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Genetic dissection of *Drosophila* adenylate cyclase

(learning mutants/cyclic AMP)

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ABSTRACT **The *Drosophila* learning mutant rutabaga is missing calcium/calmodulin activation of adenylate cyclase (EC 4.6.1.1). The mutation was mapped at a finer resolution to X chromosome bands 12F5–7. By comparing wild-type and mutant cyclase activities, the relative responsiveness of the calcium-sensitive and calcium-insensitive components to different ligands could be determined; the calcium-sensitive fraction of the total cyclase activity was significantly less responsive to guanyl nucleotides and monoamines. The results suggest that the component of cyclase activity that is stimulated by calcium/calmodulin, possibly a genetically distinct catalytic subunit, is not coupled to the G subunit or the G subunit/monoamine receptor complex.**

The *Drosophila* mutant rutabaga (*rut*) was originally isolated in a screening to select for ethyl methanesulfonate-induced X-linked mutations that block associative learning. Both the original rutabaga mutation and a chromosomal deficiency for the *rut* locus reduce basal adenylate cyclase activity (EC 4.6.1.1) and eliminate all detectable stimulation by calcium/calmodulin (1–3). Neither the mutation nor the deficiency affects the responsiveness of adenylate cyclase activity to ligands other than calcium, such as guanyl nucleotides, fluoride, and monoamines (2). The responsiveness to calcium/calmodulin in mammalian cyclase appears to reside in the enzymatically active catalytic subunit (4), whereas the responsiveness to guanyl nucleotides and hormones is mediated by protein moieties distinct from the catalytic subunit, termed "G subunits" (5). The biochemical effects of the rutabaga mutation (2) could be explained in two possible ways: (*i*) There exist at least two genetically distinct forms of the adenylate cyclase catalytic subunit; both of them contribute to the basal activity, but only one of them, the one coded for by the *rut* locus, is stimulated by calcium/calmodulin. (*ii*) The *rut* locus codes for a novel cofactor, subunit, or regulator that confers calcium/calmodulin sensitivity upon the catalytic subunit.

Two other *Drosophila* mutations, dunce and Dopa decarboxylase (*Ddc*), block learning (6, 7), and both of these mutations also affect the regulation of cyclic AMP. The dunce mutation abolishes one of two forms of the enzyme cyclic AMP phosphodiesterase (8), and subsequent studies have strongly suggested that the dunce locus is the structural gene for this enzyme (9–11). *Ddc* was isolated not as a learning mutant as such, but as a lethal mutation that affects the enzyme dopa decarboxylase (12), which is necessary for the formation of dopamine from *l*-dopa. Dopamine is essential for the tanning (hardening and darkening) of fly cuticle. Mutations at the *Ddc* locus block not only cuticular dopamine synthesis but also brain dopamine and serotonin synthesis (13). Monoamine neurohormones, including dopamine and serotonin, in flies as well as in many other species, can influence adenylate cyclase activity via specific receptors (14).

Thus three mutations that block associative learning affect the same intracellular second messenger. Several other learning or memory mutants have been isolated (1), but their biochemical alterations are not known. Although there are some nonbehavioral effects of these mutations (dunce is female sterile, and *Ddc* affects cuticle hardening) the behavioral consequences of these learning mutations are remarkably specific, affecting associative learning, some experience-dependent aspects of courtship, and two nonassociative forms of behavioral plasticity, habituation and sensitization, without affecting other complicated behaviors such as feeding, most aspects of courtship, walking, flying, phototaxis, or geotaxis (6, 7, 15, 16).

