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Microcompartmentation of cAMP in Wild-Type and Memory-Mutant dunce Strains of Drosophila melanogaster

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The "enzyme-probe" method [Solti M, Friedrich P: Eur J Biochem 95:551, 1979] has been applied to characterize the cyclic AMP pool in wild-type Canton-S and memory-mutant dunce^{M11} strains of Drosophila melanogaster. The kinetics of cyclic AMP breakdown in whole fly homogenates by endogenous cyclic nucleotide phosphodiesterase(s) indicate that the cyclic AMP pool is divided into free and bound fractions. The bound fraction in Canton-S and dunce^{M11} is 0.5 and 1.5 pmole/mg fly, respectively. Considering the total cyclic AMP content of the two strains, 1.6 and 10 pmole/mg fly, respectively, we conclude that the bulk of excess cyclic AMP in the mutant is free nucleotide.

Key words: cAMP, cAMP-dependent protein kinase, phosphodiesterase, microcompartmentation, Drosophila, dunce, memory-mutant

One type of microcompartmentation of metabolites in living cells ensues from binding to macromolecules, mostly proteins, when the metabolite pool is divided into free and bound fractions [1,2]. Such pool heterogeneities may have functional significance, especially if equilibration between free and bound species is slow: binding to a macromolecule sequesters the metabolite and thereby buffers its action. An experimental approach has earlier been devised to detect pool heterogeneities of this kind, based on the kinetics of decay of the metabolite in question under the effect of an exogenous, or liberated endogenous, degradative enzyme [3].

The dunce mutant strains of Drosophila melanogaster are deficient in associative [4–6] and nonassociative [7] learning, exhibit various degrees of female sterility [8], and have diminished activity of a cAMP-phosphodiesterase isoenzyme (PDE-II) accompanied by elevated cAMP levels [9,10]. In several other respects including behavioral, developmental, and electrophysiological parameters dunce mutants appeared to be normal [4]. While the dunce mutation seems to be localized in the structural gene of PDE-II [11,12] and it is surmised [cf 13] that the abnormal cAMP metabolism is the sole factor underlying the memory deficiency, in female sterility

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the genetic background, at least in one dunce allele, has been claimed to be more complex [14].

An intriguing question about dunce strains is how they can exist with cAMP levels six times the normal (about 10 μM bulk concentration in the whole fly) with as few phenotypic consequences as the memory and egg-producing deficiencies. To get an insight into cAMP microcompartmentation we have compared the kinetics of decay of cAMP by endogenous PDEs in homogenates of wild-type Canton-S and the amorphic mutant dunce^{M11} flies.

MATERIALS AND METHODS

Drosophila Strains

Canton-S wild-type and dunce^{M11}, amorphic with respect to cAMP-specific PDE [10], were grown on standard cornmeal medium at 25°C. The PDE isoenzyme patterns of the strains had been checked previously [15]. For the analyses wild-type and selected hemizygous (male) mutant flies were used. The olfactory learning of the two fly populations was tested as described by Quinn et al [16]. Menthol and geraniol were applied as olfactory cues and shock reinforcement on the electric grids was 70 V AC, 50 Hz. The learning index, Λ, which is defined as the fraction of flies avoiding the odorant previously associated with electric shock minus the fraction of flies avoiding the control odorant, was 0.43 $\pm$ 0.07 for Canton-S and 0.06 $\pm$ 0.04 for dunce^{M11}. These values are in agreement with earlier reports [4,9,10].

Measurement of cAMP Decay in Homogenate

Whole flies were homogenized at 100 mg fly/ml in 20 mM Tris/hydroxymethyl/aminomethane-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$ and 2 mM β-mercaptoethanol, in a motor-driven glass-Teflon homogenizer for 30 sec at 0°C. The start of homogenization was taken as 0 time and samples were withdrawn from the homogenate kept at 0°C into 6.6% trichloroacetic acid (TCA) for cAMP assay at various times. The 0 time values were obtained from flies homogenzed directly in 10% TCA. Before homogenization flies were anesthetized by a brief and mild exposure to diethylether or by nitrogen gas. Prolonged ether treatment diminished cAMP levels, especially in the mutant (unpublished observation). In our practice, mildly etherized flies had the same cAMP content (0 time value) as had flies anesthetized with nitrogen or flies directly dropped into liquid nitrogen.

cAMP determination was performed by radioimmunoassay according to Brooker et al [17]. Samples were treated with Al_2O_3 (neutral, Brockmann II) to eliminate interference by endogenous ATP, according to a batchwise modification (T. Szentendrei, P. Serfőző, unpublished) of White's [18] procedure. The standard cAMP calibration series, prepared for each experiment, was similarly treated with alumina to eliminate errors that might stem from this adsorption procedure. Very dilute cAMP samples were acetylated [17], which increased sensitivity about 80-fold. Succinyl-cAMP-tyrosyl methyl ester was purchased from Sigma and was iodinated with $Na^{125}I$ + chloramine T as described by Steiner et al [19]. Antisera against succinyl-cAMP coupled to human albumin produced in goats [17] were kindly supplied by Dr A. Seregi (Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest).

