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Signal convergence on protein kinase A as a molecular correlate of learning

(phosphorylation/Ca^{2+}-dependent proteolytic modification/acquisition/contiguity/memory)

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ABSTRACT **The response of a reaction network composed of protein kinase A, calpain, and protein phosphatase to transient cAMP and Ca^{2+} signals was studied. An essential feature of signal convergence is that the regulatory subunit of cAMP-dissociated protein kinase A undergoes limited proteolysis by the Ca^{2+}-activated proteinase calpain. A dynamic model of this system based on kinetic differential equations was built and simulated by computer. The system shows analogies to typical features of associative learning such as acquisition, contiguity detection, extinction, and memory decay, suggesting that these biochemical reactions may be part of the molecular mechanism of learning in *Drosophila*.**

The molecular mechanisms of learning and memory share several functional entities throughout the animal kingdom (1–6). In most of the systems where studies of learning, more specifically those of synaptic modulation, could be carried to the molecular level, protein phosphorylation plays a pivotal role. The kinases discussed (7–12) are rather similar in a wide spectrum of species. In *Drosophila* genetic, biochemical, and neuropharmacological evidence points to an essential role of cAMP-mediated phosphorylation in processes of learning and memory (13–18).

A fundamental question that should be asked about the molecular mechanism of a learning process is which molecular species in the reaction sequence is lastingly modified; i.e., what is the primary memory trace? It has been suggested that adenylate cyclase may serve as a target for signal convergence (19, 20), but experimental evidence for the long-term activation of this enzyme could not be obtained (21). Alternatively, type II protein kinase A (PKA) may be such a target. The autophosphorylation of the regulatory (R) subunit endows the enzyme with the property of prolonging the cAMP effect (22), or selective degradation of the R subunit can elevate kinase activity (23). The observation by Müller and Spatz (16) that the Ca^{2+}-activated neutral proteinase calpain alters some kinetic properties of *Drosophila* PKA by limited proteolysis of the R subunit offers another mechanism of long-term modification and for signal convergence by this enzyme.

In this work we have shown by computer simulation how the interactions of PKA, phosphatase, and calpain can lead to the translation of short cAMP signals into elevated kinase activity that lasts for hours. This phenomenon has features that are analogous to typical features of learning such as acquisition, contiguity detection, extinction, and memory.

DESCRIPTION OF THE MODEL

Activation of the Protein Kinase. The inactive holoenzyme of PKA consists of an R subunit dimer (R_2) and two catalytic (C) subunits. Upon binding of two cAMP molecules to each of the R subunits, the kinase dissociates and thereby becomes active. In the absence of cAMP, the C subunits reassociate with the R subunits and, consequently, PKA becomes inactive (24, 25). The type II PKA is characterized by the intramolecular phosphorylation of the R subunit (26). Dephosphorylation can only occur after dissociation of the two subunits because the C subunits protect the phosphorylated R subunits against phosphatase action. Autophosphorylation decreases the rate of reassociation of the two subunits (27, 28).

The free R subunit is a substrate for the Ca^{2+}-dependent protease calpain. This leads to a conversion of the R subunit to the truncated R subunit (R_p). The dimeric kinase comprising R_p as the regulatory subunit (16) is activated in an analogous manner. It also shows autophosphorylation. The steady-state activation curves of both R_2C_2 and R_pC in the phosphorylated state were measured (16) and a pronounced positive cooperativity was found. The Hill coefficients were 1.4 for both enzymes, suggesting that the dissociation of the two C subunits in the tetrameric R_2C_2 occurs independently and the stepwise binding of cAMP molecules alone accounts for the cooperativity. This allowed us to simplify the model by treating the R_2C_2 tetramer as two RC dimers. The activation constants (Table 1) corresponding to these mechanisms were determined by nonlinear regression with the concentration of the C subunits estimated at 0.01 μM for both sets of data. The equilibrium constants and the rate constants for the activation of the dephosphorylated enzymes were obtained by increasing only the rate constant for reassociation of the C subunit with the R subunit by a factor of 5 (27, 28), while the other rate constants remained the same. Although we considered a different mechanism, we assigned values to the rate constants, which in fact gave rates comparable to those considered by Buxbaum and Dudai (22).

