N-ETHYLMALEIMIDE INHIBITION OF THE INDUCTION OF GENE ACTIVITY BY THE HORMONE ECDYSONE

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1. Introduction

The salivary gland chromosomes of intermoult third larval instar larvae of *Drosophila melanogaster* respond rapidly to the steroid hormone ecdysone by the induction of puffing activity at a limited number of sites [1, 2]. It is currently thought that puffed sites represent active genetic units [3–6]. The response of salivary gland chromosomes of *D. melanogaster* to ecdysone is very similar *in vivo* and in glands cultured in a defined medium [1, 2]; among the earliest changes is the induction of a puff at site 74EF. This puff is inactive in intermoult (i.e. puff stage 1) animals but is one of the first puffs to appear in the larval/prepupal puffing cycle as a consequence of an increase in the animal's haemolymph ecdysone titre. In cultured glands this puff responds to ecdysone within 5 min and reaches its maximum size after 4 hr. It therefore represents a highly specific and very rapid response of the genome to the hormone. Although direct evidence is so far insubstantial [7, 8] it is probable that ecdysone, like vertebrate steroid hormones [9–12], acts via one or more protein receptors. It is, therefore, of interest to determine whether the effect of ecdysone can be influenced by agents known to react at specific sites within proteins. In addition to its intrinsic interest such information might also be of value in the design of specific affinity labels for the putative ecdysone receptor proteins [13–16]. The experiments reported in this paper demonstrate that *N*-ethylmaleimide (NEM), which irreversibly akylates sulphydryl groups of proteins and other compounds, will inhibit the action of ecdysone and that ecdysone will 'protect' cells from this effect of NEM. The interaction of vertebrate steroid hormones with their specific cytosol receptors is also blocked by NEM and other sulphydryl reacting agents in many instances, e.g. oestradiol [17, 18].

2. Materials and methods

Salivary glands were dissected from third instar larvae of the Canton-S stock of *D. melanogaster* and cultured in modified Grace's medium (Grand Island Biological Supply Co.) as described before [1, 2]. Only one lobe of the paired gland was cultured, the other was fixed, immediately after dissection, to ensure that the puffing pattern [2] was at PS1. β-Ecdysone (Rohto Pharmaceutical Co., Osaka, lot O16) was added to final concentrations detailed below. NEM was from Koch-Light, *N*-ethylmaleamic acid from Aldrich, *p*-chloromercuriphenyl sulfonic acid from Sigma and *N*-ethylsuccinimide was synthesized according to the method of Wheeler and Barnes [19].

Puff size was measured from propionic acid–orceine–carmine preparations of salivary gland chromosomes [1] and is expressed as the ratio of the diameter of the puff 3L:74EF with the diameter of an unpuffed band 3L:74A1:2 [1, 2]. 20 Measurements, 5 nuclei from each of 4 glands, were made for each determination.

All experiments were carried out at room temp (20–22°).

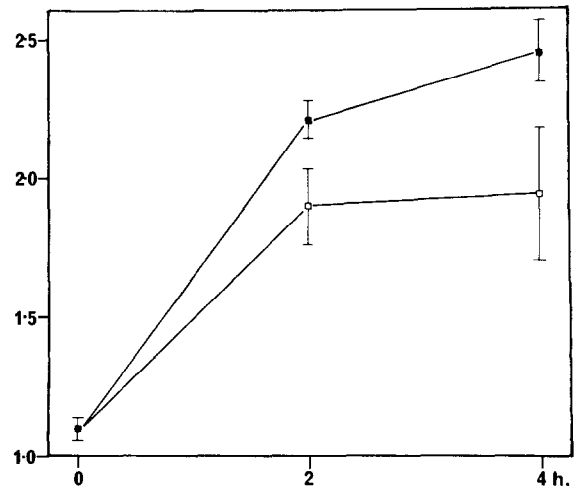

Fig. 1. Induction of 74EF in presence of β-ecdysone (1×10^{-5} M) (■—■—■) and β-ecdysone (1×10^{-5} M) plus NEM (1.6×10^{-4} M) (□—□—□). Each point on the graph is the mean (± SE) of 20 measurements.

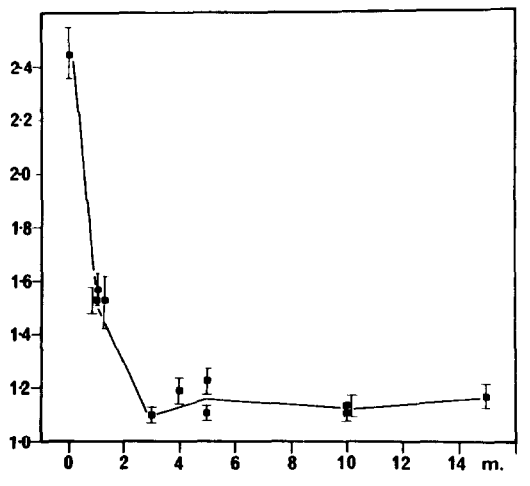

Fig. 2. Mean size of 74EF following pretreatment for varying times (in min) in medium + NEM at 3.5×10^{-4} M before transfer to β-ecdysone (1×10^{-5} M) medium for 4 hr.

