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# Acetylcholinesterase activity is affected by stress conditions in *Paracentrotus lividus* coelomocytes

**Abstract** The presence and function of acetylcholinesterase (AChE) in blood cells and plasma is well known. The enzyme activity is associated to red blood cell membranes, as well as to the perinuclear region of leucocytes, suggesting a role in the function of the latter. Recently coelomocytes, acknowledged progenitors of immune cells in vertebrate systems, have been shown to respond to stress conditions, both in laboratory experiments and field studies. Bearing in mind the similarity between coelomocytes and vertebrate blood cells, we investigated the presence of AChE activity in coelomocytes of the sea urchin *Paracentrotus lividus* characterized by specific inhibitors, in relation to cold stress induced in the laboratory. Exposure to stress was followed by electrophoretic analysis of AChE activity and Western blot analysis of AChE-like immunoreactivity. Moreover, quantitative analysis of the different molecular forms of AChE was carried out with the aid of a spectrophotometric method. The effects of stress on AChE activity were time dependent. In fact, the enzyme activity was detected by the catalytic activity in cleaving the substrate acetyl thiocholine iodide, which appears to be formed by different isoforms of the enzyme, compared to that of *Electrophorus*, which was used as a control. Western blot analyses showed the presence of different immunoreactive molecules, with molecular mass ranging from 265 kDa (corresponding to *Electrophorus* molecules) to about 180 kDa. According to quantitative analysis, the isoforms that were differentiated corresponded to an AChE with the capacity to cleave acetyl-$\beta$-methyl thiocholine iodide (the so-called true AChE, E.C. 3.1.1.7, corresponding to the 265 kDa band) and to a ChE with the capacity to cleave propionyl thiocholine iodide as well. The latter is an ancestral form of AChE, mainly present in invertebrates. Each of these forms was affected differently by varying time exposures.

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## Introduction

The presence and function of cholinesterase in blood cells and plasma is well known (Adams and Whittaker 1949; Heller and Hanahan 1974; Falugi 1985). For many years, several authors have suggested the possibility that the enzyme may play a specific role in blood cell mechanisms, such as intracellular ion change (Finin et al. 1979) and/or in the regulation of cell viability (Halbhuber et al. 1982). Authors have also hypothesized the capability of blood cells to exchange messages and to interact with immune cells to recruit macrophages for the elimination of aged or defective blood cells (Halbhuber et al. 1982). Falugi (1985) localized acetylcholinesterase (AChE) activity to red blood cell membranes, as well as to the perinuclear region of all leucocytes, suggesting a role in their function. Abnormal levels of the AChE enzyme or aberrations involving the long arm of chromosome 7, harboring the AChE gene at 7q22, occur in various diseases such as Alzheimer's, Parkinson's and leukemia (Shapira et al. 2000). Recently, a cholinergic differentiation factor, promoting the expression of cholinergic molecules in the central nervous system and specialized tissues, was also found as a molecule functional in blood differentiation; it is also called the leukemia inhibiting factor (Qiu et al. 1997).

The AChE activity present in the blood of vertebrates, as well as in humans, is affected both quantitatively and qualitatively by several factors, such as alcoholism (Chumakova and Liopo 1996), drug consumption (McGehee et al. 1995) and by interaction with the environment. In particular, neurotoxic pesticides, used as a pest control at agricultural sites, cause paralysis and death of insects and, in the case of acute intoxication in humans, a dramatic and long-lasting decrease of AChE activity in the blood (case reports, such as Romero et al. 1989). The same effect was discovered in fish blood (Balint et al. 1997). On the other hand, other pollutants, such as heavy metals (Dethloff et al. 1999) and polycyclic aromatic hydrocarbons (Den Besten et al. 2001), are suspected to cause changes in AChE activity at the level of single organs, and mainly in the blood. For this reason, the level of AChE activity in blood may be used as a biomarker for the presence of strong environmental pollution. The presence and environmental vulnerability of blood AChE are related to lymphocyte functions (Kawashima and Fuji 2000). Echinoderm immune cells, similar to vertebrate leucocytes, are represented by the so-called coelomocytes, a population of mixed cells contained in the coelomic cavity of sea urchins. These are capable of responding to injuries, host invasion and cytotoxic agents (for a review see Smith 1981; Smith 2001). Furthermore coelomocytes synthesize and release upon stimulation a variety of different molecules, such as cytokines, lectins, perforins, lysosomal enzymes, serum proteins, profilins and others, all of which are related to immune functions (Matranga and Bonaventura 2002). For all these reasons coelomocytes are considered the immune effectors of the sea urchin (Smith and Davidson 1992; Pancer et al. 1999), and can be regarded as ancestral analogues of immune cells found in the circulatory system of vertebrates. Recently coelomocytes have been shown to respond to stress conditions (temperature shock and pollution), in laboratory experiments and in field studies (Matranga et al. 2000). In addition, sea urchin coelomocytes (Matranga et al. 2002) and bivalve hemocytes (Canesi et al. 2002) have been used to monitor the effects of heavy metals on the expression of stress markers and for the assessment of functional responses, respectively.

