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EFFECTS OF DFP AND OBIDOXIME ON BRAIN ACETYLCHOLINE LEVELS AND ON BRAIN AND PERIPHERAL CHOLINESTERASES

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Abstract—The reactivator obidoxime given twice i.p. at a dose of 12·5 mg/kg shortly before and after DFP (1·5 mg/kg s.c.), or given only once at a dose of 25 mg/kg shortly after DFP, prevented the rise of rat brain acetylcholine (ACh) observed 90 min after the anticholinesterase (antiChE) agent. At 180 min and 16 hr after DFP the brain ACh levels in the DFP and DFP-obidoxime groups were similar to that of the controls. The DFP-obidoxime groups did not differ from the corresponding DFP groups in regard to brain cholinesterase (ChE) activity (about 35 per cent of normal at 90 min, about 40 per cent at 180 min and about 65 per cent at 16 hr).

The reactivation of ChE by obidoxime was complete in erythrocytes and partial in the serum. On the contrary, obidoxime did not modify DFP effects on liver, kidney and diaphragm ChE, in spite of a very high concentration of obidoxime found in the kidney.

IT IS well known that anti-cholinesterase organophosphates such as DFP† can provoke a marked alteration of central nervous system functions,[1–4] which may be attributed to the inhibition of ChE accompanied by an increase of brain ACh content.[5–7]

In spite of the fact that quaternary oximes (ChE reactivators) have little or no effect on brain ChE inhibited by organophosphates, it was shown in this laboratory that obidoxime and 2-PAM-Cl can partially antagonize the depressant effects of DFP on avoidance behavior.[8,9]

This result suggested a study of ACh levels and ChE activity in the brain of DFP-intoxicated rats, with or without additional obidoxime treatment. Since the first series of tests showed an antagonistic action of the reactivator on the rise of brain ACh caused by DFP, additional experiments were carried out on antiChE–reactivator interactions in peripheral organs and on the distribution of obidoxime in various tissues.

MATERIALS AND METHODS

Animals

Female rats of 180–220 g, from an outbred Wistar-derived strain maintained at our Institute, were used in all experiments.

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† Abbreviations used: DFP, diisopropyl phosphorofluoridate; obidoxime, bis 4-hydroxyiminoethyl-pyridinium-1-methyl ether dichloride; 2-PAM-Cl,2-hydroxyiminomethyl-*N*-methylpyridinium chloride; ACh, acetylcholine; AThCh, acetylthiocholine; ChE as used here is total capacity to hydrolyse AThCh under standard conditions.

Materials

Commercial preparations of acetylcholine chloride (Roche S.p.A., Milano, Italy), and of obidoxime (Toxogonin®, Merck A.G., Darmstadt, West Germany) were used. Reagents for the determination of ChE activity were purchased from Boehringer (A. G. Boehringer, Mannheim, West Germany) and DFP from B.D.H. (British Drug House Ltd., England).

Drug treatments and preparation of biological material for analysis

DFP dissolved in oil (1·5 mg/ml) was given I.P. at a 1·5 mg/kg dose. Control rats received an equivalent volume of oil I.P. Aqueous solutions of obidoxime (5 mg/kg) were prepared daily from new vials. The compound was administered I.P. according to two different treatment schedules. In the first (I), 12·5 mg/kg were given twice, 10 min before and 15 min after DFP. In the second (II) the total dose of obidoxime (12·5 mg/kg or 25 mg/kg) was given in a single injection 15 min after DFP. In the two experiments of the first series the rats were killed by decapitation at various time intervals after DFP administration. The heads fell directly into liquid nitrogen where they remained for 15 sec. Then the brain, without cerebellum, was removed and one hemisphere used for the determination of ACh content and the other for the determination of ChE activity. In the second series of experiments the rats, treated according to schedule I described above, were killed 90 min after DFP administration. The liver, the kidney, the diaphragm and a blood sample were used for the determination of ChE activity and of obidoxime content.