Kinetic constants are not available for the modified kinase R_pC. We made the conservative assumption that the changes in the equilibrium constants for R_pC with respect to RC are equally due to changes in the forward and reverse constants.

The intramolecular autophosphorylation of the holoenzyme and of the ternary complex containing one cAMP was described as a pseudo-first-order reaction at a constant ATP concentration of 1 mM (35, 36). The corresponding rate constants were taken to be the same for R_pC. The ratio of dephosphorylation rates of R and R_p subunits was determined in *Drosophila* head homogenates (29). The R subunit is more readily dephosphorylated than is the R_p subunit.

The total PKA concentration in the nerve cells, including its modified form was assumed to be 0.1 μM (cf. ref. 22). The kinase activity was expressed in terms of the relative concentration of the free C subunit, normalized to the total enzyme concentration.

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Abbreviations: PKA, protein kinase A; R subunit, regulatory subunit; C subunit, catalytic subunit; R_p subunit, truncated R subunit.

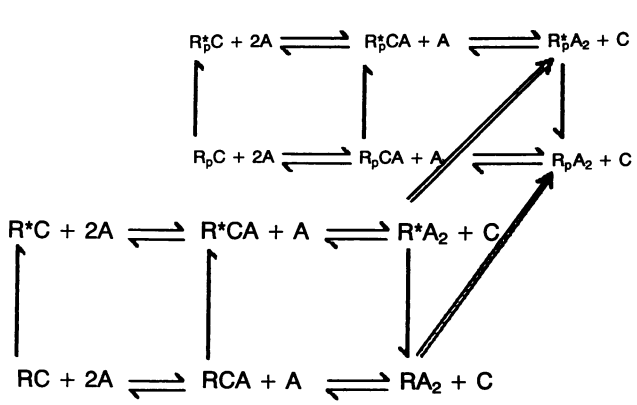
Table 1. Equilibrium and rate constants used for calculations

Reaction	K_{eq}	$k_{forward}$	$k_{backward}$	Remarks and ref(s).
1. R*C + A ⇌ R*CA	0.39 μM^{-1}	0.22 $\mu M^{-1}\cdot s^{-1}$	0.57 s^{-1}	Equilibrium constants from ref. 16, rate constants comply with refs. 22 and 46
2. R*CA + A ⇌ R*A_2 + C	0.12	0.074 $\mu M^{-1}\cdot s^{-1}$	0.6 $\mu M^{-1}\cdot s^{-1}$	
3. RC + A ⇌ RCA	0.39 μM^{-1}	0.22 $\mu M^{-1}\cdot s^{-1}$	0.57 s^{-1}	Equal to reaction 1 (27, 28)
4. RCA + A ⇌ RA_2 + C	0.024	0.074 $\mu M^{-1}\cdot s^{-1}$	3 $\mu M^{-1}\cdot s^{-1}$	$k_{backward}$ is 5 times higher than for reaction 2 (27, 28) but 2 times higher than the corresponding constant used in ref. 22
5. R_p^*C + A ⇌ R_p^*CA	0.14 μM^{-1}	0.13 $\mu M^{-1}\cdot s^{-1}$	0.94 s^{-1}	
6. R_p^*C + A ⇌ $R_p^*A_2$ + C	5.51	0.49 $\mu M^{-1}\cdot s^{-1}$	0.089 $\mu M^{-1}\cdot s^{-1}$	Fitted to experimental data (16)
7. R_pC + A ⇌ R_pCA	0.14 μM^{-1}	0.13 $\mu M^{-1}\cdot s^{-1}$	0.94 s^{-1}	Equal to reaction 5
8. R_pCA + A ⇌ R_pA_2 + C	1.10	0.49 $\mu M^{-1}\cdot s^{-1}$	0.45 $\mu M^{-1}\cdot s^{-1}$	$k_{backward}$ is 5 times higher than for reaction 6
9. RC → R*C				
10. RCA → R*CA				
11. R_pC → R_p^*C		20 s^{-1} ($k_{autophos}$)		Refs. 35 and 36
12. R_pCA → R_p^*CA				
13. R*A_2 → RA2			0.045 s^{-1} (k_{deph})	
14. $R_p^*A_2$ → R_pA_2			0.014 s^{-1} (k_{deph})	Fitted to experimental data (29)
15. → A		0.31 $\mu M/s$ (k_{AC})		Set to give a 10% R*C activation
16. → → A		40 $\mu M/s$ ($k_{AC,act}$)		Set to give a sharp cAMP pulse
17. A →		2 s^{-1} (k_{PDE})		Set to give a fast cAMP decay
18. RA_2 → R_pA_2		0.002–20 s^{-1} (k_{calp})		Same for both reactions
19. R*A_2 → $R_p^*A_2$				
20. RA_2 →				
21. R*A_2 →				
22. R_pA_2 →		1.8 × 10^{-4} s^{-1} ($k_{turnover}$)		Ref. 33
23. $R_p^*A_2$ →				
24. C →				

