Further data on expression of acetylcholinesterase during oogenesis and embryonic development of Ciona intestinalis and Ascidia malaca (Asciidiacea, Urochordata): a biochemical and histochemical study

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Abstract — The distribution of acetylcholinesterase (AChE) activity has been studied in ovary, unfertilized egg, embryos and larva of Ciona intestinalis and Ascidia malaca (Asciidiacea, Urochordata) by biochemical and histochemical methods. The biochemical results have shown that the AChE activity is detected in ovary, and in the egg before fertilization; it rises from 8-cell stage up to gastrula, neurula, and above all at swimming larva. Cholinesterase activity has been histochemically localized and the results of this study agree with the biochemical data. The distribution of the enzyme activity in ovary and in different stages of development suggests also a non-classical role on maturation of female gametes, on cell adhesiveness and interaction, on differentiation, on secretion and apoptosis process.

Key words: acetylcholinesterase activity, Ciona intestinalis and Ascidia malaca, oogenesis and embryonic development.

INTRODUCTION

Acetylcholinesterase (AChE) is an enzyme whose role is to regulate nervous transmission by reducing the concentration of acetylcholine (Ach) in the synaptic cleft through hydrolysis. This is the well known role of the enzyme, so called the classical role in order to distinguish this function from many others, usually named non classical roles. The presence of acetylcholinesterase activity in some protozoa (Schuster and Herschanov 1969), sponges (Lents 1966), in many different no-nervous tissues and cells of vertebrates (Wessler et al. 2003; Kawashima and Fuji 2003; Mayarhofer et al. 2003), as well as in some tumours (Razon et al. 1984; Zakut et al. 1990) in AChE activity in brain, in cerebrospinal fluid and blood cells and in Alzheimer’s disease (Talesa 2001; Rees et al. 2003) have been showed to suggest no classical roles for this enzyme. AChE is involved in cell proliferation, cell migration and differentiation (Vidal 2005), during osteogenesis (Grisaru et al. 1999), in neurite outgrowth (Johnson and More 2004), apoptosis (Zhang et al. 2002) and hematopoiesis (Pick et al. 2004). AChE activity appears in the nuclei of presumptive somitic mesodermal cells prior to the onset of somitogenesis in the zebrafish (Hanneman 1992). Moreover the non-classical role of AChE has been suggested also in embryonic development of different species (Ozaki 1974; 1976; Drews 1973; Küster 2005; Minganti and Falugi 1980; Aluigi et al. 2005 and Whittaker et al. 1977). Up to now a substantial body of evidence suggest that this enzyme has multiple functions, most of which “non-classical”. Since embryonic development represents a system where different cellular events occur, the study of AChE enzyme could in turn explain its involvement in several diseases. The aim of this research is to reinvestigate the occurrence and distribution of AChE activity, by coupling the biochemical and histochemical methods, in ovary, unfertilized egg, embryos and in the larva of the Ciona intestinalis and Ascidia malaca ascidia to throw light into this intriguing issue re-

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garding the function of the enzyme before brain and muscle differentiation.

MATERIALS AND METHODS

Sample Collection and Embryos Development - 200 adult specimens of *Ciona intestinalis* or *Ascidia malaca* were collected near Agrigento (Sicily). Female and male gametes were removed from the gonoducts of 200 dissected animals. Before insemination, dry sperm of 60 individuals was diluted in Millipore Filtered Sea Water (MFSW, pore size of 0.45 µm) to a final concentration of approximately 0.1 v/v (pH 7.8; salinity 37‰; T=22°C). Ovary, unfertilized eggs, embryos at 8-cell stage, gastrula, neurula and swimming larva of *Ciona intestinalis* were washed in MFSW, collected by gentle centrifugation and stored at -80°C for biochemical analysis. For each experiment 1.5 ml of packed eggs or embryos at different stages or larvae were used. All experiments were repeated three times.

Assay of enzymatic activities and total protein quantification - Eggs and embryos of *Ciona intestinalis* were homogenized with a 0.06:1 ratio (w/v) of homogenization buffer (0.1 M Tris-HCl, pH 8) and centrifuged at 2100 rpm for 15 min. The resulting pellet containing cellular debris was discarded, while the supernatant fraction was retained, stored on ice and used for immediate subsequent biochemical determinations. All procedures were carried out at 4 °C. Acetylthiocholine iodide (ASCh), was used as substrate analogues to determine acetylcholinesterase activities. Each step for determination of optimum assay conditions was carried out on pool aliquots of about 1 g of eggs and embryos. A curve depending on concentration was performed by using ASCh as substrate from 0.092 mM to 5 mM. All analyses were performed on a JASCO V 530 spectrophotometer at nm. Acetylcholinesterase activities were initially expressed as ? absorbance units min⁻¹ converted to nmol hydrolysed substrate min⁻¹ and normalised by tissue total protein content. Aliquots for total protein determination were undertaken from initial homogenate. Protein concentration was determined according to the method of Bradford (1976) (nm) and values were expressed as mg protein × ml⁻¹ homogenate.