3. Results

Fig. 1 illustrates a typical induction curve of puff 74EF when PS1 salivary glands are incubated in the presence of β-ecdysone at a concentration of 5×10^{-7} M or above. In the continuous presence of NEM (1.6×10^{-4} M) there is some inhibition of induction; the puff is only 60% of its control size.

Pretreatment of salivary glands in medium containing NEM for short periods of time before transfer to medium containing β-ecdysone but lacking NEM almost completely inhibits induction (fig. 2). At a concentration of NEM of 3.5×10^{-4} M pretreatment for 1 min inhibits induction by 77%, 2 min by 86% and 3 min by 100%. With a standard 10 min preincubation period 50% inhibition of induction by NEM occurs at a concentration of 8×10^{-5} M (fig. 3).

These experiments illustrate the protection afforded by β-ecdysone to the inhibitory effect of NEM. In a further series of experiments 10 min 'pulses' of NEM were given to glands at various stages in the normal 4 hr induction cycle. The presence of NEM (3.5×10^{-4} M) during the first 10 min of the induction inhibited to an extent ranging from 54–66% in 4 independent experiments (table 1). When the pulse period commenced 10, 20 and 30 min after addition of β-ecdysone the degree of inhibition attained decreased and no effect of a NEM pulse was seen when the pulse was given 1 hr after β-ecdysone or later. Reference to the normal induction curve (figs. 1

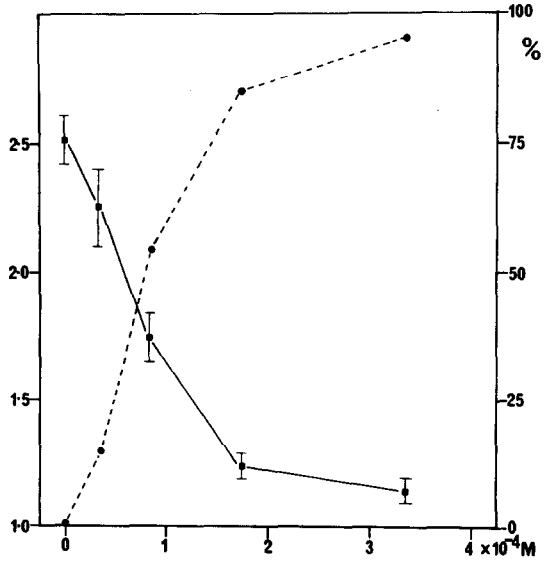

Fig. 3. Mean size (■—■—■) and % inhibition (● - - ● - - ●) of 74 EF after a 10 min pretreatment with NEM of different concentrations before transfer to β-ecdysone (1×10^{-5} M) medium for 4 hr.

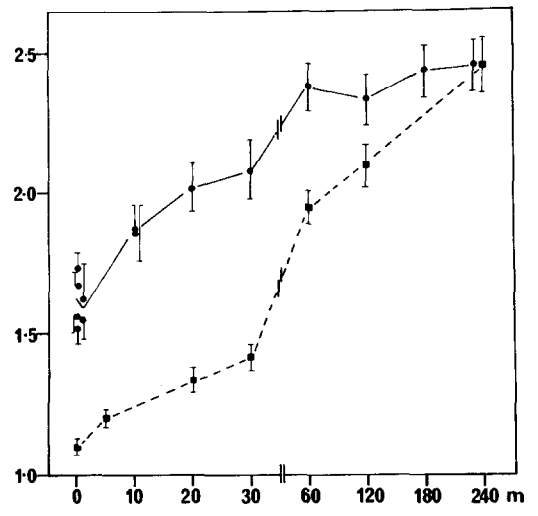

Fig. 4. Mean size (●—●—●) of 74EF, measured at 4 hr, after a 10 min pulse in NEM (3.5×10^{-4} M) beginning at times indicated. β-Ecdysone (1×10^{-5} M) was present continuously (including NEM solution) from zero time. The dashed curve (■—■—■) represents the normal induction of 74EF during a 4 hr period in β-ecdysone (1×10^{-5} M).

and 4) will be a reminder that this puff does not attain its full size until 4 hr. Thus when the NEM pulse is given during the first hour there is considerable growth of the puff yet to occur. This growth is dependent on the continued presence of β-ecdysone; if the hormone is washed out at any time during the 4 hr induction period the puff immediately and rapidly regresses (unpublished observation).

