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Histochemical localisation of acetylcholinesterase activity in ovary and embryos of *Ciona intestinalis* and *Ascidia malaca* (Ascidiacea, Urochordata) after exposition to trybutyltin-chloride

Cangialosi Maria Vittoria, Egidio Puccia*, Valentina Mansueto and Paolo D'Agati

Dipartimento di Biologia Animale "G.Reverberi", Università di Palermo, Via Archirafi, 18-90123 Palermo, Italy.

Abstract — The histological analyses have shown the major AChE activity in unfertilized egg and embryos after TBT-Cl treatment whereas no differences were recorded between the TBT-Cl treated ovary and the control group in agreement with biochemical results showed by Puccia *et al.*, (sent to publication). Moreover the localization of AChE activity in embryos at 4-8 blastomeres changes after TBT-Cl exposition: in treated groups it is on membrane and in cytoplasm, whereas in control groups is detected only on membrane. This different localization could be due to the block of cell proliferation by TBT that could promote the cell differentiation leading to the physiological death of the cell through amplification of acetylcholinesterase genes and subsequent AChE overexpression.

Key words: acetylcholinesterase activity, *Ciona intestinalis* and *Ascidia malaca*, ovary and embryonic development, tributyltin-chloride (TBT-Cl).

INTRODUCTION

The role of acetylcholinesterase (AChE) in synaptic transmission is known, whereas it is not clear during embryonic development in invertebrate and vertebrates (Layer *et al.* 1988; Layer 1990). Since the AChE appears long before synapses are functional, this enzyme is associated to non-classic functions that can influence the proliferation, differentiation, somitogenesis and neuronal outgrowth (Layer *et al.* 1988; Brimijoin S. and Koenigsberger C. 1999; Bertrand *et al.* 2001; Hanneman 2002). Also the high levels of AChE observed in non-neuronal tissues such as blood cells, notably erythrocytes and megakaryocytes (MKs) (Patinkin *et al.* 1994; Lapidot-Lifson *et al.* 1992; Layer 1990), in the development of avian cartilage (Layer 1990) and oocytes and sperm (Malinger *et al.* 1989; Sastry *et al.* 1981) let suppose the non-cholinergic AChE function (Bertrand *et al.* 2001). The non-classical role of AChE in ascidian embryonic development is not clear. In ascidians the presence of AChE has been detected histochemically at neurula stage and in the muscle cells of swimming larva, (Durante 1959; Whittaker *et al.* 1979; Minganti and Falugi 1980). Recently qualitative and quantitative distribution of enzyme during oogenesis and in developing embryos has been studied by Mansueto *et al.* (sent to publication). The authors have supposed a non classical role on oogenesis, on cell adhesiveness and interaction, on secretion and on cell migration and differentiation. Moreover Puccia *et al.* (sent to publication) utilizing the TBT pesticide have shown an overexpression of AChE activity in unfertilized egg and in the stages of 4-8 cell, gastrula and neurula, due to TBT and probably correlated to the function of this enzyme in apoptosis. Indeed JIN *et al.* (2004) had already shown that the increase of AChE activity inhibits cell proliferation and then promotes apoptosis in NRK cells. The AChE overexpression is correlated to stress as shown by Mor *et al.* (2001) in testicles mice, and to drugs or poisons exposition as shown by Farchi *et al.* (2003). There are a few researches about the effect of TBT on AChE activity (Oliveira Ribeiro *et al.* 2002; Rabitto *et al.* 2005); moreover no study has been carried out so far on AChE localization in marine organisms exposed to TBT. The aim of this work was to determine the distribution of AChE overexpression in

* Corresponding author: phone 00-39-0916230110; fax 00-39-0916230114; e-mail epuccia@unipa.it

oocytes, female gamete and embryos after TBT-Cl treatment through histochemical analysis.

MATERIALS AND METHODS

Sample collection and embryos development - 60 adult specimens of *Ciona intestinalis* and *Ascidia malaca* were collected near Agrigento (Sicily). Female and male gametes were removed from the gonoducts. Before insemination, dry sperm of 60 individuals was diluted in Millipore Filtered Sea Water (MFSW, fore 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 4-8 cell stage and late gastrula of *Ciona intestinalis* and *Ascidia malaca* were washed in MFSW.

