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Effect of heat shock on acetylcholinesterase activity in chick muscle cultures

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The effect of heat shock was studied on the acetylcholinesterase activity of chick muscle primary cultures. In cultures transferred from 37°C to 45°C, a sharp drop in activity was followed by rapid spontaneous recovery. The time of onset of recovery resembled the time needed for expression of heat shock proteins. In cultures exposed to heat shock at 45°C and allowed to recover at 37°C, reappearance of acetylcholinesterase activity did not involve de novo protein synthesis since it was not prevented by cycloheximide. Our data raise the possibility of a role for heat shock proteins as molecular chaperones in rescuing heat-denaturing acetylcholinesterase.

Acetylcholinesterase; Heat shock; Muscle culture; Protein assembly

1. INTRODUCTION

Acetylcholinesterase (AChE) exists in multiple molecular forms [1]: hydrophilic asymmetric (A) forms in which 1–3 subunit tetramers are attached to a collagen-like tail, believed to be involved in the anchoring of the enzyme to the basal lamina; and globular (G) forms of AChE which exist as monomers, dimers or tetramers and may be either hydrophobic or hydrophilic, depending upon the presence or absence of non-catalytic anchoring segments [2]. Both A and G forms of AChE exist as either cell-associated or secreted molecules [see 3]. Furthermore, subtle differences exist within a given molecular form, for example in glycosylation patterns [4]. The basis for these differences is at the mRNA-level, where alternate splicing of the product of the single AChE gene occurs [5,6]. Subsequent post-translational modifications also contribute to the enzyme's diversity [2]. Much information is available concerning the molecular biology, structure, and distribution of the various forms of AChE. Little is known, however, regarding the biosynthetic steps involved in their folding and assembly.

Most research on AChE assembly has involved use of primary muscle cultures or cell lines [7–9]. Studies involving recovery of AChE activity after application of irreversible AChE inhibitors [10–13] suggest that the complex forms of AChE are derived from simpler forms. Moreover, globular dimers and tetramers are assembled in the endoplasmic reticulum, while A forms of the enzyme are assembled in the Golgi apparatus [7–9]. However, the relationship between monomeric AChE and the more complex oligomeric forms is not a simple precursor–product relationship, as first demonstrated by Rotundo [7], who showed the existence of a large, rapidly turning-over, catalytically-inactive pool of subunit monomers in cultured chick muscle. Subsequently, inactive pools were also identified in other systems [14,15]. Conflicting reports propose either that such inactive pools become activated by an as yet unidentified mechanism or that much of their content is destined for destruction [7–9,13]. It is possible that subpopulations exist within the inactive pools and that both pathways are followed.

In recent years, much work has focused on the roles of molecular chaperones in protein biosynthesis [16,17]. Chaperones aid in the correct folding or oligomeric assembly of numerous proteins by encouraging proper folding, by discouraging undesired folding, or by unfolding existing protein aggregates [17,18]. Amongst the elements shown to act as chaperones are the heat shock proteins (hsp), proteins which are expressed by organisms ranging from *E. coli* to man in response to heat or other forms of stress [19]. Under normal conditions, cells express proteins highly homologous to the hsps, and it is assumed that these proteins fulfil chaperone functions under normal growth conditions [18,20]. The existence of diverse multisubunit forms of AChE and the presence of various pools of enzyme subunits raises the possibility that chaperones may be involved in AChE assembly. This appears to be the case for acetylcholine receptor (AChR) biosynthesis, where BiP, an endoplasmic reticulum-associated member of the 70

Abbreviations: AChE, acetylcholinesterase; hsp, heat shock protein; DFP, diisopropylfluorophosphate

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kDa hsp family, is complexed with an immature form of the AChR α-subunit [21].

In order to investigate a possible role for hsps in folding and assembly of AChE, the effect of heat shock was examined on AChE activity in chick muscle primary cultures. Our results suggest a relationship between the heat shock response and the recovery of heat-denatured AChE.