*Phosphorylated forms.

The cAMP Pulse. Unless otherwise stated, the steady state of free cAMP is determined by an influx rate (k_{AC}) of 0.31 $\mu M/s$ and a rate constant of destruction (k_{PDE}) of 2 s^{-1}. This leads to a steady-state cAMP concentration of 0.16 μM (22, 30). During stimulation k_{AC} is increased to 40 $\mu M/s$ for 1 s, while the k_{PDE} remains unchanged, giving a peak height of 20 μM. This pulse results in a maximal activation of the PKA of around 60%. We refer to this as the standard cAMP pulse. The half-width of the pulse is 1 s.

Calpain Action. The calcium-dependent neutral thiol proteinase calpain and its inhibitor calpastatin have been characterized from *Drosophila* heads (31). Whereas the holoenzyme of the neural PKA (type II) is not susceptible to calpain cleavage, the free R subunit proves to be a good substrate: the R subunit is proteolytically modified to the R_p subunit (16). The *in vivo* kinetics of the proteolytic action, in particular how long the calpain remains active after a short Ca^{2+} stimulus is not known. Here we treated the proteolytic action as a first-order reaction and assigned different rate constants to it, assuming a 5-s period of activation. The reaction scheme including the proteolytic modification can be pictured as



where an asterisk denotes the phosphorylated state, an A stands for cAMP, and the slanted arrows symbolize calpain action.

The standard calpain pulse was chosen to have a k_{calp} of 0.2 s^{-1} producing a 33% conversion of R to R_p subunits when paired to the standard cAMP pulse.

Protein Turnover. The concentration of PKA is tightly controlled in cells (32). The turnover of the enzyme seems to be determined by the turnover of the free subunits, which are much more susceptible to proteolytic degradation than is the holoenzyme. The system described so far would behave irreversibly unless protein turnover replaces R_p by R subunits, restoring the original state of PKA. An approximate estimate of the rate of turnover can be obtained from Weber and Hilz (33).

We describe the degradation of the subunits as a first-order process affecting free R_p subunits, free R subunits (regardless of the state of phosphorylation), and free C subunits at the same rate (33). The loss is immediately compensated for by synthesis of the holoenzyme RC. This way the total amount of PKA is held constant and the molar ratio of R subunits to C subunits is 1:1. It is this aspect that distinguishes our model from that of Greenberg *et al.* (23), who reported a 25% loss of R subunits in *Aplysia* after a sensitization treatment.

Numerical Treatment. To calculate chemical equilibria, we used an algorithm based on the Newton–Raphson method. Alternatively, we formulated a fifth-order polynomial, the only positive root of which gave the concentration of free C subunits available at a given cAMP concentration. From this the equilibrium concentrations of the other reactants could be expressed by back-substitution.

We designed a program that accepts up to 30 chemical reactions as an input, constructs the corresponding kinetic differential equations, and solves them using the algorithm ROW4A (37). The ROW4A is a semi-implicit fourth-order Runge–Kutta method with automatic error estimation and step-size control.

RESULTS

Kinetics of the Activation of PKA. Three states of the kinase RC were activated with a standard cAMP pulse: the fully phosphorylated state, the totally dephosphorylated state, and a state that allows dephosphorylation and autophosphorylation to proceed. The fully autophosphorylated kinase has a higher baseline activity, and the decay of activity after stimulation is 3–4 times slower for the autophosphorylated enzyme than for the dephosphorylated enzyme (cf. ref. 22). If phosphorylation/dephosphorylation is included, the model leads to intermediary kinetics.