AChE is affected by several environmental stress inductors, first of all by neurotoxic pesticides, which inhibit the catalytic activity of the enzyme. However, other altered situations may cause changes in the normal enzyme activity as well, e.g., inflammation, wound healing and general stress (Chuiko et al. 1997) may induce changes in the level of AChE activity.

Based on this similarity between coelomocytes and vertebrate blood cells, we investigated, in coelomocytes of the sea urchin *Paracentrotus lividus*, the effects of cold stress on AChE activity, characterized by specific inhibitors. Sea urchins were exposed to cold stress, and quantitative and electrophoretic changes in enzyme activity in response to the induced stress were evaluated, with the aim of assessing the possibility of using the AChE activity present in these cells as a biomarker for both field and laboratory studies on environmental stress.

## Materials and methods

Unless otherwise specified, all reagents used were obtained from Sigma (Italy).

### Exposure to stress

Adult specimens of *Paracentrotus lividus* were maintained in aquaria at a temperature of 17°C. The exposures were performed by transferring them, for different time periods (30 min, 1 h, 2 h, 4 h), to aquaria held at a temperature of 4°C. In all cases a 1-h recovery period at a temperature of 17°C was allowed for the specimens.

### Cytochemical revelation of cholinesterases

Cytochemical revelation of the different cholinesterase activities was performed to verify their presence and to choose the most well represented in the investigated cells.

Coelomocytes were obtained from adult specimens, stored in a physiological solution containing 1:1,000 EDTA/EGTA, to prevent clot formation, and fixed with cold 2% paraformaldehyde in physiological solution for 10 min. A drop of the solution containing the whole composition of coelomocyte types was placed on each polylysine-coated slide, to which the cells were permitted to adhere. The slides were then thoroughly rinsed with 0.1 M maleate buffer, pH 6, and put into the reaction mixture suggested by Karnovsky and Roots (1964), containing acetylthiocholine iodide (AcTChI), butyrylthiocholine iodide (BuTChI) and propionyl thiocholine iodide (PrTChI) as substrates. The development of the cytochemical reaction, indicated by a dark-brown precipitate was checked with a light microscope (Leica DMRB); the reaction lasted 2–4 h. Specificity controls were performed by continuing the reaction without the substrates, and/or by exposing the samples to specific inhibitors (eserin, BW284c51 and iso-OMPA).

### Electrophoretic analysis of AChE activity

Pellets from coelomocytes of unexposed and exposed sea urchins were collected at regular time intervals and homogenized in 0.5% Triton X-100 in phosphate-buffered saline, pH 7.4. Cholinesterase isozymes were separated on 8% polyacrylamide gels (Biorad mini protean apparatus). 0.5% Triton X-100 extracts were centrifuged at 13,000 rpm per 15 min in a Sigma microfuge, and 30 µl of the supernatant (containing at least 1 µg proteins µl$^{-1}$) was layered on the top of the running gels. As a control, 50 µg AChE µl$^{-1}$ from *Electrophorus electricus* (type VI) in 40% sucrose was employed. Electrophoresis was carried out at 150 V for 2 h. After electrophoresis, the gels were fixed and incubated in the reaction mixture suggested by Karnovsky and Roots (1964) for 24 h at $T = 4$–6°C. The rationale for this staining is that the AChE present in the homogenate, that is, the lythic enzyme for acetylcholine, cuts the AcTChI into an acetate and a thiocholine group. The latter causes precipitation of the copper and iron salts present in the incubation mixture, which permits identification of the bands resulting from electrophoretic migration of the enzyme by their hazel-brown color.

Controls for the specificity of the histochemical reaction were performed by pre-treatment with specific inhibitors, such as anti-cholinesterase (BW 284c51, Burroughs Wellcome, UK), as previously described (Minganti and Falugi 1980). A positive control was

supplied by using a commercial AChE, obtained either from *E. electricus* electro-plaques, or from bovine erythrocytes.

