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THE REACTIVATION BY PYRIDINIUM ALDOXIMES OF PHOSPHORYLATED ACETYLCHOLINESTERASE IN THE CENTRAL NERVOUS SYSTEM

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Abstract—The *in vivo* reactivation of phosphorylated acetylcholinesterase in the brain was studied in rats injected with a sub-lethal dose of Paraoxon (diethyl 4-nitrophenyl phosphate) and 30 min later with 0·1 m-mole/kg of a pyridinium aldoxime, e.g. N,N′-trimethylenebis(pyridinium-4-aldoxime) (TMB-4) dibromide or dichloride, or N-methyl pyridinium-2-aldoxime iodide (P-2-AM). Calculations based on acetylcholinesterase (AChE) activities of homogenates (to which both functional and non-functional AChE contribute) showed that reactivation took place but was not uniform in all parts of the brain. On the other hand, calculations based on the hydrolysis of acetyl-β-methylcholine by larger pieces of intact tissue (to which only functional AChE contributes) gave degrees of reactivation which were generally higher than those determined by the previous method, and which were similar throughout the brain. TMB-4 and P-2-AM reactivated approx. one third and one fifth, respectively, of phosphorylated functional acetylcholinesterase. The results indicate that in the brain pyridinium aldoximes can reactivate phosphorylated functional AChE, but have little effect on phosphorylated non-functional AChE. This difference is not seen with the lipoid soluble oxime monoisonitrosoacetone (MINA). The results also indicate that homogenates of the cerebellum are more useful than other homogenates for screening the *in vivo* reactivation of phosphorylated functional AChE in the brain.

The relationship between the antidotal action of oximes and their effect on phosphorylated AChE is discussed, and it is concluded that the interpretation still holds that the antidotal action of pyridinium aldoximes in mice and rats depends at least largely on the reactivation of phosphorylated AChE at peripheral sites.

PYRIDINIUM aldoximes reactivate the phosphorylated AChE formed by organophosphates which possess a dialkoxy or alkylalkoxy group, and reduce in mammals the toxicity of this type of organophosphate.[1] There is no doubt that the antidotal action of these oximes is always associated with reactivation at peripheral sites. The question of how much reactivation in the central nervous system contributes to the antidotal effect, however, is by no means settled. Assessing reactivation from the AChE activity of homogenates of the whole brain of mice or rats, it was found that hardly any reactivation takes place in the central nervous system when pyridinium aldoximes are given in amounts sufficient to produce a considerable degree of reactivation at peripheral sites. After large doses of these oximes, however, some reactivation in the central nervous system undoubtedly occurs.[2] Before any conclusions can be drawn from this, it is necessary to establish whether the reactivation calculated from the AChE activity of homogenates of the whole brain is a true measure of the reactivation

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which occurs at different sites. In addition, the effect of the oximes on phosphorylated functional AChE must be separated from that on phosphorylated non-functional (reserve) AChE. Studying the effects of some pyridinium aldoximes on diethylphosphoryl AChE in rats *in vivo* we recently described that reactivation in the cerebellum was greater than is indicated by the reactivation calculated from the AChE activity of homogenates of the whole brain.[2] In this paper we wish to present evidence which shows that in rats the reactivation calculated from the AChE activity which is observed when pieces of intact brain are incubated with substrate (i.e. reactivation of functional AChE), can be much greater than the reactivation calculated from the AChE activity of homogenates (i.e. reactivation of total AChE). This means that the reactivation by pyridinium aldoximes of phosphorylated AChE in the central nervous system has been underassessed in the past. However, in mice and rats the correlation between the antidotal activity of pyridinium aldoximes and their reactivating potency *in vitro* and at peripheral sites *in vivo* is much better than that between antidotal action and reactivation of phosphorylated AChE at central sites. This indicates that the antidotal action of the oximes in mice and rats probably depends largely on the reactivation of phosphorylated AChE at peripheral sites.

MATERIALS AND METHODS

The oximes used were N,N′-trimethylenebis(pyridinium-4-aldoxime) (TMB-4) dibromide or dichloride, N,N′-oxydimethylenebis(pyridinium-4-aldoxime) (Toxogonin) dichloride, N-methyl pyridinium-2-aldoxime iodide (2-hydroxyiminomethyl-N-methylpyridinium iodide; Pralidoxime; P-2-AM) and monoisonitrosoacetone (MINA). TMB-4 dibromide was synthesised according to Hobbiger and Sadler,[3] Toxogonin was kindly provided by Merck, Darmstadt, and TMB-4 dichloride, P-2-AM and MINA by Dr. D. R. Davies, C. D. E. E. Porton.