All experiments consisted of balanced replications, since all material of a given experiment could not be processed simultaneously. In other words, an equal number of animals of each group was treated, sacrificed and analysed in parallel, and this procedure was replicated two or three times, i.e. until the desired total N in each group was reached. Furthermore, experiment I in the first series (Fig. 1) consisted in reality of three separate experiments for the different time intervals (each with two balanced replications). Therefore, the results of Fig. 1 were analysed by t tests comparing DFP and DFP-obidoxime groups within each interval, since the use of a 2×3 variance analysis would have violated the basic assumptions underlying such treatment of data. Finally, the balanced replications in the experiments of Figs. 1 and 2 (first series, I and II) did not include untreated controls, which were studied separately and therefore not included in the direct statistical comparisons. On the other hand, the balanced replications included untreated animals in the second series of experiments (Tables 1 and 2).

Determination of ACh content in the brain

The brain was prepared for ACh determinations in a Potter homogenizer containing Ringer solution and 10% TCA (1 : 8 : 1), at 0°. The homogenate was centrifuged at 12,000 *g* for 20 min, and the supernatant brought to pH 7·2 with 0·1 N NaOH just before the bioassay. All steps were carried out at 0°.

The ACh content was bioassayed on isolated strips of terminal guinea-pig ileum,[10] suspended in a bath containing 10 ml of oxygenated Tyrode with 2 g/l. of glucose, kept at 37°. Morphine sulphate (17 μg/l.) was used to reduce spontaneous motility, diphenhydramine hydrochloride (7 μg/l.) to block responses to histamine, and neostygmine monomethylsulphate (5 μg/l.) to sensitize the organ to ACh. In a few

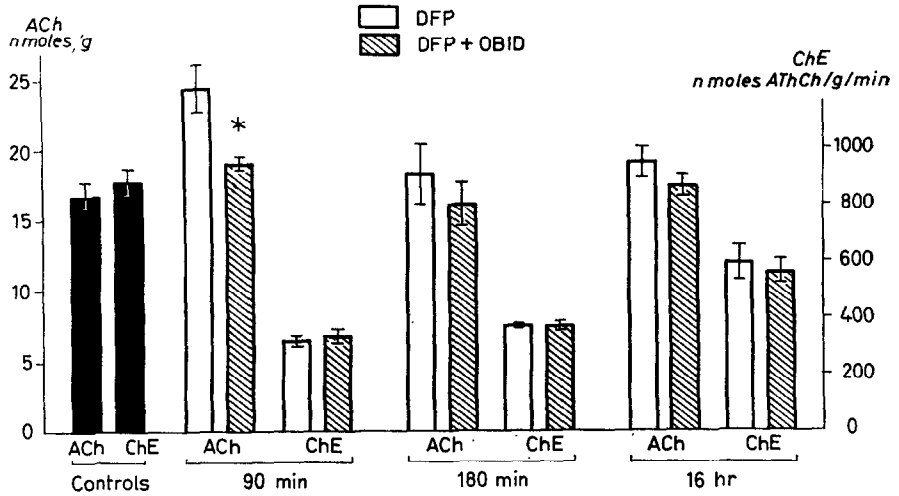

FIG. 1. Effect of obidoxime on ACh concentration and ChE activity in the brain of DFP-intoxicated rats. The rats received 1·5 mg/kg s.c. of DFP and two OBID (obidoxime) treatments (12·5 mg/kg i.p. 10 min before and 15 min after DFP). ACh was assayed on guinea pig ileum. The values are nmoles per g of fresh tissue. ChE activity, measured by a spectrophotometric method, is expressed as nmoles of AThCh hydrolysed per g of fresh tissue per min. The bars give the mean ± S.E. for groups of six rats each. The asterisk indicates a significant difference between a DFP-obidoxime group and the corresponding DFP group (two-tailed t test, $P < 0{\cdot}02$). Black bars were used to show ACh and ChE values in untreated rats since since these animals were not included in the balanced replications making up the three sections of this experiment.

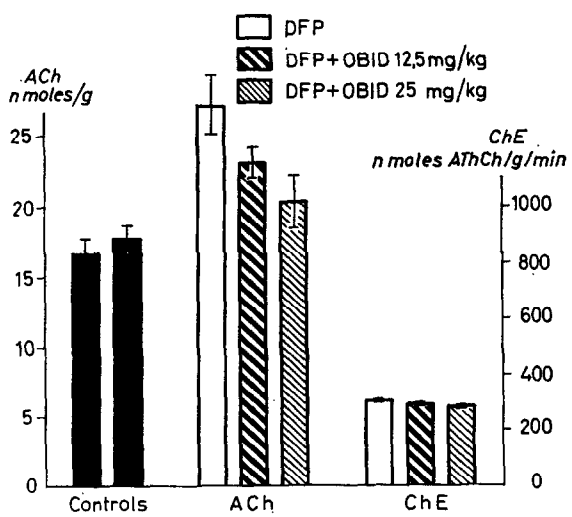

FIG. 2. Effect of different doses of obidoxime on ACh concentration and ChE activity in the brain of DFP-intoxicated rats.