### Western blot analysis of AChE-like immunoreactivity

Cytoplasm and membrane lysates of *P. lividus* coelomocytes (control and exposed sea urchins) were electrophoresed on a 12% SDS-PAGE, and transferred to nitrocellulose according to standard procedures (Biorad mini trans blot apparatus). The filter was incubated overnight at room temperature in anti-AChE monoclonal antiserum (BDA, Italy), diluted 1:1,000 in blocking solution (TBS-T 5% milk); rinsed in TBS-T; and incubated at room temperature for 90 min in anti-mouse secondary antibody, conjugated with alkaline phosphatase and diluted 1:1,000 in blocking solution. The revelation of the filter was performed by use of the NBT/BCIP system.

The molecular masses of the immuno-stained bands were evaluated by means of reference standards for molecular mass (BIOLABS, Italy), simultaneously analyzed by SDS-PAGE.

Negative controls were obtained by omitting the incubation in non-immune serums (not shown).

### Quantitative analysis of AChE

Crude homogenates and Triton X-100 extracts were employed for the biochemical analysis of AChE activity with Ellman's colorimetric method (Ellman et al. 1961).

Unfixed samples were homogenized in phosphate buffer, pH 8; 10 µl of Triton X-100 (0.5% in distilled water) extracts was incubated in the presence of 400 µl of the substrates acetyl-$\beta$-methyl thiocholine iodide (AcMTChI), PrTChI, or BTChI, respectively, in 390 $\mu$l of phosphate buffer, pH 8, and stained by 200 $\mu$l of dithiobis-nitrobenzoic acid. The reaction was carried out for 10 min at room temperature ($25 \pm 1°C$).

The different substrates were employed in order to understand which molecular form of cholinesterase (ChE) is mainly affected by the stress.

Absorption was measured by a UNIKON 930 spectrophotometer at $\lambda = 412$ nm and compared to a blank obtained by omitting the substrate. The enzyme activity in tests was expressed in ChE units ($= 1$ µmol of acetylcholine hydrolyzed min$^{-1}$) under the above conditions and referred to the protein content of the homogenates, as determined by the xanthoproteic method of Millon-Nasse (Oser 1965).

## Results

### Cholinesterase activities in unexposed samples

In normal conditions (unexposed adult specimen of *Paracentrotus lividus*), the cells showing most ChE activity were those presenting a spherule or petaloid phagocyte morphology (Fig. 1A, B). All together, the two morphologies represent >90% of the different cell types present in the total coelomocyte mixed population. The histochemical reactions carried out in the presence of different substrates showed that ChE was mainly represented by the AChE form (Fig. 1A, B); PrChE (propionylcholinesterase) activity was also present, mainly in spherule cells (Fig. 1C), while BChE (butyrylcholinesterase) activity was almost absent (Fig. 1D). Thus, AChE was chosen as a marker to investigate the stress effects.

### Electrophoretic AChE isoforms in control and exposed samples

The effects of stress on AChE isoforms were time dependent. In fact, their activity, detected by the catalytic activity in cleaving the substrate AcTChI, appeared to depend on three different isoforms of the enzyme [Fig. 2 see lane S (control: commercial AChE, obtained from the electric organ of *Electrophorus electricus*, 265 kDa) and lane A (lysates of control coelomocytes)].
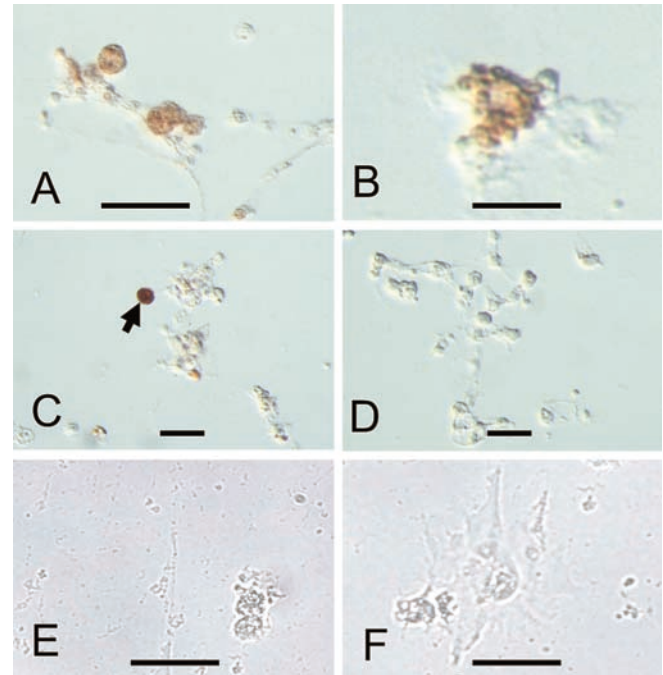