It is surprising that severe or complete defects in enzymes involved in the regulation of the ubiquitous intracellular second messenger cyclic AMP are so specific in their effects; one might have expected such mutations to be lethal or at least much more pleiotropic. One possible explanation is that, in the case of rutabaga and dunce, the affected enzyme is only one of at least two genetically distinct forms of the affected enzyme. Nevertheless, the suggestion from these genetic studies that the regulation of intracellular cyclic AMP is an important factor in associative learning is consistent with results from single-cell physiological experiments in *Aplysia*. The *Aplysia* studies suggest that both sensitization and associative learning are a consequence of alterations in the regulation of presynaptic cyclic AMP in response to extracellular serotonin (17–19). These studies further suggest that the crucial difference between associative learning and nonassociative sensitization is that, in associative learning, some consequence of neuronal activity, perhaps an influx of calcium, produces an accentuation or prolongation of the adenylate cyclase response to serotonin, whereas sensitization occurs when the serotonin receptor is activated in the absence of such an intracellular calcium increase (18, 19). The fact that the *Drosophila* learning mutant rutabaga is selectively affected in the calcium-dependent stimulation of adenylate cyclase is consistent with this idea that a critical step in associative learning may be synergism between calcium and monoamines in activating adenylate cyclase. In the simplest form of this model the locus of the synergism between calcium and monoamines would reside in the adenylate cyclase molecule itself. It would appear from biochemical studies that the adenylate cyclase complex alone should be capable of a synergistic response to monoamines and intracellular calcium, since it can be activated by both monoamines and calcium/calmodulin (5), though the effects have usually been found to be simply additive rather than synergistic (4). To test this model, I have used the rutabaga mutation to ask whether the fraction of adenylate cyclase activity that is sensitive to calcium/calmodulin is also sensitive to monoamines and whether there is any synergism

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Abbreviation: p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

between calcium and monoamine activation of adenylate cyclase.

METHODS

Fly Stocks. Wild-type flies were the Canton-Special wild-type strain. Rutabaga was originally isolated as a learning mutant, induced by ethyl methanesulfonate mutagenesis in a Canton-S stock. Stewart and Merriam generated the translocation stocks used for mapping by x-ray induced rearrangements of *y* and *y, w, f* stocks (20). *Df(1)KA9* was generated by Lefevre and is a deletion of bands 12E1–13A5. B. Ganetzky and R. Kreber (personal communication) generated the deficiency stock *Df(1)RK4* by recombination within the two overlapping inversions *In(1)2E;10F* + *In(1)13A9-B1;17C1-2, hdp-b* and *In(1)12F5-6;17C1-2,* w^{P363}, *hdp-b*, [both generated by W. R. Engels (21)]. Kenneth Livak generated the duplications of the *rut* locus by x-ray-induced deletions in $In(1)sc^{29}$ stocks.

Adenylate Cyclase Assays. Washed membranes were prepared as described previously (2); in experiments in which responses to calmodulin or monoamines were measured, the first homogenization buffer contained chlorpromazine at 0.4 mg/ml. Adenylate cyclase was assayed by the method of Salomon (22). Free calcium was varied by using a 1 mM EGTA/$CaCl_2$ buffer (23). Each result represents data from at least two separate experiments.

RESULTS

Mapping the *rut* Gene. By using conventional mapping techniques and deficiency complementation mapping, the *rut* locus has been mapped between polytene chromosome bands 12E1 and 13A5 on the X chromosome (2). To map *rut* at a finer resolution, I constructed artificial deletions of the X chromosome, using X–Y translocation stocks developed by Stewart and Merriam (20). I crossed pairs of stocks with X–Y translocation breakpoints within the 12E to 13A region and selected female progeny carrying the left half of the X chromosome from one stock and the right half of the chromosome from the other stock (over the multiply inverted X chromosome *FM7* to prevent crossing-over). These females as well as wild-type and the deficiency stocks, *Df(1)RK4* and *Df(1)KA9*, were then mated to rutabaga males, and the female progeny carrying the X chromosome to be tested and a rutabaga X chromosome were then assayed for calcium-sensitive adenylate cyclase activity. Fig. 1 shows the results: whenever the region between 12F5-6 and 12F6-7 was deleted, adenylate cyclase activity was reduced and was missing any calcium activation. This indicates that the *rut* locus lies between 12F5–6 and 12F6–7.

Gene Dosage Effects on Adenylate Cyclase. In *Drosophila* the amount of a particular protein or enzyme activity is usually proportional to the number of functional copies of its structural gene present in the genome (25). Genes on the X chromosome show different gene-dosage effects in males and females; "dosage compensation" refers to the phenomenon that, in males, X-linked genes are expressed at twice the rate per copy as in females. Thus if a particular locus on the X chromosome is thought to be the structural gene for a particular protein, then the amount of the protein would be expected to be approximately 50% of the wild-type level in females with only one wild-type copy of the gene, 100% in males with one copy of the wild-type gene or females with two copies, 150% in females with two wild-type X chromosomes plus one duplication of the locus, and 200% in males with one wild-type X chromosome plus one duplication (though dosage effects of most X-linked loci usually fall short of the expected 200% in males carrying two copies). As described previously (2), for zero or half the normal gene dosage, the amount of calcium-stimulated cyclase is proportional to the number of gene copies. In the following experiments the effects of extra doses of the *rut* gene are examined.

