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ACCELERATED COMMUNICATION

Rat Homologs of the *Drosophila dunce* Gene Code for Cyclic AMP Phosphodiesterases Sensitive to Rolipram and RO 20-1724

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SUMMARY

The *dunce* locus of *Drosophila melanogaster* codes for a low K_m, cAMP phosphodiesterase. The correct function of this gene is required for normal learning and memory activity in flies, because *dunce* mutants fail in tests of behavioral conditioning. These observations have indicated that cAMP regulation is an important aspect of the biochemistry underlying learning and memory processes in insects. To determine whether the locus is functionally conserved in mammals, we have expressed *dunce* gene homologs from the rat in a yeast expression system. We find that the rat homologs encode low K_m, cAMP phosphodiesterases similar to that coded for by the *Drosophila dunce*$^+$ gene and, more importantly, that the mammalian enzymes are inhibited by rolipram and RO 20-1724, drugs with antidepressant properties. Surprisingly, the *dunce*-encoded phosphodiesterase was not inhibited by rolipram or RO 20-1724. These findings suggest that the phosphodiesterases, through their regulation of cAMP levels, influence learning and memory in insects and mood in mammals.

The *dunce* gene of *Drosophila melanogaster* was the first identified and is the best characterized of the set of genes known to function in learning and memory processes in insects. Flies carrying mutations at the locus perform poorly in a variety of conditioning paradigms, using both negative and positive reinforcement and both visual and olfactory cues (1–5) (see Ref. 6 for a recent review). Data obtained from biochemical, genetic, and molecular analyses (7–10) have revealed that the *dunce* gene codes for a low K_m, cAMP-specific PDEase, an enzyme essential for the regulation of cAMP levels in cells. Indeed, *dunce* mutant flies have up to a 7-fold increase in cAMP levels (8). These studies have indicated that normal cAMP regulation is an indispensable part of the biochemistry underlying various types of learning and memory, a conclusion also reached from elegant studies of learning and memory in the sea snail, *Aplysia californica* (11–13). Although the significant role of cAMP metabolism in invertebrate learning and memory is clear, it remains unknown whether this function extends to vertebrates.

To study the evolutionary conservation of the *Drosophila dunce* gene, we isolated and sequenced a homologous cDNA clone named RD1 from a rat brain library, by cross-hybridization with a *Drosophila* probe (14). The amino acid sequence predicted from RD1 is strikingly homologous to the *Drosophila dunce* gene product, with 76% identical amino acids within a conserved region of approximately 275 residues found in most types of cyclic nucleotide PDEases (15, 16) and with more limited homology within a region just N-terminal to the conserved domain (Fig. 1). Independently, Colicelli *et al.* (17) isolated a second homologous cDNA clone from rat brain, named DPD, by virtue of its suppressing properties of phenotypes associated with a yeast *RAS2*^{val19} mutation. The sequence of DPD predicts a protein as homologous to the *Drosophila* sequence as that predicted from RD1 (Fig. 1). Although RD1 and DPD exhibit exceptional homology to each other, they are clearly the products of separate genes. Ninety-one percent of the amino acids are identical within the conserved domain and 67% in the upstream region, but regions of identical residues are separated by scattered amino acid differences and many of the amino acid identities use different codons (14, 17). Thus, although *Drosophila* has a single *dunce* gene, the rat has at least two and probably several other (18) *dunce*-like genes.

The remarkable homology of the predicted products of RD1 and DPD to the *Drosophila dunce*-encoded product implies that these cDNAs probably represent RNAs coding for PDEases similar in properties to the *Drosophila* cAMP PDEase. Enzymes

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ABBREVIATIONS: PDEase, phosphodiesterase; DPD, *dunce*-like phosphodiesterase; RD1, rat *dunce* 1; cG-I PDEase, cGMP-inhibited phosphodiesterase.

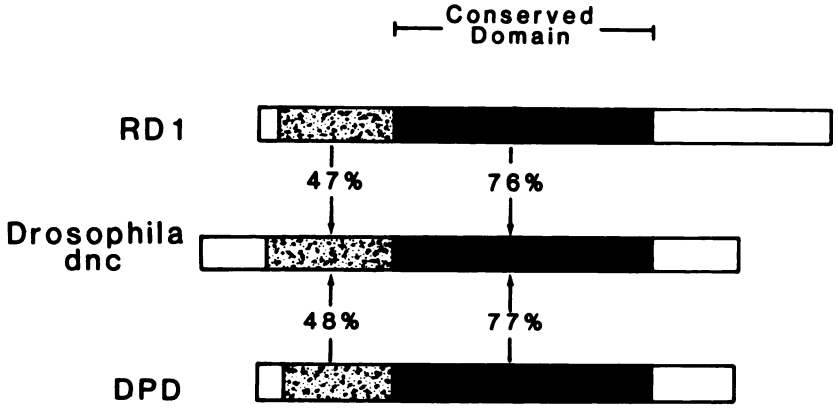

Fig. 1. Schematic diagram of the protein sequences of cAMP PDEases predicted from the nucleic acid sequences of RD1, the *Drosophila dunce* gene (*dnc*), and the rat *dunce*-like clone (DPD). Regions of homology have identical *fill patterns* and the absence of a fill pattern indicates no detectable similarity. The percentage of amino acid sequence identity of RD1 and DPD with *Drosophila dunce* is indicated for the two homologous regions, calculated from published sequences in Refs. 10, 14, and 17. The first methionine within the open reading frame of cDNA clones representing each of the three genes has been chosen as the N-terminus of the three proteins.

