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CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN LARVAL BRAIN OF WILD TYPE AND *dunce* MUTANT STRAINS OF *DROSOPHILA MELANOGASTER*: ISOENZYME PATTERN AND ACTIVATION BY Ca^{2+}/CALMODULIN

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Summary. Like adult heads and whole flies, larval brains of wild type *Drosophila melanogaster* contain two major soluble cyclic nucleotide phosphodiesterases, forms I and II. Larval brains of the learning-defective mutant strain, $dunce^{M11}$, contain only the form I enzyme. In both wild type and *dunce* strains the form I enzyme is activated by Ca^{2+}/calmodulin. A time-dependent loss of this Ca^{2+} activation was observed.

Drosophila melanogaster contains two major forms of soluble cyclic AMP phosphodiesterases: form I hydrolyzes both cyclic AMP and cyclic GMP, whereas form II is specific for cyclic AMP (1,2). The *dunce* strains of Drosophila, which carry a mutation in the X-chromosome, are deficient in phosphodiesterase II, as well as in learning and female fertility (2-5). Contradictory reports have been published about the Ca^{2+}/calmodulin sensitivity of Drosophila phosphodiesterases. Davis and Kiger (1) found no

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid

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Ca^{2+} effect, whereas Yamanaka and Kelly (6) claimed that the form I enzyme was activated by Ca^{2+}/calmodulin.

In this work the Ca^{2+} sensitivity of phosphodiesterases of the Canton-S wild type strains and of the mutant dunce^{M11} was examined. We confirm the activation of phosphodiesterase I by Ca^{2+}/calmodulin and show that this form of phosphodiesterase, but not form II, is present in the brain of dunce^{M11} larvae, whereas both forms are present in wild type larval brains.

Materials and methods

Canton-S flies were used as wild type strain. Two dunce alleles were analyzed: dunce^{2} (3) and dunce^{M11} (4), which are hypomorphic and amorphic, respectively, with regard to phosphodiesterase II (5). Stocks were grown on standard cornmeal medium at 25°C. For the analyses homozygous flies of both sexes were selected. The dunce^{M11} larvae were selected using the y and cho markers on the mutant X-chromosome.

Fly-heads were dislodged by mild sonication in liquid nitrogen and separated by sieving. Larval brains were obtained by dissection from mature 4-day-old larvae; the imaginal discs and ring-gland were removed from the brain. Dissection was made under Ephrussi-Beadle Ringer (128 mM NaCl, 4.7 mM KCl and 1.5 mM $CaCl_2$) and the brains were stored at -70°C in homogenization buffer until use. Homogenization of whole flies and heads was carried out in ice-cold 40 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM 2-mercapto-ethanol, pH 8.0, in a glass homogenizer by 20 strokes with a motor-driven Teflon pestle. Larval brains were homogenized with 20 strokes by hand in the same buffer but also containing 0.5 mg/ml bovine serum albumin.

For gel-chromatography the homogenate was centrifuged at 10^5 x g for 1 hour at 0°C and the supernatant was loaded at 4°C onto a column (95 x 0.65 cm) of Sephacryl S-200 equilibrated with 40 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.5 mg/ml bovine serum albumin pH 8.0. The flow rate was either 1.8 or 6.0 ml per hour as indicated, and the column was calibrated with rabbit muscle phosphorylase b (Mr = 190 000), aldolase (158 000) and haemoglobin (68 000).

Cyclic AMP phosphodiesterase activity was assayed according to Thompson and Appleman (7), as modified by Londesborough (8) in 40 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.5 mg/ml bovine serum albumin, pH 8.0, at 30°C, with 100 or 1.0 µM cyclic AMP. Cyclic GMP-phosphodiesterase was determined similarly, but, following the recommendation of Boudreau and Drummond (9), 200 mM formic acid was included in the Dowex 1 x 8 slurry. Under our conditions 20 % of adenosine and 30 % of guanosine remained bound to the resin, which was allowed for when calculating activities.

Phosphodiesterase activities of homogenates and supernatants were calculated from the slopes of straight lines defined by the radioactivity increments after 10, 20 and 30 min reaction times. Care was taken that less than 25 % of the initial substrate was consumed in the assays.

