# Hormonal Regulation of Acetylcholinesterase in an Epithelial Cell Line from *Chironomus tentans*

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Acetylcholinesterase (AchE) can be induced in a non-neuronal, epithelial cell line from *Chironomus tentans* by the moulting hormone 20-OH-ecdysone. Maximal response is reached after 7 days. The increase in enzymatic activity can be suppressed by simultaneous incubation with inhibitors of transcription and translation. The potency of various steroid hormones to induce AchE coincides with their affinity to the ecdysteroid hormone receptor.

AchE from *Chironomus* is tightly bound to membranes and cannot be solubilized by heparin or collagenase treatment. Incubation under conditions where phospholipase C is most active releases activity into the supernatant. If phospholipase is applied in addition, the amount of solubilized enzyme increases, but there is still membrane-bound activity left. This is in accordance with a globular form of AchE linked to membranes by a phospholipid-anchor. The enzyme sediments as a single peak of 5.4 S.

## Introduction

The presence of acetylcholinesterases (AchE) in non-neuronal tissues is well documented for a variety of species, both in invertebrates and vertebrates [1, 2]. The function of AchE in these tissues is either unknown as in mammalian red blood cells [3, 4] or it is speculated that AchE's may serve as specific proteases due to their high degree of homology to certain proteolytic enzymes [5–8]. Most convincing evidences for a special function on non-neuronal AchE is presented by Drews and his co-workers who demonstrated a transient appearance of this enzyme during vertebrate morphogenesis as part of a muscarinic cholinergic system which might be involved in cell movement during differentiation [1, 9–11]. In *Artemia* the fertilized egg shows pronounced AchE activity at the same time when intracellular movement takes place [2].

In insect cell lines from *Drosophila* and *Aedes* AchE has been demonstrated to be under the control of the moulting hormone 20-OH-ecdysone. However, the physiological relevance of this observation remained obscure [12–16]. When we detected a similar increase in AchE activity after stimulation with 20-OH-ecdysone in a cell line from *Chironomus tentans* we were able to correlate this increase in activity with the hormonally induced differentiation of these cells [17], namely a change in cell shape and cell arrangement within the multicellular vesicles after addition of ecdysteroids [18].

In a variety of morphogenetic processes in vertebrates and invertebrates [1, 19, 20] an increase in AchE activity occurs at the same time and therefore a physiological role for AchE is proposed in these processes. In the *Chironomus* cell line we are able to induce morphogenetic differentiation and AchE activity simultaneously by the same substance namely 20-OH-ecdysone. In this paper an initial characterization of the enzyme and its hormonal response is described.

## Materials and Methods

### *Cells*

The *Chironomus* cell line established by C. Wyss (1982) was obtained from Prof. M. Lezzi (ETH, Zürich, Switzerland). Cells were kept in T-flasks at 25 °C as described by Wyss [21] and subcultured after 12–20 days.

### *Enzyme preparation and measurement*

Homogenate was prepared as follows: Cells were harvested by centrifugation (11,000 × *g*, 20 sec) and washed once with phosphate-buffered

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saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 6.8). The pellet of 1 ml cell culture was dissolved in 0.2 ml buffer (50 mM Tris-HCl, pH 7.5, 2 M NaCl, 2% Triton X-100). AchE was measured essentially as described earlier [17]. In cells not treated with hormone the specific activity was around 0.1 nkat/mg protein. Determinations were done in triplicate (S.D. ≤ 5%).

*Protein determination*

Protein was determined according to Lowry *et al.* [22] using as standard bovine serum albumin containing Triton X-100 at the same concentration as the homogenate.

*Determination of the S-value*

Acetylcholinesterase was extracted from cell pellets with 50 mM Tris-HCl, pH 7.5 containing 2 M NaCl and 2% Triton X-100 according to [17]. After centrifugation at 10,000 × *g* for 5 min at 4 °C 200 µl were layered on top of a sucrose gradient (15–30%) in the same buffer and centrifuged in a swing-out rotor (TST 54, Kontron) at 40,000 rpm for 16 h at 4 °C. Aliquots (200 µl) were taken and sucrose density determined with a refractometer. Enzyme activity was determined as above.

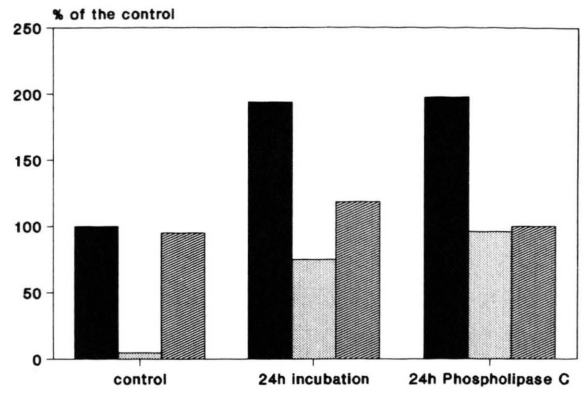


Fig. 1. Partial solubilization of acetylcholinesterase from *Chironomus tentans* cell homogenates by phospholipase C (from *Bacillus cereus*, Sigma). ■ = total activity; ▨ = AchE activity in the supernatant, ▧ = AchE activity in the pellet. Cells were homogenized in buffer (50 mM Tris, pH 7.5). Pellets were dissolved in 2 M NaCl + 2% Triton X-100 in the same buffer before activity was determined. Control: enzyme measurement immediately after homogenization. Incubation 24 h: homogenate was incubated for 24 h at 20 °C under conditions which are optimal for phospholipase C activity. Phospholipase C: incubation for 24 h at 20 °C in the presence of 0.02 units/ml phospholipase C.