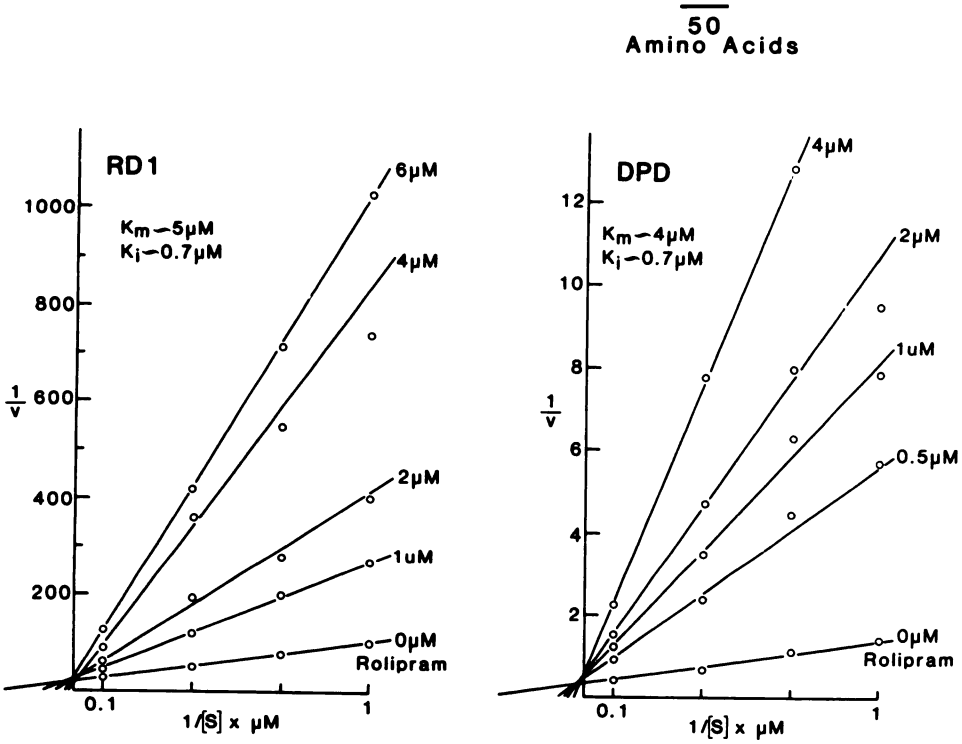

Fig. 2. Representative double-reciprocal plots of cAMP PDEase activity and its inhibition by rolipram in extracts of yeast expressing RD1 or DPD. The calculated K_m and K_i values are the average of at least three separate experiments (see Table 1). Velocities are expressed as nanomol of cAMP hydrolyzed/min of incubation/mg of protein in the yeast extracts. The DPD-containing extracts exhibited 50–100 times more activity than extracts with RD1, which may reflect differences in the catalytic efficiency of the two enzymes or their ability to be expressed in yeast.

TABLE 1

Apparent K_m and K_i values for various PDEs

In cases where three or more independent determinations were made (numbers in parentheses), the values reported are the means ± the standard errors. Other values were determined in two separate experiments.

Enzyme source	K_m		K_i			
	cAMP	cGMP	Rolipram	RO 20-1724	Fenoximone	cGMP
	μM		μM			
Drosophila cAMP PDE	2.0[a]	Undetectable at 0.1 mM[b]	>100	>100	>100	>3000[c]
RD1	4.1 ± 1.1 (4)	Undetectable at 1 mM	0.7 ± 0.1 (3)	4.2 ± 0.6 (3)	>100	>2000
DPD	2.9 ± 0.5 (4)	Undetectable at 1 mM	0.5 ± 0.1 (3)	1.7 ± 0.2 (3)	>100	>2000

[a] Ref. 9
[b] Ref. 29
[c] Ref. 25

of the low K_m, cAMP PDEase family have been identified in biochemical experiments with mammalian brain and other tissue homogenates and appear to consist of several subtypes (16, 19–21). They hydrolyze cAMP with K_m values in the low micromolar range, and at least some of these enzymes are specifically and potently inhibited by the drugs RO 20-1724 ($K_i \sim 5\ \mu M$) and rolipram ($K_i \sim 2\ \mu M$), compounds that exhibit antidepressant properties (22). To determine whether RD1 and

DPD code for RO 20-1724/rolipram-inhibited, low K_m, cAMP PDEases, we expressed the cDNA clones in yeast and evaluated the biochemical properties of the encoded PDEase activity.