2. MATERIALS AND METHODS

2.1. *Materials*

Chick embryos were obtained from Kfar Bilu hatcheries (Kfar Bilu, Israel). Horse serum was supplied by Biotek Industries (Jerusalem, Israel) and penicillin, streptomycin and neomycin by Biological Industries (Kibbutz Beit Haemek, Israel). Diisopropylfluorophosphate (DFP), bacitracin, benzamidine, aprotinin and cycloheximide came from Sigma (St. Louis, MO), and gelatin from Difco Laboratories (Detroit, MI). [^{3}H]Acetylcholine and [^{35}S]methionine were supplied by Amersham (Buckingham, UK).

2.2. *Preparation of tissue cultures*

Primary breast muscle cultures were prepared from 12-day-old chick embryos as previously described [22]. The growth medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% horse serum, 1% chicken embryo extract and 1% of penicillin (10 000 U/ml)/streptomycin (10 mg/ml)/neomycin (10 mg/ml). Cells were plated in 9-cm Nunc tissue culture plates (2×10^6 cells/plate) or in 15-cm Falcon tissue culture plates (5×10^6 cells/plate), previously coated with 0.1% gelatin. Cultures were grown in a 37°C incubator containing 8% CO_2. Medium was changed on the second and fifth days after plating. Experiments were performed on 7-day-old cultures.

2.3. *Heat shock*

Cultures were subjected to a heat shock of 45–46°C by replacing the culture medium with DMEM containing 10% horse serum, preheated to 46°C, followed by transfer to an incubator set at that temperature, for up to 100 min. In some cases, cultures were removed after 45 min, their medium exchanged for DMEM containing 10% horse serum at 37°C and then returned to the 37°C incubator.

2.4. *Metabolic labelling of cultures*

Culture plates were rinsed twice with methionine-free DMEM supplemented with 10% horse serum previously dialyzed against PBS. Cells were collected and pelleted for 10 min in an Eppendorf centrifuge. The pellet was resuspended in the above medium to which [^{35}S]methionine (5 μCi) was added. After a 45 min incubation at 37°C, lysates were prepared as previously described [23]. The lysates were left at 4°C overnight before being frozen in liquid N_2 and stored at −70°C. Labelled lysates were subjected to SDS-PAGE on 5–15% gels [24]. Autofluorography was performed as described [25], except that 0.4% in acetic acid/xylene/ethanol (1.8:1:2, v/v/v) replaced 20% PPO in DMSO.

2.5. *Inhibition of AChE*

Cultures were washed with 3 × 5 ml Hank's balanced salt solution (HBSS) on ice and incubated with 10^{-4} M DFP in HBSS, at room temperature, for 20 min. Under such conditions, >95% inhibition of AChE activity is achieved [11]. The cultures were rinsed with 4 × 10 ml HBSS, to remove free DFP. Medium was reapplied to the cultures, which were returned to the incubator.

2.6. *Inhibition of protein synthesis*

Protein synthesis was inhibited by including 100 μg/ml cycloheximide in the culture medium [11].

2.7. *Determination of AChE activity*

Plates were washed with 3 × 5 ml cold HBSS. This was performed on ice to diminish AChE secretion. Cells were scraped, collected and pelleted. The pellet was homogenized in a glass homogenizer in AChE extraction buffer (1 M NaCl, 1% Triton X-100, 0.01 M EDTA, 0.01 M Tris, pH 7.0, 0.1 mg/ml bacitracin, 1 mM benzamidine and 1.1 U/ml aprotinin). The homogenate was centrifuged (100 000 × g, 10 min) in a Beckman airfuge. AChE activity in the supernatant was determined radiometrically [26]. In some cases, the homogenate was frozen in liquid N_2 and stored at −70°C overnight before centrifugation. This did not affect AChE activity.

3. RESULTS

Cultures were subjected to a continuous heat stress by transfer to 45.5 ± 0.5°C, and their AChE activity was monitored at 20 min intervals. Fig. 1 shows the activities obtained, expressed as a percentage of the activity in cultures maintained at 37°C. An initial small increase in activity was followed by a marked decrease to below 60% of control levels 40 min after the temperature had been raised. This was followed by a significant recovery to 80–85% of control levels. This situation was maintained for up to 60 min, after which enzyme activity began to fall again, presumably due to maintained heat stress. The observed increase in AChE activity was not an artifact associated with the introduction of fresh medium into the cultures, since control experiments comparing AChE activity in cultures fed with fresh medium with that of cultures taken from a 37°C stock revealed no difference in AChE activity.