During a 10 min pretreatment with NEM at 2.5×10^{-4} M the extent of protection increases with increasing β-ecdysone concentrations present simultaneously with the NEM (table 2). In this experiment the β-ecdysone was present at concentrations ranging from $1 \times 10^{-8} - 2 \times 10^{-5}$ M during the pretreatment period. Subsequently all series of glands were transferred to medium plus β-ecdysone at 1×10^{-6} M for the 4 hr induction period.

Pretreatment (10 min) of glands with either *N*-ethylsuccinimide or *N*-ethylmaleamic acid had no effect on subsequent induction by β-ecdysone (table 3). With *p*-chloromercuriphenyl sulfonic acid (PCMPS) continuous treatment, at a concentration of 5.2×10^{-5} M, inhibited induction to an extent of 62%. Higher concentrations of this chemical were lethal. On the other hand a 10 min pretreatment with PCMPS at 8.7×10^{-4} M had no effect on subsequent induction (table 3). Unlike NEM the reaction between sulphydryl groups and PCMPS is readily reversible.

Table 1
Size of 74EF, at 4 hr, after NEM (3.5×10^{-4} M) pretreatment.

Treatment	Size
None	2.441 ± 0.121
10 min control pretreatment	2.651 ± 0.066
10 min NEM Exp. 1	1.148 ± 0.048
2	1.129 ± 0.025
10 min NEM + 1×10^{-5} M β-ecdysone Exp. 1	1.730 ± 0.051
2	1.668 ± 0.046
3	1.525 ± 0.037
4	1.562 ± 0.047

Table 2
Size of 74EF, at 4 hr, after 10 min treatment with NEM (2.5×10^{-4} M) + β-ecdysone at varying concentrations before transfer to β-ecdysone at 1×10^{-6} M for 4 hr.

β-Ecdysone concentration during pretreatment (M)	Size	Inhibition (%)
0	1.163 ± 0.036	92
1×10^{-8}	1.273 ± 0.057	84
1×10^{-7}	1.366 ± 0.079	78
1×10^{-6}	1.504 ± 0.060	68
2×10^{-5}	1.605 ± 0.067	60

4. Discussion

Despite the fact that sulphydryl groups of many proteins probably react with NEM in these experiments (and that NEM alkylates other amino acids, e.g. histidine, lysine), the effect of this agent on the induction of specific gene activity by β-ecdysone is remarkably clear cut. Pretreatment of glands for relatively short times (2–3 min) almost completely

Table 3
Size of 74EF, at 4 hr, after miscellaneous treatments.

Treatment	Size
PCMPS (8.7×10^{-5} M) 10 min pretreatment	2.469 ± 0.082
PCMPS continuous (4 hr): 0.87×10^{-5} M	2.089 ± 0.075
1.74×10^{-5} M	1.925 ± 0.069
5.22×10^{-5} M	1.610 ± 0.154
N-Ethylmaleamic acid (4.4×10^{-4} M) 10 min pretreatment	2.476 ± 0.077
N-Ethylsuccinimide (6.6×10^{-4} M) 10 min pretreatment	2.896 ± 0.114

β-Ecdysone at 1×10^{-6} M.

inhibits the effect of the hormone. Simultaneous treatment of the glands with hormone and NEM has a considerably less effect while treatment of glands after 30 min in hormone has virtually no effect on the induction pattern. During NEM pretreatment higher β-ecdysone concentrations cause greater protection than do lower β-ecdysone concentrations. These results are to be expected if a step in the reaction mechanism of β-ecdysone involves complexing with one or more specific protein receptors and that the proteins' SH groups are important in this interaction. Once β-ecdysone has complexed further steps in the reaction sequence leading to specific gene induction appear little effected by NEM. The protection afforded by β-ecdysone would occur if after β-ecdysone binding SH groups essential for such binding were no longer accessible to NEM. It must be stressed, however, that attractive as this interpretation may be, the experiments reported are by no means rigorous proof of its correctness. Much depends upon an evaluation of the rate of NEM inhibition. The argument that the inhibition of β-ecdysone action by NEM is a consequence of the inhibition of some general metabolic function which must remain intact for β-ecdysone to be effective would, for example, rely on the proposition that the rate of inhibition of this function is considerably slower than the rate of β-ecdysone–receptor complex formation. The rate of NEM inhibition is rapid, 50% within 45 sec, even faster than that reported for the reaction of NEM and bovine serum albumin [20]. The first detectable response of the genome to β-ecdysone is after 5 min. It is these considerations which make me favour the interpretation given above – that one of the actions of NEM is to interact with the β-ecdysone receptor protein(s) and block β-ecdysone binding. If further experiments substantiate this conclusion it may be worthwhile designing affinity labels for the β-ecdysone receptor protein(s) as has been done, for example, for acetylcholine receptors [15], oestradiol [16], and other proteins [21].

Acknowledgements

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