**Fig. 1 A–D** Histochemical detection of enzyme activity, revealed by a brown precipitate, analyzed by Nomarsky optics, **E–F**: unstained, contrast phase microscopy: **A** spherule cell type, true AChE (acetylcholinesterase) activity; **B** petaloid phagocyte cell type, true AChE; **C** mixed cells, PrChE (propionylcholinesterase) activity, mainly present in spherule cells, *arrow* points to a spherule-positive cell; **D** mixed cells, BChE (butyrylcholinesterase) activity almost absent; **E** typical morphology of spherule cell type; **F** typical morphology of petaloid phagocyte. True AChE activity was identified by use of AcMTChI (acetyl-ß-methyl thiocholine iodide) as a substrate; PrChE activity was identified by use of PrTChI (propionyl thiocholine iodide) as a substrate. Controls were performed by pre-incubating cells with $10^{-6}$ M BW284c51. *Scale bars*: 15 µm
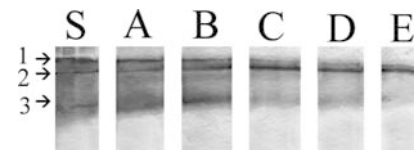


**Fig. 2** Non-denaturing electrophoretic pattern of the isoforms of AChE at pH 6 present in cold-exposed and control coelomocytes (*lane S* control performed by use of commercial *Electrophorus electricus* acetylcholinesterase; *lane A* control performed with the homogenate of coelomocytes not exposed to cold stress). Cells were obtained from specimens exposed to cold stress for 30 min (*lane B*), 1 h (*lane C*), 2 h (*lane D*) and 4 h (*lane E*)

These molecular forms were affected differently by treatment, as shown in Fig. 2, which corresponds to the electrophoretic analysis of enzyme activity: the third form (from the top of the gel) was strongly inactivated in an exposure-dependent way, while the first two forms appeared more resistant to the exposure (see lanes C, D and E, corresponding to 1, 2 and 4 h of exposure, respectively); the coelomocytes exposed for 30 min maintained the activity of all the AChE isoforms.

## Western blot analysis of AChE molecular forms

Western blot analysis also showed the presence of different molecular forms: a heavier one (about 265 kDa molecular mass) corresponding to the commercial AChE (E.C. 3.1.1.7.) from either *E. electricus* or bovine erythrocytes, and other lighter forms (Fig. 3).

Following exposure, the amount of the heavier AChE progressively decreased, while lighter forms appeared, probably as a result of increased fragility to SDS treatment of the enzyme present in the exposed cells. Through comparison with the electrophoretic results, these molecular forms maintained their isoelectric features and their enzyme activities, except the lighter one, which did not show catalytic activity.

## Quantitative analysis of AChE activity

According to quantitative analysis, the enzyme activity found by histochemical and electrophoretic analysis corresponded to an AChE capable of cleaving AcMTChI (the so-called true AChE, E.C. 3.1.1.7, corresponding to the 265 kDa band found by Western blot analysis) and to an AChE capable of cleaving propionyl thiocholine iodide as well, which is an ancestral form of AChE, mainly present in invertebrates (Fig. 4).

These molecular forms are differently affected by different exposure times (Fig. 4).

The effect on AChE cleaving PrTChI was represented by a time-dependent increase of enzyme activity, while the "true" AChE showed a peak of enzyme activity after
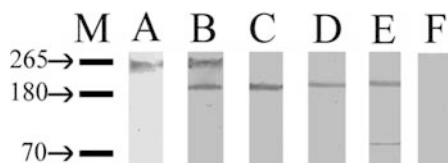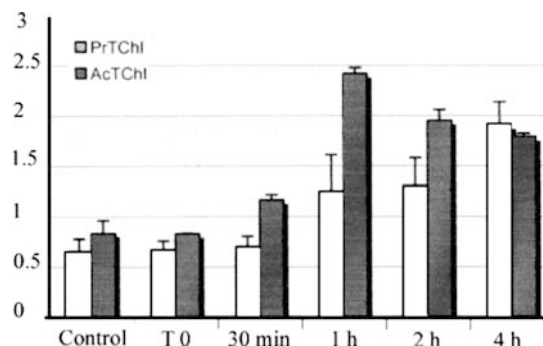
**Fig. 4** ChE (cholinesterase) enzyme activity, in control and cold-stressed coelomocytes; quantitative spectrophotometer assay. ChE activity corresponded both to an AChE able to cleave AcMTChI (the so-called true AChE, E.C. 3.1.1.8, corresponding to 265 kDa molecular mass: *open bars*) and to a ChE able to also cleave PrTChI (*filled bars*) (*Y*-axis, units of ChE mg$^{-1}$ protein; *X*-axis, times of exposure)

