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RAT BRAIN ACETYLCHOLINESTERASE TURNOVER IN VIVO : USE OF A RADIOACTIVE METHYLPHOSPHONOTHIATE IRREVERSIBLE INHIBITOR

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A new organophosphorus compound was used in its non radioactive and tritiated forms in order to study rat brain acetylcholinesterase. We measured the activity recovery of the total enzyme and of its two main molecular forms (4 S and 10 S) as a function of time following the inhibition. The radioactive compound allowed us to study the disappearence of the inhibitor irreversibly bound to the enzyme in the main cholinergic areas. Both approaches gave similar results : acetylcholinesterase turn-over proceeds in two steps, a rapid one of about 30 mn and a slow one of about 2 days. Our results suggest an in vivo reactivation process concerning a fraction of the bound inhibitor.

Acetylcholinesterase (AchE : EC 3.1.1.7.) plays a key role in cholinergic mechanisms of nerve transmission. Its blockade by irreversible inhibitors (for example organophosphorus pesticides) causes severe and even lethal troubles in mammals and particularly in man. The therapeutics of these troubles is difficult to perform and it seems essential to know the spontaneous recovery mechanisms which leads to a return to normal AchE levels. This aspect has been little studied, particularly in the brain. The existence of two phases in the recovery kinetics has been observed since 1955 by Davison (1) and attributed to different rates of synthesis of the brain molecular forms of the enzyme (2-5). However in all these works the inhibitors used lack of specificity and the kinetics of disappearance of the inhibited enzyme and of enzyme activity recovery were never conducted simultaneously. Thus we used a new organophosphorus compound O-ethyl-S-(2,diisopropylaminoethyl)-methylphosphonothionate, (abbreviated MPT), whose high specificity and irreversible inhibition properties for AchE have been previously demonstrated (6,7,8). This compound has been used in its normal and tritiated forms in order to study in vivo the recovery of whole rat brain AchE activity and of its molecular forms, as well as the time-course of the inhibitor disappearance in the different regions of the brain where it was bound.

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MATERIAL AND METHODS

The MPT inhibitor was synthetized according to Ley and Sainsbury (9). Its radioactive form was obtained by incorporating tritiated ethanol at the first step of the process (Commissariat à l'Energie Atomique, Pr.Pichat and Dr.Audinot). A 10 mM stock solution of cold MPT in ethanol was diluted in saline before injection. The radioactive compound was stored in toluene (specific activity 20 Ci/mM) in liquid nitrogen. Toluene was evaporated under nitrogen at room temperature, and the compound diluted in saline at the desired concentration just before use.

Ninety Sprague-Dawley rats weighing about 260g were injected either in the left carotid or in the right heart ventricule under ether anesthesia. The mean MPT lethal doses, calculated according to Reed and Muench (10) were respectively 15 µg/kg and 20 µg/kg for the intracarotid and intracardiac injections. Cold MPT was given by intracardiac route (19 µg/kg) and the animals were sacrificed at various time intervals afterwards. Brains were immediately homogenized with a Potter apparatus, the homogenates were centrifuged at 1000 x g for 10 min, and AchE activity measured in the supernatant by the Ellman method (11). In our conditions, AchE activity was 385 + 36 nmoles of acetylthiocholine hydrolyzed per 10 mn and per mg of protein at 20°C.

Molecular forms of the enzyme were separated by sedimentation in continuous sucrose gradient (5-20%) according to Rieger et al (12). The activity of each form was measured by the Ellman method and expressed as percentage of the total activity of the homogenate.

In order to label specifically and intensively brain AchE, the tritiated inhibitor was injected in the left carotid at a dose of 15 µg/kg (200 µCi) in 6 rats. At various time intervals after injection, animals were decapitated, the brains freezed in liquid nitrogen and sliced at - 20°C with a PMV cryomicrotome. The slices were exposed on LKB films for 40 days. After processing, the radioactive brain regions were identified and the darkening of the film was quantified with a Vernon densitometer. The radioactivity present in the slices was also measured with a SL 30 Intertechnique liquid scintillation counter.

RESULTS

1) Kinetics of AchE activity recovery

- Whole brain AchE: after an intracardiac MPT injection of 19 µg/kg, 95% of the AchE activity is inhibited in the first three minutes. AchE activity recovery begins within 15 min (16% of control level at 15 min) and occurs with an initial rapid increase during the first 3 hours and then a slow one afterwards, allowing the return at the control level around the seventh day (figure 1).

