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Expression of cholinesterase gene(s) in human brain tissues: translational evidence for multiple mRNA species

Hermona Soreq*, Dina Zevin-Sonkin and Nissim Razon[1]

Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, and [1]Department of Neurosurgery, Ichilov Hospital, Tel Aviv Medical Center, Israel

*To whom reprint requests should be sent
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To resolve the origin(s) of the molecular heterogeneity of human nervous system cholinesterases (ChEs), we used *Xenopus* oocytes, which produce biologically active ChE when microinjected with unfractionated brain mRNA. The RNA was prepared from primary gliomas, meningiomas and embryonic brain, each of which expresses ChE activity with distinct substrate specificities and molecular forms. Sucrose gradient fractionation of DMSO-denatured mRNA from these sources revealed three size classes of ChE-inducing mRNAs, sedimenting at ~32S, 20S and 9S. The amounts of these different classes of ChE-inducing mRNAs varied between the three tissue sources examined. To distinguish between ChEs produced in oocytes and having different substrate specificities, their activity was determined in the presence of selective inhibitors. Both 'true' (acetylcholine hydrolase, EC 3.1.1.7) and 'pseudo' (acylcholine acylhydrolase, EC 3.1.1.8) multimeric cholinesterase activities were found in the mRNA-injected oocytes. Moreover, human brain mRNAs inducing 'true' and 'pseudo' ChE activities had different size distribution, indicating that different mRNAs might be translated into various types of ChEs. These findings imply that the heterogeneity of ChEs in the human nervous system is not limited to the post-translational level, but extends to the level of mRNA.
Key words: acetylcholinesterase-mRNA/glioma/meningioma/molecular form heterogeneity/pseudocholinesterase-mRNA/*Xenopus* oocytes

Introduction

The enzyme acetylcholinesterase (acetylcholine hydrolase, AChE, EC 3.1.1.7) plays an essential role in cholinergic mechanisms by rapidly hydrolysing acetylcholine (ACh). AChE is relatively abundant in the nervous system and muscles. It exists in multiple molecular forms, differing in their sedimentation in sucrose gradients, tissue localization, mode of association with the surface membrane, regulation and probable physiological function (e.g., see Hall, 1983; Massoulie and Bon, 1982). In most vertebrate tissues, AChE is accompanied by a related enzyme, pseudocholinesterase (acylcholine acylhydrolase, pseudo-ChE, EC 3.1.1.8), which differs from AChE in its substrate specificity and sensitivity to inhibitors (Austin and Berry, 1953). Pseudo-ChE exists in molecular forms corresponding to each of the known molecular iso-forms of AChE (Vigny *et al.*, 1978). There is conflicting evidence in the literature, some suggests a coordinate or parallel regulation of pseudo-ChE and AChE (Lyles *et al.*, 1979; Silman *et al.*, 1979; Jedrzejczyk *et al.*, 1981), other findings support the opposite view (Graybiel and Ragsdale, 1982; Edwards and Brimijoin, 1982; Layer, 1983).

There is no direct information as to whether ChEs of different substrate specificities and sedimentation properties are produced by different genes or are the result of post-transcriptional and/or post-translational processing. A possible precursor-product relationship has been suggested for pseudo-ChE and AChE (Koelle *et al.*, 1977). Others have contended this view because of immunological evidence and because of the existence of parallel sets of multiple forms of both enzymes. Thus, it has been suggested that the monomeric and dimeric forms of the cholinesterases serve as precursors to the more complex forms (Wilson and Walker, 1974; Rieger *et al.*, 1976). The possible metabolic relationships among different cholinesterase forms have been approached by analysis of the recovery of ChE activity after irreversible inhibition of pre-existing enzyme by diisopropylfluorophosphate (DFP), of the effects of transcription inhibitors, or of the pattern of appearance of the molecular forms in developing cells *in vivo* and in culture (e.g., see Wilson and Walker, 1974; Rieger *et al.*, 1976; Rotundo and Fambrough, 1980, 1982). However, the non-selective DFP and transcription inhibitors may affect various biosynthetic processes in the cells; and the pattern of appearance of the different ChE forms during differentiation does not initially distinguish between the different possibilities. Moreover, it is difficult to distinguish between enzyme interconversion and sequential appearance and breakdown of ChE activities. Some of these difficulties may be overcome by direct measurements of ChE synthesis in *Xenopus* oocytes microinjected with mRNA from ChE-producing tissue (Soreq *et al.*, 1982a; Meedel and Whittaker, 1983) followed by radiometric measurement of the induced ChE activity (Johnson and Russell, 1975).