The rats received 1·5 mg/kg of DFP and either 12·5 mg/kg or 25 mg/kg of OBID (obidoxime) 15 min after DFP. For other explanations see Fig. 1. The controls (same as in Fig. 1) were not included in the balanced replications making up the experiment.

TABLE 1. EFFECTS OF OBIDOXIME ON ChE ACTIVITY IN THE TISSUES OF DFP-INTOXICATED RATS*

	Control (nmoles/g/min)	DFP (nmoles/g/min)	DFP (% of control)	DFP + Obidoxime (nmoles/g/min)	DFP + Obidoxime (% of control)
Serum	2847·9 ± 331·3	207·1 ± 20·7	7·3†	507·4 ± 82·8	17·8‡
Liver	1553·4 ± 196·7	745·6 ± 165·6	48·0	642·0 ± 186·4	41·3
Kidney	621·3 ± 10·3	445·3 ± 41·4	71·7	455·6 ± 31·0	73·3
Diaphragm	538·5 ± 62·1	331·3 ± 31·0	61·5	331·3 ± 20·7	61·5
Erythrocytes	120·0 ± 14·6	70·0 ± 12·2	58·3	130·0 ± 11·8	108·3‡

* Animals treated as in the experiments illustrated by Fig. 1, and sacrificed 90 min after DFP. Values are presented as means ± S.E. of nmoles of substrate hydrolyzed per g of fresh tissue per min ($N = 6$). The ChE activity was measured by a spectrophotometric method (substrate AThCh) in the case of the serum, liver, kidney and diaphragm and by a titrimetric method (substrate ACh) in the case of erythrocytes.

† All values for animals treated with DFP alone were significantly different from those for control animals.

‡ Significantly different from the corresponding DFP group (two-tailed test, $P < 0{\cdot}02$).

TABLE 2. OBIDOXIME CONTENT OF THE TISSUES OF DFP-OBIDOXIME TREATED RATS*

Tissue	Obidoxime content (μg/g)
Brain	<1
Serum	1·90 ± 0·13
Liver	1·8 (1·0–2·7)
Kidney	14·92 ± 1·12
Diaphragm	1·7 (1·2–2·1)

* Same animals as in Table 1. Obidoxime was measured by a spectrophotometric method (sensitivity threshhold 0·2 μg/g). The values are (a) means ± S.E. in the case of measurements well above the threshold of the method, and (b) means and ranges of approximate values in the case of measurements close to the threshold of the method (due to low content of obidoxime and 5-fold dilution of the material). ($N = 6$.)

experiments atropine sulphate was found to be equally active in antagonizing both the contraction caused by a certain amount of an active sample and that caused by an equipotent dose of standard ACh. Additional experiments showed that obidoxime (0·1–100 mg/l.) did not modify the contractions caused by ACh.

Determination of ChE activity

The enzymatic hydrolysis of AThCh in the brain and other tissues, except erythrocytes, was measured as in previous experiments carried out in this laboratory,[11,12] i.e. according to the procedure of Ellman *et al.*[13] with a Beckman DK-2 spectrophotometer. The concentrations of the reagents were 0·039 M phosphate buffer, pH 7·2, 5·6 mM acetylthiocholine iodide and 0·21 mM 5,5′-dithiobis-2-nitrobenzoic acid. The tissues were homogenized in 0·2 M phosphate buffer, pH 7·8 (1 : 9) on ice and the homogenates centrifuged at 12,000 *g* (brain) or 23,500 *g* (other tissues). The serum was used without dilution. The reaction was initiated by adding the enzyme preparation (0·1 ml in the case of the brain, 0·05 ml in the case of other tissues and serum) to a cuvette containing all the reagents in a total volume of 1·4 ml. The values of extinction at 412 nm were measured once-a-minute from the second to the sixth minute of the reaction. The difference in extinction between the second and the third minute was used to calculate the enzymatic activity. The nonenzymatic hydrolysis of AThCh was found to be negligible.

