Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 557–560, January 1991
Neurobiology

A cyclic AMP-activated K^+ channel in *Drosophila* larval muscle is persistently activated in dunce

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Communicated by Georgio Weber, October 11, 1990

ABSTRACT **Single-channel recording from longitudinal ventrolateral *Drosophila* larval muscle reveals the presence of a potassium-selective channel that is directly and reversibly activated by cAMP in a dose-dependent fashion. Activation is specific and it cannot be mimicked by a series of agents that include AMP, cGMP, ATP, inositol trisphosphate, and Ca^{2+}. Channel current records obtained from larval muscle in different dunce mutants possessing abnormally high levels of cAMP show that, in the mutants, the channel displays an increased probability of opening.**

To elucidate the role of ion channels in neuronal modulation and behavior it is of special interest to study those that are regulated by second messengers. *Drosophila* neurons and muscle cells provide excellent preparations in which the modulation of electrical activity can be investigated at the single-channel level (1–4). Mutants showing altered excitability have been identified (5–9) and in some cases the mutation has been shown to be restricted to one class of channel (4, 10–12). The *Drosophila* mutant dunce is a single-gene mutant showing poor levels of associative learning and rapid short-term memory losses (13, 14). The mutant dunce lacks a form of phosphodiesterase leading to abnormally high intracellular levels of cAMP (15, 16). Although this mutant has been well characterized from a biochemical and a genetic point of view (15–18), there are no studies linking the dunce defect to changes in the electrical properties of excitable cells. We report here our finding that *Drosophila* larval muscle possesses a K^+-selective channel that is activated directly and reversibly by cAMP. Single-channel recording from muscles of different dunce mutants demonstrates that in dunce this K^+ channel displays a much increased probability of opening compared to the wild type.

MATERIALS AND METHODS

Experiments were performed at room temperature (20°C–24°C) in longitudinal ventrolateral muscle from male *Drosophila* larvae by using standard patch clamp techniques (1, 19). The patch pipette contained standard saline buffer (SSB) consisting of 128 mM Na^+, 2 mM K^+, 4 mM Mg^{2+}, 1.8 mM Ca^{2+}, 140 mM Cl^-, 5 mM Hepes, and 36 mM sucrose (pH 7). In the cell-attached configuration, the experimental chamber contained SSB. In studies in which excised patches (inside-out configuration) were used, the bath was filled with a pseudointracellular buffer made of 5 mM Na^+, 150 mM K^+, 4 mM Mg^{2+}, 9 mM Cl^-, 150 mM acetate, and 5 mM Hepes (pH 7). Current records were stored in a digital tape and the fraction of open time (P_o) was obtained from records filtered at 1 KHz by using an eight-pole Bessel filter. Sample points were taken at 20 KHz. Membrane potentials were measured as described (1). The wild-type strain of fly was Oregon-RC.

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Table 1. Fraction of time in the open state (P_o), average number of cAMP-activated K^+ channels per patch, and resting potential in wild-type *Drosophila* and in dunce mutants

Mutant	P_o	Average no. of channels per patch*	Resting potential,† mV
Oregon	0.0074 ± 0.0004	(6)	48.6 ± 0.6 (112)
$dunce^1$	0.0377 ± 0.016	2.5 (4)	51.8 ± 0.4 (72)
$dunce^2$	0.0956 ± 0.025	2.5 (5)	
$dunce^{M11}$	0.1185 ± 0.060	3.5 (3)	
$dunce^{M14}$	0.1600 ± 0.050	3.0 (15)	55.6 ± 0.7 (48)
Oregon + 8-BrcAMP	0.1580 ± 0.007	4.0 (6)	

The number of channels per patch was obtained assuming independence and using a binomial distribution (e.g., see ref. 21).
*Number in parentheses is the number of different patches studied.
†Number in parentheses is the number of different measurements done.

The dunce mutants were $ydnc^{M14}cvvf$, $ydnc^{M11}cvvf$, dnc^2, or dnc^1. Mutants were kindly supplied by Ronald Davis (Department of Cell Biology, Baylor College of Medicine, Houston). These mutations were induced by ethyl methanesulfonate (15, 16). The dnc^{M14} stock was balanced with *FM3* and *FM7a*. Therefore, two types of male larvae will be present—$ydnc^{M14}cvvfY$ and *FM7a/Y* (*FM3* is lethal). Two types of males will also be present in the dnc^2 and dnc^{M11} stocks since these were balanced with *FM7b* and *FM7a*, respectively. In dnc^2, dnc^{M11}, and dnc^{M14}, inspection of the adult flies obtained from the same cultures used in patch-clamp studies revealed that ≈50% of the male adults ($n \approx 200$ for each mutant) were dunce. The two types of males can be distinguished by the color of their malpighian tubules, since *FM7a* carries a white eye mutation (colorless tubules) versus the pale yellow tubules of the $ydnc^{M14}cvvf$ chromosome. For the dnc^1 stock all larvae are dunce since the stock is homozygous. All patch clamp experiments were done in male larvae that were recognized under the microscope by inspection of their gonads. Earlier experiments done with $dunce^{M14}$ cultures kindly provided by Linda Hall (Department of Genetics, Albert Einstein College of Medicine, Bronx, NY) and Tim Tully (Department of Biology, Brandeis University, Waltham, MA) gave essentially the same results.