We have injected *Xenopus* oocytes with size-fractionated mRNA from brain tissues expressing different ChEs, including embryonic human brain, gliomas and meningiomas (Razon *et al.*, 1984; Libermann *et al.*, 1984). We also examined the ability of various size fractions of human brain mRNA to induce defined ChE activities in microinjected oocytes. The results suggest that heterogeneity of mRNA contributes to the formation of the various ChEs in the human brain.

Results

Biosynthesis of ChE in oocytes injected with unfractionated human brain mRNAs

We used the following tissues: embryonic human brain tissue, the poorly differentiated embryonal tumors of gliogenous origin, glioblastoma multiforme, and the mesenchymal meningiomas (Gillespie and Mahaley, 1981; Razon *et al.*, 1984; Libermann *et al.*, 1984). The specific activity of ChE in extracts from these tissues ranged between 300 and 500 nmol of ACh degraded/min/g tissue. In both normal forebrain and meningiomas, acetylcholinesterase accounted for almost all

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Table I. Properties of ChEs and of mRNA from frozen human tissues

Tissue	Embryonic brain (17 weeks)	Meningioma	Glioblastoma multiforme	Epidermoid carcinoma
1 Specific activity of total ChE	480	308	352	60
2 % of BW284C51-insensitive ChE	10 ($n=12$)	4 ($n=7$)	26 ($n=14$)	6 ($n=3$)
3 Major ChE molecular forms	10S, 4S	4S	10S, 4S	n.d.
4 RNA yields				
Total	700	1300	600	3000
poly(A)$^+$	16.4	39.7	16.7	80.0
5 Translational activity *in vitro*	4.3	3.9	3.6	13.3
6 ChE mRNA activity *in ovo*	1400 ($n=6$)	500 ($n=3$)	300 ($n=5$)	200 ($n=4$)

1. The specific activity of ChE, in nmol [^{3}H]acetate released/min/g tissue, was determined radiometrically (Johnson and Russell, 1975), as detailed in Materials and methods, for each of the tissue samples, prior to RNA preparation.
2. Percent inhibition by 10^{-5} M BW284C51 was determined in separate homogenates from several individual tumors and tissue samples (no. of samples is noted in parentheses) (Razon *et al.*, 1984).
3. Sedimentation properties of ChE activities were determined by sucrose gradient centrifugation of several homogenate samples from each type of tissue (Razon *et al.*, 1984). S values of the major activity peaks are noted.
4. Preparation of RNA and of polyadenylated RNA was as described (Soreq *et al.*, 1982b). Yields of RNA are presented at µg/g tissue.
5. mRNA-directed incorporation of [^{35}S]methionine (730 Ci/mmol, Radiochemical Centre, Amersham) into polypeptides was performed in a nuclease-treated reticulocyte lysate as described (Soreq *et al.*, 1982b). Reaction mixtures of 12.5 µl contained ~0.1 µg of mRNA, and were incubated for 60 min at 30°C. TCA-precipitable radioactivity was measured on samples of 4 µl. Background incorporation (~10^4 c.p.m./sample) was subtracted. Data are presented as net incorporation directed by 1 µg of polyadenylated RNA x 10^{-6}.
6. Unfractionated polyadenylated RNA (1 mg/ml) from the various tissues was injected into oocytes, and ChE activity was determined radiometrically in oocyte homogenates and incubation medium, as detailed in Materials and methods. Average activity induced in injected oocytes is presented as pmol [^{3}H]acetate released/h by oocyte samples representing 1 µg of injected mRNA. (No. of separate microinjection experiments is noted in parentheses.) Background activity in control, Barth medium-injected oocytes was subtracted.