The modified kinase R_pC under the same circumstances shows a longer-lasting activation as compared to RC due to its lower rate constants of reassociation. Important for the model is the shift in the baseline from 5% activation for RC to 21% for R_pC with ongoing phosphorylation/dephosphorylation in both cases. The term baseline is used to describe the activity after the system has relaxed to an equilibrium of holoenzymes RC and R_pC and the dissociated subunits.

Effect of Calpain and Pairing of the Two Stimuli. Activation of calpain leads to the conversion of free R subunits to R_p subunits and consequently a decrease in the rate of reassociation and an elevation in the concentration of free C subunits, hence persistent activation of part of the kinase. Since the amount of free C subunits is also a function of the steady-state cAMP concentration, it is appropriate to characterize the behavior of the system both in terms of R_p subunit production and the concentration of free C subunits.

Rather than converting R to R_p subunits in a single step, the reaction can be carried out in a number of consecutive steps with intermediate calpain activities (Fig. 1). Both the amount of R_p subunits and the baseline of kinase activity rise with every pairing.

The consequences of separating the calpain and the cAMP pulse in time are shown in the contiguity plot (Fig. 2). If the calpain pulse is given more than 5 s—the assumed activation period—before the cAMP pulse, it remains nearly ineffective because too little free R subunit is available. For significant R_p subunit production the cAMP pulse must coincide with or precede the calpain pulse. The optimal conversion of R to R_p subunits is observed if the calpain pulse starts 1 s after the cAMP pulse. Upon further separation the conversion decreases, because more and more free subunits reassociate to give the holoenzyme, which is not susceptible to calpain action.

Protein Turnover. After stimulus pairing, the long-term behavior of the system was determined by protein turnover. Since free R_p, R, and C subunits are consumed and RC is slowly replenished, the net effect of protein turnover is a replacement of R_p subunits by R subunits. This in turn leads to a decrease in the concentration of free C subunits (Fig. 3). This process can be accelerated to a small extent by a series of cAMP pulses that dissociate R_pC and thereby increase the degradation of R_p subunits (Fig. 3 *Inset*).

cAMP Turnover. It is of particular interest to study the consequences of changed steady-state levels of cAMP. Reducing the efflux rate of cAMP in our system, as in the dunce mutant (17), gives a flatter contiguity curve (Fig. 4). Elevated cAMP levels also yield an increased proteolytic modification of R subunits even without a cAMP pulse. This is in agreement with the observation that the amount of R_p subunits is higher in dunce than in wild-type flies (16). Since the rate of turnover of PKA is determined by the availability of free subunits, higher cAMP levels promote "memory decay" (Fig. 5, curves A and dunce).

In another learning mutant rutabaga[1], the steady-state levels of cAMP are only slightly reduced. The mutant is characterized by a loss of calcium/calmodulin activation of adenylate cyclase (18, 20) and diminished activation by