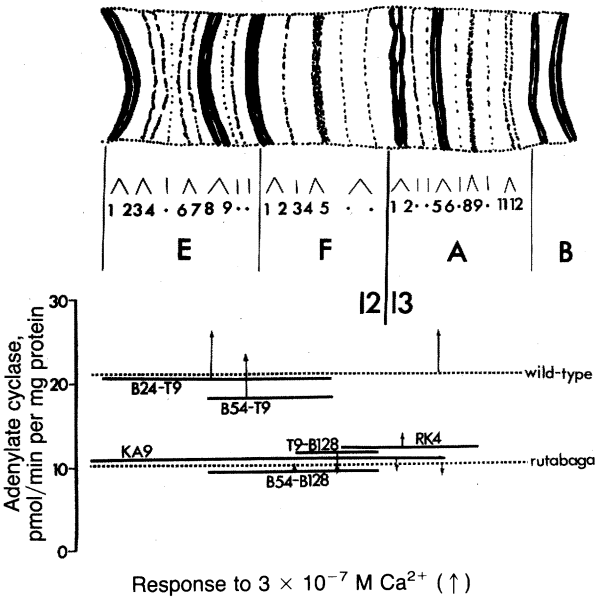

FIG. 1. A portion of the *Drosophila* polytene X chromosome, showing cytological extent of deficiencies assayed for abdominal adenylate cyclase activity. The maximum estimated extent of each deletion is represented by a solid horizontal line, whose height, relative to the scale on the left, indicates for each stock the basal level of adenylate cyclase in 10^{-10} M calcium; the lengths of the arrows indicate the response to 3×10^{-7} M calcium. Each stock was tested as a heterozygote: *rut*/test chromosome. Wild-type/*rut* and *rut/rut* are shown as dotted lines. *Df(1)KA9* is a deletion of bands 12E1–13A5, and *Df(1)RK4* is a deletion of bands 12F5–6 to 13A9–B1; several other deletions in this region were tested with results consistent with the location determined. The estimated breakpoints for the X–Y translocations used to make artificial deficiencies are B24, 12E; B54, 12E8–12F1; T9, 12F2–5; and B128, 12F6–7. All the translocation stocks themselves had wild-type cyclase activity. The chromosomal banding pattern is from ref. 24.

The two duplications used in this study were *Dp(1:f)LJ4* and *Dp(1:f)LJ9* (26). These two duplications contain a small inverted region of the X chromosome between regions 12 and 13, attached distally to the tip of the X chromosome, which carries the marker y^+, and proximally to the centromere. *DpLJ9* covers all of *Df(1)KA9* and garnet (*g*), and thus extends at least from 12C to 13A2-5. *DpLJ4* does not cover all of *Df(1)KA9* or *g* but does cover narrow abdomen, and thus probably extends only from 12E to 13A2-5.

When either of these two duplications were crossed into a rutabaga stock (marked with *y* so that the presence of the duplication could be determined), both compensated for the mutation's effect on adenylate cyclase, supporting the earlier conclusion that the *rut* locus lies between 12E and 13A5. Rutabaga males carrying either of these duplications had almost as much of the calcium-sensitive adenylate cyclase as wild-type males: *rut;DpLJ4* males had 70% of the wild-type calcium activation and *rut;DpLJ9* males had 81%. Rutabaga females carrying either duplication had about half as much calcium-sensitive adenylate cyclase as wild-type females: *rut/rut;DpLJ4* females had 55% of the wild-type calcium activation and *rut/rut;DpLJ9* females, 53%. This result suggests that the X chromosome-derived material on these small free duplications does carry the wild-type *rut* gene and is probably subject to dosage compensation. Furthermore, wild-type females carrying either of these duplications had approximately 150% as much calcium-sensitive cyclase activity as normal wild-type females: 156% for +/+;*DpLJ4* females and 139% for +/+;*DpLJ9*. Wild-type males carrying

a duplication for *rut* also had about 150% of the wild-type activity: 142% for +;*DpLJ4* males and 134% for +;*DpLJ9* males, instead of the expected 200%. As described above, this type of gene-dosage effect is consistent with the possibility that the *rut* locus is the structural gene for a calcium-sensitive catalytic subunit of adenylate cyclase. See Fig. 2 for calcium responses in rutabaga, wild-type, and +; *DpLJ4* males.