the ChE activity, but in almost all gliomas elevated levels of BW284C51-insensitive pseudocholinesterase could be detected. The cholinesterase activity of both normal brain and gliomas sedimented on sucrose gradients as a major component of 10S together with a minor component of 4S. In meningiomas, the 4S component was predominant (Table I; see also Razon *et al.*, 1984). In epidermoid carcinoma tumors (Miskin and Soreq, 1981), which served as a ChE-deficient control, the apparent specific activity of the enzyme was as low as 60 nmol/min/g tissue (Table I). Polyadenylated RNA prepared from these frozen tissues induced the incorporation of [^{35}S]methionine into TCA-insoluble protein in reticulocyte lysates. Both the yield of mRNA and its translation efficiency were lower from brain tissues than from the carcinoma, but close to the values observed using fresh rat cerebellum tissues (Soreq *et al.*, 1982b; Eliyahu and Soreq, 1982). We therefore concluded that all the mRNA preparations were both intact and translatable. When injected into *Xenopus* oocytes, these mRNA preparations reproducibly induced the biosynthesis of active ChE with different efficiencies. The mRNA from embryonic brain was most active; 1 µg RNA induced synthesis of ChE which degraded 1400 pmol of acetylcholine/h.

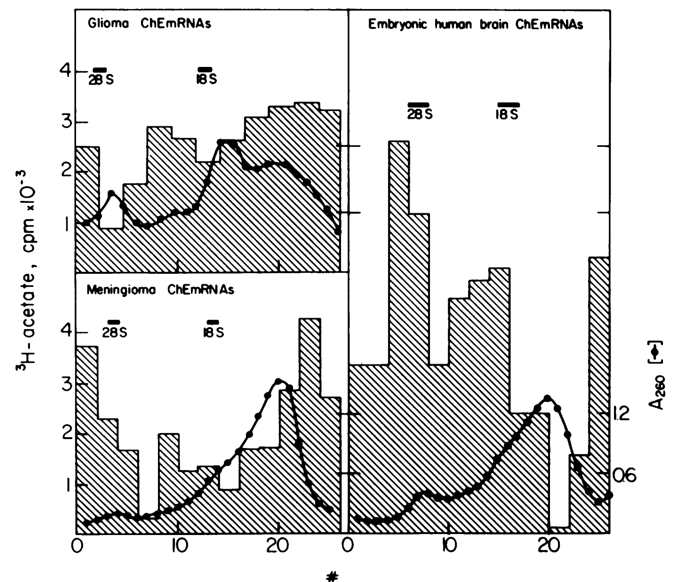

Fig. 1. Three size classes of mRNAs from human brain tissues induce the biosynthesis of cholinesterase activity in microinjected *Xenopus* oocytes. Poly(A)-containing RNA was prepared from embryonic human brain (17 weeks), glioblastoma multiforme and meningioma, and fractionated by sucrose gradient centrifugation. 200–400 µg of ethanol-precipitated polyadenylated RNA were suspended in 60 µl of DMSO (Eastman, Spectrograde) and incubated at 37°C for 20 min, to avoid aggregation of mRNAs. 10 µl of double distilled sterile H_2O was added and the samples were further incubated for 20 min at 37°C, brought to a final volume of 250 µl each with H_2O and layered on top of the gradient. Linear sucrose gradients (11.4 ml, 15–30%) were run in a SW41 rotor of a Beckmann centrifuge and contained 5 mM Tris-HCl, pH 7.4, 0.5 mM EDTA and 0.1% SDS. Centrifugation was for 20 h at 20°C, at 35 000 r.p.m. 0.4 ml fractions were collected, using a peristaltic pump, and A_{260} (—●-) determined in a Bausch and Lomb spectrophotometer in each fraction. A parallel gradient was loaded with poly(A)-free RNA, and the migration of 28S and 18S rRNAs is noted for each gradient. 30 µl of 5 N NaCl and 1 ml of ethanol were added to each fraction and RNA precipitated overnight at –20°C. One third of each fraction was then injected into 10 oocytes. Columns represent cumulative ChE activity, radiometrically determined in triplicate in oocyte homogenates and incubation medium. Spontaneous degradation of acetylcholine and activity in Barth-injected oocytes (5000, 7000 and 6000 c.p.m. for the three experiments, respectively) were subtracted.