A similar experiment was performed in vitro in cellular extracts prepared by homogenization in AChE extraction buffer. These extracts, containing almost all of the AChE activity, were transferred to a 45°C incubator. Their AChE activity decreased rapidly, falling to

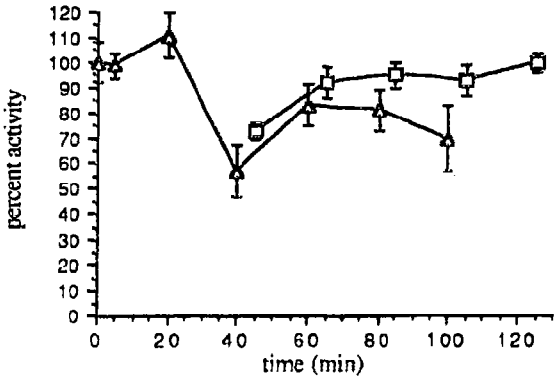

Fig. 1. AChE activity in heat shocked myocultures. 7-day-old chick embryo muscle cultures were subjected to a 45°C heat shock. After 45 min, the culture dishes were either maintained at 45°C or allowed to recover at 37°C. AChE activity is expressed as a percentage of the activity of control cultures. Each point represents the average (±SE) of 4–6 experiments involving duplicate or triplicate samples. (△) AChE activity in cultures maintained at 45°C; (□) AChE activity in cultures allowed to recover at 37°C after 45 min at 45°C.

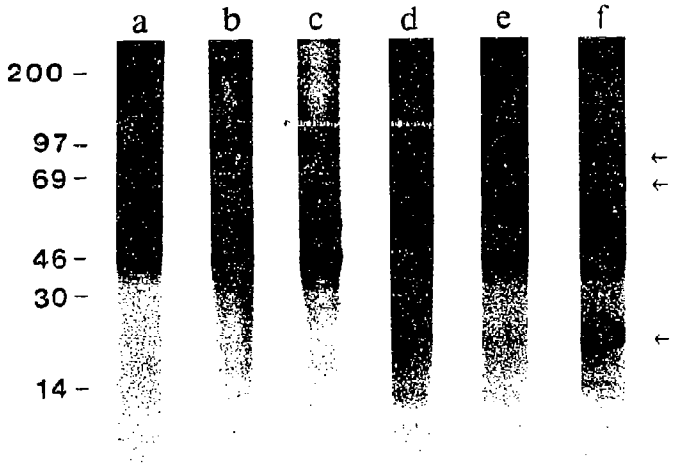

Fig. 2. Metabolic labelling of muscle cultures subjected to heat shock. Cultures were maintained at 37°C or exposed to a 45°C heat shock for various times, metabolically labelled with [^{35}S]methionine, and examined by SDS-PAGE. In heat-shocked cultures, proteins of 81, 66 and 23 kDa are preferentially expressed, beginning at 30 min (shown by arrows). Each lane contains 20 000 cpm: (Lane a) 37°C culture; (lanes b–f) cultures experiencing heat shock for 5, 15, 30, 60, or 100 min, respectively. Molecular weight markers are on the left.

half its initial value within 60 min (not shown). Thus, it appears that some intact intracellular machinery is required for the observed recovery of AChE activity in heat-shocked cultures.

In a second set of experiments, the effect of transient heat shock on AChE activity was examined. Cultures were transferred to 45.5°C for 45 min and then returned to 37°C (Fig. 1). A time of 45 min was selected for the transient heat shock since this period caused maximum heat-induced reduction of AChE activity. Furthermore, this is a sufficient time for expression of heat shock

