml of ACh-buffer-salt mixture reagent. This reagent was composed of 8 volumes of 1/15 M phosphate buffer and 1 volume of each 0.5 M ACh and salt mixture containing 4.2 g of MgCl₂ and 0.2 g of KCl per 100 ml. After incubation of the sample for 1 hr at 37°C, the content of ACh remaining in the sample was determined colorimetrically by the method of Hesterin (8). For the assay of nordrenaline, the method of Anton and Sayre was used (9). Concentrated perchloric acid was added to a part of the brain homogenate together with ice-cold saline so that the final concentration of the acid in the sample was 0.4 N.

2) Recording of EEG and EMG

Male Wistar rats, weighing approx. 250 g were anesthetized with pentobarbital and the head of the animal was fixed in a stereotaxic instrument. The skull was exposed and a silver ball-tipped electrode was placed on the surface of the motor cortex which is located 2 mm frontal and 2 mm lateral to the bregma. A small opening was made in the skull and bipolar stainless steel electrodes were inserted into the ipsilateral hippocampus (A: 2.6, L: 4.0, V: 1.0 to -2.0) with the aid of a brain atlas of König and Klippel (10). An indifferent electrode was placed on the occipital cranium. EMG was recorded from the dorsal neck muscle. A 6-pin microminiature female plug (Amphenol, 221 Series) was mounted to the midline of the skull. After connecting the plug pins to the electrodes, the space beneath and around the socket was filled with acrylic acid cement (Yata Chemical, Poliset M 18). These animals were allowed at least 7 days for recovery after which they were placed in a sound-attenuated box with a floor area of 30 cm x 30 cm and a height of 28 cm. A 6-lead cable contained in plastic tubing was connected to a 3-channel electrograph (San′ei Sokki, EB 102). Recording was done 8 hr/day (10 a.m.-6 p.m.) every day.

3) Drug treatments

Chlorfenvinphos (Shell Kagaku) dissolved in soya bean oil was administered p.o. by an oesophageal tube in a volume of 0.2-0.3 ml/100 g body weight. Atropine sulfate and physostigmine salicylate were injected i.p. and i.m., respectively. The statistical significance of the results was determined by Student's t-test.

RESULTS

1) Effects of chlorfenvinphos on ChE and noradrenaline

As shown in Fig. 1, ChE activity in the brain was unaffected 3 hr after 1 mg/kg of chlorfenvinphos. Oral doses of 2 and 4 mg/kg produced a marked decrease in the brain
ChE activity to 62% and 18% of the control, respectively. The inhibitory effect of the drug was not modified by supplement of atropine (2 mg/kg i.p.) 2 hr later. Fig. 2 shows the time course of change in the brain ChE activity following 4 mg/kg of chlorfenvinphos. The maximum inhibition was found 3 hr after the administration, after which the ChE activity elevated gradually. Activity in the brain at the 72nd hr, however, still remained 67% that of the control.

ChE activity in the RBC also decreased after 4 mg/kg of chlorfenvinphos (Fig. 3). The lowest level (20%) was attained 3 hr after the treatment, and the activity returned to the control level at the 72nd hr.

**Fig. 1.** ChE activity in whole brain 3 hr after chlorfenvinphos (1, 2 and 4 mg/kg p.o.). Atropine (2 mg/kg) was given i.p., 2 hr after chlorfenvinphos (4 mg/kg). Results are expressed as mean±s.e. Number of animals: Control=6, Treated=4. *: p<0.001 (Significantly different from the control).

**Fig. 2.** Time course of ChE activity in whole brain after chlorfenvinphos (4 mg/kg p.o.). Results are expressed as mean±s.e. Number of animals: Control=6, Treated=4. *: p<0.01, **: p<0.001 (Significantly different from the control).

**Fig. 3.** Time course of ChE activity in the red blood cells after chlorfenvinphos (4 mg/kg p.o.). Results are expressed as mean±s.e. Number of animals: Control=6, Treated=4. *: p<0.05, **: p<0.01, ***: p<0.001 (Significantly different from the control).
Noradrenaline content in the whole brain was not significantly affected during 72 hr after 4 mg/kg of chlorfenvinphos, although a slight decrease (84% of the control) was observed 3 hr after the treatment. (Fig. 4).

