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Loss of Calcium/Calmodulin Responsiveness in Adenylate Cyclase of *rutabaga*, a Drosophila Learning Mutant

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Summary

We have isolated and mapped an X-linked recessive mutation in Drosophila that blocks associative learning, and have partially characterized it biochemically. The mutation affects adenylate cyclase activity. Cyclase activity from mutant flies differed from the wild-type enzyme in that it was not stimulated by calcium or calmodulin. Mutant cyclase activity did respond to guanyl nucleotides, fluoride, and monoamines, which suggests that the defect is neither in the hormone receptor nor in either known GTP-binding regulatory protein. The mutation possibly affects the catalytic subunit directly. We postulate that there is at least one other type of adenylate cyclase activity that is unaffected by the mutation and insensitive to calcium/calmodulin.

Introduction

We isolate Drosophila melanogaster mutants that cannot learn and then try to use them to dissect genetically the key biochemical steps involved in learning and memory. Drosophila is the most sophisticated organism in which specific mutations can easily be generated and selected. Fruit flies show associative learning (Quinn et al., 1974) as well as simpler forms of behavioral plasticity (Duerr and Quinn, 1982). Five single gene X-linked mutations have been isolated that either block associative learning or shorten memory (Dudai et al., 1976; Quinn et al., 1979; Aceves-Piña et al., 1983). These mutants not only fail to learn in the negatively reinforced olfactory discrimination test used to select the mutants, but they also show deficient learning or memory in an olfactory discrimination test using positive (sucrose) reinforcement (Tempel et al., 1983), in an operant leg-flexion task (Booker and Quinn, 1981), in a visual discrimination task (Folkers, 1982; see, however, Dudai and Bickers, 1978), and in tests of experience-dependent effects on courtship (Siegel and Hall, 1979; Gailey et al., 1982, 1984). Furthermore, the learning mutants show abnormal habituation, sensitization, or both (Duerr and Quinn, 1982), suggesting that these simpler nonassociative forms of behavioral plasticity involve at least some of the mechanisms involved in associative learning since they require the same gene products.

Studies in many systems have strongly implicated one biochemical pathway as important for synaptic plasticity: that which regulates intracellular cyclic AMP and the consequent protein phosphorylations (Greengard, 1978; Thompson et al., 1983; Hawkins et al., 1983; Walters and Byrne, 1983). The information from Drosophila mutants has thus far been consistent with this. The first learning mutant isolated, *dunce,* is missing one form of the enzyme cyclic AMP phosphodiesterase (Byers et al., 1981). Preliminary results on a second learning mutant, *turnip,* suggest that it has defective monoamine receptors secondary to an alteration in a GTP-binding protein (Choi, Smith, Sziber, and Quinn, unpublished data). Mutants at a third locus, *Ddc,* originally isolated as cuticular mutants, have greatly reduced levels of the enzyme dopa decarboxylase (Wright et al., 1982) and cannot synthesize either dopamine or serotonin (Livingstone and Tempel, 1982). *Ddc* mutant alleles show a learning defect proportional to the defect in brain enzyme (Tempel et al., 1984). Since in Drosophila, as in most other organisms, monoamines affect adenylate cyclase activity (Uzzan and Dudai, 1982), *Ddc* mutations should indirectly alter cyclic AMP metabolism. Here we describe a fourth mutation, *rutabaga,* isolated as a learning mutant, that also affects cyclic AMP metabolism, specifically calcium-dependent stimulation of cyclic AMP synthesis.

Results

Isolation of the *rutabaga* Mutation and Characterization of its Behavior

Wild-type males were fed the mutagen ethyl methanesulfonate (Lewis and Bacher, 1968). Seven hundred and fifty stocks with male progeny isogenic for the mutagenized X chromosomes were bred from them using attached-X females and then tested for learning ability in the negatively reinforced odor-discrimination task of Quinn et al., 1974. *rutabaga (rut)* was the 511th such stock tested. Homozygous *rutabaga* flies showed no learning in the standard negatively reinforced task, but heterozygous *rut*/+ flies showed only a slight reduction in learning (Table 1).

Even though this is the first formal description of the *rutabaga* mutation, it was isolated eight years ago and has been used in several behavioral studies. Tempel et al. (1983) found that *rutabaga* flies do show some learning if positive reinforcement is used ($\lambda = .13 \pm .04$ for *rutabaga;* $\lambda = .34 \pm .02$ for wild type), but this learning decays at least 25 times faster than in wild-type flies, suggesting that the mutation may affect memory. Duerr and Quinn (1982) found that *rutabaga* flies are also abnormal in nonassociative forms of learning; they habituate less than wild-type flies to repeated sucrose presentation, and the sensitization of the proboscis-extension reflex after application of a concentrated sucrose solution decays abnormally rapidly.

Adenylate Cyclase Levels

We measured adenylate cyclase in *rutabaga* and other previously isolated learning mutants both to screen for defects in monoamine receptors and to screen for defects

in the enzyme itself or its multiple regulatory subunits. We first assayed basal levels of adenylate cyclase in crude homogenates of heads and abdomens of learning mutants and several other behaviorally interesting mutants (Table 2). Only one of the mutants, *rutabaga,* had strikingly abnormal adenylate cyclase, and the levels were most abnormal in the abdomens. This tissue distribution was a surprising but consistent finding and may explain why *rutabaga* adenylate cyclase levels were previously reported to be essentially normal (Uzzan and Dudai, 1982). It would be highly counterintuitive to find that the primary effect of a mutation that blocks learning was restricted to the abdomen or nón-neuronal tissues, but, as we will describe later, a similar qualitative defect can be seen in both the head and the abdominal enzyme activity.

When we measured separately the supernatant and pellet from a 10 min 178,000 g centrifugation, the *rutabaga* flies had normal levels of supernatant activity but reduced levels of particulate activity, especially in the abdomen (Table 3). The reduction in particulate activity was largest in the abdomen, but there was also a small but reproducible decrease in the particulate activity in the head and thorax. The effect of the mutation did not seem to be restricted to any specific tissue in the abdomen, because separately assayed gonads, intestines, and abdominal wall from *rutabaga* flies all showed consistently lower levels of adenylate cyclase than the same tissue from wild-type flies.

Table 1. Learning in *rutabaga* and Wild-Type Flies

Genotype	Learning Index (λ)
+/+	.31 ± .02 n = 15
rut/rut	0.00 ± .02 n = 15
rut/+	.21 ± .03 n = 8

Learning was tested exactly as described by Quinn et al., 1974.