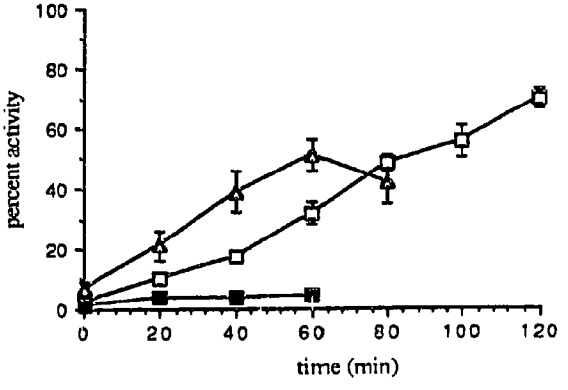

Fig. 3. Recovery of AChE activity following DFP inhibition. Cultures were treated with 10^{-4} M DFP to block AChE activity. Reappearance of AChE activity in the cultures was followed at 37°C or 45°C. AChE activity is expressed as a percentage of the AChE activity of control cultures. AChE activity in cultures maintained at 45°C after DFP treatment; (□) AChE activity in cultures allowed to recover at 37°C after DFP treatment; (■) AChE activity in cultures allowed to recover in the presence of cycloheximide (100 μg/ml). Each point represents the average (±SE) of 2–3 experiments involving duplicate or triplicate samples.

proteins, the suspected mediators of recovery of AChE activity, as shown by metabolic labelling of cultures undergoing heat shock (Fig. 2). In these cultures, proteins of 81, 66 and 23 kDa are preferentially expressed within 30 min of onset of the heat stress [23]. As in the previous experiment, samples were collected every 20 min for AChE determination. Following the 45 min heat shock, activity in the cultures had dropped to 70% as compared to cultures no subjected to heat stress. However, within 20 min of incubation under non-stress conditions, AChE activity had returned to >90% of control levels, where it remained for the duration of the experiment.

To test whether the rapid recovery of AChE activity could be explained by de novo synthesis, cultures were treated with DFP and the kinetics of reappearance of AChE activity monitored at 37 and 45°C (Fig. 3). In cultures recovering at 45°C, 93% of the original AChE activity was blocked by DFP treatment at the onset of recovery. After 60 min, activity had reached 51% of that in control cultures, after which it began to slowly decline, reaching 42% at 80 min. At 37°C, 97% of the AChE activity was inhibited by DFP. After 2 h, activity had returned to 70% of the original value. This rate of recovery is in excellent agreement with previous studies [8,10]. Confirming earlier observations, it was shown also that cycloheximide (100 μg/ml) completely prevented recovery of activity, clearly showing that reappearance of AChE was entirely due to de novo synthesis [7,11]. The somewhat higher initial rate of synthesis observed at 45°C might be due to a general temperature-induced stimulation of cellular activities. Nonetheless, the rates of de novo synthesis measured here are not adequate to account for the recoveries of AChE activity observed in cultures subjected to heat shock.

Although our data showed that both transient reappearance of AChE activity during prolonged heat shock, and permanent recovery subsequent to transient heat shock occurred faster than might be expected from the rates of de novo synthesis, the data of Fig. 1 could not exclude some contribution of de novo synthesis to the reappearance of AChE activity. To examine this possibility, cultures were subjected to heat stress and either maintained at 45°C or allowed to recover at 37°C, in both cases in the presence of cycloheximide (Fig. 4). Cultures experiencing constant heat stress at 45°C, in the presence of cycloheximide, maintained their AChE levels for the first 20 min, following which rapid loss of AChE activity occurred. It is possible that cycloheximide not only prevented de novo synthesis of AChE, but also interfered with the enhanced expression of heat shock proteins, the suspected mediators of heat shock-associated rescue of AChE activity. If the hsp's are in fact responsible for the observed recovery of enzyme, existing levels of constitutively-expressed hsp homologues may not suffice to cope with the large amount of heat-denatured AChE resulting from the maintained

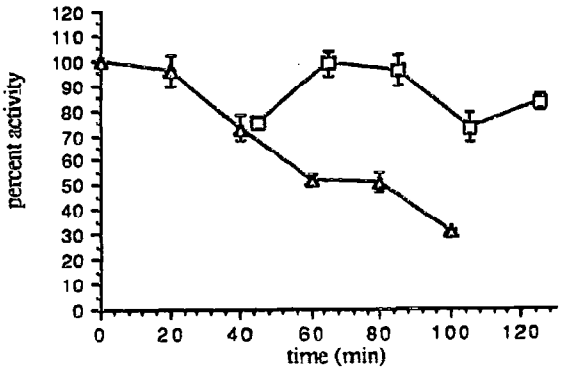

Fig. 4. Effect of cycloheximide on recovery of AChE activity in heat shocked muscle cultures. Muscle cultures were maintained at 45°C in the presence of 100 μg/ml cycloheximide or allowed to recover at 37°C, in the presence of cycloheximide, from a 45 min heat shock at 45°C. In both cases, AChE activity is expressed as a percentage of the activity of control cultures. (△) AChE activity in cultures maintained at 45°C in the presence of cycloheximide; (□) AChE activity in cultures subjected to a 45 min heat shock and then allowed to recover at 37°C in the presence of cycloheximide. Each point represents the average (±SE) of 2–3 experiments involving duplicate or triplicate samples.