Adenylate cyclase activity was measured by determining cAMP by radioimmunoassay. The original procedure [17] was modified in that the fly homogenate described above served as the basis of reaction mixture and steady-state ATP level (~1 mM) was maintained, instead of the creatine phosphate-creatinephosphokinase system, by continuous input from a concentrated ATP-Mg^{2+} solution, with vigorous stirring. Basal adenylate cyclase activity of the homogenate was ~0.4 pmole cAMP/mg of protein at 30°C. At 0°C the activity was practically nil. Protein was determined according to Lowry et al [20].

RESULTS

The time course of cAMP decay in fresh homogenates of Canton-S and $dunce^{M11}$ flies is shown in Figure 1. The initial cAMP concentration was 1.6 ± 0.3 and 10.3 ± 1.7 pmole/mg fly for Canton-S and $dunce^{M11}$, respectively. For both types of fly

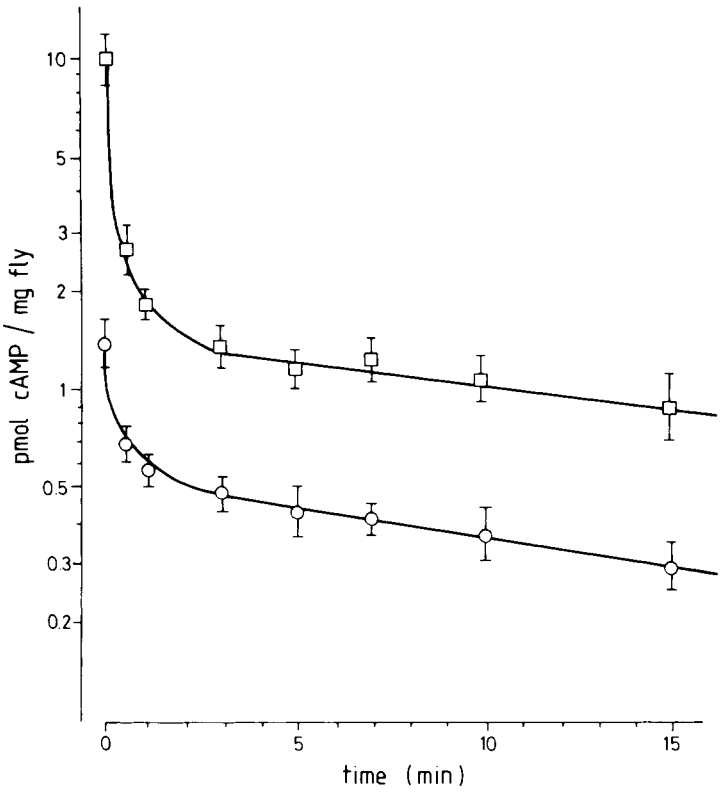

Fig. 1. Time course of cAMP decay in homogenates of Canton-S and $dunce^{M11}$ flies. The homogenate (100 mg fly/ml) was made and incubated at 0°C; at the times indicated samples were withdrawn for cAMP radioimmunoassay as described in Methods. The mean ± SD of seven independent experiments is shown for both Canton-S (○) and $dunce^{M11}$ (□). In the individual experiments each point was obtained by averaging the cAMP contents of three different volumes (as a rule: 10, 20, and 30 μl), in duplicates, of the Al_2O_3-treated supernatant.

cAMP decrease was biphasic, consisting of a rapid phase, too fast to be analyzed, and a much slower one. The slow phases were kinetically of apparently first order with rate constants k^I of 4.5 ($\pm$ 0.5) $\times$ 10^{-2} min^{-1} and 4.2 ($\pm$ 0.6) $\times$ 10^{-2} min^{-1} for Canton-S and $dunce^{M11}$, respectively; ie, they agreed within the limits of error. The decline of rapid phase was not due to inactivation of PDE in the homogenate as witnessed by the fast disappearance of exogenous cAMP added in the slow phase (Fig. 2A). Furthermore, the observed time course could not follow from the kinetic properties of PDEs, as indicated by the first-order decay of exogenous cAMP in a 20-fold diluted fly homogenate down to cAMP concentrations well below the onset of slow phase in Figure 1 (Fig. 2B). The slope in the slow phase could not be influenced by cAMP production in the homogenate, since 1) if homogenization was performed in the presence of 20 mM theophylline, a PDE inhibitor, cAMP level remained constant over the time period examined, and 2) there was no detectable adenylate cyclase activity in the homogenate at 0°C (data not shown).