Histochemical study - A lot of unfertilised eggs have been dechorionated by steel needles. Oocytes, female gamete and embryos were incubated in TBT-Cl solution for 3 hours. After incubation the oocytes, gametes and embryos were washed in MFSW and fixed in alcool 80% for three minutes. Afterwards they were incubated in the mixture of Karnovsky and Roots (1964) for 40 minutes and observed in toto under Leitz Diaplan microscope and photographed without filters. Others oocytes, female gametes and embryos were stained with Karnovsky and Roots mixture and used as controls. Some samples were preincubated for 30 min in the specific inhibitor BW 284c5 (1 5-Bis 4-allyldimethylammoniumphenyl pentan-3-one dibromide) 10^{-4} M solutions in sea water.

RESULTS

The results do not show differences of AChE activity between the two species studied:

Ovary - The staining in the TBT- Cl treated oocytes is like that of controls (Figures 1-2-3-4).

Unfertilized egg - (Figures 5-7). In TBT-Cl exposed egg the cytoplasm is divided in to clear and pigmented parts; the follicle cells of eggs have been detached from the chorion. The staining is

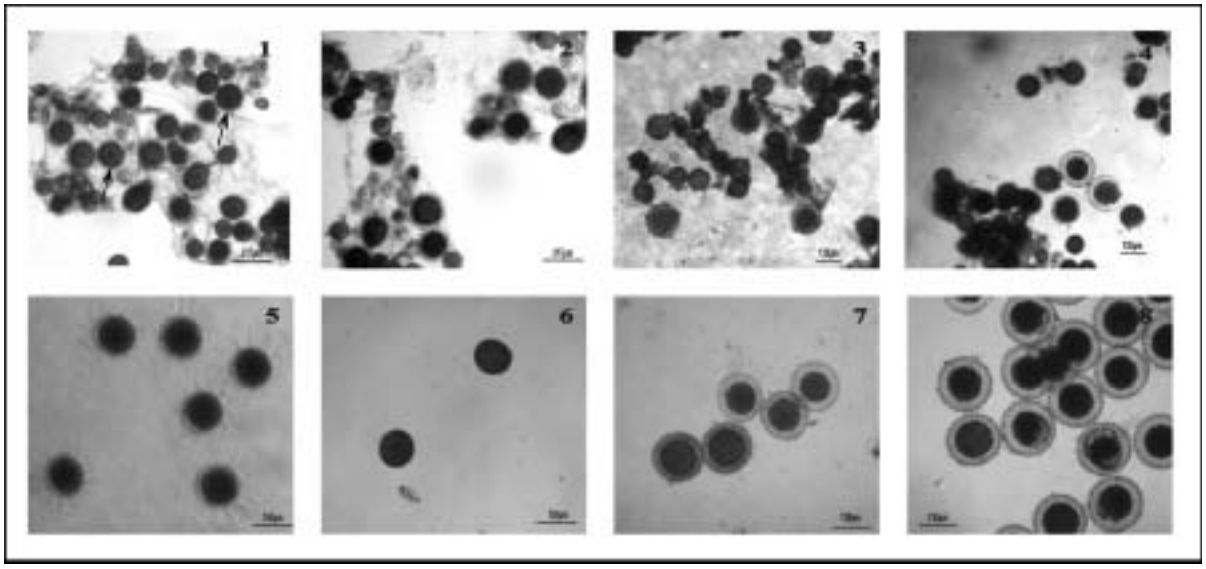

Fig. 1 — *Ascidia malaca* ovary control - The histochemical AChE reaction is shown by dark staining in the germinal vescicle on plasma membrane of vitellogenetic oocytes.
Fig. 2 — *Ascidia malaca* TBT-Cl treated ovary - There is no difference with the controls.
Fig. 3 — *Ciona intestinalis* ovary control - The histochemical AChE reaction is shown by dark staining in the germinal vescicle and on plasma membrane of vitellogenetic oocytes.
Fig. 4 — *Ciona intestinalis* ovary after treatment with TBT-Cl - There is no difference with the controls.
Fig. 5 — *Ciona intestinalis* unfertilized eggs control - The staining is on whole the egg.
Fig. 6 — *Ciona intestinalis* unfertilized eggs after TBT-Cl treatment - The reaction is more evident than controls, the follicular and the test cells are detached from the egg.
Fig. 7 — *Ascidia malaca* eggs control – There is the staining on all the egg.
Fig. 8 — *Ascidia malaca* unfertilized eggs after TBT-Cl treatment - The staining is heavier in the pigmented area of eggs.

stronger than controls (Figures 6-8) and it is localized in the pigmented portion of the egg (Figures 8).