Table 2. Adenylate Cyclase Activity in Some Behavioral Mutants

	Adenylate Cyclase Activity	
Genotype	Heads	Abdomens
Canton-S (wild type)	3.86 ± .08 (100%)	.40 ± .02 (100%)
rutabaga	2.67 ± .04 (69%)	.15 ± .01 (38%)
dunce	3.26 ± .13 (84%)	.42 ± .02 (105%)
amnesiac	4.30 ± .30 (111%)	.51 ± .06 (128%)
cabbage	3.56 ± .11 (92%)	.36 ± .05 (90%)
turnip	3.01 ± .05 (78%)	.35 ± .02 (88%)
hyperkinetic	3.45 ± .30 (89%)	.40 ± .01 (100%)
per^0	3.78 ± .13 (98%)	.33 ± .09 (83%)

Adenylate cyclase activity was measured in crude homogenates of heads or abdomens from male flies in the presence of 0.2 mM ATP without any added guanyl nucleotides or other ligands. Activity is expressed as pmoles of cyclic AMP formed/min/mg protein and as a percentage of the wild-type level.

The enzyme activity in the supernatant fraction appears to be soluble since the activity could not be further fractionated by a 3-hr centrifugation at 178,000 g: 93% of the activity in the supernatant fraction from a 10 min centrifugation was found in the supernatant fraction after a 3 hr centrifugation at 178,000 g. This soluble activity differs from the Drosophila particulate activity, which we will describe below, and from the soluble adenylate cyclase from rat testis (Braun and Dods, 1975; Neer, 1978) in that it was not stimulated by guanylyl imidodiphosphate (5×10^{-5} M), forskolin (50 μM), or manganese (5 mM). Since this activity was not affected by the *rutabaga* mutation, we have not characterized it further.

Since *rutabaga* flies had abnormally low particulate adenylate cyclase activity but normal soluble activity, we suspected that the mutation might be producing a generalized membrane defect. We therefore measured the activities of three other membrane-bound enzymes: sodium/potassium-dependent ATPase, magnesium-dependent ATPase, and guanylate cyclase. All three of these enzymes were normal in *rutabaga* flies in both heads and abdomens.

In crude homogenates the *rutabaga* adenylate cyclase showed altered enzyme kinetics, namely, a 3-fold lower affinity for the substrate ATP as compared to the wild type. This result appears, however, to have been an artifact of using crude homogenates. The particulate enzyme had a higher affinity (Km = 72 μM) for the substrate ATP than did the soluble enzyme (Km = 420 μM). Crude homogenates of *rutabaga* abdomens have less particulate activity and thus proportionately more soluble activity. When the particulate and soluble enzymes were measured separately, the *rutabaga* enzymes had the same affinities as the wild type, but there was a much lower Vmax for the abdominal particulate fraction (V_{max} for *rutabaga* = 0.9 pmoles/min/mg protein; V_{max} for wild type = 3.1 pmoles/min/mg protein).

Table 3. Adenylate Cyclase Distribution

	Head		Thorax		Abdomen	
	Particulate	Soluble	Particulate	Soluble	Particulate	Soluble
Wild type	.64 ± .05	.028 ± .004	.84 ± .10	.1 ± .05	.61 ± .05	.16 ± .02
rutabaga	.52 ± .07	.023 ± .001	.70 ± .08	.1 ± .06	.13 ± .03	.14 ± .02

Adenylate cyclase activity was measured at 0.2 mM ATP in the absence of any added ligands and is expressed as pmoles cyclic AMP formed/min/fly. Soluble and particulate activities were separated by a 10 min centrifugation at 178,000 g.

Table 4. Mapping the *rutabaga* Mutation

Class	Learning Index
Parental chromosomes	
rut	.00 ± .02 n = 15
y cv v f car	.19 ± .04 n = 8
Recombinant chromosome markers	
y	.01 ± .01 n = 4
y cv	.00 ± .02 n = 4
y cv v	Two-populations:
	.04 ± .01 n = 6
	.22 ± .02 n = 5
y cv v f	.23 ± .02 n = 4
y cv v f car	.22 ± .03 n = 8
cv v f car	.21 ± .02 n = 4
v f car	.24 ± .02 n = 6
f car	Two-populations:
	.06 ± .02 n = 5
	.22 ± .02 n = 5
car	.05 ± .01 n = 6
No markers	.03 ± .02 n = 4

Each chromosome was tested over *rutabaga* in heterozygous females, to eliminate the effects of markers. Learning was measured as described in Quinn et al., 1974.

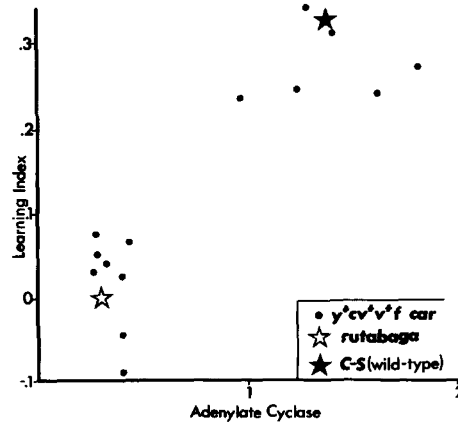

Figure 1. Cosegregation of the Failure to Learn Phenotype and the Reduced Abdominal Adenylate Cyclase Phenotype in Recombination Stocks from the Mapping Study of Table 4

The stocks in which the crossover event had occurred between *vermilion* and *forked* fell into two distinct groups: those with normal abdominal cyclase and normal learning and those with reduced cyclase and no learning. Adenylate cyclase activity is expressed as pmoles cyclic AMP formed/min/mg protein.

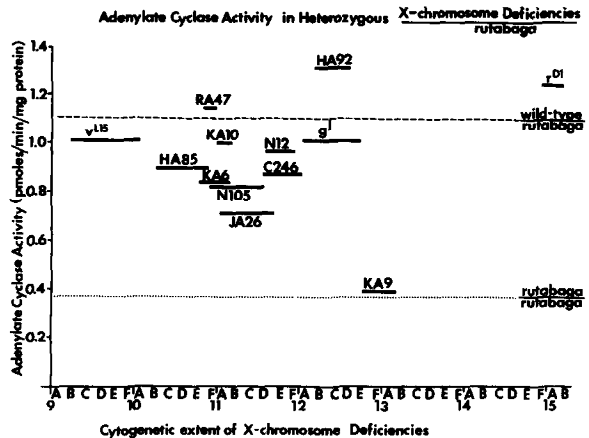

Figure 2. Adenylate Cyclase Activity in Various X Chromosome Deletions between *vermilion* and *forked* (and one deletion distal to *vermilion*)

Each deletion was tested as a heterozygote in combination with *rutabaga*. Crude homogenates of female abdomens were assayed in the absence of any added ligands. The only deletion that reduced adenylate cyclase levels as much as the *rutabaga* mutation did was *Df(1)KA9*, a deletion of bands 12E1 to 13A5 of the X chromosome.

