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Biochemical Pharmacology, Vol. 25, pp. 353–354. Pergamon Press, 1976. Printed in Great Britain.

Effect of oximes upon inhibition of bone marrow acetylcholinesterase by neostigmine

(*Received* 24 *January* 1975; *accepted* 2 *October* 1975)

The anti-cholinesterase agent, neostigmine, has been used clinically and experimentally for many years [1]. *In vivo*, in the mouse, neostigmine inhibits femoral marrow acetylcholinesterase (AChE) activity. It also triggers marrow haemopoietic stem cells into DNA synthesis [2]. The demonstration that, *in vitro*, cholinergic agents initiate DNA synthesis in the bone marrow stem cell [3], suggests that the *in vivo* effect of neostigmine may be secondary to a build up of acetylcholine. The oximes 2-PAM (1-methyl pyridinium-2-aldoxime) and toxogonin (*bis*[4-hydroxy iminomethyl pyridinium-1-methyl] ether dichloride) are used clinically to reverse the effects of organophosphorus cholinesterase inhibitors [4, 5]. There is also evidence that, in some systems, the effects of neostigmine can be reversed by oximes [6, 7]. It was thought that the oximes may provide a useful tool in the elucidation of the mechanisms of stem cell triggering by neostigmine, and may also provide a means of reversing the stem cell effect of the anticholinesterase agent. The present study therefore investigated the effects of toxogonin and the chloride of 2-PAM (2-PAM-Cl), *in vitro*, upon inhibition of bone marrow AChE activity by neostigmine.

B_6AF_1/J female mice aged $2\frac{1}{2}$–$4\frac{1}{2}$ months were used as donors of femoral bone marrow. Suspension cultures of marrow cells were set up in Fischer's medium (approx $5–6 \times 10^6$ cells per ml), as previously described [8]. The cells were allowed to equilibrate in a shaking water bath at 37° for 15 min, then the drugs were added and the incubations were carried out, as indicated in the results. At the end of incubation AChE activity was measured by the spectrophotometric method of Ellman *et al.* [9]. It has been shown [10] that oximes accelerate non-enzymic hydrolysis of acetylthiocholine, the substrate for AChE in the Ellman assay. In the present experiments dilution of the oximes for AChE assay was not sufficient to prevent significant non-enzymic hydrolysis. A filtration method was therefore devised to remove the oximes at the end of incubation. Suspensions from all cultures were filtered in those experiments where oximes were used. Aliquots of suspension containing the required cell numbers were passed through Millipore filters (0·45 μm pore size) in Swinnex filter units. Air (12 ml) was passed through each filter and the filters were then dropped into assay bottles containing phosphate buffer. The AChE activity of the cells on the filters was then measured [9]. Throughout the assay the bottles containing the filters were shaken at room temperature to ensure mixing of the solutions above the filters. Blanks containing no cells were also shaken. For the spectrophotometer readings the solutions were poured off the filters, the readings were made, and the solutions immediately poured back onto the filters. Readings were taken every 3–4 min for periods of up to 1 h. In all experiments a marrow sample that had been cultured with oxime only was included, to check that the drug had been removed by the filtration procedure.

Readings taken during the first 30 min of assay were used in the calculations. Using the method of least squares, the slope of the line of best fit was calculated. This gave the change in absorbance units per min. Values obtained by subtraction of blank values from sample values were used to calculate the percentage protection or reversal.

$$\%\ \text{Protection or reversal*} = [(z - y)/(x - y)] \times 100$$

x = control (no treatment) value
y = value from sample treated with neostigmine only
z = value from sample treated with neostigmine plus oxime

As 10^{-6} M neostigmine completely inhibited AChE activity, but 10^{-10} M neostigmine had no effect upon the enzyme activity, 10^{-8} M neostigmine was used in most of the experiments.