Glioma and meningioma mRNAs had intermediate activities, degrading 300 and 500 pmol acetylcholine/h, respectively. The lowest activity was observed with mRNA from the ChE-deficient carcinoma, which induced the synthesis of ChE that degraded ~200 pmol of acetylcholine/h/µg mRNA (Table I).

Size fractionation of ChE-inducing mRNAs from human brain tissues

Polyadenylated RNA preparations from pooled samples of embryonic human brain, gliomas and meningiomas were denatured by DMSO and fractionated on linear 15–30% sucrose gradients. Each gradient fraction was monitored for its ability to induce ChE activity in microinjected oocytes (Figure 1). The total activity induced by size-fractionated embryonic brain mRNA was significantly higher than that induced by mRNAs from gliomas and meningiomas, as expected from injecting unfractionated mRNA (Table I). The major peak of ChE-inducing mRNA in embryonic brain sedimented at 32S, with two additional peaks of 20S and 9S (Figure 1, right). Similarly sedimenting size groups of ChE-inducing mRNAs, as verified by ethidium bromide staining of

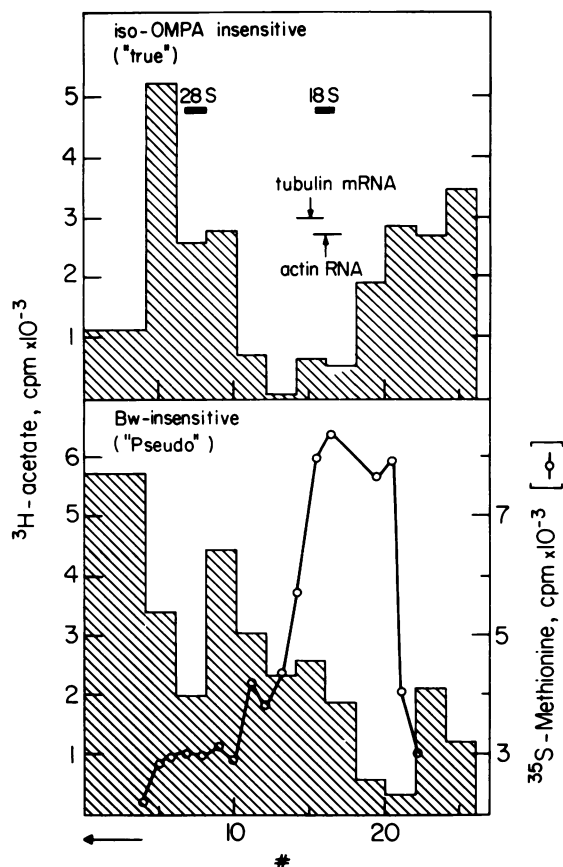

Fig. 2. Human brain mRNAs inducing the synthesis of iso-OMPA and BW284C51-insensitive ChE activities exhibit different sedimentation patterns. ChE activities (columns) were separately determined in the presence of iso-OMPA and of BW284C51 in the same oocyte homogenates and incubation medium referred to in the right panel of Figure 1. Parallel samples (1/10 of each fraction) were translated *in vitro* and the incorporation of [^{35}S]methionine into TCA-insoluble protein measured (—○—). Translation products were analysed by polyacrylamide SDS gel electrophoresis, followed by autoradiography, and migration of tubulin and actin mRNA noted. Spontaneous release of [^{3}H]acetate and activity of Barth medium injected oocytes (5000 and 6500 c.p.m./sample for iso-OMPA and BW284C51 insensitive ChEs, respectively) were subtracted.