Materials and Methods

For the expression of the RD1 sequence, the RD1 cDNA was inserted by blunt-end ligation into the *Sac*II site of the yeast expression vector pADNS (17), between the yeast alcohol dehydrogenase promoter and terminator sequences. This RD1 construct, the DPD cDNA in pADNS (17), and the pADNS vector were each used to transform the yeast strain 10DAB, [10DAB = MATα *leu2 his3 ura3 ade8 pde1::ADE8 pde2::URA3 ras1::HIS3*]. This strain contains insertional mutations in each of its two PDEase genes (17), which allows for the assay of plasmid-encoded PDEase activity in the absence of any interfering host cell activity. The transformed yeast cells were incubated at 30° overnight in 4 ml of minimal medium plus amino acid supplements (23). These small cultures were then used to innoculate 500-ml cultures, which were grown for 36 to 42 hr. The yeast were washed and resuspended in 30 ml of homogenization buffer used for PDEase assays (24) plus 50 μl of 1 M phenylmethylsulfonyl fluoride. The resuspended cells were passed through a French press at 20,000 psi and the broken cells were centrifuged at 25,000 × *g*. The supernatants were aliquoted and stored at −70° until needed. PDEase assays were performed as previously described (24). Fenoximone was a gift from J. Beavo (University of Washington), rolipram was a gift from Schering AG (Berlin), and RO 20-1724 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

Results and Discussion

Cyclic AMP PDEase activity was readily detected in extracts of yeast containing an expression plasmid with RD1 or DPD inserts, but no activity was detected in control extracts containing the plasmid without an insert (Fig. 2). The activity detected was of the high affinity type as analyzed by double-reciprocal plots of the assays, with K_m values for cAMP of approximately 4 and 3 μM for extracts containing RD1 and DPD, respectively (Fig. 2). Our estimate of the K_m for DPD is in good agreement with that determined by Colicelli *et al.* (17). Cyclic GMP PDEase activity was not detected in either extract with up to 1 mM substrate, at the enzyme concentrations used for cAMP PDEase determinations (Table 1). Most importantly, the drugs rolipram and RO 20-1724 inhibited competitively both enzymes with submicromolar K_i values (Fig. 2; Table 1). Thus, RD1 and DPD both code for low K_m, cAMP-specific PDEases that are inhibitable by the drugs rolipram and RO 20-1724.

The inability of the RD1- and DPD-encoded enzymes to hydrolyze cGMP argued against the possibility that the clones coded for another type of low K_m PDEase known as the cG-I PDEase, an enzyme with both cGMP and cAMP hydrolytic activity (16). This is important because the cG-I PDEase and the RO 20-1724/rolipram-inhibited forms have been unknowingly co-isolated in past biochemical purifications, and subsequent characterization of the mixed activities has produced some confusion about the individual properties of each. To eliminate any doubt regarding the nature of the enzymes, we tested the sensitivity of the cAMP PDEase activities to cGMP and to fenoximone, a drug known to specifically inhibit the cG-I PDEases with a K_i of approximately 2.0 μM (16). Neither fenoximone nor cGMP inhibited the cAMP PDEase activity present in the extracts (Table 1), showing conclusively that the enzymes do not belong to the cG-I family.

To determine whether the cAMP PDEase encoded by the *Drosophila dunce* locus shares the rolipram/RO 20-1724 sensitivity of its mammalian counterparts, we measured cAMP PDEase activity in crude fly homogenates in the presence of the drugs (Table 1). These assays were performed in the presence of excess cGMP, which inhibits the Ca^{2+}/calmodulin-regulated cyclic nucleotide PDEase in the homogenates and allows for the specific determination of the cAMP-specific form (24, 25). Surprisingly, neither rolipram nor RO 20-1724 inhibited the *Drosophila* enzyme. Therefore, rolipram and RO 20-1724 do not inhibit the low K_m, cAMP-specific PDEases of all species. An attractive hypothesis to explain this difference is that the drug sensitivity was acquired by sequence changes of the vertebrate progenitor gene after the separation of vertebrates from invertebrates (approximately 600 million years ago) but before the expansion of the progenitor gene into a gene family, represented currently by the two known members RD1 and DPD.

We conclude that the mammalian homologs of the *Drosophila dunce* gene, RD1 and DPD, code for low K_m, cAMP PDEases with kinetic properties similar to those of the *Drosophila* PDEase. In addition both of the rat brain PDEases are inhibited by rolipram and RO 20-1724, drugs with antidepressant properties. In clinical trials, rolipram has been evaluated as a good to very good drug for the treatment of depression, even though many of the patients tested were refractory to other types of antidepressant therapy (26, 27). It is intriguing that the genetic elimination of cAMP PDEase in *Drosophila* causes learning and memory dysfunction, whereas its pharmacological inhibition in humans may elevate mood. If the antidepressant effects of rolipram prove to be due to PDEase inhibition, as has been suggested (22, 26–28), this would imply that both the *Drosophila* cAMP PDEase and its mammalian counterparts are significant players in the biochemistry underlying learning/memory in insects and the regulation of mood in mammals.

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