Mapping the *rutabaga* Mutation

The approximate genetic locus of *rutabaga* was determined by conventional recombination mapping using the visible X chromosome markers *yellow* (*y*), *crossveinless* (*cv*), *vermilion* (*v*), *forked* (*f*), and *carnation* (*car*) following the procedures used for *dunce* and *amnesiac* (Dudai et al., 1976; Quinn et al., 1979). As shown in Table 4, when the original *rutabaga* chromosome was present either to the right of *vermilion* or to the left of *forked*, the flies failed to learn. Thus the *rutabaga* learning defect maps between the markers *vermilion* and *forked* on the X chromosome. When we measured the abdominal adenylate cyclase in the same recombinant stocks, the biochemical defect also mapped between *vermilion* and *forked*.

In order to rule out the possibility that the two phenotypes, the failure to learn and the reduced abdominal adenylate cyclase levels, might be because of two separate mutational events, we did the experiment shown in Figure 1. Fourteen identically marked recombinant stocks from the mapping study in Table 4 in which the crossover event had occurred between *vermilion* and *forked* (phenotype: *forked carnation*) were tested for learning ability and abdominal adenylate cyclase levels. The biochemistry and behavior were tested blindly with respect to each other. Figure 1 shows that flies with low learning ability all had low adenylate cyclase levels whereas flies with high learning had high adenylate cyclase. Thus the two phenotypes, failure to learn and reduced adenylate cyclase, cosegregated, suggesting that they are both due to the same mutation or to two independent mutations that are closely linked (at 95% confidence limits, two such mutations would be within 4 map units of each other; Stevens, 1942). Furthermore, as we described below, both phenotypes are uncovered by a single, relatively small, deletion.

We then mapped *rutabaga* at a finer resolution by measuring abdominal adenylate cyclase in a series of genetic deficiencies between *vermilion* and *forked*. When assayed as heterozygotes in combination with *rutabaga* only one of these deficiencies reduced adenylate cyclase activity as much as the *rutabaga* mutation (Figure 2). This deficiency was *KA9*, a deletion of bands 12E1 to 13A5 on

the X chromosome. The *Df(1)KA9/rut* heterozygote failed to learn (λ = .07 ± .02), even though *rutabaga* heterozygotes with neighboring deficiencies did learn. Thus the reduction in adenylate cyclase and the failure to learn were both uncovered by the *KA9* deletion. *Df(1)KA9* is homozygous lethal, but it does appear to affect the ventral nerve cord formation earlier or more severely than it affects other structures in development (Campos-Ortega and Jiménez, 1980).

Activation of Adenylate Cyclase by Various Ligands

In order to ask which subunit of the enzyme was affected by the mutation, we used various ligands known to alter the activity of mammalian adenylate cyclase via different subunits. Adenylate cyclase in mammalian systems is a complex enzyme consisting of a catalytic subunit and at least four other proteins that can regulate the activity of the catalytic subunit (for review see Ross and Gilman, 1980). Two of these regulatory subunits bind GTP, one, N_s, activates the enzyme while the other, N_i, inhibits it (Pfeuffer and Helmreich, 1975; Yamamur et al., 1977; Ross et al., 1978; Cooper et al., 1979; Hildebrandt et al., 1983; Jakobs et al., 1983). Various hormone receptors, including monoamine receptors, alter the activity of the catalytic subunit indirectly by binding to a GTP-binding subunit and modifying its interaction with the catalytic subunit (see Ross and Gilman, 1980 for refs.). The catalytic subunit can also be stimulated, probably directly (Salter et al., 1981), by the small calcium-binding protein calmodulin (Brostrom et al., 1975; Cheung et al., 1975).

We examined the stimulatory effectiveness of guanyl nucleotides on Drosophila adenylate cyclase by measuring the stimulation by a poorly hydrolyzable analog of GTP, 5′ guanylyl imidodiphosphate (Lefkowitz, 1974). As shown in Figure 3, although the maximal level of activity of the *rutabaga* abdominal cyclase produced by guanylyl imidodiphosphate was substantially lower than the maximal level of the wild-type enzyme, both were clearly stimulated by a similar amount and showed the same concentration dependency. 10^{-5} M guanylyl imidodiphosphate produced a 90% stimulation of the wild-type activity and a 140% stimulation of the *rutabaga* activity. Both wild-type and *rutabaga* cyclases were also increased by roughly the same absolute amount by sodium fluoride (which activates N_s, see Ross and Gilman, 1980), and had similar concentration dependencies (not shown).

In mammalian systems, the inhibitory effects of guanyl nucleotides can best be seen by adding a guanyl nucleotide to enzyme previously activated by forskolin (Seamon and Daly, 1982). We did not see such an effect with the Drosophila enzyme (adding 3×10^{-5} M guanylyl imidodiphosphate did not alter the activity of forskolin-activated cyclase), but we could see an inhibitory effect of guanylyl imidodiphosphate on manganese-activated adenylate cyclase. We measured the inhibitory effects of guanylyl nucleotides by adding 3×10^{-5} M gunaylyl imidodiphosphate to abdominal enzyme activated with 5 mM manganese. The guanylyl imidodiphosphate, at a concentration which normally stimulated the enzyme (Figure 3), inhibited the manganese-activated enzyme; it reduced the *rutabaga* and the wild-type activity similarly (by 65% and 73%, respectively). In mammalian systems, manganese can potentiate the inhibition of hepatic adenylate cyclase (Londos and Preston, 1977) but not brain adenylate cyclase (Neer, 1979; Seamon and Daly, 1982). From these experiments we conclude that the residual adenylate cyclase activity from *rutabaga* flies responds to ligands that act via the guanyl nucleotide-binding proteins.

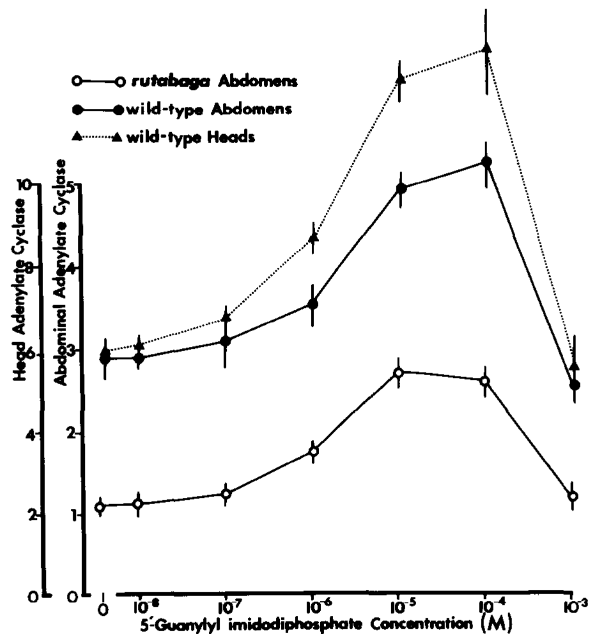

Figure 3. Response of Adenylate Cyclase in Washed Membrane Fractions to Various Concentrations of 5′Guanylyl Imidodiphosphate

Adenylate cyclase is expressed as pmoles cyclic AMP formed/min/mg protein. Both *rutabaga* and wild-type enzyme show the same dose-response to the ligand.

