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Inhibition of rat cerebrum acetylcholinesterase isoenzymes after acute administration of soman*

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Recent work in our laboratory has been involved with characterization of the acetylcholinesterase isoenzymes in rat cerebrum. It has been reported that rat brain acetylcholinesterase (AChE, EC 3.1.1.7) exists in multiple molecular forms [1–5], some of which are less susceptible to inhibition by diisopropyl phosphorofluoridate (DFP) than others [1]. Using isoelectric focusing in polyacrylamide gels as a means of protein separation and characterization [5–9], we have been able to separate several isoenzymes of acetylcholinesterase. We now wish to report some results that demonstrate that acetylcholinesterase isoenzymes in rat brain cerebrum are inhibited at different rates by soman (pinacolylmethylfluorophosphonate acid), an irreversible inhibitor.

Materials and methods

Materials. Sprague–Dawley rats (180–200 g) were bred in-house.† Triton X-100 was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Ampholytes (Ampholine) were obtained as 40% solutions from LKB Produkter, Bromma, Sweden. Soman was obtained from the Chemical Systems Laboratory, Aberdeen Proving Ground, MD, U.S.A. and was 92 per cent pure. It was diluted in saline immediately prior to use and administered intramuscularly to rats (90 μg/kg). Control animals received saline injections. Iso-OMPA was from K & K Chemicals, and BW284C51 was a gift of Burroughs Wellcome Co., Research Triangle Park, NC.

Enzyme assay. Cholinesterase was measured by the method of Groff *et al.* [10], employing acetylthiocholine as substrate. Using rat cerebrum as an enzyme source, hydrolysis due to nonspecific cholinesterase (butyrylcholinesterase) has been reported as being negligible [11, 12].

Homogenization of tissues. Rats were killed by exsanguination and perfused with three times their body volumes of ice-cold saline. Brain tissue was ground in a glass homogenizer with a teflon pestle in 9 vol. of ice-cold physiologic saline containing 1% (v/v) Triton X-100. Homogenates were sequentially centrifuged at 15,000 *g* for 10 min and at 75,000 *g* for 30 min at 5°. The resultant supernatant fractions were used immediately or stored frozen at −20° for subsequent analysis. Frozen samples were stable for at least 30 days.

Isoelectric focusing. Isoelectric focusing was carried out on multiple samples from groups of three to four rats at five time points over a 15-min interval after soman administration. Isoelectric focusing was conducted in polyacrylamide gel (5% total gel 2.6% cross-linked with *N,N'*-methylene bis acrylamide) at pH 4.0 to 5.5, carrier ampholine concentration of 1% (w/v). Both carrier ampholytes and homogenates containing acetylcholinesterase were added prior to polymerization. *N,N,N',N'*-Tetraethylenediamine [0.3% (v/v)] was the catalyst and ammonium persulfate [0.06% (w/v)], the initiator. Focused gels were washed with 0.4 M sodium acetate and 0.1 M sodium acetate, pH 6.0, sequentially, to remove excess ampholytes. They were then stained for acetylcholinesterase activity by the method of Karnovsky and Roots [13] for 7 hr. Stained gels were scanned at 480 nm using a Gilford spectrophotometer equipped with a linear transport gel scanner.

pH measurements. After focusing, an unstained gel was frozen and then sliced into 3-mm thick slices. Each slice was then placed in 0.5 ml of freshly distilled water, allowed to soak, capped and stored overnight at 4° and the pH was measured with a Beckmann model 1019 research pH meter.

Results and discussion

When protein that had been solubilized from rat brain homogenate by Triton X-100 was subjected to isoelectric focusing and subsequently visualized for acetylcholinesterase activity, four isoenzymes were consistently identified on the basis of their pI values: $pI_1 = 4.78 \pm 0.05$; $pI_2 = 4.65 \pm 0.04$; $pI_3 = 4.54 \pm 0.08$; and $pI_4 = 4.47 \pm 0.08$. To ensure that the isoelectric focused material was acetylcholinesterase, the inhibitors iso-OMPA (specific for butyrylcholinesterase) and BW284C51 (specific for acetylcholinesterase) were used. The latter inhibitor at 10^{-5} M abolished all staining, whereas gels incubated with iso-OMPA at 10^{-5} M for 45 min prior to staining showed no decrease in staining intensity. From this we concluded that all the activity in the focused gels was due to acetylcholinesterase.

Within 15 min all the cholinesterase activity in rat cerebrum was completely inhibited following a dose of 90 μg/kg of soman. Similar rates of inhibition of total acetylcholinesterase were found for midbrain, cerebellum, and medulla.