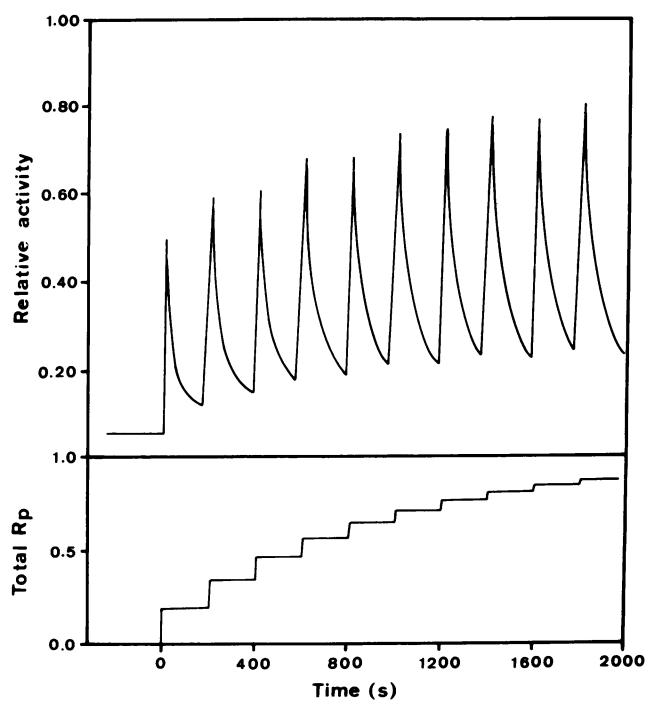


FIG. 1. Repeated application of cAMP and Ca^{2+} pulses. The system, initially containing 0.1 μM RC and no R_pC, was repeatedly and simultaneously stimulated with a standard cAMP pulse and calpain activation (k_{calp} = 0.1 s^{-1}, duration = 5 s). The time interval between the stimulations was 200 s. (*Lower*) Total R_p subunits produced. (*Upper*) Concentration of free C subunits. (Data are relative to the total enzyme concentration.)

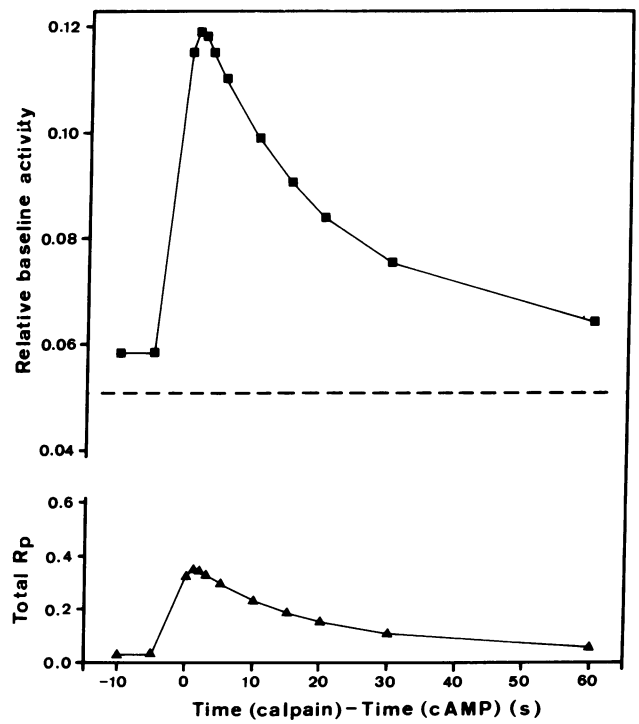


FIG. 2. Contiguity of calpain and cAMP pulses. The two pulses were given separately in a system initially containing 0.1 μM RC. The concentration of R_p subunits produced (▲) (*Lower*) and the baseline concentration of free C subunits relative to the total enzyme concentration (■) (*Upper*) were plotted as a function of time separating the two pulses. The baseline concentration of free C subunits without stimulation is indicated by the broken line.

forskolin (34, 38). Reducing the rate of cAMP influx under stimulation in our system depresses the peak of the contiguity curve to 60% of the wild type (Fig. 4), analogous to the poor learning performance of rutabaga[1]. Memory decay in the simulated rutabaga[1] (Fig. 5 *Inset*), though starting from a lower level, proceeds with approximately the same rate constant as in the simulated wild type.

DISCUSSION

Simulation of the kinetic behavior of three interacting enzymes, PKA, phosphatase, and calpain, indicates that this enzyme system in *Drosophila* may underlie both signal extension and signal convergence, two essential features in memory mechanisms. This is an extension of the analysis done by Buxbaum and Dudai (22), who have pointed out that the properties of PKA enable it to translate a transient cAMP signal into a prolonged kinase activation. Our model invokes the calpain-catalyzed proteolytic modification of the R subunit of PKA (16), which provides us with a further mechanism to prolong kinase activity. Recall that in *Aplysia* the persistent activation of PKA, and consequential phosphorylation of a K^+ channel, brings about a change of synaptic strength and hence behavior (39).

Aszódi and Friedrich (40) have devised hypothetic enzymatic reaction networks that are capable of mimicking associative learning. The present analysis of a real system reveals several aspects that bear close analogies to typical features of learning processes. Two stimuli, cAMP and Ca^{2+}, converge on PKA, leading to a covalent modification of the R subunit to the R_p subunit, which can be regarded as an elementary memory trace. The appearance of the R_p subunit alters the properties of the kinase, resulting in long-term activation. Small concomitant pulses of the two stimuli lead to the buildup of the modified subunit R_p and, consequently, of free C subunit, a process akin to acquisition (Fig. 1).