[^{3}H] cyclic AMP (specific radioactivity: 36.4 Ci/mmol) and [^{3}H] cyclic GMP (s.r.: 34.5 Ci/mmol) were the products of NEN. All chemicals were reagent grade commercial preparations. Protein was determined by the Folin method (10) with bovine serum albumin as standard. Calmodulin from human erythrocytes was prepared according to Sarkadi et al. (11).

Results and discussion

The gel-chromatographic profiles for cyclic AMP-phosphodiesterase activity of whole fly extracts are shown in Fig. 1. The wild type Canton-S strain has two activity peaks (forms I and II) of about equal size. In the hypomorphic mutant *dunce*^{2}, the second peak (form II) is smaller, while in the amorphic mutant *dunce*^{M11} it is essentially absent. These results accord with earlier data from other laboratories (1,2,5). We obtained 178,000 and 70,000 for the molecular weights of phosphodiesterases I and II, which are close to the values (168,000 and 68,000) determined by Davis and Kiger (1) using a similar technique.

Activation by Ca^{2+}/calmodulin was tested in assay mixtures containing 1.5 mM $CaCl_2$, 4 µg/ml calmodulin and 1 mM EGTA compared with mixtures

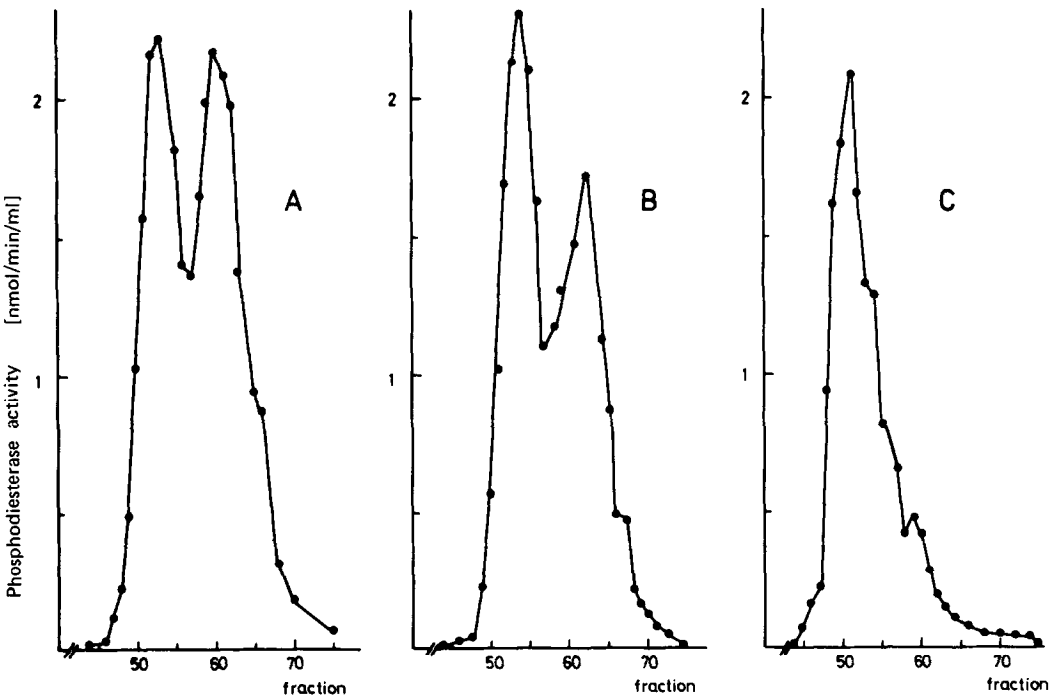

Fig. 1. Sephacryl S-200 gel-chromatographic profiles of cyclic AMP phosphodiesterase activity in extracts of whole flies. About 180 flies of each strain were homogenized in 2 ml buffer (giving 10 mg of protein/ml of homogenate) and 1 ml of the 10^5 x g supernatant was loaded onto the column as described in Methods. Flow rate: 1.8 ml/hour; fraction volume: 0.27 ml. Each point is the mean of duplicate activity assays at 100 µM cyclic AMP. A, Canton-S; B, *dunce*^{2}; C, *dunce*^{M11}. Recoveries of enzyme activity from the column were 78, 89 and 72 % for A, B and C, respectively.