The organophosphates diethyl 4-nitrophenyl phosphate (E 600; Paraoxon) and diisopropyl phosphorofluoridate (DFP) were also kindly provided by Dr. D. R. Davies, C. D. E. E. Porton.

All experiments were carried out on male Wistar rats weighing 200–250 g. The rats were given an i.p. injection of 0·015 m-mole atropine/kg to protect them against excessive muscarinic actions of accumulating endogenous ACh. Ten minutes later a sublethal dose of Paraoxon, i.e. 0·002 m-mole/kg, or DFP, i.e. 0·005 m-mole/kg, was injected subcutaneously (in the middle of the back) and after a further 30 min either 0·9% NaCl or an oxime were given i.p. A volume of 0·1 ml/100 g was used for all injections, and dilutions were made in 0·9% NaCl. Two hours after the injection of an oxime or NaCl the rats were killed with ether and the blood vessels in the brain were washed free of blood by perfusing them with 0·9% NaCl before the removal of the brain from the cranial cavity.

Acetylcholinesterase activity was determined in the Warburg apparatus at 37° in a medium consisting of 0·15 M NaCl and 0·025 M $NaHCO_3$, and using an atmosphere of $N_2 + CO_2$ (95:5), pH 7·45, and 0·03 M acetyl-β-methylcholine as substrate. The total volume of fluid in each Warburg vessel was 3 ml. Reactivation was determined as follows.

a. *Homogenates*

Homogenates were prepared in 0·025 M $NaHCO_3$ in an analytical mixer mill. The final concentration of brain tissue in homogenates was adjusted to 100 mg/ml for

homogenates of the cerebellum and to 25 mg/ml for homogenates of other parts of the brain or for homogenates of whole brain. For the determination of acetylcholinesterase activity 1 ml of homogenate was used per Warburg vessel and reactivation was calculated as follows.

$$\text{Reactivation} = 100 \times \frac{\frac{B}{B'} - \frac{A}{A'}}{1 - \frac{A}{A'}} \%$$

where A is the acetylcholinesterase activity of a homogenate from a rat injected with Paraoxon and NaCl, and A' the AChE activity of the same homogenate incubated with 0·1 mM TMB-4 for 30 min at 37° before the addition of substrate. A' represents the maximum reactivation obtainable. B and B' are the acetylcholinesterase activities corresponding to A and A' for a homogenate from a rat injected with Paraoxon and an oxime.

b. *Intact brain*

Reactivation was also assessed from the acetylcholinesterase activity of larger pieces of brain, which for simplicity will be referred to as intact cerebral hemispheres, intact residual telencephalon, and intact cerebellum. The tissue referred to as cerebral hemisphere consisted of that part of the brain which can be isolated as follows. A horizontal cut is made a few mm above the corpus callosum. This opens up the lateral ventricle and the dissection is then completed by keeping the knife vertically to the brain surface and cutting along the anterior, inferior and posterior part of the lateral ventricle. The term residual telencephalon is used for that part of the forebrain which after removal of the cerebral hemispheres is separated from the rest of the brain by a section between midbrain and pons. The preparations of cerebellum used consist of the isolated cerebellum divided into four approximately equal parts by sagittal and transverse cuts, unless otherwise stated. The weights of the intact cerebral hemispheres, residual telencephalon and cerebellum were of the order of 250–350, 200–300 and 200–250 mg respectively.

The CO_2 output after addition of substrate to an individual tissue, previously rinsed with the $NaHCO_3$-NaCl buffer, was recorded over two consecutive periods of 20 min. TMB-4 in a final concentration of 0·1 mM was then added. This speedily caused maximum reactivation and if half of the CO_2 output recorded before addition of TMB-4 is divided by the CO_2 output during a 20 min period after addition of TMB-4 we get a percentage AChE activity for the pre-TMB-4 period. From this reactivation can be calculated as follows.

$$\text{Reactivation} = 100 \times \frac{A - B}{100 - B} \%$$

where A is the percentage activity of a tissue from a rat injected with Paraoxon and an oxime, and B the percentage activity of the same tissue from a rat injected with Paraoxon and NaCl.

Using this method, which is illustrated in Table 1, we get a result which is largely independent of the spontaneous release of acid from the tissue (which is quite considerable and in the experiment shown in Table 1 amounted to 12–14 μl/20 min),

and the spontaneous hydrolysis of substrate.The spontaneous release of acid varied only very little between different preparations of the same tissue and treatment of rats with Paraoxon and NaCl or Paraoxon and oxime had no consistent effect on it.