When the isoenzyme pattern obtained after isoelectric focusing was examined at various times after administration of soman, it was seen that not all forms were affected to the same extent at a given time (Fig. 1). If the areas under the curve for a given time were plotted against total acetylcholinesterase activity determined by biochemical assay (from samples from the same animal), a correlation of $r = 0.98$ was obtained. Thus, if small samples of acetylcholinesterase are available, isoelectric focusing could be used to quantitate total enzymatic activity and for estimates of the rates of inhibition of various molecular forms.

Our findings revealed that not all forms of acetylcholinesterase in rat cerebrum were inhibited at the same rate after soman administration (Fig. 2). In fact, isoenzymes 1 and 2 were inhibited 30 per cent more rapidly than isoenzymes 3 and 4. This suggests that in mammals certain acetylcholinesterase isoenzymes may be critical to cholinergic function in a manner similar to that observed for housefly acetylcholinesterase [14, 15].

It is not clear why there are differing rates of inhibition for the isoenzymes of acetylcholinesterase. Davis and Agranoff [1] reported that there was an isoenzyme of acetylcholinesterase that was synthesized with a $T_{1/2}$ of 3 hr. However, even an isoenzyme with a turnover as rapid as this cannot account for the results reported here. Although DFP and paraoxon administered *in vivo* were reported

* The opinions or assertations contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

† In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.*

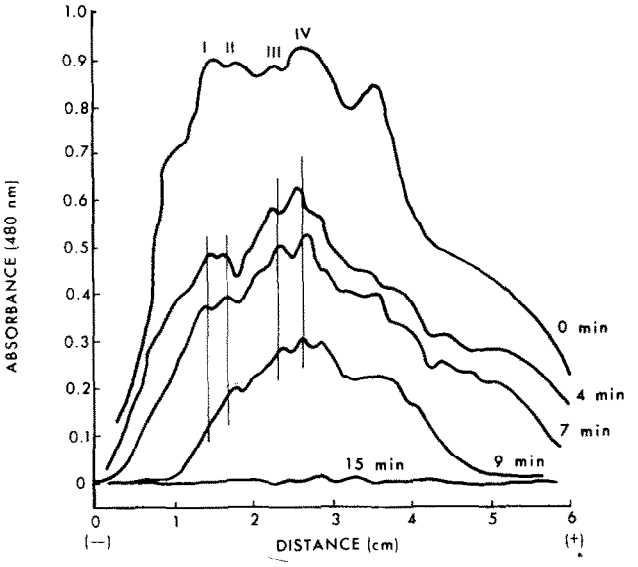

Fig. 1. Scans of gels showing cerebral acetylcholinesterase isoenzyme levels as a function of time after soman administration. Gels were scanned at 480 nm; specific activity for uninhibited enzymes was 125.5 ± 19.9 $\mu M \cdot (g\ protein)^{-1} \cdot min^{-1}$. The total per cent activity of the acetylcholinesterase isoenzymes at the various times was: t_0 = 100%, t_4 = 71%, t_7 = 48%, t_9 = 35%, and t_{15} = 5%. Peaks I, II, III and IV correspond to those identified in the text. Vertical lines aid in following the decrease in peak height. The unidentified peak next to IV did not appear reproducibly, but it may have been another acetylcholinesterase isoenzyme.

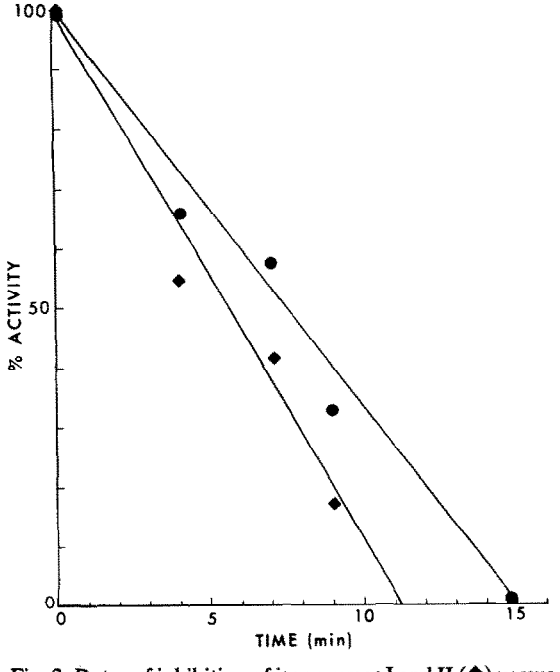

Fig. 2. Rates of inhibition of isoenzymes I and II (◆) versus isoenzymes III and IV (●). All points are the average activity of isoenzymes I and II or III and IV for a given time, as determined from Fig. 1. The slopes for the two lines, determined by the method of least squares, were statistically different at the 95 per cent confidence level ($F = 8.86$, $P < 0.01$, $df = 16$). There is no 15-min point for (●) because complete inhibition of these isoenzymes occurred between 9 and 15 min.