gel-separated RNA samples, were also observed in samples from the two other sources. These observations suggest that multiple classes of ChE-inducing mRNAs exist in these human brain tissues, and that the regulation of ChE properties in the human brain is not limited to post-translational modifications. Three distinct size-classes of ChE-inducing mRNAs were also seen when sucrose gradient fractionated mRNA from embryonic and mature rat brain, or from *Drosophila* larvae were injected. In the latter, the size of the 9S mRNA was also verified by Northern blot hybridization to identified markers (not shown). The proportion of enzymatic activities attributable to the different size groups of ChE-inducing mRNAs was different in the mRNA preparations from the three human tissue sources examined.

Selective inhibition of oocyte-produced ChEs

ChE activities in oocyte homogenates and incubation medium were determined in the presence of the pseudo-ChE-specific inhibitor, iso-OMPA, or the AChE-specific inhibitor, BW284C51 (Austin and Berry, 1953). Two broad major peaks (of 23 – 30S and 8 – 15S) of mRNA-inducing iso-OMPA-insensitive ChE (AChE), and two major and different peaks (≥32S and 20 – 25S), and a minor peak (8 – 9S), of mRNA-inducing BW284C51-insensitive ChE (pseudo-ChE) were observed in embryonic brain extracts (Figure 2). The size distribution of total translatable mRNA, assayed in the reticulocyte lysate, differed from both patterns of ChE-inducing mRNAs (Figure 2). The proportion of oocyte-induced pseudo-ChE was significantly higher than that in the original brain tissue. In addition, the sums of iso-OMPA-insensitive and of BW284C51-insensitive mRNA-induced ChE activities were, in some fractions, different from the values obtained in the absence of inhibitors (compare with Figure 1, right panel). Both phenomena were reproducible, and appeared also in oocytes injected with sucrose gradient fractionated mRNA from gliomas (not shown). This could result from translation of pseudo-ChE-inducing mRNA with better efficiency, from interaction of the inhibitors with proteins other than ChE, which affect the two catalytic activities differently, or from production of ChE forms which react differently with these inhibitors (see Razon *et al.*, 1984).

Molecular forms of ChEs induced in the oocytes by the various mRNAs

Homogenates and incubation medium of oocytes which were injected with the peak fractions of embryonic brain mRNA that sediment as 32S, 20S and 9S were centrifuged on linear sucrose gradients, and ChE activities were examined in triplicate in each gradient fraction in the presence of iso-OMPA or of BW284C51. The limited amounts of resolved enzyme activities could only be separated into a small number of fractions. In spite of this limited resolution and low activity, it was clear that oocytes injected with different 'peaks' of ChE-inducing mRNAs displayed both 'true' and 'pseudo' ChE activities, and that each RNA fraction produced somewhat different patterns of multisubunit forms of the enzyme.

In control oocytes injected with Barth medium, there were no peaks of activity in the presence of either inhibitor. Iso-OMPA-insensitive ('true') activity, produced in oocytes injected with 32S mRNA, segregated on the sucrose gradient as three discrete peaks, one heavier than the co-centrifuged catalase (with sedimentation coefficient of 11.4S), and two lighter peaks, of ~8 – 9S and 4 – 5S. The BW284C51-insensitive activity in these oocytes was separated into two major peaks, a heavy (>11.4S) and a lighter (8 – 9S) one. The major peak of activity in oocytes injected with 20S mRNA was iso-OMPA-insensitive and appeared to migrate as 8 – 9S. A similar sedimentation pattern could be observed for ChE induced in oocytes injected with rat brain mRNA (not shown). Oocytes injected with 9S mRNA displayed relatively low ChE activities, with two distinct BW284C51-insensitive peaks, both sedimenting slower than 11.4S (Figure 3). Thus, several multisubunit molecular forms of ChE were detected in oocytes injected with any of the size fractions of brain mRNA, and the proportion of these different ChE forms appeared to depend on the sedimentation properties of the injected mRNA. This implies that different mRNA species contribute to the formation of the various molecular forms of ChE. Incomplete fractionation on the sucrose gradient, combination of subunits of the oocyte enzyme with ones directed by mRNA (see also Labarca and Paigen, 1977) and/or assembly of single polypeptides into different ChE molecular forms might explain the complex patterns of ChE isoforms that were observed in this experiment.