It follows that in the slow phase cAMP decay is limited not by PDE action but rather by the release of cAMP from binding site(s). To test this explanation, Canton-S flies were homogenized in buffer containing a 20-fold molar excess of cAMP over endogenous cAMP and then the decay was followed (Fig. 3). It is seen that exogenous cAMP elevated the ordinate intercept of the slow phase but did not change its slope. This finding corroborates the notion that in the slow phase a reversibly cAMP-binding component of the homogenate is being stripped of the cyclic nucleotide.

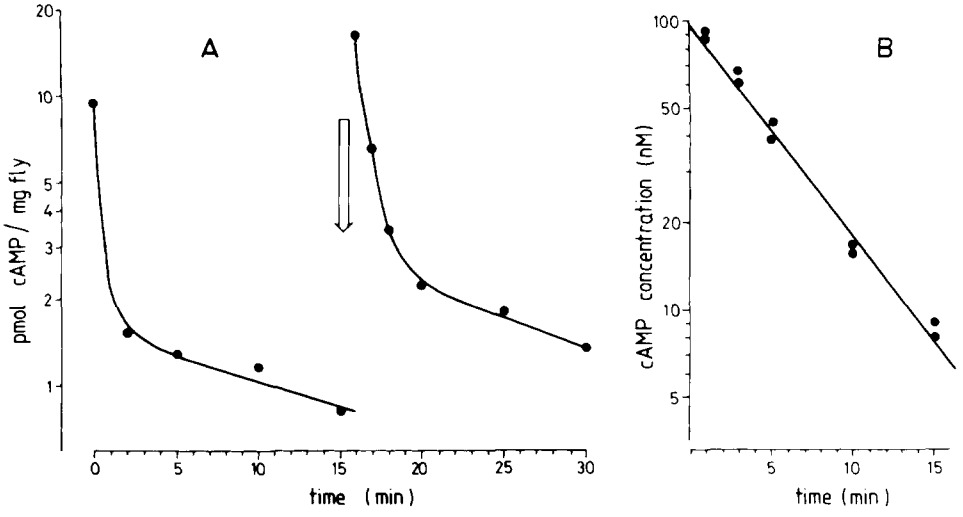

Fig. 2. Checking of phosphodiesterase activity in the homogenate by exogenous cAMP. A) The experiment, with $dunce^{M11}$ flies, was the same as in Figure 1, but at 15 min (arrow) exogenous cAMP (20 pmole/mg fly) was added to the homogenate. B) Decay of 100 nM exogenous cAMP by 20-fold diluted fly homogenate (5 mg fly/ml). Note that in A, as well as in Figure 1, 1 pmole/mg fly corresponds to 100 nM cAMP concentration.

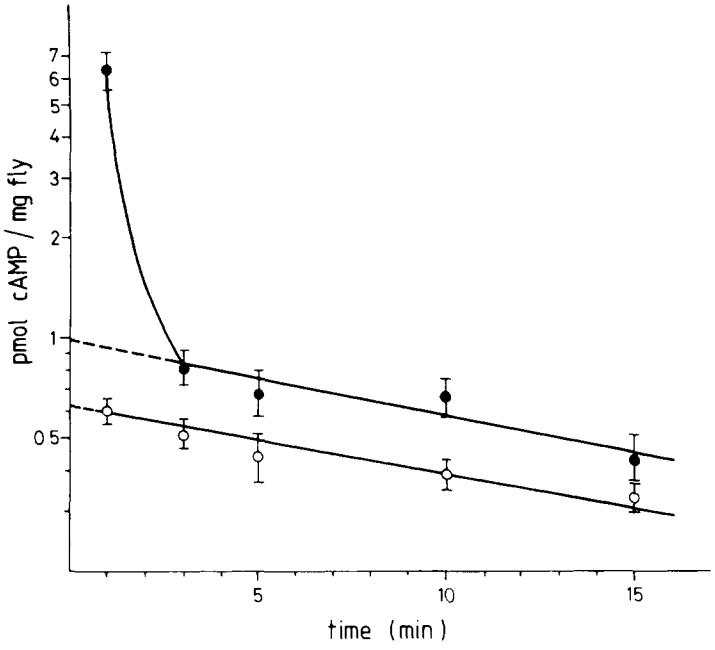

Fig. 3. Effect of exogenous cAMP on the slow phase of cAMP decay in homogenate of Canton-S flies. One homogenate (○) was prepared as in Fig. 1. The other homogenate (●) was the same except that 30 pmole of cAMP/mg fly was included in the homogenizing buffer. The mean ± SD of three independent experiments is shown.