## Results and Discussion

According to their kinetic behaviour the cholinesterase activity in the *Chironomus* cell line is a true AchE [17]. The enzyme is not secreted into the medium (unpublished observation). Immediately after homogenization in buffer without detergent only neglectable amounts of enzymatic activity are found in the supernatant. The tightly membrane-bound enzyme can be extracted with 2% Triton X-100 and 2 M NaCl. However, after prolonged incubation at pH 7.4 in buffer without detergent and salt at room temperature, considerable amounts of enzyme are solubilized (Fig. 1). Even more enzyme is obtained in the supernatant, if phospholipase C is added. This indicates the presence of a phospholipid anchor in the molecule which is described for the globular form of AchE in a variety of nerve and non-neuronal cells of vertebrates and invertebrates [23–25]. Phospholipase C treatment does not solubilize all the enzymatic activity even after enzyme treatment for 24 h, which might be due to inhibition by the lipid environment [23]. The total amount of enzymatic activity recovered from supernatant and pellet after phospholipase C treatment is considerably higher than the activity obtained by extraction with Triton X-100 and 2 M NaCl. Acetylcholinesterase can be absorbed to WGA- and Con A-sepharose and is eluted with N-acetylglucosamine, respectively *a*-methyl-mannoside, which means that the enzyme is linked to a carbohydrate moiety. However, addition of heparin (data not shown) [26] to solubilize the enzyme or treatment with collagenase [27] (Table I) were not successful. This means that the asymmetric form of AchE, which is typical for the electric

Table I. Influence of collagenase (2 mg/ml; type II, 190 units per mg, Cooper Biomedical Co.) on the solubilization of acetylcholinesterase from *Chironomus tentans* cell homogenates. Samples were incubated for 2 h at 25 °C.

| | % total activity | % of the total activity in supernatant | pellet |
|---|---|---|---|
| Control | 100 | 17.6 | 82.4 |
| +Collagenase | 91.8 | 19.2 | 80.8 |

organ of the eel or the endplate in muscle, is absent in *Chironomus* cells. In insects only several globular forms were found [28, 29] which can be detected by differences in their S-value [28]. Sedimentation analysis of AchE in a high salt buffer with Triton X-100 revealed only a single peak at 5.4 S (Fig. 2). This coincides fairly well with the S-value of 5.0 determined for AchE from brain of *Tenebrio molitor*, extracted with the same detergents under high salt conditions [28]. In *Chironomus* cells only one molecular form is found. The reason is the absence of a soluble form immediately after homogenization in buffer. Whether the soluble form of AchE is already present in intact tissues or might be an artefact during preparation is still a matter of debate [24, 28], since usually enzyme preparations were conducted with buffer using protease inhibitors which do not affect endogenous phospholipase activity [29].

The increase in AchE activity during permanent presence of 20-OH-ecdysone is shown in Fig. 3. Maximal values are reached after 7 days. If hormone is withdrawn earlier the response is weakened and corresponds to the length of hormone treatment (Fig. 3).

The inhibition of transcription by α-amanitine and of translation by cycloheximide prevents the ecdysteroid-induced increase in enzymatic activity. This indicates, that transcription and translation are necessary for AchE induction (Fig. 4). This resembles the situation of "late genes" as first described for the hormonally induced puffs in *Chironomus tentans* [30] and later on extensively studied in *Drosophila melanogaster* by Ashburner and co-workers who also demonstrated that the induction of late puffs was inhibited by cycloheximide [31, 32].

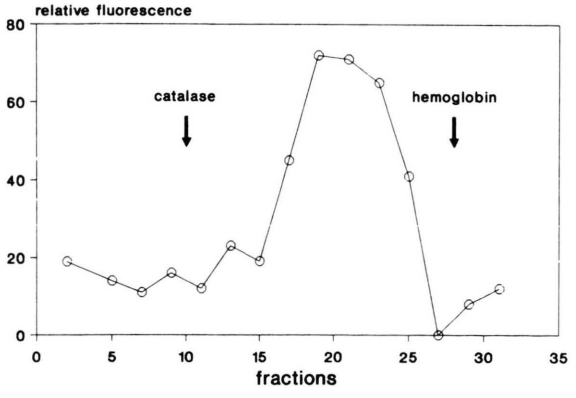


Fig. 2. Determination of the S-value of acetylcholinesterase from *Chironomus tentans* cells. Cells were homogenized in 50 mM Tris, pH 7.5 with 2% Triton X-100 and 2 M NaCl and layered on top of a 15–30% sucrose gradient in the same buffer.