The Drosophila cyclase enzyme is strongly stimulated by the monoamine octopamine (Uzzan and Dudai, 1982). Both *rutabaga* and wild-type abdominal adenylate cyclase were stimulated by 10^{-5} M octopamine (*rutabaga* was stimulated by 3.2 ± .3-fold and wild type by 2.8 ± .2-fold). Both showed the same dosage dependency for octopamine stimulation.

The residual adenylate cyclase activity in *rutabaga* flies thus probably has both a normally functioning monoamine receptor and a normal N_s regulatory subunit. In order to learn whether the catalytic subunit itself might be defective, we tried to determine if the *rutabaga* enzyme responds normally to ligands thought to act directly on the catalytic subunit. In mammalian systems, the divalent cation manganese can activate the isolated catalytic subunit in the absence of any GTP-binding subunits (Ross et al., 1978; Strittmatter and Neer, 1980). The addition of 5 mM manganese stimulated both *rutabaga* and wild-type enzymes

by 2- to 3-fold (see Figure 8 for typical data). In contrast to guanyl nucleotides and sodium fluoride, which stimulated both the wild-type and the *rutabaga* enzyme by approximately the same absolute amount, manganese stimulated both enzymes by the same percentage of their basal level. The diterpene forskolin (50 μM), which also can stimulate the isolated catalytic subunit in mammalian systems (Seamon and Daly, 1981; Bender and Neer, 1983), similarly produced a 4- to 5-fold stimulation of both *rutabaga* and wild-type abdominal cyclases (*rutabaga* cyclase activity was stimulated by 5.8 ± 1.5-fold and wild type was stimulated by 5.5 ± 1.3-fold).

Calcium Activation of Adenylate Cyclase

The one ligand that did produce a qualitatively different response in *rutabaga* was calcium. Wild-type Drosophila adenylate cyclase, like mammalian brain cyclase (Cheung et al., 1975), showed a biphasic response to added calcium, being stimulated by low (<10^{-6} M) concentrations of calcium and inhibited by higher concentrations. The *rutabaga* enzyme, in contrast, did not show any stimulation by calcium; only inhibition (Figure 4). Consistent with this, the calcium chelator EGTA, in concentrations from .25 to .5 mM, produced a decrease in the wild-type activity but an increase in *rutabaga* activity (Figure 5). These differences between mutant and wild-type responses to calcium and EGTA were also seen, although to a lesser extent, in the cyclase from heads (Figure 4). The smaller stimulation of the head enzyme by calcium is consistent with the smaller effect of the *rutabaga* mutation in the head.

Measurement of Endogenous Calmodulin

The calcium stimulation of mammalian brain adenylate cyclase requires the calcium-binding protein calmodulin (Brostrom et al., 1975; Cheung et al., 1975). Yamanaka and Kelly (1981) showed that calmodulin is present in Drosophila and that it mediates the calcium activation of phosphodiesterase. They found further that Drosophila calmodulin was functionally interchangeable with mammalian calmodulin: it could activate rat brain phosphodiesterase, and porcine calmodulin could activate Drosophila phosphodiesterase.

We wondered whether *rutabaga's* insensitivity to calcium might be due to a defect or an insufficiency in calmodulin. We measured the amount of calmodulin by radioimmunoassay and the activity of calmodulin by a bioassay. Radioimmunoassays showed that *rutabaga* and wild-type abdomens contained similar levels of calmodulin-like immunoreactivity (2.5 ± .3 ng/mg abdomen in *rutabaga;* 2.1 ± .2 ng/mg abdomen in wild type).

We assayed calmodulin function by measuring the ability of Drosophila extracts to activate calmodulin-depleted rat brain phosphodiesterase (Yamanaka and Kelly, 1981). Boiled extracts of both *rutabaga* and wild-type abdomens were able to activate the rat brain phosphodiesterase. Half-maximal activation required 1.6 mg of wild-type abdomens/ml and 1.9 mg of *rutabaga* abdomens/ml.

Effect of Exogenous Calmodulin on Adenylate Cyclase Activity

Even though the inability of the *rutabaga* cyclase to be stimulated by calcium did not seem to be due to a deficiency in calmodulin activity, we nevertheless tried to determine whether the *rutabaga* defect could be corrected by exogenous calmodulin. Simply adding bovine calmodulin at concentrations ranging from 1 to 100 μg/ml to the washed membrane fraction did not affect the *rutabaga* adenylate cyclase, but it did, in some experiments, produce a small (<10%) increase in the wild-type activity, suggesting that the mutant might be defective in its responsiveness to calmodulin.

In order to test the ability of Drosophila adenylate cyclase to be stimulated by calmodulin, we needed to strip off

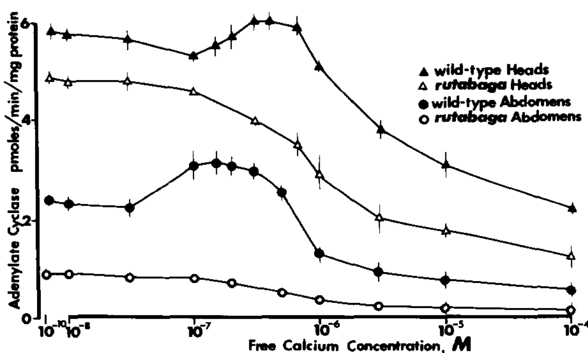

Figure 4. Response of Particulate Adenylate Cyclase to Calcium

The various calcium concentrations were generated using a calcium/EGTA buffer (1 mM EGTA) as described by Caldwell (1976).