Table 1
Loss of Ca^{2+}-activatability of cyclic AMP phosphodiesterase during aging at 0°C of *dunce*^{M11} head extract

Age (hours)	Ca^{2+}/Calmodulin activity (%)	
	Homogenate	10^5 x g Supernatant
0.3	330	-
2	161	152
24	143	130
46	98	108

About 100 heads (10 mg fresh wt.) of *dunce* M^{11} flies were homogenised in 1 ml of buffer (giving 1.2 mg of protein/ml of homogenate) and a portion centrifuged at 10^5 x g, as described in Methods. The homogenate and supernatant were then stored at 0°C. Their Ca^{2+}/calmodulin activities were measured in assay mixtures containing 1.5 mM $CaCl_2$, 4 µg/ml calmodulin and 1 mM EGTA, and are expressed as percentages of the control activities measured in the presence of 1 mM EGTA only. Cyclic AMP concentration: 1 µM. The control activities were initially 172 pmol/min/ml of homogenate and 162 pmol/min/ml of supernatant and decreased by about 20 % in 46 h.

containing 1 mM EGTA only. In fresh extracts of whole flies or heads from both Canton-S and *dunce* mutants a 1.5 to 4-fold Ca^{2+}-activation was always apparent, but after separation of the two phosphodiesterase forms by gel-chromatography, as in Fig. 1, activation was no longer detectable. Since the removal by gel-chromatography of some substance other than calmodulin needed for the Ca^{2+}-effect seemed unlikely in view of the mechanism of activation of mammalian cyclic AMP phosphodiesterase (12,13), there remained the possibility that the enzyme(s) became desensitized during the time required for gel-chromatography. This assumption is borne out by the data of Table 1, which shows a decrease in the Ca^{2+}/calmodulin activatability of phosphodiesterase-I in *dunce*^{M11} head extract as a function of the age of the extract. The initial 3-4-fold activation practically disappeared by 46 h after cell disruption. The ability to be activated could not be restored by the addition of 10 mM dithioerythritol nor preserved by the inclusion of 2 mM phenylmethylsulfonyl fluoride in the homogenization buffer. Loss of Ca^{2+}/calmodulin activatability is therefore caused either by a structural change in the enzyme or by proteolysis not