Table 1 also shows that the CO_2 output of tissues from control rats slightly falls throughout the period of measurements (see columns 5 and 6 of Table 1). The same

TABLE 1. METHOD FOR CALCULATING REACTIVATION

	μl CO_2					
	Rat treated with Paraoxon and NaCl		Rat treated with Paraoxon and 0·1 m-mole TMB-4/kg		Control rat	
Time after transfer of vessels into water bath (min)	247 mg 1	247 mg 2	265 mg 3	272 mg 4	254 mg 5	269 mg 6
30–50	26·2	26·8	37·2	41·0	54	65·5
50–70	26·2	23·2	36·0	37·7	49·5	62·9
85–105	54·4	45·1	52·7	56·6	48·8	55·7

Percentage activity

uncorrected: 51% } (Means for vessels 1 and 2) 70% } (Means for vessels 3 and 4)

corrected: 46% } 63% }

Reactivation calculated from uncorrected percentage activity: $\frac{70 - 51}{100 - 51}\% = 39\%$

Reactivation calculated from corrected percentage activity: $\frac{63 - 46}{100 - 46}\% = 31\%$

Thus reactivation: $\frac{31 + 39}{2}\% = 35\%$

The tissues used were the isolated cerebral hemispheres of the rat. Vessels 1, 3 and 5 contained the left hemisphere and vessels 2, 4 and 6 the right hemisphere. Substrate was added 20 min and TMB-4 75 min after transfer of vessels into the water bath. Calculations of percentage activities (see text) are based on the combined CO_2 outputs of both hemispheres of each rat. Corrected percentage activities were obtained by multiplying the CO_2 outputs of vessels 1–4 for the 50 to 70 and 85 to 105 min periods with:

$1{\cdot}06\left(\text{derived from } \frac{54 + 65{\cdot}5}{49{\cdot}5 + 62{\cdot}9}\right)$ and $1{\cdot}14\left(\text{derived from } \frac{54 + 65{\cdot}5}{48{\cdot}8 + 55{\cdot}7}\right)$, respectively.

usually applied to the tissues from rats treated with either Paraoxon and NaCl, or Paraoxon and oxime. Thus the method of calculation described above slightly overassesses reactivation. To correct this the individual CO_2 outputs (of tissues from rats treated with Paraoxon and NaCl or Paraoxon and oxime) for the periods 50–70 and 85–105 min are multiplied by the factors by which the corresponding CO_2 outputs of tissues of untreated control rats differed from that during the 30–50 min period. Calculating reactivation from the percentage activities based on corrected CO_2 outputs we obtain a second figure for reactivation which represents a slight underassessment. The two values for reactivation derived from uncorrected and corrected percentage activities are combined, therefore, and divided by 2. This gives the final figures for reactivation which are quoted under Results and in Table 2.

Comparisons between the AChE activity of an intact tissue with that of the corresponding homogenate showed that in the case of intact tissues only a fraction of the

total acetylcholinesterase present contributes to the hydrolysis. In the case of the intact cerebral hemispheres the AChE activity was 5–10 per cent of that of homogenates containing the same amount of tissue. With the cerebellum the activity of intact tissue was of the order of 25 per cent of that of the homogenate. These differences are at least partly attributable to a failure of acetyl-β-methylcholine to penetrate through the entire depth of the various tissues, since variations in the size of a tissue were not linearly related to the rates of hydrolysis observed.

After removal of tissues from the Warburg vessels at the end of an experiment, the CO_2 production by the residual incubation medium was only slightly above that attributable to spontaneous hydrolysis of the substrate. Thus the leakage of enzyme into the incubation medium was too small to affect results. Furthermore, there was no gain in enzyme activity during the 40 min preceding the addition of TMB-4 to the tissue. This excludes that the observed reactivation represents an *in vitro* artifact.

RESULTS

When rats were injected first with Paraoxon s.c. and 30 min later with 0·1 m-mole TMB-4/kg i.p. the phosphorylated AChE formed by Paraoxon in the central nervous system was partly reactivated by the oxime. The degree of reactivation recorded depended on the method by which it was determined.

Reactivation assessed from the AChE activity of homogenates of the total brain was relatively low, as shown in Table 2, and was not a guide to the reactivation which occurred in different areas of the brain. Assessing reactivation from the AChE activities of homogenates of different parts of the brain, it was found that reactivation in some parts was more pronounced than in others, e.g. it was much greater in the cerebellum than in the cerebral hemispheres as shown in Table 2.