The ChE activity in erythrocytes was measured according to the procedure of Glick[14] with a pH-meter (Amel, model 331), using a combined microelectrode (Ingold, model 401/M5) and ACh as a substrate. The reagents were 15 ml of 11·1 mM ACh chloride and 0·002 N NaOH for titration. The erythrocytes were prepared

by washing with 0·9% NaCl, followed by dilution with distilled water (1 : 5). The reaction, carried out at 37°, was initiated by adding 1 ml of the enzyme preparation. The ml of 0·002 N NaOH necessary to maintain the pH at 7·8 ± 0·1 for the first 5 min of the reaction, when linear, were used to calculate the enzymatic activity. The value for nonenzymatic hydrolysis of ACh was subtracted from that obtained in the presence of the enzyme preparation.

Determination of the obidoxime content of tissues

The tissue content of obidoxime was measured according to the procedure of Erdmann and Okonek[15] with a Beckman DK-2 spectrophotometer. The tissues were homogenized in 0·2 M phosphate buffer pH 7·8 (1 : 5) on ice and centrifuged at 12,000 *g* (brain) and 23,500 *g* (other tissues). The serum was used without dilution. The supernatant was treated with 50% trichloroacetic acid so as to obtain a final concentration of 5% trichloroacetic acid, centrifuged and subsequently brought to pH 10·0 ± 0·1 with 1 N and then 0·1 N NaOH. The differences in values of extinction at 351 nm between the samples obtained from obidoxime treated and untreated rats were used to calculate the obidoxime content of tissues.

RESULTS

Brain ACh and ChE

The effects of obidoxime administered shortly before and after DFP (schedule I) on brain ACh content and ChE activity are shown in Fig. 1. Rats treated with DFP alone exhibited an elevated ACh level when killed 90 min after injection. On the other hand, normal values were found in animals killed 180 min or 16 hr after DFP treatment. The brain ChE, measured in the same groups of animals, was always below normal, i.e. 36 per cent of the control activity after 90 min, 42 per cent after 180 min and 67 per cent after 16 hr. Obidoxime prevented the rise of the ACh content in rats killed 90 min after antiChE treatment (two-tailed *t*-test, $P < 0{\cdot}02$), and did not significantly affect ACh levels in the brain of animals killed 180 min or 16 hr after DFP. The inhibition of brain ChE activity in the DFP-obidoxime groups was indistinguishable from that of the corresponding DFP groups.

The effects of obidoxime administered only after DFP injection (schedule II) are shown in Fig. 2. Also in this case the reactivator antagonized the DFP action on brain ACh content, apparently in a dose-dependent fashion. An analysis of variance showed that the groups differed significantly ($P < 0{\cdot}05$). *t*-Tests, however,—two-tailed, with probability correction for multiple comparisons—failed to point out more precisely which particular group difference(s) had caused this significant treatment effect. As in the previous experiment, the brain ChE activity was equally depressed both in the presence and in the absence of the reactivator.

ChE in peripheral organs

Table 1 contains the data on the ChE activity of serum, liver, kidney, diaphragm and erythrocytes for untreated, DFP-treated and DFP-obidoxime treated animals. A marked inhibition of ChE activity 90 min after DFP was observed in the serum, showing only 7 per cent of the control activity. In the liver, kidney and diaphragm the values were 50–70 per cent of normal. Similarly, the enzyme activity in erythrocytes,

measured by a titrimetric method, was about 60 per cent of control. The effect of obidoxime consisted of a partial reactivation of ChE in the serum and a complete reactivation in erythrocytes. In both instances, the difference between DFP and DFP-obidoxime groups was statistically significant (two-tailed *t*-tests, $P < 0{\cdot}02$). Obidoxime did not modify the effect of DFP on ChE activity in the liver, kidney or diaphragm.

Obidoxime content of tissues

The results of the studies on the obidoxime content of different tissues in DFP-treated animals killed about 90 min after the administration of obidoxime are shown in Table 2. The highest concentration of the reactivator was found in the kidney while much lower levels were found in the serum, liver and diaphragm. It must be emphasized, however, that the values for the liver and diaphragm are not as reliable as those for serum. In fact, the 5-fold dilution of the material in the case of the former organs resulted in determinations made at the lower level of the sensitivity range. Obidoxime levels were not measurable in the brain, i.e. they must have been below 1 μg/g of fresh tissue.