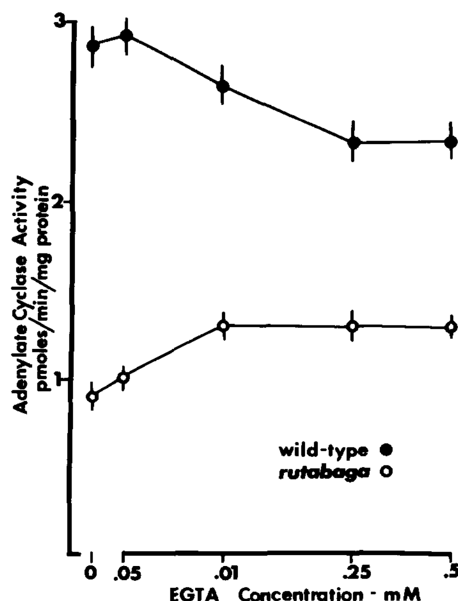

Figure 5. Response of Particulate Abdominal Adenylate Cyclase to Different Concentrations of EGTA

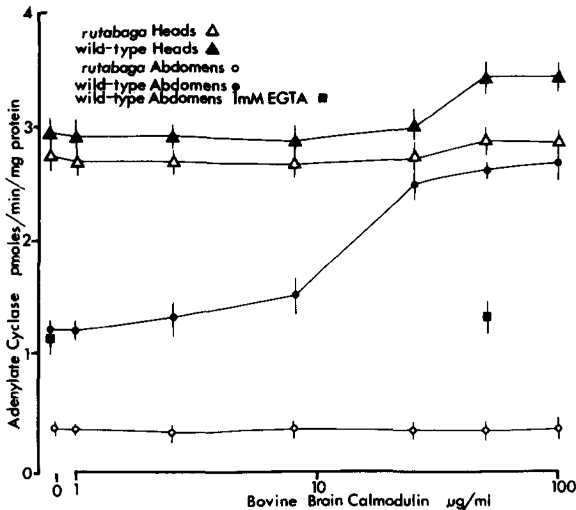

Figure 6. Response of Particulate Adenylate Cyclase, Stripped of Endogenous Calmodulin by Washing with Chlorpromazine, to Various Concentrations of Added Bovine Calmodulin at a Constant Calcium Concentration (3×10^{-7} M)

Wild-type abdominal enzyme was also assayed in 1 mM EGTA without any added calcium. There is a possibility that residual chlorpromazine may have decreased the effectiveness of the added calmodulin and thereby shifted the dose-response curve to the right, but we do not think that this is a major problem because we saw a similar dose response in the EGTA-treated enzyme.

endogenous calmodulin. We first used the technique of Lynch et al. (1977), homogenizing tissue in EGTA, then washing the particulate enzyme by repeated centrifugation and resuspension. After such EGTA treatment, the wild-type abdominal enzyme was stimulated 14% ± 2% by 50 µg/ml bovine calmodulin, whereas the *rutabaga* enzyme was not stimulated by adding calmodulin (3% ± 1% decrease).

We were able to obtain an enzyme preparation with a greater responsiveness to calmodulin by using a different stripping procedure. We homogenized the tissue in buffer with .1 mg/ml chlorpromazine, a potent calmodulin-binding agent (Weiss and Levin, 1978), and then washed the membrane fraction by repeated centrifugation and resuspension in the Tris assay buffer. After chlorpromazine treatment, the wild-type abdominal enzyme activity was increased by 86% ± 9% by 50 µg/ml bovine calmodulin whereas the *rutabaga* enzyme was slightly inhibited (4% ± 2% decrease) by the same concentration of calmodulin. Dose-response curves for the chlorpromazine-stripped enzymes are shown in Figure 6. A similar though less pronounced effect was seen in the enzyme from heads. As shown, the addition of 1 mM EGTA to the wild-type abdominal preparation eliminated the calmodulin stimulation, showing that the stimulation requires calcium. Thus in both heads and abdomens of *rutabaga* flies the calcium/calmodulin-sensitive component of total adenylate cyclase activity seems to be completely, or almost completely, missing.

Gene Dosage Effects on Calmodulin Responsiveness

The alteration in the adenylate cyclase activity associated with the *rutabaga* mutation is easily seen biochemically, but its genetic explanation is not necessarily straightforward. Any valid explanation of the lesion must account for the observation that most of the enzyme activity from heads and some of the activity from abdomens was unaffected by the *rutabaga* mutation. The fact that the mutation affected only the calmodulin responsiveness of the adenylate cyclase activity, not its responsiveness to guanyl nucleotides, sodium fluoride, or octopamine suggests that the mutation does not affect any of the known regulatory subunits of adenylate cyclase and must therefore affect either the catalytic subunit itself or some novel regulatory subunit. A loss of calmodulin responsiveness is consistent with a defect in the catalytic subunit, since, at least in mammalian brain cyclase, the calmodulin-binding site is probably on the catalytic subunit itself (Salter et al., 1981). It is nevertheless possible that there is yet another regulatory protein, which confers calmodulin sensitivity upon the catalytic subunit, and that this is the molecule affected by the mutation.

In Drosophila the amount of a particular protein or enzyme activity is usually proportional to the number of normal copies of the relevant structural gene present in the genome (O'Brien and MacIntyre, 1978). Consequently, given the availability of deletions or null mutations, which abolish gene function, one can often distinguish the primary function of a gene.

We looked at the biochemical effect of zero, one, and two copies of the wild-type (*rutabaga*$^+$) gene and the mutant *rutabaga* gene hoping that the results would allow us to distinguish among possible explanations for the *rutabaga* defect. Figure 7 shows the effect of altered gene dosage on particulate abdominal cyclase activity, assayed in the presence of various ligands. In all four assay conditions tested, the *Df(1)KA9/rut* flies had approximately the same amount of activity as the homozygous *rut/rut* flies. The fact that the deficiency failed to reduce enzyme activity more than the mutation suggests that *rutabaga* is a null mutation; that is, it eliminates rather than alters the gene function. Furthermore, the deficiency/+ heterozygotes showed a selective reduction in calmodulin (CAM) responsiveness, which indicates that the loss of calmodulin responsiveness in the mutant is also probably due to an elimination, rather than an alteration in the protein function.

We are then left with three possible explanations for the altered biochemistry in *rutabaga* flies: One, the *rutabaga*$^+$ gene codes for a previously unidentified factor that mediates the calmodulin responsiveness of the cyclase; two, there is more than one form of cyclase catalytic subunit and the *rutabaga* locus is the structural gene for the form of the catalytic subunit that is responsive to calmodulin; three, the *rutabaga*$^+$ gene regulates the synthesis or activity of a calmodulin-sensitive catalytic subunit. All three of these explanations are consistent with the observation that