Statistical analysis - All determinations were performed in quadruplicate for each pool aliquot and results were expressed as mean values ± standard deviation. Statistical significance between the means was determined by using one-way analysis of variance (ANOVA) for parametric data. Differences with p<0.05 were considered as significant. Statistical analyses were carried out using Statgraphics 5.1 software (StatSoft, USA).

Histochemical study - Observations have been made on *Ciona intestinalis* and *Ascidia malaca* oocytes, female gametes, embryos and larva. *Ascidia malaca* eggs and embryos are less pigmented than those of *Ciona intestinalis* and the staining may be visualized more clearly.

Histochemical staining was performed by using the thiocholine method of Karnovsky and Roots (1964) with prefixation in cold 80% ethanol for three minutes. The reaction was carried out at 10° C for 40 min. Some examples were pre-incubated for 30 min in the specific inhibitor BW 284 C5 (1 5-Bis 4-allyldimethylammoniumphenyl pentan-3-one dibromide) 10⁻⁴ M solutions in sea water. This inhibitor has been proved to block enzyme activity (Meedel and Whittaker 1979; Minganti and Falugi 1988) in *Ciona intestinalis* embryos. For each set of experiments a control was performed by rearing about 200 gametes or embryos or larvae in sea water, for the whole duration of the experiments. Some unfertilised eggs have been dechorionated by steel needles.

Light Microscopy - After the enzyme reaction, samples of oocytes, unfertilized eggs, embryos and larvae were observed in toto under a Leiz Diaplan microscope and photographed without filters.

RESULTS

Biochemical analysis - The AChE activities in ovary, unfertilized egg, gastrula and neurula and larva increase trough the use of the ASCh substrate concentration from 0.092 mM to 0.277 mM and decrease at higher concentrations. Whereas AChE activity in larvae increases though the use of ASCh substrate concentration from 0.092 mM to 0.461 mM, it decreases at higher concentrations (Table 1).

The AChE activity is present in ovary, unfertilized egg, gastrula, neurula and larva stages (Table 2). The ChE vs ASCh activity increases significantly from ovary neurula embryos and, heavily, from neurula to larva (p<0.05).
Histochemical analysis - Ovary - The ovary of ascidians is composed by oogonia, young basophilic oocytes, that is oocytes where the first vitelline yolk granules are present, and vitellogenetic oocytes. The AChE staining is present in oocytes at the beginning of yolk deposition and in the vitellogenetic ones. The positive reaction is especially localized on the membrane and in the germinal vesicle (Figures 1-2). The inhibitor completely or almost completely suppresses the staining (Fig. 3).

Unfertilized Egg - In unfertilized egg with envelopes and also in dechorionated egg, the staining is localized on the whole surface (Figures 4-5). After treatment with the inhibitor, there is no staining (Fig. 6).

4,8-Cell Stage - The staining is present on the blastomeres membranes (Fig. 7) of the embryos deprived of their envelopes and it is not present in the embryos that have been pretreated with the inhibitor.

Gastrula - Some cells of gastrula are more stained, especially the presumptive neural cells and notochord cells appear to be much stained (Fig. 8).

Neurula And Tail-Bud Stages - In these stages the muscle cells appear to be heavily stained (Figures 9-10)

Larva - In the swimming larva after 1 hr of incubation in Karnovsky and Roots reagent, the staining is particularly strong in the muscle cells of tail (Fig. 11) and in the two clusters of cells near the base of the tail, that are situated bilaterally. In the metamorphosing half-tail larvae the reaction is localized in the muscle cells of their reduced tails and in some of them whose tails have already been absorbed in the cephalenteron near the tail; a positive reaction is also in adhesive papillae and in the cells around them (Fig. 11a). After the inhibitor treatment the staining is not evident (Fig. 12).