The contiguity curve of Fig. 2 bears nontrivial resemblance to those of classical conditioning. The enzyme system as outlined can discriminate the temporal order of the two stimuli. We observe the same asymmetry and the same characteristic times in the contiguity curve as found in learning experiments (41, 42). In contrast, a cellular model of associative learning in *Aplysia* (43), where the two stimuli are proposed to converge on the adenylate cyclase, predicts considerably shorter interstimulus intervals.

Protein turnover leads to a loss of R_p subunits and their replacement by R subunits and, consequently, to a decrease in the baseline level of free C subunits (Fig. 3). This can be regarded as analogous to memory decay. Additional cAMP pulses speed up the replacement of R_p by R subunits (Fig. 3 *Inset*), producing a phenomenon analogous to extinction, but to a smaller extent than for behavioral experiments on *Drosophila* (42, 44).

Moderate agreement is found between the consequences of changes in cAMP turnover in our model and the behavior of *Drosophila* learning mutants. Low olfactory learning scores and accelerated memory decay are characteristic for the dunce mutant (17) and also for rutabaga[1] (42). It has to be borne in mind that the differences in memory decay between

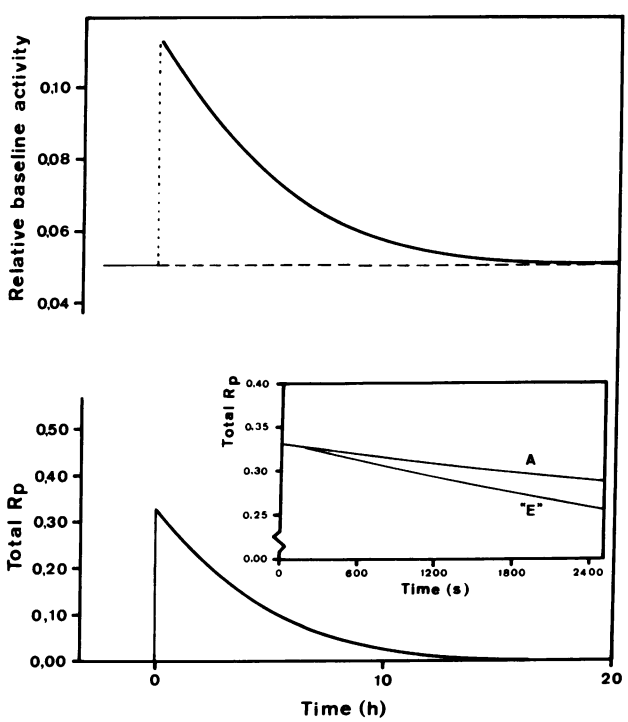


FIG. 3. Memory decay of the system. A cAMP pulse and a Ca^{2+} pulse were applied simultaneously as described above. The disappearance of R_p subunits (*Lower*) and the return of free C subunits to the original baseline (*Upper*) due to protein turnover are shown. The transient rise in the free C subunit level for the first 10 min after activation is not shown. The broken line is the baseline level before proteolytic modification. (*Inset*) Extinction. The elimination of R_p subunits is slightly faster when repeated cAMP pulses (20 pulses over 40 min, without Ca^{2+}) transiently elevate the free R_p subunit level. Curves: A, memory decay without extra cAMP pulses; E, memory decay with extra cAMP pulses.