1 h of treatment, and a successive decrease for longer treatments. No change was found for BChE.

## Discussion

Our results show that cholinesterase activity, mainly in the form of molecules able to cleave either acetylcholine (ACh) or propionylcholine (PrCh), is present in coelomocytes of the sea urchin *Paracentrotus lividus*, with especially high activity in the petaloid phagocytes and spherule cell types. This may be due to the active function of the two cell types; the petaloid phagocyte cell type has a phagocytic function, while the spherule cell type has a secretive function (Matranga 1996). In both cases, the function may be linked to cytoskeletal dynamics, which follow changes in intracellular ion concentrations. This has been observed in sea urchin zygotes, as a result of the action of molecules related to the cholinergic system (Harrison et al. 2002). This activity is dependent on temperature, and is affected by stress due to the exposure of *P. lividus* specimens to cold conditions. The specificity of the enzyme activity was demonstrated by use of different substrates, such as AcMTChI, specifically hydrolyzed by true AChE (E.C. 3.1.1.7.), BuTChI, specifically hydrolyzed by BChE (E.C. 3.1.1.8.), and PrTChI, specifically hydrolyzed by an aspecific cholinesterase, generally found in primitive organisms (Falugi et al. 2002) instead of BChE. The specificity of the reaction was also shown by the effect of the anti-AChE BW284c51, which specifically inhibits AChE.

Heat shock generally induces a loss in AChE activity in mammals (Lallement et al. 1998) and a tendency to decrease activity as temperature increases in marine invertebrates (*Nereis diversicolor*) kept in tanks with stable salinity (Scaps and Borot 2000). On the contrary, our results show that cold temperatures induced an increase in the activity of the enzyme,

**Fig. 3** Western blot analysis of the molecular forms of AChE in control and exposed samples. *Lane M* column corresponds to the molecular masses; *lane A* (control coelomocytes) shows a high band, corresponding to the heavier *M*r, lanes *B*, *C*, *D*, *E* correspond to the immunoreactive bands obtained by running the SDS extracts of coelomocytes obtained from sea urchins exposed to cold stress for 30 min, 1 h, 2 h and 4 h, respectively; *lane F* is a negative control, obtained by omitting the primary antibody

dependent on the exposure time. This effect is similar to the increase in enzyme activity observed during wound healing in vertebrates (Falugi 1993), cnidarians (Falugi et al. 1994), and in vertebrates exposed to heavy metals, where the percentage of lymphocytes consistently decreased, while percentages of neutrophils and monocytes increased (Dethloff et al. 1999). An increased AChE activity was also found in tumor cells (Falugi et al. 1983, 1986).

Our results are consistent with the fact that AChE activity is performed by different molecular forms of the enzyme (see Massoulié et al. 1993, for a review), which can polymerize and assemble together to obtain heavier molecular forms, faster and more active in their catalytic activity when membrane depolarization is increased (De la Porte et al. 1984). Thus, membrane potential may be modulated by different parameters, both normal and abnormal (e.g. wound potential as well as action potential), including stress.

PrChE represents an ancestral form of cholinesterase, able to cleave both ACh and other choline esters. This enzyme may replace AChE activity in cases of cold stress, because the activity of cleaving ACh is still high, even when the Western blot analysis shows the absence of high-molecular-weight forms of AChE.

Other stress markers, such as hsp70 and metallothioneins, have been used to monitor stress induced by heavy metals (Müller et al. 1998; Matranga et al. 2000, 2002) or other pollutants (Schröder et al. 1999) in marine invertebrates.

The ease of analyzing the stress effects on the AChE activity of coelomocytes suggests its use as a marker of stress, and qualifies the coelomocyte model as a cytological sentinel for the monitoring of environmental stress both in field and in laboratory studies.

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