DISCUSSION

The results of the effects of substrate concentration from 0.092 to 5 mM on acetylcholinesterase activity in unfertilized eggs of *Ciona intestinalis* show that the highest rates of hydrolysis were found at 0.277 mM substrate concentration in ovary, unfertilized egg, gastrula and neurula, whereas at 0.461 in larva. The following hydrolysis decrease suggests an inhibition of enzyme activity by higher concentration of substrate; this confirms the substrate specificity in invertebrate cholinesterase as showed by Moralev and Rozengart (2000). These data have permitted to found the optimale substrate concentration for subsequently analyses to determine the AChE activity in significative number of ovaries, embryos and larvae (n=200). The above reported biochemical results indicate that AChE activity in *C. intestinalis* occurs very early in embryonic development and it is already present in ovary and unfertilized egg. Its activity begins to increase at gastrula stage, it rises at late neurula stage and above all at swimming larva. The occurrence of AChE activity during embryonic development in the stages before the appearance of muscle and cer-
The main function of AChE at the neuromuscular junction is well known; however, the role of enzyme in cells and tissues that are not innerved by cholinergic nerves is unclear. There is increasing evidence that AChE has functions unrelated to neurotransmission (cfr. Small et al. 1996): it may have a function in the regulation of neurite outgrowth (Layer et al. 1988 a;b), and as cell-cell or cell-substrate adhesion molecules (Krejci et al. 1991). Moreover acetylcholine has been shown proved to influence cell proliferation and migration (Layer and Sporns 1987; Mizoguti and Miki 1985). A strong AChE activity has been found in cells undergoing morphogenetic movements, such as primary mesenchyme cells in gastrulating sea urchin embryo (Ozaky 1974; Drews 1975). In osteoblastic stem cells AChE activity has been hypothesized to be involved in the interaction between the matrix and differentiating stem cells (Inkson et al. 2004). In the chick embryonic development a strong AChE activity has been found from gastrula stage in the notochord cells and in neural floor plate (Aluigi et al. 2005). To better focalise the histochemical localization of ascidian AChE during embryonal development, both C. intestinalis and Ascidia malaca were analysed because the ovary, egg, embryos and larvae are less pigmentated in the latter. The AChE presence in female gamete before fertilization and in embryos where cholinergic sites are not present, suggests some hypothesis about its role: the localization in oocytes might be in relation to maturation events of female gametes as Guraya (1995) have hypothesized for the Graafian follicle during ovulation in the rat. It has been suggested that cholinestarese may play a role for cell-cell adhesion. We detected AChE activity biochemically in unfertilized egg and in the first stages of the development of Ciona intestinalis, and histochemically on the cell membranes of the blastomeres of the two species of ascidians. These results could indi-
cate the involvement of enzyme in cell-cell adhesiveness like that of egg-sperm and of blastomeres. Other authors have detected AChE on cell membranes of cultured human cells (Falugi et al. 1983a;b) and blastomeres of early embryos of Ciona intestinalis (Minganti and Falugi 1980), of sea urchin (Ozaki 1974; 1976) and of a teleost (Fluck 1982). In gastrula stage the AChE activity is quantitatively more than activity in unfertilized egg and some territories are more interested in its expression. Gastrula is a central stage of development when cell migration, changes in cellular adhesiveness, cellular interactions and recognition lead to the basic organism pattern. It is generally accepted that specific cell surface molecules primarily mediate recognition and adhesion events: AChE could play an important role at this stage. A new peak of AChE amounts has been detected at late neurula stage as already shown by Durante (1956) and Whittaker (1973). This activity occurs not only on the cell membrane, but also into the cytoplasm. At this stage muscle cells begin their differentiation and the movement to the larval tail. The highest level of AChE activity has been detected at swimming larva in the muscle cells of tail, and during metamorphosis in the adhesive papillae and in clusters of cells in the posterior cephalenteron. The new role of the enzyme may be related to neuromuscular transmission as the larva actively moves for two or three days. It has been shown that in the larva, all the muscle cells in a band are coupled electrically by gap junctions (Mackie and Bon 1976) and neuromuscular transmission is cholinergic (Ohmori and Sasaki 1977). After a period of swimming the larva finds the idoneous substrate to fix by means of adhesive papillae where AChE activity is detected as other Authors have shown (Minganti and Falugi 1980; Coniglio et al. 1998). Sticky substances for adhesion of the larva to the substrate and nervous structure were observed and described in the adhesive papillae of Phallusia...
mammillata (Gianguzza et al. 1996; DeBernardi et al. 1996). In our experiments some cells, near adhesive papillae, are positive to AChE staining mixture: they could be the cells secreting the adhesive substances. Other cells in the cephalonteron are AChE positive: they are mesenchymal cells located in the posterior region of the larval head which migrate and will give rise to many muscle structures of the adult (Clooney 1990). Finally, in larva the AChE expression may be induced during apoptosis. The cellular death is an integrant part of embryonic development, with organized and regulated biochemical events, in response to a variety of stimuli, when various structures no longer needed, must be removed. Apoptosis has been revealed in larva of ascidians by Chambon et al. (2002) and by Jeffery (2002). Zhang et al. (2002) have found a relation between expression of AChE and programmed cell death. In this respect AChE could play a role in remodelling and cellular homeostasis occurring at the end of development. The presence of enzyme activity not only on the membranes, but also in the cytoplasm of muscle cells could be in relation to apoptotic events of these cells.

From this research it is possible to hypothesize that the embryonic development can be an interesting example of modulation of the AChE functions on maturation of female gametes, on cell adhesiveness, proliferation, migration, differentiation, secretion and finally on neurotransmission and apoptosis events in late development.

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