DISCUSSION

The present results confirm the data of earlier investigators[16–18] showing that the effect of a DFP treatment on brain ChE cannot be antagonized by the administration of obidoxime (Figs. 1 and 2). This data was obtained by using a spectrophotometric method with AThCh as substrate. The estimates of enzyme rates determined with this ester may be considered good estimates of the rate for ACh hydrolysis.[13] Furthermore, AThCh has been considered the substrate of choice for the standardization of ChE preparations.[19]

The lack of action of obidoxime on DFP-inhibited brain ChE seems to be due to the difficulty with which the reactivator, as all *N*-quaternary oximes, crosses the blood–brain barrier. For example a recent study[20] carried out with ^{14}C-obidoxime showed that only negligible amounts of the drug can be detected in the rat brain (about 1 μg/g 20 min after the i.v. administration of 50 mg/kg). Our findings, showing that the brain concentration of obidoxime about 90 min after the i.p. administration of 25 mg/kg must be below 1 μg/g, are in agreement with the above result.

DFP-inhibited ChE's in certain peripheral tissues can be reactivated partially (serum) or completely (erythrocytes) by obidoxime (Table 1). These results are in agreement with those of Erdmann and v. Clarmann[21] on whole blood and of Ohnesorge *et al.*[18] on erythrocytes. The latter investigator also found some reactivation in cardiac muscle, while our data show no reactivation in the diaphragm. Furthermore, the available data indicate that the penetration of obidoxime into a tissue is a necessary but not sufficient condition for ChE reactivation. In fact, in our own as in previous experiments,[20] a very high obidoxime concentration was found in the kidney, in which ChE reactivation was absent.

In spite of the inability of obidoxime to antagonize DFP effects on brain ChE, the reactivator was shown to prevent the rise of brain ACh found in DFP-treated animals 90 min after injection. This phenomenon was observed both when obidoxime was administered twice, before and after DFP, and when the same total dose was administered in a single injection 15 min after DFP. The differences between groups with or without reactivator were in the same direction, but slighter and non significant,

when the animals were sacrificed at longer intervals after DFP treatment (180 min or 16 hr, Fig. 1). This seems to be in relationship with the fact that, in spite of low ChE levels especially at the 180 min interval, the brain ACh content had returned to normal or near-normal levels also in the absence of reactivator treatment.

These data are apparently in contrast with some of those recently reported by Milosević,[22] showing that the rise of brain ACh observed 60 min after the injection of a sublethal dose of paraoxon is not prevented by obidoxime. However, these results were obtained with a 10 min interval between the i.v. administration of the antiChE and the i.p. administration of 12·5 mg/kg of obidoxime. Our experiments with the latter dose of the reactivator, given only after DFP, did not show a clear antagonism of the rise of ACh concentration. Furthermore, it is possible that the s.c. administration of DFP in our experiments be the equivalent of a longer interval between the two treatments, leading to more favourable conditions for the demonstration of an antagonistic action of the reactivator.

Our data may explain why, in certain experimental conditions, the administration of obidoxime can accelerate the recovery from the behavioral depression induced by DFP.[8] The neurochemical and behavioral results confirm that a raised ACh level in the brain, rather than a reduced ChE activity, is the critical factor leading to an alteration of central nervous system functions in the course of antiChE intoxication.

It remains to be explained how a reactivator can antagonize the rise of brain ACh without modifying antiChE effects on brain ChE. Such antagonsim may be due to an effect of the oximes on ACh synthesis, perhaps at the level of a negative feedback mechanism between ACh concentration and the rate of ACh synthesis, suggested by Sharkawi and Schulman.[23] On the other hand, our data bring some support to the hypothesis that ACh accumulated in the brain diffuses to the periphery and the erythrocyte ChE plays an important role in hydrolyzing the ACh which has crossed the blood–brain barrier. In fact a significant reactivation of peripheral ChE's by obidoxime was found in erythrocytes and serum. If this explanation is correct, the conclusion would be that reactivators, in spite of their inability to antagonize directly antiChE effects on the brain, can modify the course of central neurochemical events through their peripheral action.

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