When reactivation was determined from the rates of hydrolysis of acetyl-β-methylcholine by larger pieces of intact tissues the results differed markedly from those obtained with homogenates, i.e. reactivations in the cerebral hemispheres, residual telencephalon and cerebellum were all of a similar order, as shown in Table 2. In the case of the cerebral hemispheres the reactivation calculated from the AChE activities of intact tissues was much greater than that calculated from the AChE activities of homogenates. This did not apply to the cerebellum.

Toxogonin, 0·1 m-mole/kg i.p., produced an effect comparable to that of TMB-4, 0·1 m-mole/kg i.p., according to results obtained with intact cerebellar tissues and homogenates of the total brain or cerebellum.

The monoquaternary oxime P-2-AM, 0·1 m-mole/kg i.p., produces little or no reactivation in the brain as assessed from the AChE activities of homogenates of the whole brain.[4,5] However, when calculations were based on the rates of hydrolysis of acetyl-β-methylcholine by larger pieces of intact tissue, the reactivating effect of P-2-AM could be established clearly (Table 2). On the other hand, if the lipoid soluble oxime[5] MINA was given instead of a pyridinium aldoxime the difference between the percentage reactivation calculated from the AChE activities of intact tissues and that calculated from the AChE activities of homogenates was very small, as shown in Table 2.

Since the reactivation calculated from the AChE activities of cerebellar homogenates is similar to that obtained by the much more cumbersome method involving the use of intact tissues, the former might be used as a rough guide for the latter. We have

previously shown that in Paraoxon poisoned rats TMB-4 in a dose of 0·01 m-mole/kg produces 6–7 per cent reactivation, as assessed from the AChE activities of cerebellar homogenates.[2] Comparable studies have now been carried out in rats injected with DFP, since reactivation of diisopropylphosphoryl AChE requires a much higher oxime

TABLE 2. REACTIVATION OF DIETHYLPHOSPHORYL ACHE IN THE BRAIN OF RATS

Experiment number	Dose of oxime/kg	Percentage reactivation					
		Cerebellum		cerebral hemisphere			
		Homogenate	Intact	Homogenate	Intact	Intact telencephalon	Homogenates of total brain
1	0·1 m-mole TMB-4						13
2							11
3		35	34				
4		31	36				
5		26	40				
6				12	41		
7				15	38		
8			29		31		
9			27		26		
10			42		37		
11					32	30	
12					35	28	
13	0·1 m-mole P-2-AM				24	18	
14					22	19	
15	0·25 m-mole MINA			17	23		
16				24	26		
17			20		24		

Expts. 1 *and* 2. For each experiment four rats were injected with Paraoxon and TMB-4 (group 1) and four rats with Paraoxon and NaCl (group 2). The homogenates from rats receiving the same treatment were pooled before assay.

Expts. 3–5. For each experiment four rats were used in groups 1 and 2. One half of the cerebellum of all rats in each group was used to make a pooled homogenate and the other halves were each divided into two parts, thus giving eight parts. For assay four of these parts were used per Warburg vessel and the CO_2 outputs of the two vessels were combined for calculating reactivation. Correction factors in these and all other experiments were obtained by including in each assay two Warburg vessels with tissues from untreated controls.

Expts. 6, 7, 15 *and* 16. For each experiment, two rats were used in groups 1 and 2. In each group the left hemisphere of one rat and the right hemisphere of the other rat were used to make a pooled homogenate. The remaining hemispheres were assayed in separate Warburg vessels and the combined CO_2 outputs of the hemispheres of rats receiving the same treatment were used for calculations.

Expts. 8–14 *and* 17. For each experiment, two rats were used in groups 1 and 2. The cerebellum was divided into four approx. equal parts before assay. For assays involving hemispheres the left hemisphere of one rat and the right hemisphere of the other rat in the same group were assayed. Calculations of reactivation were based on the combined CO_2 outputs of two Warburg vessels containing the tissues from rats receiving the same treatment.

Note: in each individual experiment reactivation listed under different headings refers to reactivation in the same animals.

concentration than that which suffices for reactivation of diethylphosphoryl AChE.[1] Using the pooled cerebellar homogenates of six rats which had been given 0·1 m-mole TMB-4/kg i.p. 30 min after the injection of 0·005 m-mole DFP/kg s.c., and comparing its AChE activity with that of the pooled cerebellar homogenates of six rats which had been given DFP only, a reactivation of 3 per cent was found.