Effects of Guanyl Nucleotides and Monoamines on *rut* and non-*rut* Cyclase Activity. Even though it is not clear whether the *rut* locus is indeed the structural gene for the calcium-sensitive adenylate cyclase, this mutation can nevertheless be used to differentiate functionally between calcium-sensitive and calcium-insensitive subfractions of the total cyclase activity. An earlier study comparing the effects of p[NH]ppG on rutabaga and wild-type cyclase activity had suggested that the adenylate cyclase activity affected by the *rut* mutation was stimulated by guanyl nucleotides much less than was the non-*rut* cyclase activity (figures 3 and 7 of ref. 2). Here I have extended these studies to ask whether guanyl nucleotide stimulation is affected by calcium and whether the calcium-sensitive fraction is insensitive to monoamines as well as to guanyl nucleotides. Fig. 2 shows comparisons of adenylate cyclase activity from rutabaga, wild-type, and *DpLJ4* over a range of calcium concentrations with and without p[NH]ppG. In wild-type flies there is a biphasic response to calcium: the cyclase activity is stimulated by low (1–3 $\times 10^{-7}$ M) concentrations of calcium and is inhibited by higher concentrations. The activity from rutabaga flies does not show any stimulation by calcium but does show the inhibition, and the inhibition by high concentrations of calcium is accentuated by p[NH]ppG. Second, as described above in the gene-dosage section, both heads and abdomens of flies carrying an extra copy of the wild-type *rut* locus show a larger calcium response relative to wild-type flies. It would appear from this experiment that the calcium-stimulated cyclase and the p[NH]ppG-stimulated cyclase are completely independent, because (*i*) the absolute amount of stimulation by calcium is the same in the presence or absence of p[NH]ppG, (*ii*) the stimulation by p[NH]ppG is the same size in rutabaga and in wild-type, and (*iii*) the p[NH]ppG response is not affected by the calcium concentration.

Since all known responses of adenylate cyclase to hormones, including monoamines, seem to be mediated by G subunits (5), the result in Fig. 2 predicts that monoamine responses, like the p[NH]ppG response, should be independent of the calcium-sensitive activity. As shown in Fig. 3, the response of the cyclase to monoamines is independent of the calcium activation: the magnitude of the stimulation in response to serotonin, dopamine, or octopamine is the same in rutabaga as in wild-type and is independent of the calcium concentration.

The experiments shown in Figs. 2 and 3 indicate that the wild-type adenylate cyclase activity is stimulated by low concentrations of calcium and inhibited by higher concentrations, whereas the activity from rutabaga flies shows only the inhibition by high concentrations, implying that the stimulation and inhibition by calcium might be due to two different types of processes. Adenylate cyclase activity from mammalian brain similarly shows a biphasic response to calcium, and the stimulation by low concentrations of calcium requires calmodulin (27, 28), whereas the inhibition by higher concentrations of calcium does not require calmodulin (29, 30). As shown in Fig. 4, wild-type *Drosophila* adenylate cyclase similarly requires calmodulin for the stimulation by low concentrations of calcium but not for the inhibition by higher concentrations.