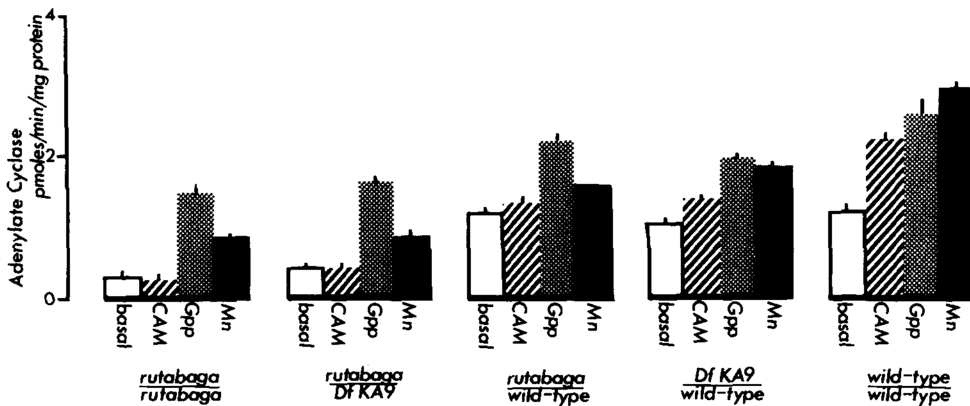

Figure 7. Responses of Particulate, Calmodulin-Stripped Abdominal Adenylate Cyclase from Flies of Different Genotypes to 50 μg/ml Bovine Calmodulin (CAM), 5 × 10^{-5} M Guanylyl Imidodiphosphate (Gpp), and 5 mM Manganese (Mn)

flies homozygous for a null allele have only a partial reduction in enzyme activity, but only the second explanation would be expected to show strong gene-dosage effects on enzyme activity. If the *rutabaga* mutation or a deletion of the *rutabaga* locus really decreased the number of functional adenylate cyclase catalytic subunits, then one would expect a decrease in cyclase activity, particularly if measured in the presence of manganese ion, which, in mammals, apparently circumvents all the known regulatory subunits and directly activates catalytic subunits (Neer, 1978, 1979). Figure 7 shows this to be the case: about 70% of the wild-type manganese-activated (Mn^{++}) cyclase activity is missing in *rutabaga/rutabaga* and *rutabaga/ Df(1)KA9*. Furthermore, the heterozygotes, *rutabaga/+* and *Df(1)KA9/+* (with one rather than two copies of the normal *rutabaga*$^{+}$ gene), respectively showed 40% and 30% reductions in Mn^{++}-dependent activity, approximately half the 70% deficit observed in the homozygous mutant and in *rutabaga/Df(1)KA9*. Basal activities measured without Mn^{++} did not, however, show the same correlation. Such basal activity probably depends on activation by regulatory proteins and should therefore be a less faithful reflection of "raw" catalytic activity than activity measured in Mn^{++}.

The above gene-dosage effects are consistent with a model in which the *rutabaga*$^{+}$ gene codes either for calmodulin-activable adenylate cyclase or for some other molecule that is rate limiting for calmodulin-activable cyclase activity. If the wild-type *rutabaga*$^{+}$ gene codes for a calmodulin-sensitive form of the catalytic subunit of adenylate cyclase, this form should account for 70% of the activity in abdominal tissue. The other, non-*rutabaga*-coded cyclase or cyclases would account for the remaining 30% of the activity in the abdomen and most of the activity in the head and would not be sensitive to calcium/ calmodulin.

If the *rutabaga* gene instead codes for a calmodulin-mediating factor, then this factor would have to be rate-limiting for enzyme activity in the abdomen in order to produce the gene-dosage effects shown in Figure 7, yet its loss produces only a small effect in the enzyme activity from the head. We suspect therefore that the *rutabaga* gene does not code for such a calmodulin-mediating factor.

The other possibility is that the *rutabaga*$^{+}$ gene product controls the synthesis or activity of a calmodulin-sensitive cyclase catalytic subunit. In this case, the amount of the catalytic activity would have be rather precisely titrated to the amount of the *rutabaga* gene product. With only these data on gene dosage we cannot conclusively determine whether the *rutabaga* locus is the structural gene for one form of adenylate cyclase catalytic subunit or if it codes for some factor that is rate-limiting for calmodulin-sensitive cyclase activity.

The absolute magnitude of the guanylyl imidodiphosphate response, in contrast to the Mn^{++} response (which was reduced by the mutation), was roughly the same in *rutabaga,* in both heterozygotes, and in the wild type (an increase of approximately 1.5 pmoles/min/mg protein over the basal level for each genotype). For example, in Figure 7, the guanylyl imidodiphosphate-stimulated activity ("Gpp") was greater than the Mn^{++}-stimulated activity in stocks containing the *rutabaga* mutation, whereas in the wild type the Mn^{++}-stimulated activity was greater. This suggests that the *rutabaga*$^{+}$ gene product is less sensitive to guanyl nucleotides than the non-*rutabaga* enzyme. This result is reminiscent of the result of Brostrom et al. (1977), who found that the calmodulin-sensitive component of rat brain adenylate cyclase was stimulated by sodium fluoride to a lesser extent (50%–100%) than was the calmodulin-insensitive component (4- to 6-fold).

Interactions between a Phosphodiesterase Mutation and an Adenylate Cyclase Mutation

The *dunce* (*dnc*) mutation and the *rutabaga* mutation both directly affect cyclic AMP metabolism, but in opposite directions. We thought therefore that the two mutations might compensate for each other when both were present in the same fly.

There is strong evidence that the *dunce* locus is the

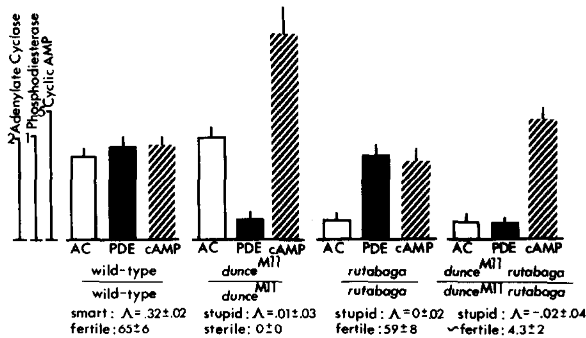

Figure 8. Particulate Abdominal Adenylate Cyclase Activity (pmoles cyclic AMP formed/minute/mg protein), Soluble Abdominal Cyclic AMP Phosphodiesterase Activity (nmoles cyclic AMP hydrolyzed/minute/mg protein), and Endogenous Abdominal Cyclic AMP Levels (pmoles/mg protein) in Different Genotypes

All three enzyme activities were assayed in aliquots of the same homogenate. Fertility is expressed as the number of progeny/female/10 days.

structural gene for the enzyme cyclic AMP phosphodiesterase form II (Kiger and Golanty, 1977, 1979; Byers et al., 1981; Kauvar, 1982; Shotwell, 1983). There are several mutant alleles of the *dunce* locus; all fail to learn and have reduced female fertility. For these experiments we used *dunce*^{M11} because it is a null allele and removes cyclic AMP phosphodiesterase II activity completely. *dnc*^{M11}/*dnc*^{M11} females are virtually completely female-sterile (Mohler, 1977). Using linked visible genetic markers we generated flies carrying both the *dnc*^{M11} mutation and the *rutabaga* mutation on the same X chromosome. We assayed phosphodiesterase activity, adenylate cyclase activity, and endogenous cyclic AMP in the abdomens of four genotypes: +/+, *dnc*^{M11}/*dnc*^{M11}, *rut*/*rut*, and the double mutant, *dnc*^{M11} *rut*/*dnc*^{M11} *rut* and measured the learning index and the fertility of each genotype (Figure 8). The *dnc*^{M11}/*dnc*^{M11} flies had about 25% as much abdominal cyclic AMP phosphodiesterase activity as the wild type, but normal levels of adenylate cyclase. The endogenous abdominal cyclic AMP level was about double that of the wild type. In contrast, the *rut*/*rut* flies had reduced abdominal adenylate cyclase, normal levels of phosphodiesterase, and slightly reduced levels of cyclic AMP. The double mutant *dnc*^{M11} *rut*/*dnc*^{M11} *rut* had reduced adenylate cyclase and reduced phosphodiesterase. The endogenous cyclic AMP level was intermediate, lower than that of *dnc*^{M11}/*dnc*^{M11} but higher than *rut*/*rut*. Neither the *dunce*^{M11} nor the *rutabaga* flies showed any learning. The double mutant flies also failed to learn.