In order to quantify this recovery kinetics, we assumed that the experimental data could be represented by the sum of two decreasing exponentials according to the relation :

$$I(t) = I_1 \exp(-t/T_1) + I_2 \exp(-t/T_2)$$

where I(t) is the inhibition level at time t, I_1 and I_2 being the extrapolated inhibition levels at time 0 (fig.2A) respectively for the rapid and slow

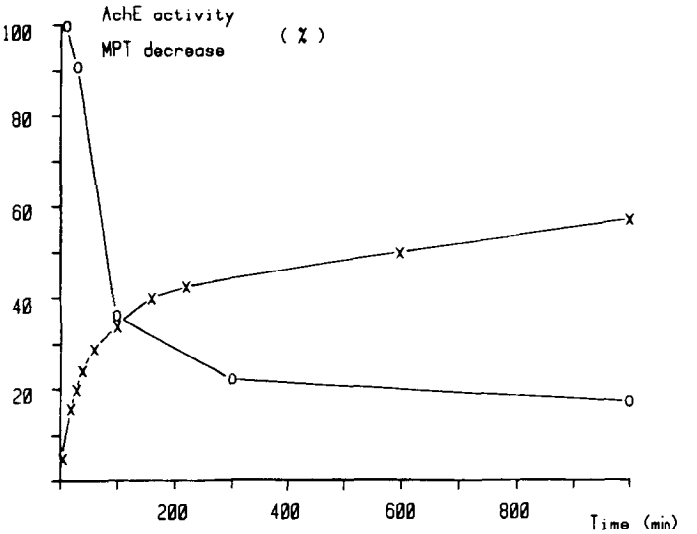

Figure 1 : Acetylcholinesterase activity recovery (X—X) and tritiated MPT disappearance (O—O) in whole rat brain as a function of time after a single injection of the cold inhibitor (19 µg/kg, intracardiac) or of the radioactive compound (15 µg/kg, intracarotid). Enzyme activity is given in per cent of the initial activity before inhibition. Radioactivity is expressed in per cent of the initial maximum value of brain labeling.

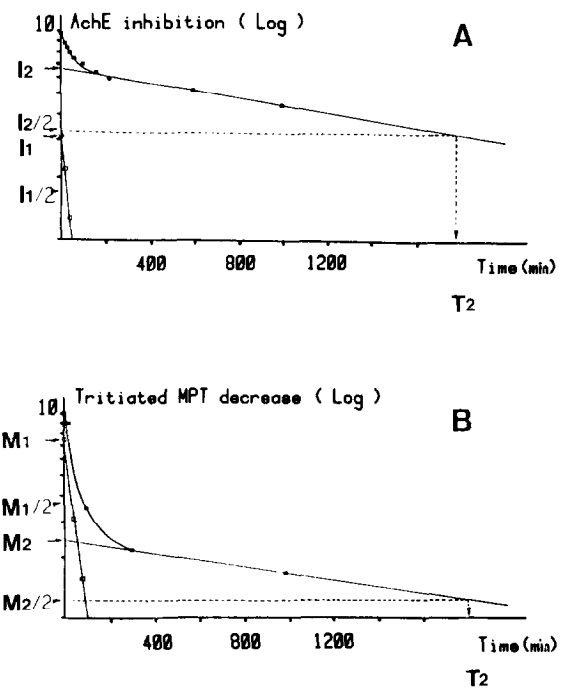

Figure 2 : A) Semilogarithmic plot of the calculated AchE inhibition values as a function of time.
B) Semilogarithmic plot of the bound tritiated MPT radioactivity decay.
In both cases, the graphical determination of the time-constants is indicated.

TABLE 1

Time-constants of acetylcholinesterase activity recovery and tritiated MPT decay in the rat brain. These values were calculated by the graphical method shown in figure 2.

	AchE RECOVERY			TRITIATED MPT DECAY					
	TOTAL AchE	4 S form	10 S form	TOTAL brain	Striatum	Amygdala	Habenula	Hippo-campus	Cortex
Rapid period (min)	30 ± 12	30	24	37 ± 17	31	26	31	25	32
Slow period (days)	1.9 ± 0.5	1.8	1.4	1.3 ± 0.7	2.9	0.5	1.5	2.1	1.1

decays characterized by the time-constants T_1 and T_2. I(t) is calculated from the measured activities by the relation :

$$I(t) = \frac{Ao - A(t)}{Ao - Amin} \times 100$$

where Ao, A(t) and Amin are respectively the AchE activities of the control, at time t and at the time of maximum inhibition (minimum activity). In fact, a good fit is observed (fig.2A) and this classical graphical method allowed us to determine the apparent time-constants of this two-step kinetics (Table I).

- AchE molecular forms : only two AchE forms characterized by the sedimentation constants 4S (19.2 ± 3.5%) and 10S (80.8 ± 3.5%) are found in rat brain (12). Biphasic recovery kinetics are also observed (fig.3). For both forms, the graphically determined time-constants are very close to those measured for whole AchE (Table I).