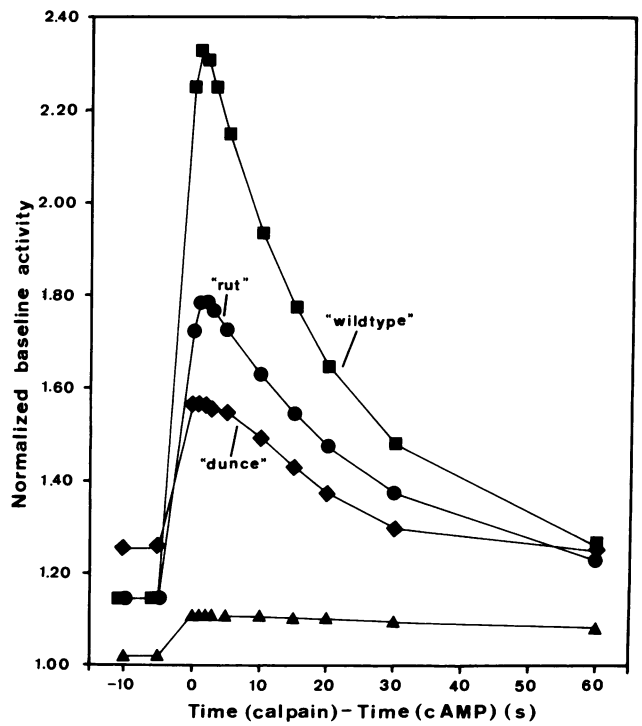


FIG. 4. Contiguity curves at various cAMP levels. Changes in baseline concentrations of free C subunits are plotted versus the time difference between calpain and cAMP pulses as in Fig. 2. ■, Wild type; ●, cAMP influx ($k_{AC,act}$) 2 times reduced during stimulation only, resembling mutant rutabaga[1]; ◆, cAMP efflux (k_{PDE}) 10 times reduced, resembling mutant dunce; ▲, cAMP influx (k_{AC}) 10 times reduced both in steady state and during activation. To facilitate comparison, the effect of pairing was described with the new baseline concentrations of free C subunits, normalized to the level before proteolytic modification. It is assumed that the change in baseline activity due to the altered cAMP levels is compensated for in the mutants and that during stimulus pairing only the change relative to the original baseline provides physiologically relevant information.

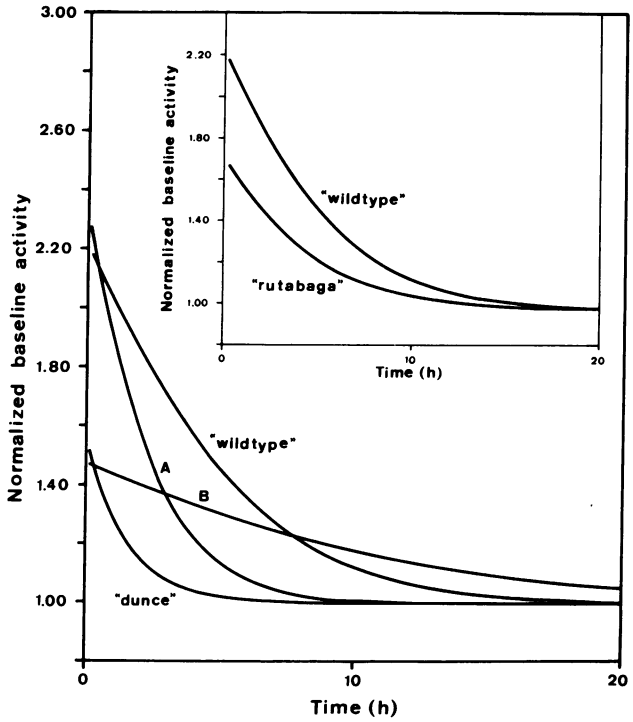


FIG. 5. Effect of cAMP turnover on memory decay. The systems were simulated as in Fig. 3 and are characterized in terms of baseline concentrations of free C subunits, normalized to the level before proteolytic modification. Curves: dunce, cAMP efflux (k_{PDE}) 10 times reduced; A, cAMP efflux (k_{PDE}) 3 times reduced; B, cAMP influx (k_{AC}) 3 times reduced at the baseline and during stimulation. (*Inset*) cAMP influx rate ($k_{AC,act}$) reduced by a factor of 2 during stimulation but the turnover rates are otherwise unchanged. The rutabaga system starts from a lower relative C subunit level but the decay kinetics are approximately the same as in the wild-type system.

wild type and mutants is less pronounced in visual learning experiments (45).

Although our model describes only part of the chain of molecular events that can lead to changes in synaptic transmission and although we had to assume values for some of the rate constants and had to use others from non-*Drosophila* studies, the analogy of the response of our simple enzyme system to learning behavior of *Drosophila* imagoes suggests that modification of PKA by calpain plays a key role in the mechanism that accounts for plasticity in nervous systems.

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