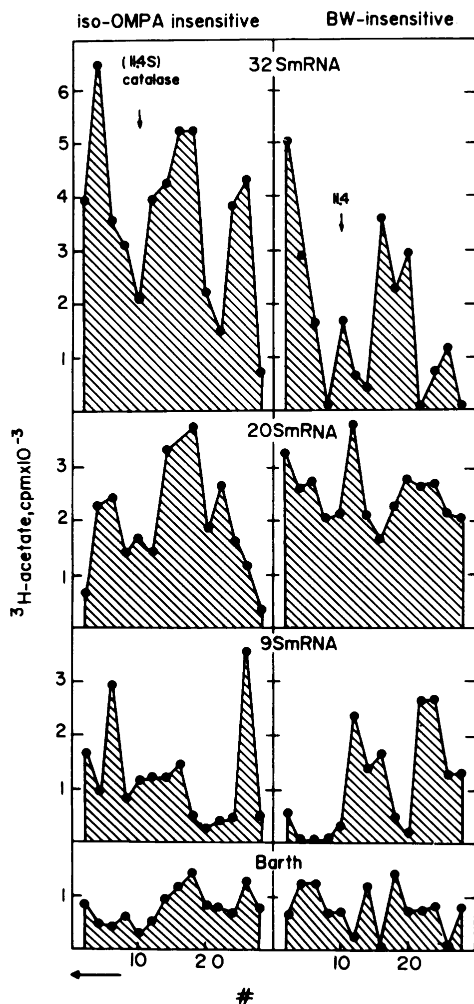

Fig. 3. Various size classes of human brain mRNAs induce the formation of differently sedimenting ChE activities. Homogenates and incubation medium of oocytes injected with the indicated size fractions of human brain mRNA, and with Barth medium, were fractionated on linear sucrose gradients. Gradients (5–20%) were prepared on a 0.5 ml cushion of 60% sucrose in 12 ml polyallomer tubes for an SW 40 Ti rotor. Sucrose solutions were made up in 1 M NaCl–0.1% Triton X-100–0.5 M Tris chloride, pH 7.5. Samples were applied in 200 μl aliquots and centrifuged for 18 h at 35 000 r.p.m. in a Beckmann L8-70 ultracentrifuge. ChE activities were separately determined in each gradient fraction in the presence of iso-OMPA (left) or of BW284C51 (right). Bovine catalase (11.4S) was co-centrifuged as a sedimentation marker.

Discussion

The substrate specificities and the molecular iso-form composition of ChE activities in both glioblastoma multiforme tumors and in mesenchymal meningiomas differ from brain ChE (Razon *et al.*, 1984). The different ChEs could have originated from a single ChE mRNA species, with the tissue-characteristic properties of the various ChEs determined at the post-translational level, by protein(s) translated from other, tissue-specific mRNA(s). This possibility would be in line with the proposed precursor-product relationships between the different ChE forms (Wilson and Walker, 1974; Rieger *et al.*, 1976; Koelle *et al.*, 1977). Alternatively, each of the different ChEs could be translated from a distinct, tissue-specific mRNA species. A third possibility could be a combination of the first two, namely, that multiple ChE mRNA species encode the formation of several polypeptides, each of which may be further modified by additional tissue or cell type-specific protein(s) to yield a higher variability in the final composition of brain ChEs. Our findings appear to favor this third situation.