2) Kinetics of disappearance of MPT binding

- whole brain radioactivity: liquid scintillation counting or integration of the densitometric values obtained from the brain autoradiographies gave similar results. The disappearance of the tritiated inhibitor occurs again according to a two-phase kinetics. The curve shown in fig.1 is a mirror image of the one obtained for the AchE activity recovery. We applied the same graphical treatment as previously used (fig.2B) and found again a good fit with the experimental data. The calculated time-constants for

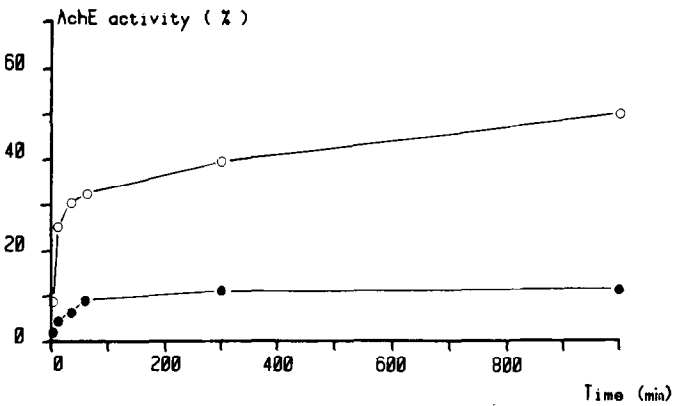

Figure 3 : Activity recovery of the 4 S (●) and 10 S (○) brain AchE molecular forms following an intracardiac injection of MPT (19 µg/kg). The values are given in per cent of the total AchE activity in the brain homogenate before inhibition.

brain radioactivity decrease are close to the corresponding values found for AchE recovery (Table I).

- radioactivity of the principal cholinergic brain areas: densitometry of the principal labeled brain areas allowed us to determine with a reasonable accuracy the kinetics of disappearance of the radioactivity due to MPT binding in the striatum, amygdala, cortex, hippocampus and habenula (figure 4). In each case, a two-step kinetics is again observed, the

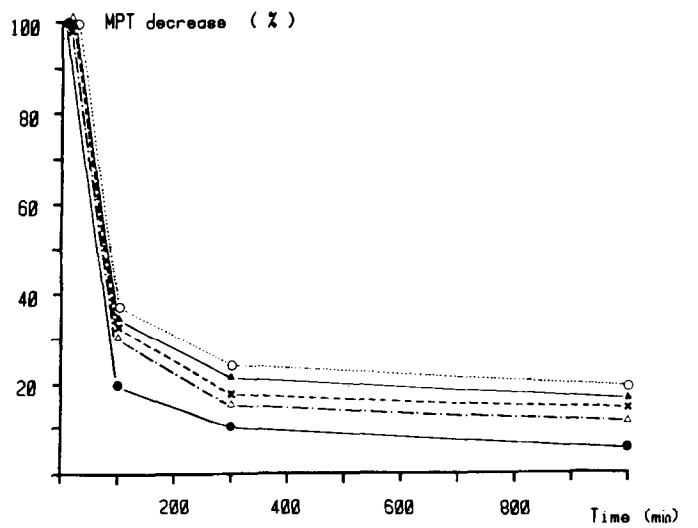

Figure 4 : Variation of the radioactivity (% of the maximum initial values) as a function of time in different brain areas following the intracarotid injection of radioactive MPT : △---△ : striatum; ●——●: amygdala; ×— —×: parietal cortex; ▲——▲ : hippocampus; ○-----○ : locus niger.

time-constants of which can be evaluated respectively to 25-32 min for the rapid one and 0.5-2.9 days for the slow one (table I).

DISCUSSION

The organophosphorus compound MPT used in this study presents in our opinion the following advantages comparatively to more classicaly used inhibitors like DFP, paraoxon or sarin. It is very specific for acetylcholinesterases from various sources (6), and does not bind on other esterases since its inhibition mechanism involves an enzymatic hydrolysis of the molecule at the level of the P-S bond. Its affinity for "true" acetylcholinesterase is about 50 times higher than for butyrylcholinesterases (7). This inhibitor is not subject to the phenomenon of ageing nor to spontaneous reactivation in vitro (6,8). Furthermore, it is very stable in aqueous solution and it can be relatively easily synthetized in its tritiated form with a high specific activity (20 Ci/mM).

In order to study the mechanisms of AchE metabolism after irreversible MPT binding, it was necessary to obtain a maximum level of inhibition as quickly as possible and a rapid disappearance of the circulating free inhibitor. This was achieved by intracardiac or intracarotid injections, showing the very rapid binding of the compound and the early starting of AchE activity recovery.

The striking fact which we want to point out from the present results is the parallelism and the synchronism between the recovery kinetics of total brain AchE activity and of its molecular forms on one hand and the disappearance of the bound radioactive inhibitor in total brain and in the main cholinergic areas on the other hand (figures 1-4). Both kinetics can be characterized by two phases, a rapid one with a time-constant of abCut 30 min and a slow one with a time-constant of about 2 days. This observation indicates the existence of close relationship between the recovery of AchE activity and the disappearance of bound tritiated MPT.

Such a biphasic recovery kinetics has also been observed with other organophosphorus inhibitors, like DFP or Sarin (2,5,13,14). Although the binding mechanism of these compounds on AchE is different from that of MPT, a P-O bond is formed on the serine in the active site of the enzyme. This bond

has been shown to be irreversible *in vitro*, particularly for MPT (6-8). However, the parallelism observed between AchE activity recovery and tritiated MPT disappearance strongly suggests a reactivation process occuring *in vivo*, which concerns at least a fraction of the brain enzyme and explains the initial rapid phase, the slow phase being due to new active enzyme resynthesis. This partial reactivation mechanism is presently unknown and might be related to the AchE metabolism and to the location of the enzyme inside the cells.

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