Since the double mutant flies still had cyclic AMP levels that were higher than normal, we also generated flies with two copies of the *rutabaga* mutation and one copy of the *dunce*^{M11} mutation (*dnc*^{M11} *rut*/+ *rut*), but these flies also failed to learn ($\lambda = -.05 \pm .09$).

There was some physiological compensation generated by the two mutations in that the homozygous double mutant females were not sterile. They were much less fertile than wild-type females, but were more fertile than homozygous *dnc*^{M11}/*dnc*^{M11} females. The ability of the *rutabaga* mutation to suppress *dunce's* female sterility was dominant: *dnc*^{M11} *rut*/*dnc*^{M11}+ flies were also partially fertile (1.6 ± .2 progeny/female/10 days). The deficiency *Df(1)KA9* was also a dominant suppressor of the *dunce*^{M11} sterility (2.0 ±.7 progeny/female/10 days). Another suppressor of *dunce* sterility besides *rutabaga* has been isolated (Salz et al., 1982), but this mutation maps elsewhere, and its biochemical consequences have not been determined.

Discussion

The *rutabaga* mutation, isolated on the basis of its failure to show associative learning, affects adenylate cyclase activity, specifically its ability to be stimulated by calcium/calmodulin. The simplest, though not the only, explanation for our findings is that the *rutabaga*$^{+}$ gene codes for only one of two or more forms of the adenylate cyclase catalytic subunit, specifically the one that is responsive to calmodulin. Brostrom et al. (1977) have found that the adenylate cyclase from rat brain consists of two kinetically distinguishable components, one that is calcium-dependent and another that is calcium-independent. Our results suggest that flies similarly have at least two genetically distinct types of adenylate cyclase, only one of which is calcium-dependent. In Drosophila, the distribution of calcium-sensitive and calcium-insensitive forms of adenylate cyclase differs from the mammalian distribution, in which calcium-sensitive activity is found predominantly, if not exclusively, in brain and other neural tissues and the calcium-insensitive form is found in both neural and nonneural tissues (see Ross and Gilman, 1980 for refs.).

The studies of Drosophila learning-deficient mutants have produced two surprising results. The first is that four of them—*dunce, rutabaga, Ddc,* and *turnip*—all affect some aspect of cyclic AMP metabolism. The second surprise is the remarkable specificity of the behavioral lesions, considering the ubiquity of the enzyme systems that are affected. The mutants *dunce, Ddc,* and *rutabaga* all have gross biochemical defects, yet, although there are some nonbehavioral effects of the mutations, such as the *dunce* female sterility, these mutants are all behaviorally normal except in tests that look at behavioral plasticity (Dudai et al., 1976; Wright et al., 1982; Quinn et al., 1979; Byers et al., 1981; Duerr and Quinn, 1982; Livingstone and Tempel, 1982; Aceves-Piña et al., 1983). One might have expected that flies with a major defect in an important enzyme such as phosphodiesterase or adenylate cyclase would be dead; instead they are practically normal. They walk, fly, feed, mate, phototax, and geotax normally, and they respond normally to the odors and the reinforcements used in the learning tests. In the case of both *dunce* and *rutabaga,* however, even though one enzyme seems to be completely missing, it is only one of at least two forms of the same enzyme. The question then arises whether these two mutants are missing the most dispensable form

of the enzyme, perhaps one particularly concerned with behavioral plasticity, or whether loss of any subtype of adenylate cyclase or phosphodiesterase would produce the same phenotype. If the mammalian S-49 lymphocyte cell line also has multiple forms of adenylate cyclase catalytic subunits, this might explain why no mutations in the catalytic subunit have yet been found (Johnson et al., 1980); since the mutants are selected on the basis of inability to increase cyclic AMP synthesis, the loss of only one form might not reduce cyclase levels enough to result in differential survival.

It does not seem simply that some critical level of cyclic AMP is necessary for learning, since the effect of the *rutabaga* mutation on cyclic AMP levels is rather small and since the *dunce* mutation's effect on basal levels is in the opposite direction, yet both mutants fail to learn. Furthermore, attenuating the *dunce* mutations's effect on cyclic AMP levels by adding one or two doses of the *rutabaga* mutation did not produce a fly that could learn. Rather we suspect that it is important to be able to modulate cyclic AMP levels appropriately and/or in critical neurons.

The biochemical defect we observe in the learning mutant *rutabaga* fits with recent work by Hawkins et al. (1983) and Walters and Byrne (1983) on classical conditioning of the Aplysia siphon- and tail-withdrawal reflexes. Their electrophysiological results suggest that during associative learning a cyclic AMP response is amplified by some direct concomitant of spiking activity, most likely an elevation of intracellular calcium (Kandel et al., 1983). Our finding that a learning mutant is specifically deficient in calcium/calmodulin stimulation of adenylate cyclase is consistent with this model. The ability of adenylate cyclase to respond to two different types of signals, namely extracellular monoamines and intracellular calcium levels, makes it potentially capable of acting as the essential temporal integrator of environmental stimuli in associative learning, so it is illuminating that the loss of one of these responses should block learning.

Results from both Aplysia and Drosophila link associative learning to alterations in intracellular cyclic AMP levels, perhaps to a synergistic effect on cyclic AMP synthesis produced by the close temporal coupling of the presence of extracellular monoamines and a rise in intracellular calcium. Such a model would be powerful in relating psychology to some concrete biochemistry. The biochemistry involved would have to have unusual kinetics to account for even the simplest temporal specificity known to govern conditioned stimulus–unconditioned stimulus interactions in associative learning. The biochemistry of the regulation of intracellular cyclic AMP is in fact kinetically complex, with multiple regulatory features for each of the enzymes involved. Even if learning can be tied to this particular system, it is an unusually complicated biochemical system, and our understanding of it is still profoundly incomplete. The use of genetic mutants in the cyclic AMP pathway in S-49 cells has already contributed to the understanding of cyclic AMP biochemistry and its functions (Johnson et al., 1980). We expect the Drosophila mutants will also help in unravelling this complex problem because flies are behaviorally more entertaining than lymphocytes and doing genetics on them is much easier.