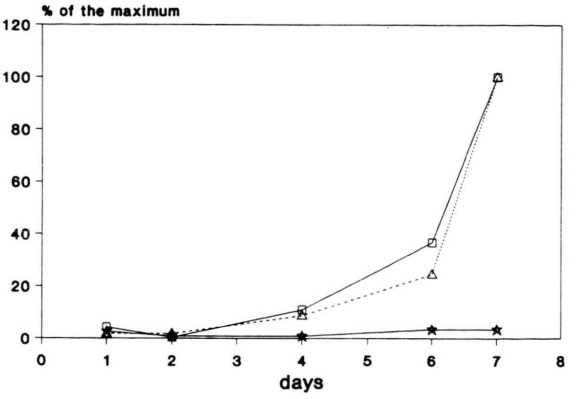


Fig. 3. Increase in acetylcholinesterase activity in the cell line from *Chironomus tentans* in the absence (☆) or the permanent presence of $10^{-6}$ M 20-OH-ecdysone (△). The data are redrawn from [17]. In (□) $10^{-6}$ M 20-OH-ecdysone was present for the time indicated and then withdrawn. Enzymatic activity was measured always at day 7.

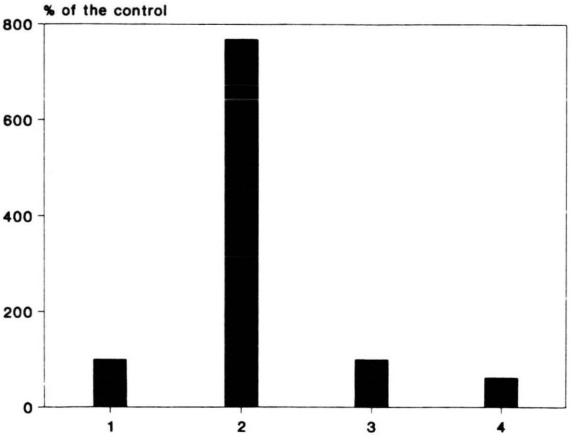


Fig. 4. Influence of α-amanitine and cycloheximide on acetylcholinesterase induction by 20-OH-ecdysone in *Chironomus* cells. 1 = control, 2 = 20-OH-ecdysone ($1 \times 10^{-6}$ M), 3 = α-amanitine ($1 \times 10^{-6}$ M) + 20-OH-ecdysone ($1 \times 10^{-6}$ M), 4 = cycloheximide ($1 \times 10^{-6}$ M) + 20-OH-ecdysone ($1 \times 10^{-6}$ M). The inhibitors alone do not influence enzymatic activity significantly.

The increase in activity is specific for ecdysteroids (Fig. 5) and corresponds with the strength of the morphological response [18] as well as with the ability of these steroids to compete for the ecdysteroid receptor from this cell line [33, 34]. The weak reaction of ecdysone is in accordance with the fact that ecdysone is not converted to 20-OH-ecdysone in this cell line, even after 7 days of incubation [35]. These data suggest that both the hormonally induced morphogenesis and the increase in AchE activity are ecdysteroid receptor-mediated events. This is also stressed by the fact, that the non-steroidal ecdysteroid agonist RH 5849 [36] is able to replace 20-OH-ecdysone not only in *Drosophila* [36] but also in the *Chironomus* cell line where it is about 4-fold less active in inducing AchE as well as about 4 times less effective in competing for [$^3$H]ponasterone A-binding as compared to 20-OH-ecdysone [34].

The easy and sensitive test system and the considerable increase after stimulation with 20-OH-ecdysone make this enzyme a suited tool for screening of ecdysteroid analogs which was successfully demonstrated by Wing [36]. The physiological relevance of this reaction, especially its function as part of a non-neuronal cholinergic muscarinic system [37] which is active during morphogenetic events is currently investigated.

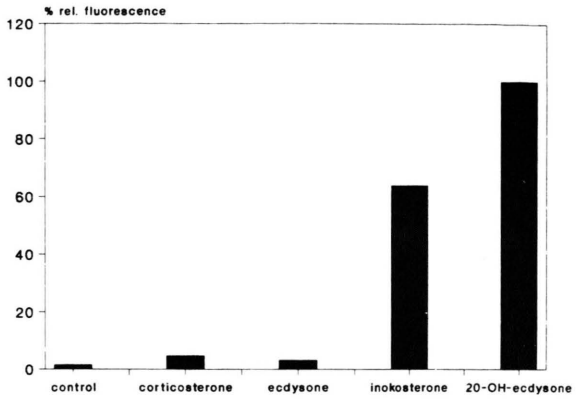


Fig. 5. Influence of various steroids on acetylcholinesterase activity. *Chironomus tentans* cells were treated for 7 days with steroid hormones in a final concentration of $10^{-6}$ M.

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