DISCUSSION

The strongly biphasic cAMP decay curves of Figure 1 indicate that part of the cAMP pool is in the bound state. The most likely candidate for a cAMP-binding species is the regulatory (R) subunit of cAMP-dependent protein kinase (cAMP-PK). In fact, Tsuzuki and Kiger [21] have estimated the dissociation rate constant of the R.cAMP complex from Drosophila to be 6.6×10^{-2} min^{-1} at 0°C. The good agreement between this value and those found in our experiment for the slow phase supports the assumption that the cAMP-binding component responsible for the slow phase in the R subunit of cAMP-PK. (Dissociation of cAMP from the holoenzyme is apparently rapid [21] and therefore the ternary complex cAMP.R.C cannot be distinguished from free nucleotide in the present analysis.) If this holds true, then extrapolation of the straight lines in Figure 1 to 0 time gives the amount of R.cAMP complex in the intact flies. The ordinate intercepts for Canton-S and $dunce^{M11}$ are 0.55 and 1.5 pmole/mg fly, respectively. Consequently, in the mutant there is about three times as much R.cAMP complex as in the wild type.

It should be mentioned that Tsuzuki and Kiger [22] have detected a cAMP-binding protein other than the R subunit in Drosophila extract. This protein had two orders of magnitude lower affinity toward cAMP than did the R subunit. The involvement of this enigmatic protein in the slow phase observed by us cannot be

excluded. However, its markedly lower affinity toward cAMP makes it unlikely that it would contribute significantly to the binding capacity offered by R subunits in our experimental setup.

The observation that exogenous cAMP elevated the ordinate intercept of the slow phase of Canton-S (Fig. 3) is in keeping with the expectation that more cAMP will produce more R.cAMP complex. It should be noted that the elevation of slow phase in Figure 3 is hardly twofold, less than the difference found between Canton-S and dunce^{M11} strains. This may look controversial since in the experiment of Figure 3 about twice as much cAMP was added in the homogenate as is present in dunce^{M11} flies. However, it must be recalled that PDEs rapidly depress the initial high cAMP level. What we observe is, we think, the outcome of two opposing processes: binding to the cAMP-PK holoenzyme and decay by PDEs of cAMP. Tsuzuki and Kiger [21] have deduced from the estimated rate constant ratios that about 95% of the ternary complex RC.cAMP, where C stands for the catalytic subunit of cAMP-PK, reverts to RC + cAMP rather than giving rise to R.cAMP and C. Therefore, even if the concentration of ternary complex is markedly increased by exogenous cAMP, only a small fraction of the holoenzyme will break up; hence the moderate upward shift of the slow phase in Figure 3. The fact that in dunce^{M11} a smaller excess of endogenous cAMP causes a greater upward shift in the slow phase than in the model experiment with Canton-S points to the importance of cellular compartmentalization in cAMP metabolism. Indeed, the latter must impose severe diffusional restrictions; otherwise, already at the "bulk" cAMP concentration existing in Canton-S flies (about 2 μM), the cAMP-dependent protein kinase would be fully activated (K_m for cAMP: 0.05 μM; K_d for R.cAMP: $\sim$ 1 nM [21]).

The raised level of bound cAMP in dunce^{M11} probably reflects the greater degree of activation of cAMP-PK in this strain. This would be in accord with our earlier finding that the labeling in vivo of some phosphoproteins is higher in dunce strains relative to Canton-S [23]. However, the same phenomenon may represent a compensatory mechanism in dunce^{M11}: by synthesizing more R subunit the organism may adapt to a permanently elevated cAMP level. Nevertheless, sequestration by binding of cAMP can only be part of such a mechanism, as it copes merely with a small fraction of extra cAMP. Therefore it seems probable that the neutralization of excess of cAMP also occurs at other levels, possibly through the control of protein dephosphorylation. If high cAMP level is indeed responsible for the dunce phenotype, it remains to be elucidated why the compensatory mechanism is insufficient only in ovarian cells and certain neurons.

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