2) Effects of chlorfenvinphos on awake-sleep cycle

According to the change in spontaneous EEG and EMG, awake-sleep patterns of the rats were classified into the following 4 stages: 1) wakefulness, 2) drowsiness, 3) slow wave sleep, and 4) parasleep. Fig. 5 illustrates typical patterns of each stage. Wakefulness was characterized by low-voltage fast wave in the cortical EEG, mainly synchronized arousal wave in the hippocampal EEG and high activity of EMG. In the stage of drowsiness, the cortical EEG mainly showed a low-voltage fast wave and was occasionally intermingled with slow or spindle waves. The hippocampal EEG exhibited an alternative appearance of synchronized and desynchronized patterns, and muscle activity was reduced in this stage as compared with wakefulness. Slow wave sleep was characterized by high-voltage slow waves with spindle bursts in the cortical EEG and high-voltage desynchronized

![Fig. 4. Time course of noradrenaline content in whole brain after chlorfenvinphos (4 mg/kg). Number of animals: Control=6, Treated=4. Mean±s.e.](image)

![Fig. 5. Four stages of awake-sleep pattern in the rat. MC: Motor cortex, HPC: Hippocampus, EMG: Electromyogram recorded from dorsal neck muscle.](image)
pattern in the hippocampal EEG. Muscle activity was retained despite the low amplitude. In the stage of parasleep, the cortical EEG showed a low-voltage fast wave, the hippocampal EEG synchronized completely and EMG disappeared. In the 8 hr-recording of 27 control animals, the mean percentage of these 4 stages was 29.8, 19.3, 41.4 and 9.5\% respectively.

Oral administration of chlorfenvinphos up to 1 mg/kg did not interfere with the normal awake-sleep cycle. A dose-dependent increase in duration of wakefulness and concomitant decrease in slow wave sleep and parasleep were found in doses over 2 mg/kg, as shown in Table 1. Fig. 6 illustrates the effects of 2, 4 and 8 mg/kg of chlorfenvinphos on awake-sleep cycle, when percentage of these 4 stages was calculated every 1 hr for 8 hr after the administration. With doses over 4 mg/kg, the duration of wakefulness increased more than twice that of the control for a period of 5 hr. The slow wave sleep and para-

### Table 1. Effects of chlorfenvinphos on awake-sleep patterns of the rat.

| Oral doses | Day    | Wakefulness | Drowsiness | Slow wave sleep | Parasleep |
|------------|--------|-------------|------------|-----------------|-----------|
| 2 mg/kg    | Control| 29.7±4.6\%  | 17.6±1.1\% | 43.0±4.6\%      | 9.8±2.0\% |
|            | 1st day| 43.6±5.8\%  | 29.1±2.1** | 25.2±4.2*       | 2.2±0.2*  |
|            | 2nd    | 23.8±3.6    | 20.2±1.4   | 44.5±1.7        | 11.6±1.0  |
|            | 3rd    | 29.5±3.1    | 17.0±1.2   | 43.2±3.0        | 10.2±1.4  |
|            | Control| 28.2±1.8    | 21.9±1.5   | 40.4±4.0        | 9.3±0.8   |
|            | 1st day| 64.7±5.4**  | 27.7±3.7   | 7.3±1.9**       | 0.2±0.1***|
|            | 2nd    | 32.7±1.8    | 26.6±3.5   | 32.8±2.5        | 7.9±0.9   |
|            | 3rd    | 23.1±3.6    | 15.6±2.4   | 45.7±3.3        | 15.5±2.4  |
|            | 4th    | 23.6±6.4    | 19.4±2.5   | 43.5±2.5        | 13.6±2.0  |
| 4 mg/kg    | Control| 31.4±1.1    | 18.4±1.7   | 40.9±0.8        | 9.4±0.9   |
|            | 1st day| 75.1±1.9*** | 20.1±1.6   | 4.7±0.5***      | 0.1±0.1***|
|            | 2nd    | 29.4±2.3    | 22.5±0.3   | 37.6±5.6        | 10.5±2.9  |
|            | 3rd    | 26.0±1.3*   | 18.9±0.1   | 41.1±1.7        | 14.0±1.3* |
|            | 4th    | 27.6±2.0    | 16.0±1.0   | 42.2±1.6        | 14.0±0.4* |
| 8 mg/kg    | Control| 31.4±1.1    | 18.4±1.7   | 40.9±0.8        | 9.4±0.9   |
|            | 1st day| 75.1±1.9*** | 20.1±1.6   | 4.7±0.5***      | 0.1±0.1***|
|            | 2nd    | 29.4±2.3    | 22.5±0.3   | 37.6±5.6        | 10.5±2.9  |
|            | 3rd    | 26.0±1.3*   | 18.9±0.1   | 41.1±1.7        | 14.0±1.3* |
|            | 4th    | 27.6±2.0    | 16.0±1.0   | 42.2±1.6        | 14.0±0.4* |