The three different size classes of ChE-inducing mRNAs which we observed in microinjection experiments are unlikely to be aggregates, co-sedimenting with other RNA species, since: (i) the mRNA preparations were denatured by DMSO prior to sucrose gradient centrifugation; (ii) there appear to be similarly sedimenting ChE-inducing mRNAs in all of the three tissue sources employed, in spite of the very different translational activities and absorbance patterns of poly-adenylated RNAs; and (iii) the size distribution of AChE-inducing mRNAs is different from that of pseudo-ChE-inducing mRNAs. This indicates that there are multiple ChE mRNA species in the human brain, and strongly supports the notion that the regulation of the heterogeneity of ChEs is not limited to the post-translational level. We observed these three peaks of ChE-inducing mRNAs in all of the tissue sources examined, in spite of the differences between ChEs in gliomas, meningiomas and embryonic brain. This implies that the various ChE mRNAs can all be transcribed, although possibly to different extents, in various cell types of the human brain. Further experiments (such as injection of mixed mRNA fractions) will be required to resolve this issue.

In evaluating the results of these microinjection experiments, several points should be taken into consideration. (i) Injected ChE mRNAs compete with other injected mRNAs, and with oocyte mRNAs, for polysomes, since the amount of polysomes for mRNAs for secretory or membrane-bound proteins is rather limited in the oocytes (Richter *et al.*, 1983). Hence, the different size fractions of the sucrose gradient centrifuged mRNA might not compete for polysomes equally. (ii) It is possible that more than a single mRNA species is required to produce catalytically active ChEs. This appears to be the case for the biosynthesis of epidermal growth factor (Burmeister *et al.*, 1984). The additional mRNA(s), which may be either an oocyte mRNA(s) or brain mRNA species, could then become the limiting factor in ChE biosynthesis. (iii) It is possible that the oocyte-produced enzyme is not identical to the fully functional brain protein due to improper post-translational event(s) (for review, see Lane, 1983). In this case our calculations would be underestimates. We therefore refrain from using these experiments to quantify the absolute levels of the different size classes of ChE-inducing mRNA species.

An interesting finding is the activity of the relatively short, 9S RNA in inducing cholinesterase activity in the oocytes. The length of this RNA is only sufficient to produce a polypeptide of ~17 000 daltons. A small cholinesterase of this size has been reported in *Drosophila melanogaster* (Zingde and Krishman, 1980) but not in mammalian brain. Possibly this 9S mRNA-induced polypeptide is not a human brain cholinesterase, but only induces the formation of such activity in the oocytes. Although we cannot exclude this at present, the issue may be clarified once a single mRNA species, selected by hybridization to a ChE DNA probe, is injected into oocytes and its translation product(s) characterized. This issue is under investigation.

Differently sized mRNAs can be produced from a single gene, either by differential splicing or by alternative transcription, e.g., calcitonin and human growth hormone mRNAs (Rosenfeld *et al.*, 1981; Evans *et al.*, 1982) or *Aplysia* neuropeptides mRNAs (Scheller *et al.*, 1983). Alternatively, the various ChEs might be encoded by a multigene family (for

review, see Long and Dawid, 1980). It is not clear whether the ChEs produced from the different mRNAs differ in their amino acid sequences, are differently glycosylated and/or interact with other proteins (like collagen) in a specific manner. Molecular cloning and sequencing of genomic ChE DNA sequences, as well as further analysis of the oocyte-produced ChEs, will be required to answer these questions. Such experiments are in progress. Finally, it would be interesting to learn what the biological role(s) of the various mRNAs for the same enzyme might be. ChEs appear to be transiently expressed early in the development of a variety of embryonic tissues, always correlated with morphogenetic migrations of cells, where their expression pattern appears not to be related to that of ChEs in cholinergic tissues (Graybiel and Ragsdale, 1982; Layer, 1983). Studies on the appearance of the various ChE-inducing mRNAs during development and in various tissues and brain regions might yield further insight into the function of these mRNAs and their cholinesterase products, as related to pattern formation and/or cholinergic functions.