Both the calmodulin-depleted wild-type activity (Fig. 4) and the *rut* activity (Figs. 2 and 3) are missing the stimulation by low concentrations of calcium but exhibit a strong inhibition by calcium between 3 $\times 10^{-7}$ M and 10^{-6} M. By

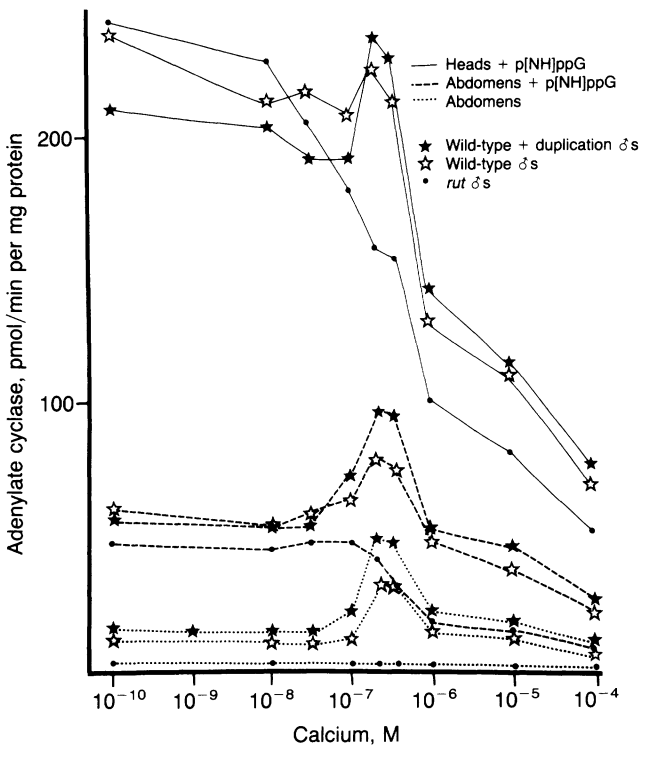

FIG. 2. Adenylate cyclase activity in washed membranes from heads and abdomens of wild-type, rutabaga, and +*DpLJ4* males at different calcium concentrations. Guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG), when indicated, was at 10 μM.

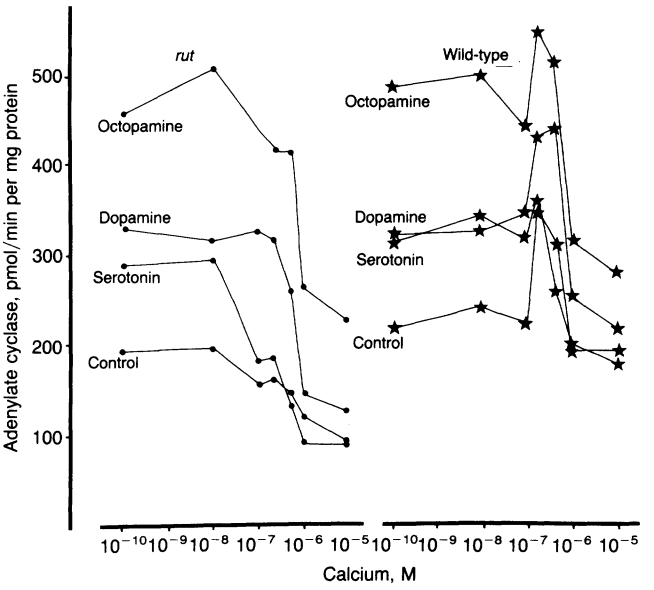

FIG. 3. Adenylate cyclase activity in chlorpromazine-treated washed membranes from rutabaga and wild-type heads at different calcium concentrations in the presence of 10 μM p[NH]ppG, bovine calmodulin at 50 μg/ml, and 2 mM dopamine, 2 mM serotonin, or 20 μM octopamine. [These are saturating concentrations of monoamines; the calcium concentration did not affect the dose response of monoamines (data not shown)]. An experiment using GTP instead of p[NH]ppG gave a similar result but with much smaller monoamine responses. The small stimulation of the *rut* cyclase at 3 $\times 10^{-7}$ M calcium in the presence of dopamine was not seen in other experiments.