Experimental Procedures

Fly Stocks

We used the *Canton-Special* wild-type strain and single gene mutants in *Canton-S* genetic backgrounds. *rutabaga, dunce, turnip, amnesiac,* and *cabbage* were isolated from ethyl methanesulfonate-mutagenized *Canton-S* stocks. The circadian rhythm mutant, per^{o} was isolated by (and kindly supplied by) Konopka and Benzer (1971) from ethyl methanesulfonate-mutagenized *Canton-S* stocks. The neurological mutant $hyperkinetic^{1}$ was isolated by Kaplan and Trout (1969) from ethyl methanesulfonate-mutagenized *Canton-S* stocks and was obtained from the Pasadena Stock Center. $dunce^{M11}$ was isolated by Mohler (1977) as a female sterile mutant from stocks carrying the visible X chromosome markers *yellow, crossveinless, vermilion,* and *forked*. It was later shown to be allelic to $dunce^{1}$ (Byers et al., 1981), a *Canton-S*-derived learning mutant (Dudai et al., 1976), and was made approximately coisogenic with *Canton-S* by Byers et al. (1981). Stocks with X chromosome deficiences used to map *rutabaga* were isolated by G. Lefevre from a56i stocks or by M. Green from Oregon-R stocks and were provided by L. Cramer from the Pasadena Stock Center. Flies were maintained at 25°C and 40% relative humidity on standard cornmeal medium (Lewis, 1960) or Carolina Biological Supply instant fly food.

Learning ability was measured in an olfactory discrimination task with electric shock reinforcement, exactly as described by Quinn et al. (1974). Groups of 40–50 flies were exposed alternately to each of two odorants, one of which was accompanied by an aversive electrical shock. Their avoidance of each odor was then tested in fresh chambers. The numerical index of learning performance we used, λ, is defined as the fraction of flies avoiding the shock-associated odor minus the fraction of flies avoiding the non-reinforced control odor, averaged for reciprocal halves of the experiment. Results are reported here as mean values $\pm$ standard error of the mean.

Fertility was measured by putting single virgin females in vials with 5 males for 8 days. The parents were then removed and the number of adult progeny emerging over the next 10 days were counted.

Biochemical Assays

We used female flies for biochemical assays unless stated otherwise. Adult flies 4–10 days after eclosion were dissected with forceps under CO_2 anesthesia. Results are expressed as mean values $\pm$ standard error of the mean. All incubations were done at 25°C. For all the biochemical assays the amount of activity was proportional to time and the amount of tissue. All comparisons are made between assays run in parallel in the same experiment. Each result represents data from at least three separate experiments.

Adenylate Cyclase Assay

Adenylate cyclase was assayed by the method of Salomon (1979). Each assay tube contained 30 μl of tissue homogenate (10–20 heads/ml, 20 thoraxes/ml, or 20 abdomens/ml) or a washed membrane preparation (the pellet from a 178,000 g $\times$ 10 min centrifugation, washed twice by resuspension and centrifugation in assay buffer, 25 mM Tris HCl [pH 7.6], 1 mM dithiothreitol; 50 abdomens, thoraxes, or heads/ml final volume) in a total assay volume of 90 μl. The assay mixture contained a final concentration of 5 mM creatine phosphate, 50 U/ml creatine phosphokinase, 25 mM Tris-HCl acetate (pH 7.6), 10 mM $MgCl_2$, .05 mM cyclic AMP, 1 mM dithiothreitol, .1 mg/ml BSA, 0.1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 0.2 mM ATP, and 0.2–5 μCi/assay tube α-^{32}P-ATP (New England Nuclear). Free calcium was kept at a concentration of 3 $\times$ 10^{-7} M, unless stated otherwise, using a 1 mM EGTA/$CaCl_2$ buffer (Caldwell, 1976). Each tube was incubated for 10 min and the reaction stopped by the addition of 100 μl 2% sodium lauryl sulfate, 45 mM ATP, 1.3 mM 3′5′-cyclic AMP. ^{3}H-labeled 3′5′ cyclic AMP was added to monitor column recovery. Cyclic AMP was separated from ATP by sequential chromatog-

raphy on Dowex and alumina columns and counted on a scintillation counter with double label windowing. For assaying the responsiveness of adenylate cyclase to calmodulin, membrane fractions were homogenized in Tris-HCl buffer (25 mM [pH 7.6]) containing 1 mM dithiothreitol and either 5 mM EGTA (Lynch et al., 1977) or 0.1 mg/ml chlorpromazine and then washed four times by centrifugation for 10 min at 178,000 g and resuspension in Tris/dithiothreitol buffer. Bovine calmodulin was obtained from Sigma.

Cyclic AMP Phosphodiesterase Assay

Cyclic AMP phosphodiesterase activity was measured by a technique similar to that of Kiger and Golanty (1979). Each assay tube contained, in a final volume of 50 μl, 30 μl of eight abdomens/ml, 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 20 mM mercaptoethanol, 10^{-5} M unlabeled cyclic AMP, and 0.5 μCi ^{3}H-cyclic AMP (New England Nuclear). Each tube was incubated for 20 min. The reaction was stopped by boiling for 5 min and unlabeled carrier containing excess adenosine, cyclic AMP and 5′AMP was added. Adenosine and 5′AMP were separated from cyclic AMP by high voltage electrophoresis (42 volts/cm for 3 hr) at pH 1.9. The adenosine and 5′AMP spots were identified under a UV light, cut out, eluted from the paper with 0.3 ml 0.01 m HCl and counted in a scintillation counter.

Cyclic AMP Assay

Endogenous cyclic AMP content of fly abdomens was measured with a New England Nuclear 3′5′cyclic AMP radioimmunoassay kit. Aliquots (50 μl) of homogenates containing four abdomens/ml 0.05 M sodium acetate buffer (pH 6.2) were acetylated and assayed according to the instructions in the handy kit.

Calmodulin Assays

Endogenous calmodulin was assayed both by radioimmunoassay (New England Nuclear) and also by its ability to activate rat brain calmodulin-depleted phosphodiesterase (Ho et al., 1976) (Sigma). Calmodulin was extracted from fly tissues as described by Yamanaka and Kelly (1981), except that homogenates were centrifuged for 10 min at 178,000 g before boiling, to separate soluble from membrane-bound calmodulin.

Guanylate Cyclase Assay

Guanylate cyclase activity in washed membrane preparations was measured using α-^{32}P-GTP as described by Garbers and Murad (1979).

ATPase Assays

Sodium/potassium-dependent and magnesium-dependent ATPase activities in washed membrane preparations were measured as described by Goldin (1977).

Protein Assay

Protein was assayed by the method of Lowry et al. (1951).

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