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SHORT COMMUNICATION

Paraoxon Reversibly Inhibits Neurotoxic Esterase

Paraoxon Reversibly Inhibits Neurotoxic Esterase. CARRINGTON, C. D., AND ABOU-DONIA, M. B. (1985). *Toxicol. Appl. Pharmacol.* 79, 175–178. It has recently been reported that two paraoxon-insensitive carboxylesterases may be distinguished by their sensitivity to mipafox. However, we have not been able to reliably detect two components under the conditions of the widely used assay for neurotoxic esterase (NTE). We have determined that this discrepancy is a result of differences in the technique of preinhibition by paraoxon and mipafox. We report here that paraoxon is apparently able to reduce the rate of inhibition of both neurotoxic esterase isozymes by mipafox in a concentration-dependent manner. As a result, the rate of inhibition of NTE by mipafox is greater when sequential, rather than concurrent, preinhibition is utilized. An apparently greter reduction in the inhibition rate of the more sensitive minor component may make the two isozyme species indistinguishable in the presence of paraoxon at concentrations at or above 40 μM. © 1985 Academic Press, Inc.

Neurotoxic esterase (NTE) is a protein which has been proposed to be the site at which organophosphorus-induced delayed neurotoxicity (OPIDN) is initiated (Johnson, 1969b, 1982). NTE is characterized by its ability to bind labeled diisopropyl phosphorofluoridate (DFP; Johnson, 1969a), and its esterase activity (Johnson, 1969b, 1977) following preinhibition with nonneurotoxic organophosphorus compounds, but not following preinhibition with neurotoxic compounds. NTE may be assayed by comparing phenylvalerate hydrolysis following preinhibition either with paraoxon alone or with paraoxon and mipafox (Johnson, 1977). Mipafox is a potent delayed neurotoxicant, while paraoxon is not.

Chemnitius *et al.* (1983) reported that two paraoxon-insensitive, mipafox-sensitive esterases are present in hen brain which may be distinguished by their sensitivity to mipafox, with a smaller component (about 20%; NTE_A) being irreversibly inhibited by mipafox at a faster rate than the major species (about 80%; NTE_B). We have been unable to detect two species of NTE under the conditions of the standard assay (Johnson, 1977) which utilizes concurrent inhibition by mipafox and paraoxon rather than the sequential preinhibition technique used by Chemnitius *et al.* (1983). In this paper, we present data which suggest that this discrepancy is due to a difference in the ability of paraoxon to reversibly inhibit the irreversible binding of mipafox to NTE_A as compared to NTE_B in a reversible manner.

METHODS

Animals and materials. White leghorn hens, weighing 1.5 to 2.0 kg, were obtained from Featherdown Farms, Raleigh, North Carolina. *O,O*-Diethyl-*O*-4-nitrophenyl phosphate (paraoxon) was obtained from Sigma Chemical Company, St. Louis, Missouri. *N,N'*-Diisopropylphosphorodiamidic fluoride (mipafox) was synthesized by Midwest Research Institute, Kansas City, Missouri.

Tissue preparation. Following termination of the animals with sodium pentobarbital, the brains were removed and homogenized in 0.32 M sucrose–50 mM Tris buffer, pH 8.0. The homogenate was spun at 100,000*g* for 10 min at 25°C, the supernatant fraction was discarded, and the pellet was resuspended before preincubation with paraoxon.

Preinhibition with organophosphorous compounds. Before assay, homogenates were preincubated with paraoxon and mipafox at various concentrations in 50 mM Tris–HCl, pH 8.0, for 40 min at 25°C. Paraoxon concentrations used were 640, 160, 40, and 10 μM/liter. Mipafox concentrations of 600, 400, 300, 200, 100, 80, 60, 40, 30, 20, 10, 8, 6, 4, 2, 1, and 0 μM were used. Preinhibition of the esterases was conducted either sequentially or concurrently. Sequential inhibition was executed by incubating with paraoxon for 30 min and then centrifuging for 10 min at 100,000*g*, so that the total incubation time