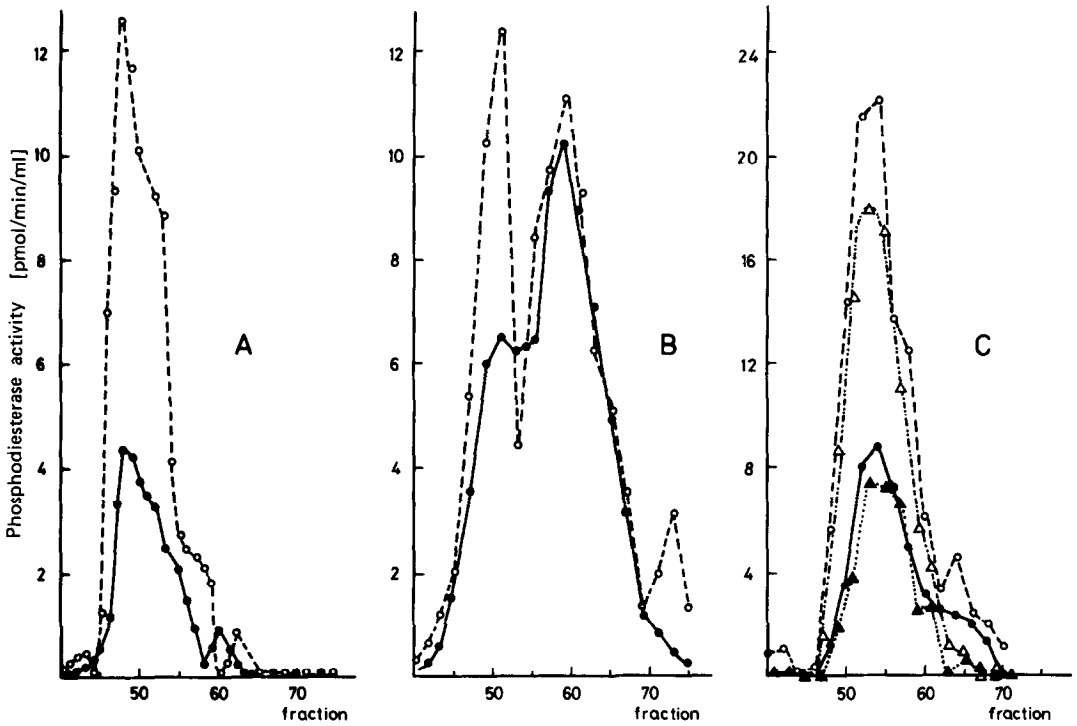

Fig. 2. Activation by Ca^{2+}/calmodulin of the form I phosphodiesterase of dunce^{M11} adult heads and larval brain, and Canton-S larval brain. Heads (10 mg) in 0.7 ml buffer and about 300 larval brains in 0.6 ml buffer were homogenized. Protein in the homogenates was about 1.6 and 0.8 mg/ml for heads and brains, respectively. The head homogenate was diluted fourfold with elution buffer and 1 ml of the 10^5 x g supernatant was loaded on the Sephacryl S-200 column. With larval brains 0.4 ml of the 10^5 x g supernatant was put on the column without dilution. Flow rate: 6.0 ml/hour; fraction volume 0.30 ml. Each point is the mean of duplicate activity assays at 1 µM cyclic nucleotide concentration. Full symbols: the assay buffer also contained 1 mM EGTA; open symbols: the assay buffer also contained 1.5 mM $CaCl_2$, 4 µg/ml calmodulin and 1 mM EGTA. A, dunce^{M11} heads, cyclic AMP hydrolysis; B, Canton-S larval brain, cyclic AMP hydrolysis; C, dunce^{M11} larval brain, ●, ○: cyclic AMP hydrolysis, ▲, △: cyclic GMP hydrolysis. Recoveries of enzyme activity from the column were (without calcium activation): 55, 60 and 87 % for A, B and C in cyclic AMP hydrolysis and 70 % in C for cyclic GMP hydrolysis.

arrested by phenylmethylsulfonyl fluoride. It should be mentioned that a time-dependent decrease in the degree of calcium activation has also been observed with bovine brain phosphodiesterase, and is accompanied by an oligomer-monomer transition (12).

In light of the above data the strategy for detecting the Ca^{2+}/calmodulin sensitivity of the two phosphodiesterase isoenzymes was at hand. The gel-chromatography was accelerated so that activity profiles could be measured within 5 hours after homogenization. Fig. 2 demonstrates

that, indeed, phosphodiesterase-I (but not form II) is activated by Ca^{2+}/calmodulin. The apparent molecular weight of the activatable form I enzyme was very similar to that of the non-activatable form I enzyme seen in Fig. 1. In Fig. 2A the calcium activation is evident in head extract of dunce^{M11} flies, which contain only phosphodiesterase-I. Fig. 2B and C show the profiles from larval brain extracts of Canton-S and dunce^{M11} strains, respectively. The poor resolution with Canton-S is due to the high flow rate and resembles the sucrose density patterns obtained by others (5). Nevertheless, it is clear that two phosphodiesterases are present in the brains of wild type larvae and that only form I is activated by calcium. Furthermore, the larval brain phosphodiesterase-I of dunce^{M11} is equally active toward cyclic GMP, like the enzyme from whole animals (1,2), and cyclic GMP hydrolysis is also enhanced by Ca^{2+}/calmodulin.

While completing this manuscript, we noticed the very recent paper by Kauvar (14) on Drosophila phosphodiesterases. He has detected a labile form III enzyme that is specific for cyclic GMP. More importantly, in the present context, he also observed the loss of Ca^{2+}-activatability, described above, but attributed it to irreversible activation by proteolysis. We observed a decrease, rather than an increase, of basal activity during aging of the form I enzyme (Table 1). The source of this discrepancy is not yet clear: possibly an irreversible activation (by Ca^{2+}-desensitization) was superimposed on an inactivation under our conditions.

Our demonstration of the two forms (I and II) of cyclic nucleotide phosphodiesterase in the actual brain tissue of Drosophila melanogaster larvae encourages attempts to define the exact ultrastructural location of the form II enzyme within the insect's nervous system, in view of the impaired learning ability of Drosophila mutants lacking this enzyme.

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