[16–18] to cause additional inhibition of acetylcholinesterase during homogenization, when soman [19] or sarin [20] has been administered, respectively, at doses up to 1 LD_{50}, no additional tissue inhibition during homogenization was reported. Therefore, our results do not represent effects that would occur upon homogenization of the brain tissue prior to assay. Our method of solubilizing acetylcholinesterase was identical to the one reported by Rieger and Vigny [21] who found that 90 per cent of the acetylcholinesterase of rat brain is membrane-bound. Thus, the various isoenzymes reported here do not result from being isolated from two different sources, membrane and cytoplasm.

We were interested in determining the generality of the appearance of four acetylcholinesterase isoenzymes. To address this, we examined, by isoelectric focusing, purified 11S acetylcholinesterase from electric eel. Four isoenzymes with the same pI values as those reported above were identified. This observation leads us to conclude that our findings with rat cerebrum acetylcholinesterase are not attributable to the presence of various molecular weight forms of the enzyme.

The results of our present study agree well with the recent observations of Bajgar [22] who concluded that there are isoenzymes of acetylcholinesterase present in rat brain under physiological conditions. We have been able to amplify on those findings by demonstrating that there are four isoenzymes which fall into two groups having different kinetic properties *in vivo* after the administration of the potent, nonreversible anticholinesterase inhibitor soman.

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Sodium ion and the effect of acetylcholine on phospholipid and phosphoprotein phosphate turnover in the rabbit iris smooth muscle*

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The responses of a variety of tissues to neurotransmitters, neurohumors, neuropharmacological agents and electrical pulses involve increases in the phosphate turnover of certain phospholipids, such as the phosphoinositides [1], and certain phosphoproteins [2]. Neither the molecular mechanism nor the physiological significance of the "phosphoinositide effect", which may be defined as a change in the rate of metabolism or turnover of the inositol-containing phospholipids when the tissues in which they occur are stimulated, is well understood. It is well established that the phosphoinositide effect is mediated through muscarinic cholinergic and α-adrenergic receptors [1]. Although activation of these receptors is known to lead to changes in the intracellular ionic environment, changes in intracellular concentration of a second messenger such as cyclic AMP or cyclic GMP, or changes in relevant enzymes, there has been little work done to show that these changes could mediate the increase in phosphoinositide turnover in response to receptor activation [1]. This has lead Michell and his colleagues [1, 3] to postulate that the agonist-stimulated breakdown of phosphatidylinositol (PI) in target tissue leads to the opening of Ca^{2+} gates and thus, to increased intracellular Ca^{2+} which, in turn, triggers the observed cellular responses. In previous communications from this laboratory, we reported on the characteristics of the phosphoinositide effect in the rabbit iris (for summary see Refs. 4 and 5). A key finding in these studies was the observation that in iris muscle, which was prelabeled with $^{32}P_i$ and in which the Ca^{2+} content was depleted with EGTA,† the acetylcholine (ACh)-stimulated breakdown of triphosphoinositide (TPI) and the ^{32}P-labeling of phosphatidic acid (PA) and to a much lesser extent PI are dependent on the presence of extracellular Ca^{2+} [6]. More recently the dependence of the phosphoinositide effect on the presence of extracellular Ca^{2+} has been demonstrated in synaptosomes [7, 8].

Activation of muscarinic cholinergic and α-adrenergic receptors in smooth muscle also leads to an increase in cell-surface permeability to Na^+ and K^+ in addition to Ca^{2+} [9]. In the present study, the effects of Na^+ and other cations on ^{32}P-labeling of phospholipids and phosphoproteins in the absence and presence of ACh were investigated.

Materials and Methods. Irises for the following experiments were obtained from albino rabbits at a local slaughterhouse. In general, whole irises were incubated singly (of the pair, one was used as control and the other as experimental) in isosmotic medium that contained 10 μCi $^{32}P_i$ (carrier free, New England Nuclear Corp., Boston, MA), at 37° for 1 hr in the presence and absence of the pharmacological agent as indicated. ACh was added 30 min after preincubation of the tissue with $^{32}P_i$. When the Na^+ concentration was varied, isosmolar substitution of NaCl was made by sucrose. The reaction was ended by addition of ice-cold trichloroacetic acid (TCA) to a final concentration of 10%. Phospholipids were extracted from the tissue with chloroform–methanol–HCl (400:200:1.5, by vol.), then separated into individual phospholipids by means of two-dimensional t.l.c. with silica gel H, and their radioactive contents determined as described previously [10]. After extraction of the lipids, phosphoproteins were determined in the residual-insoluble precipitate as alkali-labile phosphate by a modification of the procedures of Ahmad and Judah [11]. Briefly, the residual-insoluble precipitate was washed three times with 3 ml of 5% TCA containing 0.1 M KH_2PO_4. The tubes were centrifuged after each

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† EGTA, ethyleneglycolbis (amino-ethylether)tetra-acetate.