Results are mean±s.e. (n=3), * : P<0.05, ** : P<0.01, *** : P<0.001 (Significantly different from the respective control).

Fig. 6. Effect of chlorfenvinphos (2, 4 and 8 mg/kg p.o.) on awake-sleep patterns. Percentage of 4 stages (□ : Wakefulness, ◊ : Drowsiness, ▼ : Slow wave sleep, □ : Parasleep) Calculated every hour×8. Results are the mean of 3 animals.
sleep patterns completely disappeared for 5 hr. The awake-sleep cycle almost returned to normal on the 2nd day, and a rebound elevation of parasleep was observed in doses over 4 mg/kg on the 3rd and 4th days.

As can be seen in the lower panel of Fig. 7, earlier appearance of slow wave sleep occurred with 2 mg/kg of atropine administered 2 hr after chlorfenvinphos (4 mg/kg). Parasleep also appeared 3 hr after atropine, whereas such were rarely observed in the animals treated with chlorfenvinphos alone.

3) Effects of physostigmine on awake-sleep cycle

Physostigmine in doses of 0.05 and 0.1 mg/kg produced an increase in wakefulness during the first 1 hr (Fig. 8). Thereafter, the arousal pattern was reduced and slow wave sleep and parasleep patterns increased as compared with the control 3 to 5 hr after the administration.
DISCUSSION

Chlorfenvinphos in oral doses of more than 2 mg/kg produced a dose-dependent inhibition of ChE activity in the brain. The maximum inhibition of ChE activity was obtained 3 hr after the treatment in both the brain and RBC. Spontaneous EEG also showed the arousal pattern shortly after the administration in doses above 2 mg/kg, and the duration of wakefulness was proportional to the doses. Among drowsiness, slow wave sleep and parasleep, the most remarkable change was observed in parasleep after the treatment. There are many reports implicating cholinergic mechanism with EEG activation. EEG and behavioral arousal signs following ACh were prevented by pretreatment of animals with muscarinic antagonists (11). Jasper and Tessier (12) have described a diminished release of ACh from the surface of cerebral cortex during slow wave sleep in unanesthetized cats. In the present study, atropine reduced the EEG arousal pattern induced by chlorfenvinphos without affecting ChE activity in the brain. These results suggest that the arousal pattern after the organophosphate may be derived from cholinergic receptor activation in the central nervous system. This arousal pattern, however, did not persist for even 2 days, and the rebound phenomenon which was characterized as an increase in parasleep was found on the 3rd and 4th days, despite the fact that the brain ChE activity was still significantly reduced. An adaptation to the decreased ChE activity may occur in animals treated with chlorfenvinphos, as reported for other organophosphates (13, 14). It is, however, still obscure whether the remarkable decrease and the following rebound increase in parasleep after the treatment of chlorfenvinphos correlate directly to the cholinergic function in the sleep mechanism or secondary phenomena to the cholinergic arousal manifestation.

Physostigmine, a reversible ChE inhibitor, also manifested an EEG arousal pattern, however, the duration of the pattern was very short. Varagic and Krstic (15) have suggested physostigmine produces a central adrenergic as well as cholinergic receptor activation. Bhatnager (16) has reported that physostigmine inhibits a loss of brain noradrenaline induced by α-methyl-p-tyrosine. In the present study, however, noradrenaline content in the brain after chlorfenvinphos did not significantly differ from that of the control.

In conclusion, the EEG arousal signs produced by chlorfenvinphos correlated well with the decreased activity of ChE in the brain and RBC and concomitant activation in the central cholinergic system.

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