Materials and methods

Specimens from brain tissues were taken at the time of surgery, frozen in liquid nitrogen and stored at −70°C until use (Razon *et al.*, 1984; Libermann *et al.*, 1984). Polyadenylated RNA was prepared by phenol-cresol extraction from 2 to 10 g of pooled tissue specimens. The integrity of the various RNA preparations was examined by testing their ability to be translated *in vitro* in the reticulocyte lysate system (Soreq *et al.*, 1982b), and their capacity to induce ChE synthesis was measured using a modification of the oocyte microinjection bioassay (Soreq *et al.*, 1982a). Since *Xenopus* oocytes secrete proteolytic activities (Soreq and Miskin, 1981), and to improve the reproducibility of the ChE mRNA bioassay, we developed an appropriate mixture of protease inhibitors, which protects ChE activity in oocyte incubation medium and homogenates. In the presence of these inhibitors, the enzymatic release of acetate in the ChE assay was found to be linear in time and with the quantity of oocytes added, and reproducible from experiment to experiment. To test the effect of protease inhibitors, purified eel AChE was added to oocyte homogenates under these conditions. The added enzyme was fully expressed, and a linear increase in ChE activity was determined with increase in the amount of added AChE.

Injected oocytes were incubated in groups of 10 in microtiter plates (Falcon) in the presence of 2 μg/ml leupeptin, 4 μg/ml pepstatin, 2 mg/ml bacitracin, 0.04 U/ml aprotinin and 2 mg/ml EGTA (we designated this mixture lpbaE). This combination of inhibitors was found to protect secreted ChEs without interfering with the rate of protein synthesis in the oocytes. Following 24 h incubation at 20°C, injected oocytes were removed from the incubation medium. Ten μl of 10% Triton X-100 (Sigma) and 20 μl of 5 M NaCl were added to the incubation medium in the original incubation well, to solubilize the enzyme and release it from the plastic well. Each group of oocytes was homogenized in 100 μl of 0.01 M Tris-HCl, pH 7.0, 1.0 M NaCl, 1% Triton X-100, containing lpbaE. Oocyte homogenates were centrifuged for 2 min in an Eppendorf centrifuge, and transparent interphase was collected for determination of ChE activity. Medium and homogenate interphase were stored at −20°C until assayed.

For radiometric measurements of ChE activity and inhibition specificity, reaction mixtures (100 μl) contained 50 mM Tris-HCl, pH 7.0, 0.12 M NaCl, 1 mM *p*-chloromercurophenyl sulfonic acid, which preferentially inhibits the activity of oocyte ChE but not that of the brain enzyme (Soreq, 1984), 4 mM EDTA, 1 mM KCN, 1 mM [^{3}H]acetylcholine (Amersham, 1 x 10^5 c.p.m./100 μl mixture), oocyte homogenates or incubation medium samples and the appropriate inhibitor to differentiate 'true' and 'pseudo' ChE: 1,5-bis(4-allyldimethylammonium-phenyl) pentan-3-one dibromide (BW284C51, inhibits AChE); or tetraisopropyl pyrophosphamide (iso-OMPA, inhibits 'pseudo' ChE) at 10^{-4} M. The reaction mixture was incubated for 24 h at 21°C and stopped by addition of 100 μl stop mixture (1 M chloroacetic acid, 2 M NaCl, 0.5 M NaOH) (Johnson and Russell, 1975) and vigorous shaking. 4 ml of scintillation cocktail (xylene:xylofluor:isoamyl alcohol, 81:9:10) were then added and mixed, and released [^{3}H]acetate was determined in a liquid-scintillation counter. Enzyme assays were carried out in triplicate and the deviation did not exceed 7%. Assays were also routinely conducted without tissue extracts or in the presence of 10^{-4} M eserine, to evaluate reagent backgrounds. Oocyte extracts or incubation medium samples were treated separately, and the values obtained were pooled for each group of oocytes.

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