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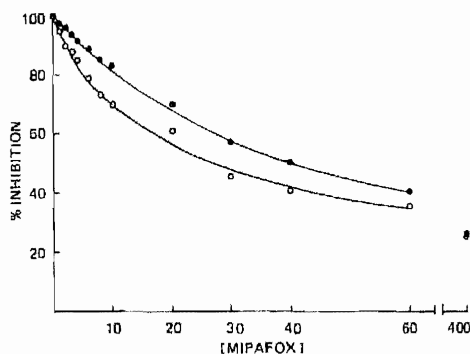

FIG. 1. Inhibition of phenylvalerate hydrolysis by 40 μM paraoxon and various concentrations of mipafox with either concurrent (solid circles) or sequential (open circles) preinhibition. The units for the mipafox concentrations are μM/liter. The lines illustrate computer-generated fits of the data based on a three-component (IRE, NTE_B, and NTE_A) model.

with paraoxon was about 40 min. The supernatant fraction was then resuspended in buffer before preincubation with mipafox. For concurrent preincubation, mipafox and paraoxon were added simultaneously.

Phenylvalerate hydrolysis. Hydrolysis of phenylvalerate was assayed by the method of Johnson (1977) with three variations: the assay was conducted at 25°C, the phenylvalerate concentration was increased to 2 mM, and the incubation period was increased to 30 min.

Calculations. The stripping method used by Chemnitius *et al.* (1983) to analyze organophosphorus (OP) concentration curves has several disadvantages. (1) Since the regression line is fitted to a log plot of the data, the points at the low end of the curve are given more weight. (2) The points used to calculate the quantity and rate of inhibition of each component must be chosen arbitrarily. (3) Since the variables for each component are calculated individually, the error associated with the estimates may be unevenly distributed. All of these problems may be overcome through the use of an optimization technique (Swann, 1969). This procedure involves the use of a computer to find a nonlinear solution to the equation. We used a program based on the direct search method of Hooke and Jeeves (1961) which evaluated the fit of the generated estimates on a least-squares basis. The eighteen-point curves were fitted with either two (one NTE component)- or three (two NTE components)-component models. The two-site and three-site fits of the data were compared statistically by the extra sum of squares principle (Draper and Smith, 1966; Munson and Rodbard, 1980). Differences in the two preincubation techniques were compared either by comparing the estimates of the rate constants or reaction velocities by a Student's *t* test, or by comparing the ability of a three-site model to improve the computer generated fit of the data.

RESULTS AND DISCUSSION

A plot of percentage inhibition vs mipafox concentration with either sequential or concurrent preinhibition with 40 μM paraoxon is given in Fig. 1. The inhibition curve is

TABLE 1

ESTIMATES OF THE ACTIVITIES OF IRE, NTE_B, AND NTE_A

	Paraoxon concentration			
	6.4×10^{-4}	1.6×10^{-4}	4.0×10^{-5}	1.0×10^{-5}
IRE				
Concurrent	6.6 ± 0.8	7.0 ± 0.8	9.9 ± 1.0	19.8 ± 3.8
Sequential	5.5 ± 0.6	6.3 ± 1.0	9.0 ± 2.1	19.4 ± 6.5
NTE_B				
Concurrent	11.8 ± 1.1	16.1 ± 1.8	17.0 ± 2.6	14.5 ± 2.5
Sequential	11.2 ± 0.6	13.7 ± 0.7	15.6 ± 1.5	14.0 ± 1.0
NTE_A				
Concurrent	2.6 ± 0.7	2.5 ± 0.1	2.9 ± 0.9	2.2 ± 0.4
Sequential	2.8 ± 0.3	3.0 ± 0.3	3.3 ± 0.5	3.7 ± 0.2

Note. Phenylvalerate hydrolysis due to inhibitor-resistant esterase (IRE), NTE_B, and NTE_A following preincubation with four different concentrations of paraoxon. The upper value is derived from concurrent preincubation with mipafox and paraoxon, while the lower value resulted from sequential preinhibition. All values are the average of three determinations (±SE). All values are nM phenylvalerate hydrolyzed per min/mg protein.