DISCUSSION

The AChE in the central nervous system consists of two fractions, a surface-located functional fraction and an intracellular non-functional (reserve) fraction.[6] This must be taken into consideration in experiments designed to elucidate the part which reactivation of phosphorylated AChE plays in the antidotal action of oximes against organophosphates. Our experiments show that reactivation calculated from the AChE activity of homogenates of the whole brain or the cerebral hemispheres (to which functional and non-functional AChE contribute), is considerably less than the reactivation of phosphorylated functional AChE if the oxime used is a pyridinium aldoxime. The reason for this is that pyridinium aldoximes, like other quaternary compounds, are fully ionized and do not cross cell membranes easily; thus the effect on the surface-located phosphorylated functional AChE is greater than that on phosphorylated non-functional AChE, which is located inside the cells.[7,8] A second factor which plays an important part in the reactivation by pyridinium aldoximes of phosphorylated AChE in the central nervous system is the blood brain barrier which limits the access of quaternary ammonium compounds to neuronal structures.[9–11] Our results show that in rats injected with Paraoxon (which forms diethylphosphoryl AChE), the subsequent injection of the pyridinium aldoxime TMB-4, given in a dose of 0·1 m-mole/kg, reactivates approximately one third of the phosphorylated functional AChE in brain. Such an effect is comparable to that produced by a dose of 0·001 m-mole/kg TMB-4 at peripheral sites.[2] With the monoquaternary pyridinium aldoxime P-2-AM the hindrance posed by the brain barrier, like that posed by the intestinal barrier,[12] is less marked than with the bisquaternary pyridinium aldoxime TMB-4. As shown by our experiments 0·1 m-mole P-2-AM/kg reactivates approx. 20 per cent of the functional AChE in the central nervous system although its reactivating potency at peripheral sites is only 5 per cent of that of TMB-4.[3] This is in agreement with recent findings of Milošević and Andjelković, who obtained reactivation of phosphorylated functional AChE in rats injected with Paraoxon and 20 min later with 0·1 m-mole P-2-AM/kg i.v.[13]

The question which arises now is, to what extent does the reactivation of phosphorylated AChE in the central nervous system play a part in the antidotal action of pyridinium aldoximes against lipoid soluble organophosphates which form a reactivatable phosphorylated AChE. A comparison of the data obtained in previous work shows that in mice a good correlation exists between the reactivating potencies of P-2-AM and TMB-4 *in vitro*[14] and in the blood *in vivo*,[3] and the antidotal activities of these oximes against Paraoxon.[2,4] In rats a reasonably good correlation exists between the reactivating potency of pyridinium aldoximes *in vitro* and their antidotal effect (in the presence of atropine) against tetraethyl pyrophosphate (TEPP) which like Paraoxon forms diethylphosphoryl AChE.[15] On the other hand, the results presented in this paper indicate that the correlation between the reactivation in the brain obtained with P-2-AM and TMB-4 and the antidotal action of these oximes is poor. It might be concluded from this that the antidotal action of pyridinium aldoximes in mice and rats must depend largely on the reactivation of phosphorylated AChE at peripheral sites. In reaching this conclusion the assumption has to be made that reactivation at central sites controlling respiration and blood pressure is similar to that obtained in our studies. It is known, however, that the permeability of the blood brain barrier is not uniform at all sites[16] and thus the findings described in this paper

could be misleading. Quantitative studies of the reactivation of phosphorylated functional AChE at vital sites of respiration and circulation, therefore, are urgently needed. Larger animals would be best suited for this but if they are used it must be remembered that the cause of death from poisoning by a given organophosphate varies between species[17] and results obtained in one species are thus not always generally applicable.

Additional evidence which supports the interpretation that reactivation of phosphorylated AChE at peripheral sites is the most important factor in the antidotal action of TMB-4 and P-2-AM in organophosphate poisoning of mice and rats is provided by Erdmann,[18] who observed that in Paraoxon-poisoned rats, both oximes fail to restore what is thought to be respiratory paralysis due to phosphorylation of AChE at central sites. He further found that the "central" respiratory paralysis produced by Paraoxon could be reversed by Toxogonin, an oxime which according to our results[2] behaves like TMB-4 as regards reactivation of phosphorylated AChE at different sites and antidotal activity. This indicates that antidotal action and reactivation of phosphorylated acetylcholinesterase in the brain are not related. In addition, Tong and Way[19] reported that the intracerebral injection of P-2-AM has little effect on the LD_{50} of mice poisoned with TEPP, although oximes applied by this route should readily reach the phosphorylated functional AChE in the central nervous system, according to results obtained with quaternary organophosphates.[6] Similarly, Brown[20] reported that the intracisternal injection of N-methylpyridinium-2-aldoxime methansulphonate (P2S), which as far as reactivation is concerned behaves like P-2-AM, fails to reverse the respiratory paralysis produced in dogs by isopropyl methylphosphonofluoridate (Sarin), an organophosphate which also forms an oxime-reactivatable type of phosphorylated AChE.

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