Table 1 shows the effect of adding 10^{-3} M or 10^{-4} M 2-PAM-Cl or toxogonin to the cultures 10 min before addition of neostigmine. A high level of protection is seen when 10^{-3} M 2-PAM-Cl is added before 10^{-8} M neostigmine, and the level of protection is similar whether the cultures are incubated for 30, 90 or 120 min. 10^{-3} M 2-PAM-Cl is much less effective when 10^{-7} M or 10^{-6} M neostigmine is added. 10^{-4} M 2-PAM-Cl provides some degree of protection against 10^{-8} M neostigmine when incubated for 30 or 60 min, but when the incubation is extended to 90 or 120 min the protective effect disappears. 10^{-3} M toxogonin partially protects against 10^{-8} M neostigmine and to a lesser extent against 10^{-7} M neostigmine, but 10^{-4} M toxogonin has no protective effect against 10^{-8} M neostigmine.

* Protection when oxime added before neostigmine, reversal when added afterwards.

Table 1. % Protection against neostigmine by oxime

Oxime	Incubation time					
	10^{-8} M neostigmine				10^{-7} M neostigmine	10^{-6} M neostigmine
	30 min	60 min	90 min	120 min	90 min	90 min
10^{-3} M 2-PAM-Cl	94·1*	—	90·6	85·0	29·4	9·9
	64·8		41·3	76·0	23·8	27·2
			70·2			
10^{-4} M 2-PAM-Cl	42·2	48·6	4·4	1·7	—	—
	37·3	11·9	0·0	9·0		
10^{-3} M Toxogonin	—	—	59·5	—	24·6	—
			37·0		17·0	
10^{-4} M Toxogonin	—	—	0·0	—	—	—
			0·0			

The oximes were added to culture 10 min before neostigmine. Incubation times shown are the periods of incubation after neostigmine addition.

* As the number of experiments per point was too small for calculation of means, the results from individual experiments are shown.

Table 2. % Reversal of neostigmine effect by oxime

Oxime	Neostigmine	
	10^{-8} M	10^{-7} M
10^{-3} M 2-PAM-Cl	64·3*	26·9
	76·5	52·4
10^{-3} M Toxogonin	72·9	—
	70·8	
10^{-4} M Toxogonin	1·5	—
	0·0	

The oximes were added to culture 10 min after neostigmine. Incubation times shown are the periods of incubation after neostigmine addition.

* As the number of experiments per point was too small for calculation of means, the results from individual experiments are shown.

The effect of addition of the oximes to the cultures 10 min after neostigmine is shown in Table 2. Incubation with 10^{-3} M toxogonin or 2-PAM-Cl results in approx 70% reversal of the inhibitory effect of 10^{-8} M neostigmine. 10^{-3} M 2-PAM-Cl is less effective against 10^{-7} M neostigmine. 10^{-4} M toxogonin is completely ineffective in reversing the inhibitory effects of 10^{-8} M neostigmine.

The results indicate that, *in vitro*, 2-PAM-Cl and toxogonin reduce the inhibitory effects of neostigmine upon bone marrow AChE. The oximes may be added before or after the anti-cholinesterase agent, but a high degree of protection or reversal is achieved only when the oximes are added in at least a 10^5-fold excess over the level of neostigmine. Further study is required to determine whether it will be possible, or necessary, to achieve this excess of oxime *in vivo*, to reverse or protect against the inhibitory effects of neostigmine on bone marrow cholinesterase.

It is well known that inhibition of AChE by organophosphorus compounds involves phosphorylation of the enzyme at its esteratic site [1]. In order to do this, some of the organophosphates combine with both the anionic and esteratic sites of the enzyme, whereas others combine only with the esteratic site. Oximes such as 2-PAM-Cl protect against this inhibition by interaction of the quaternary nitrogen of the oxime molecule with the anionic site of the enzyme. Reactivation of phosphorylated AChE involves nucleophilic attack on the phosphorus at the esteratic site, freeing the active enzyme.

It is likely that cholinesterase inhibitors such as neostigmine combine with both the anionic and esteratic sites of AChE, resulting in carbamoylation at the esteratic site [11]. It can therefore be envisaged that, in the present experiments with neostigmine, the oximes may protect and reactivate bone marrow AChE by interaction with the anionic site and nucleophilic attack on the dimethyl carbamoyl group at the esteratic site of the inhibited enzyme.

Acknowledgements—Toxogonin was kindly supplied by Dr. F. R. Sidell, Edgewood Arsenal, Maryland 21010, U.S.A.

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