clearly shallower when concurrent preinhibition is employed, particularly in the beginning of the curve. This difference is apparently due to a reduction in the rate of phosphorylation of NTE_A by mipafox (Table 1). The estimated rate constants for the inhibition of the high affinity components are significantly less ($p < 0.001$; two-way ANOVA, $F(1,16) = 21.5$) when concurrent preinhibition was used. Differences in the rate of phosphorylation of the main component (NTE_B) could also be observed with high concentrations of paraoxon (Table 1). There was a concentration-dependent reduction in the NTE_B rate constant when concurrent preinhibition was used ($p < 0.05$; ANOVA, $F(3,11) = 3.7$), but not when suquential preinhibition was used ($p > 0.50$, $F(3,11) = 0.2$).

Three- and two-component fits of the data (with or without NTE_A) resulting from sequential or concurrent preinhibition were compared. Four of six of the curves using sequential preinhibition were fit significantly better ($p < 0.05$) by a three-component than by a two-component model (five of six with criteria of $p < 0.10$). However, none of the six curves generated with concurrent preinhibition were fit better ($p > 0.10$) if a model including a third component was used.

The calculated hydrolytic activity of NTE_A, NTE_B, and the residual activity following either concurrent or sequential preinhibition with four concentrations of paraoxon are given in Table 2. There were no significant or notable differences in the estimated reaction velocities of any of the three components between the two preincubation procedures at any of the four paraoxon concentrations. The high variability in the measurements for NTE_A are a result of the difficulty of dissecting out a small component from a large one. Most of the variability in the measurements for the rate of inhibition of NTE_B by mipafox occurred between experiments. This indicates that the two procedures are equivalent with respect to the rate of inhibition by paraoxon.

The results presented here concur with the contention of Chemnitius *et al.* (1983), that there are two components to the activity measured by the neurotoxic esterase assay of Johnson (1977). The data also appear to demonstrate that paraoxon can act as a reversible inhibitor of both components, and that the nonneurotoxic compound is better

TABLE 2

INHIBITION RATE CONSTANTS OF MIPAFOX FOR IRE, NTE_B, AND NTE_A

	Paraoxon concentration			
	6.4×10^{-4}	1.6×10^{-4}	4.0×10^{-5}	1.0×10^{-5}
IRE				
Concurrent	19 ± 6	14 ± 6	8 ± 4	11 ± 6
Sequential	17 ± 2	14 ± 1	16 ± 1	15 ± 3
NTE_B				
Concurrent	604 ± 35	882 ± 66	1174 ± 58	1520 ± 426
Sequential	1345 ± 241	1406 ± 303	1380 ± 243	1573 ± 154
NTE_A				
Concurrent	1460 ± 595	1910 ± 908	2596 ± 1220	4378 ± 1863
Sequential	5771 ± 760	9284 ± 2654	6744 ± 2631	9691 ± 309

Note. Reaction rates of IRE, NTE_B, and NTE_A with mipafox either in the presence of (concurrent) or following (sequential) preincubation with four different concentrations of paraoxon. All values are the average of three determinations (±SE). All values are liters per min/mol.

able to compete with mipafox for the higher activity component, NTE_A. Although the understanding derived from the data is important from a technical standpoint for the purpose of differentiating the NTE subspecies, it is perhaps more interesting from a mechanistic point of view. There are two mechanisms by which paraoxon could act as a reversible inhibitor. First, paraoxon may be acting as a competitive inhibitor by occupying the active site without phosphorylation. Second, the adduct formed on either NTE species may be unstable, so that instead of aging and forming an irreversibly inhibited esterase, the diethyl phosphate adduct left by paraoxon may be more likely to be released. The reversible inhibition of acetylcholinesterase by carbamates is an example of this mechanism. Further research will be required to determine which mechanism is operating and whether or not the case of paraoxon can be generalized to other nondelayed neurotoxic OPs.

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