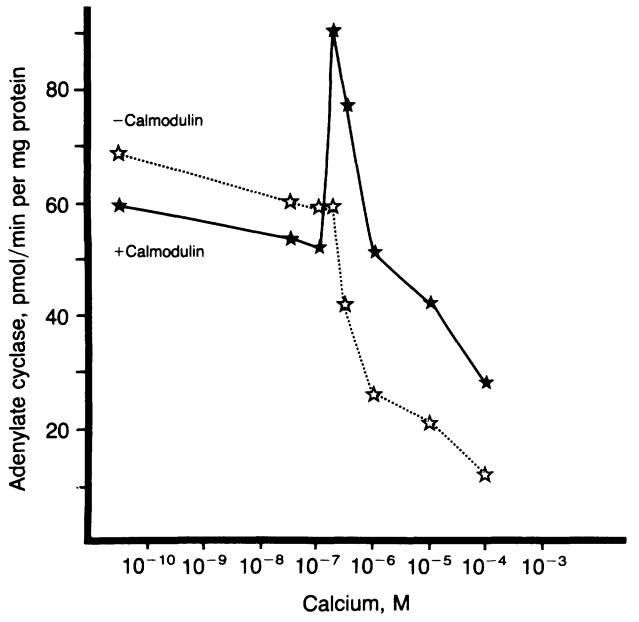

FIG. 4. Adenylate cyclase activity in chlorpromazine-treated washed membranes from wild-type abdomens in the presence and absence of bovine calmodulin at 50 μg/ml.

inspection, the inhibition of the calmodulin-independent, or non-*rut*, activity could account for most, if not all, of the calcium-dependent inhibition of the total activity up to 10^{-5} M calcium. These results therefore suggest that the biphasic response to calcium of the total activity is due to the sum of the stimulation of the *rut* cyclase by low concentrations of calcium and the inhibition of the non-*rut* activity by only slightly higher concentrations.

DISCUSSION

On the Biochemical Nature of the *rut* Gene Product. Evidence presented in a previous paper suggested that there were two likely possibilities for the gene product of the *rut* locus: (*i*) the *rut* locus codes for one of at least two genetically distinct forms of the adenylate cyclase catalytic subunit, in particular, one that is stimulated by calcium/calmodulin, (*ii*) the locus codes for some novel cofactor or subunit that confers calcium/calmodulin sensitivity upon the cyclase catalytic subunit. The results of the present study support the first possibility over the second but do not rule out the second possibility. If the *rut* gene product is such a cofactor, then it must be stoichiometrically rate limiting for calcium-sensitive activity, since it shows gene-dosage effects. Moreover, since the effects of calmodulin and p[NH]ppG are simply additive, the effect of this putative cofactor must be largely independent of the effect of the G subunit. If this putative cofactor and the G subunit act on the same catalytic subunit, then their effects would have to be simply additive instead of multiplicative or synergistic. The localization of the *rut* gene to one or two bands of the X chromosome should make it possible to clone the gene and possibly establish the identity of the gene product.