heat stress. When cultures exposed to 45°C for 45 min were treated with cycloheximide only after being returned to 37°C, the rate of recovery of activity was similar to that observed for cultures allowed to recover from heat shock in the absence of cycloheximide.

4. DISCUSSION

For many years, it was widely accepted that the information required for realization of a protein's final structure is contained in its primary structure [27]. However, this concept of self-folding and assembly has recently been challenged by the identification of molecular chaperones which aid in protein assembly by mediating the folding of polypeptides and, in some cases, assist their assembly into oligomers [16,17]. Members of the chaperone family include the heat shock proteins, a group of proteins expressed in response to various stress stimuli [19]. Due to its large subunit size and complex oligomeric diversity, it is reasonable to assume that AChE might enlist the aid of chaperones at various stages of its folding and assembly. In this study therefore, the effect of heat shock on AChE activity was investigated.

When the AChE activity of chick muscle cultures was examined under conditions of maintained or transient heat shock, it was seen that after an initial decrease, the cultures quickly recovered a substantial percentage of the lost AChE activity. This recovery could not be explained solely by synthesis of new AChE molecules. Indeed, recovery occurred even in the absence of protein synthesis. Still, intact intracellular structural elements were required for the recovery of AChE activity, as reflected by the fact that when extracts of the cultured cells were exposed to elevated temperatures, AChE activity dropped off rapidly, and no recovery was observed.

It has been suggested that chaperones possess the ability to disentangle protein aggregates, either naturally-occurring or formed in response to cellular stress [18,20,28,29]. Our results may reflect the ability of stressed cells to rescue AChE activity lost as a result of aggregation caused by heat-induced denaturation. Such a recovery has been reported by Bensaude et al. [30], who monitored the activity of reporter enzymes, luciferase and β-galactosidase, in heat shocked cells. Obvious candidates for mediating these effects are the heat shock proteins. These entities, or closely related homologues, have previously been implicated in disaggregation reactions, namely in the in vitro reactivation of RNA polymerase following heat-induced aggregation [29], in disassembly reactions involved in the initiation of λ-bacteriophage replication [28], and in disruption of clathrin-coated vesicles [20].

Up to 70% of the cultures' AChE content is intracellularly localized, sequestered in a variety of intracellular organelles including the endoplasmic reticulum, Golgi apparatus and within clathrin-coated vesicles [7,8]. Heat shock proteins or their homologues can also be localized to some of these organelles [16,18,19]. Thus, the opportunity for the two proteins to encounter one another in situ exists. The mechanism by which hsp's, the putative mediators of the reaction, might enhance restoration of lost AChE activity is, however, open to speculation. The fact that AChE activity is partially recovered even under conditions of maintianed heat stress suggests that some form of permanent manipulation of the enzyme has occurred. Such change(s) must be minor, as no difference is observed in the sucrose density gradient sedimentation patterns of AChE in the cultures before or after recovery (not shown).

It is still unclear whether the observed return of AChE activity results from refolding of heat-denatured enzyme, or whether heat shock-associated moieties, such as hsp's, might be involved in activation of the large inactive pool of chick AChE previously referred to [8,15]. It was recently reported that BiP, the hsp70 homologue resident in the endoplasmic reticulum, plays a role in assembly of another oligomeric protein involved in cholinergic transmission, the acetylcholine receptor [21]. BiP was shown to bind preferentially to unassembled, conformationally-immature α-subunits, and as such assists in AChR assembly either by preventing aggregation of these immature subunits, or by participating in a maturation step. With these results in mind, we are currently investigating a putative relationship between hsp's and inactive chick AChE.

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