4-8-16-Cell stage - In TBT-Cl the embryos interrupts the division; on the contrary the controls develop up to 64-cell stage. The staining after Karnovsky and Roots treatment is more evident on the membranes of blastomeres (Figures 9-11). In the embryos treated with TBT-Cl, the AChE is also localized into the cytoplasm (Figures 10-12).

Late gastrula stage - After three hours of incubation in the TBT solution the gastrulae are anomalous with a chaotic distribution of blastomeres. The staining of the normal gastrulae is more evident in some cells (Figures 13-15); in the anomalus embryos it is heavily present in all the blastomeres (Figures 14-16).

DISCUSSION

Previous biochemical results of MANSUETO *et al*. (sent to publication) had shown that the AChE activity was present in all the stages of the embryonic development of *Ciona intestinalis*, also during oogenesis. The higher amount had been found at gastrula, neurula and above all at larva stage. Our histochemical study performed on embryonic development of *Ciona intestinalis* and *Ascidia malaca*, has shown the localization of the enzyme in the nuclei and around the cell membrane of oocytes, on membranes of 4-8 cell stage, on some territories of gastrula, in muscle cells of neurula and in the muscle cells of larva tail. The staining is present also in the trunk ventral cells and in the adhesive papillae of larva (MANSUETO *et al.* sent to publication). The results show a darker staining after TBT-Cl treatment than all controls, in all studied stages, such as unfertilised egg, 4-8-16 cell, late gastrula stages, except for ovary. All these results are in agreement with the overexpression that has been biochemically detected in all these stages previously recorded by PUCCIA *et al.* (sent to publication) who have suggested that in ascidian cytoplasmic determinans are somehow anchored to the cytoscheletal network in accord with MEEDEL AND WHITTAKER (1984), ZUMBE *et al.* (1982) and SAWADA *et al.* (2005). The TBT-Cl