On Calcium/Calmodulin and Hormone/G Subunit Interactions. Several reports in the literature suggest, on the basis of pharmacological or kinetic data, that there are two components to the adenylate cyclase activity of mammalian brain, a calcium/calmodulin-sensitive component and a calcium/calmodulin-insensitive component, and that the calmodulin-sensitive component is much less responsive to guanyl nucleotides and fluoride than is the calmodulin-insensitive component (29, 31, 32). The results presented here suggest that in flies as well there exist two forms of the adenylate cyclase catalytic subunit that can be distinguished genetically. The form that is sensitive to calcium/calmodulin (the *rut* cyclase) is much less responsive to guanyl nucleotides and monoamines, at least under these conditions, when the other cyclase component is responsive. The results are not precise enough to determine whether the calcium-sensitive cyclase is completely unresponsive to monoamines or guanyl nucleotides or only relatively less responsive than the calcium-insensitive cyclase; if the calcium-sensitive activity were an order of magnitude less responsive to guanyl nucleotides, as found by Brostrom *et al.* (29), its stimulation would not have been apparent in these experiments. Since in all other systems the monoamine and hormone responsiveness is mediated by G subunits (5), the fact that the cyclase affected by the *rut* mutation is apparently unresponsive to monoamines (Fig. 3) is consistent with the result that it is also unresponsive to p[NH]ppG (Fig. 2). These were surprising results, since it has been postulated that a synergism between calcium and monoamines should occur at the level of adenylate cyclase itself, a justifiable assumption since adenylate cyclase can respond to both monoamines and calcium. For example, Kandel *et al.* (18) suggest that a key molecular step in classical conditioning might be "that Ca^{++} might bind to ... the catalytic subunit of the cyclase ... so that the cyclase subsequently generates more cAMP in response to 5-HT [serotonin]." The results presented here suggest that the genetically distinguishable fraction of the cyclase activity that is responsive to calcium is unresponsive to monoamines and vice versa. Though it is certainly possible that the calcium-sensitive cyclase activity would be responsive to monoamines under some different assay conditions, its apparent unresponsiveness under these assay conditions nevertheless provokes one to consider the possibility that the capacities of the cyclase to respond to two types of activators (monoamines and calcium) might reside in two genetically distinct types of adenylate cyclase molecules rather than in the same molecule. This possibility suggests a slightly different model for associative learning that is consistent with both the *Drosophila* and the *Aplysia* data. I propose that the synergism between calcium and monoamines is a two-step process rather than a simple synergistic activation of one type of cyclase catalytic subunit. In this model, calcium, entering the neuron as a result of action potentials, would activate a calcium-dependent adenylate cyclase (the *rut* gene product), producing an elevation of intracellular cyclic AMP. The cyclic AMP in turn, and/or the calcium itself, would activate a protein kinase, which would modify the responsiveness of the monoamine receptor to its monoamine. This last step is not at all unlikely, since there are several reports in the literature of effects of cyclic AMP-dependent (as well as non-cyclic-AMP-dependent) phosphorylations that affect the affinity of monoamine receptors for their ligands (33–35). One particularly attractive aspect of a two-step model is that it automatically imposes order dependence on the calcium/monoamine interaction. Associative learning is characterized by a strong order dependence: the conditioned stimulus must precede the unconditioned stimulus, usually by a critical length of time. As first described by Pavlov (36), if the unconditioned stimulus precedes the conditioned stimulus no learning occurs. Consistent with this, Kandel *et al.* (18) found that to elicit activity-dependent amplification of monoamine responses, the neuronal activity must precede the monoamine release by 1 second or less, and there is no amplification if the monoamine precedes the neuronal activity. Fig. 5 shows two models for the biochemical basis for associative learning: in the model proposed by Kandel *et al.*, on the left, a synergistic response occurs in the adenylate cyclase enzyme if it is activated both by calcium that enters the terminal during an action potential and by the monoamine

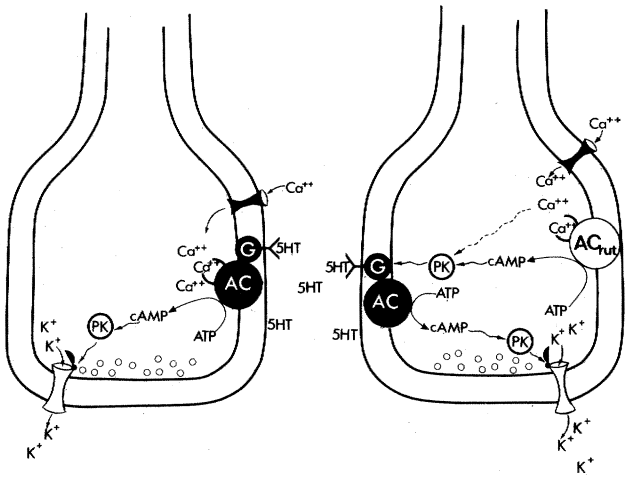

FIG. 5. Models proposed for the biochemical basis for activity-dependent amplification of monoamine responses at presynaptic nerve terminals. (*Left*) Model of Kandel *et al.* (18). (*Right*) A two-step model in which two genetically different adenylate cyclase catalytic subunits respond sequentially to calcium influx and to serotonin. AC, adenylate cyclase; PK, protein kinase; G, G subunit; 5HT, serotonin (5-hydroxytryptamine).

serotonin acting via a monoamine receptor coupled to a G subunit. In the second model, on the right, the synergism between calcium and serotonin is a two-step process: calcium that enters the nerve terminal during an action potential first activates a calcium-dependent (*rut*) cyclase, producing cyclic AMP, which in turn, via a cyclic AMP-dependent protein kinase, alters the serotonin receptor (or the receptor/G subunit interaction) so that it is more responsive to serotonin. A similar or synergistic role for a calcium-sensitive protein kinase is also indicated. In both models, the elevation of cyclic AMP in response to serotonin is postulated to act on a protein kinase, which phosphorylates a protein, either an ion channel or something that modifies an ion channel. In the case of the particular reflex described in *Aplysia*, this is likely a potassium channel (37).

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