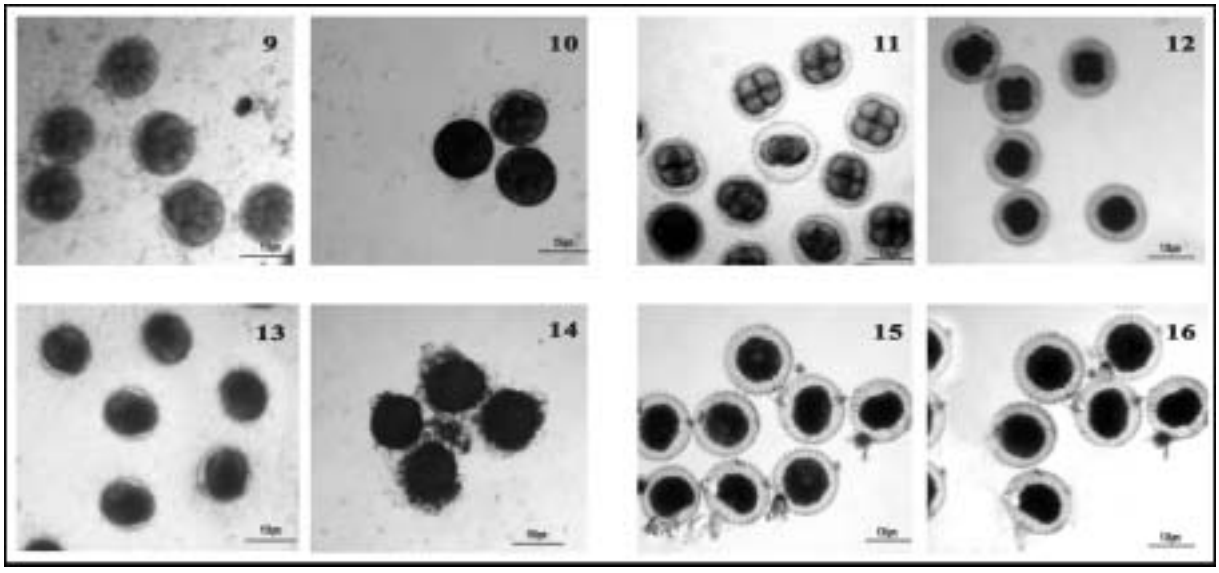

Fig. 9 — *Ciona intestinalis* 8-16 cell stage control - The staining is on the membranes of the blastomeres.
Fig. 10 — *Ciona intestinalis* 8-16 cell stage after TBT-Cl treatment - The staining is around the blastomeres and also in the cytoplasm.
Fig. 11 — *Ascidia malaca* 4-8-cell stage control - The staining is on the membrane of the blastomeres.
Fig. 12 — *Ascidia malaca* 4-8-cell stage after TBT-Cl treatment - The staining is stronger on the membranes and in the cytoplasm.
Fig. 13 — *Ciona intestinalis* late gastrula control - The staining is darker in some cells.
Fig. 14 — *Ciona intestinalis* late gastrula after TBT-Cl treatment - The dark staining is heavily detected in all the blastomeres.
Fig. 15 — *Ascidia malaca* late gastrulae control – The staining is more evident in some cells.
Fig. 16 — *Ascidia malaca* late gastrulae after TBT-Cl treatment - The staining is on all the embryos and stronger than in controls.

treatment could destroyed cytoscheleton and the regulatory molecules could initiate the switch on of specific genes activity very early. The histochemical data have permitted not only the localization in the different developmental stages, but also in the cellular compartment. The unfertilized eggs of two species after TBT-Cl treatment have shown AChE staining on whole the egg; in particular in the *Ascidia malaca* egg, the treatment with TBT-Cl divides the cytoplasm in clear and pigment parts; AChE staining is found only in the pigmented one. Unfortunately we do not know the composition of pigmented zone of unfertilized egg in order to detect the exact localization of enzyme activity, but it looks as if the localization was related to the cytoplasmic organules. The embryos of 4-8-16 cell stage in the TBT-Cl solution stop cleaving and the blastomeres have an irregular spatial position. In the controls the staining is localized only on the membranes of blastomeres.

Other authors have found AChE activity on membranes of cultured human cell (Falugi *et al.* 1983), in blastomeres of early embryos of *Ciona intestinalis* (Minganti and Falugi 1980), of sea urchin (Ozaki 1974; 1976) and of a teleost (Fluck 1982). In TBT-Cl treated late gastrula the staining is darker than the controls and localized in all the cells, while in controls it is localized in the cytoplasm of muscle cells (Durante 1956; Whittaker *et al.* 1973; Mansueto *et al.* sent to publication). The gastrula is the stage when the cells through process as migration, cell recognition and communication give rise to normal pattern of development. In these gastrula controls, some territories are more stained than others. In TBT-Cl treated gastrulae these processes are anomalous and they are blocked by the pollutants: the staining is darker than controls and localized in whole the cell. According to Whittaker *et al.* (1973; 1977), the enzyme is considered a cytoplasm marker for muscle differentiation. The link between AChE and differentiation is also demonstrated by other authors. Fitzpatrick-Mcelligott And Stent (1981) have detected histochemical localization of AChE in the polar plasms and in the D blastomere of the leech *Helobdella triserialis*. At later stages AChE is present in the meso- and ectoteloblasts and their daughter stem cells that form the germinal bands. Recently Mansueto *et al.* (sent to publication) have detected AChE in the trunk ventral cells of *Ascidia malaca* and *Ciona intestinalis* swimming larva, which give rise to structures of the adults (Cloney 1990). From these results it appears that AChE activity is present in cells that undergo to a specific differentiation through a migration and cell-cell communication. The overexpression that we have found in cytoplasm of unfertilized egg, 8-16 cells and gastrula stages, could be an event like that of other biological systems: overexpression of cholinesterases has been correlated with tumoriogenesis and abnormal megakaryocytopoiesis, in the brain of the Alzheimer, in systemic lupus erythemathosis and in autoimmune diseases (Small *et al.* 1996). We can suggest that TBT-Cl could act on AChE activity as a factor of stress which impedes cell proliferation as Mansueto *et al.* (1989) have shown in the first stages of embryonic development of *Ciona intestinalis*. Afterward it could switches on a beginning of differentiated state of cell leading to cell physiological death through the amplification of acetylcholinesterase genes and subsequent overexpression of the enzyme.

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