RESULTS

In cell-attached patches we have detected a channel that opens very infrequently (Fig. 1*A*). The same channel can be recorded in inside-out patches in which the bath solution contains pseudointracellular buffer (Fig. 1*B*). In this condition, the current–voltage relationship for the open channel is nonlinear (Fig. 1*C*, solid circles) and a fit to the current–voltage data using the constant field equation (Fig. 1*C*) yields a permeability ratio of $P_K/P_{Na} = 10$. The channel slope conductance measured at voltages >40 mV is 47 pS and is reduced to 23 pS by addition of tetraethylammonium to the

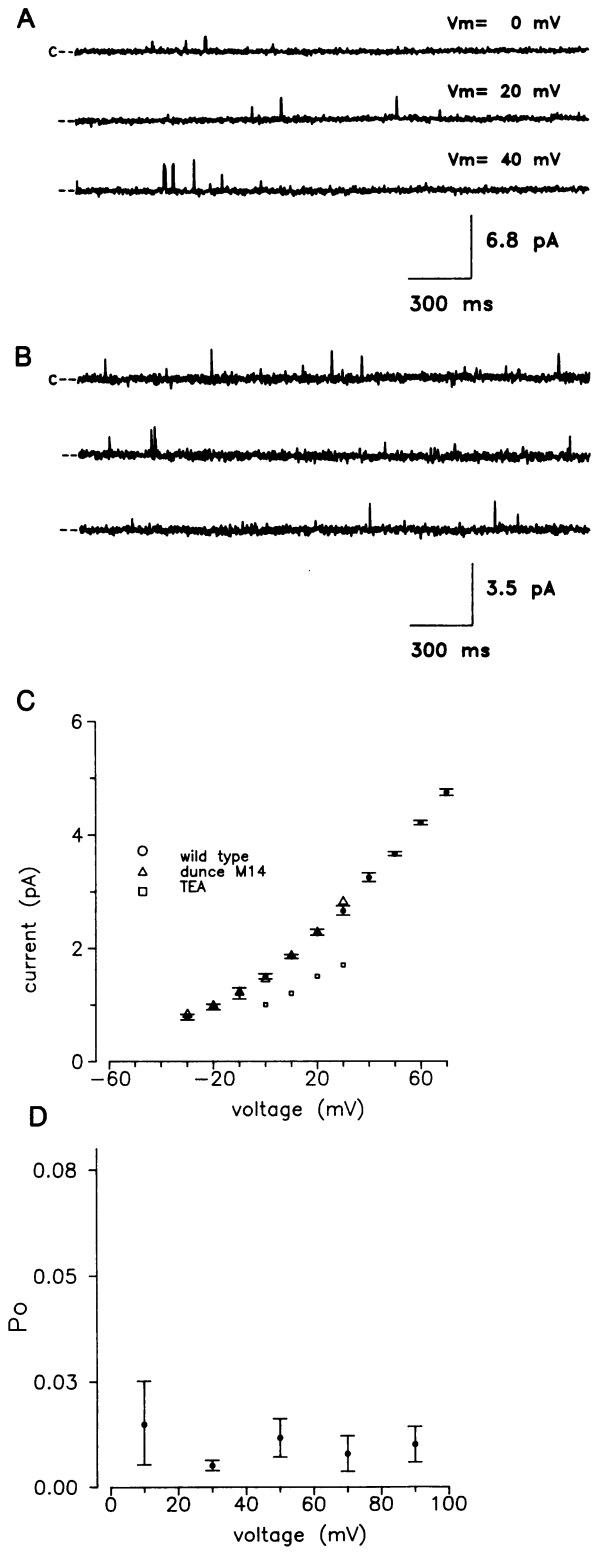


FIG. 1. Single K^+ channels in cell-attached and excised patches in dorsal longitudinal *Drosophila* larval muscle. (*A*) Single-channel current records in cell-attached patches in wild-type larval muscle at different voltages. c, Closed state. Channel opening is represented by upward deflections. V_m, applied voltage (pipette negative). (*B*) Single-channel currents after excising the patch shown in *A*. $V_m = 10$ mV. (*C*) Current–voltage relationship for the open channel in excised patches of wild-type muscle (solid circles). Each point represents the average current from the measurement of the amplitude of at least 30 current fluctuations at the indicated voltages and the bars are SEMs. Solid line is a fit to the data using the constant field equation (20). Triangles represent the current–voltage relationship obtained under the same experimental conditions but in excised patches from dunce mutants. Open squares are channel current amplitudes measured in an excised patch in the presence of 100 μM tetraethylammonium (TEA) in the external buffer. (*D*) Channel open state probability vs. applied voltage in excised patches in wild-type muscle.

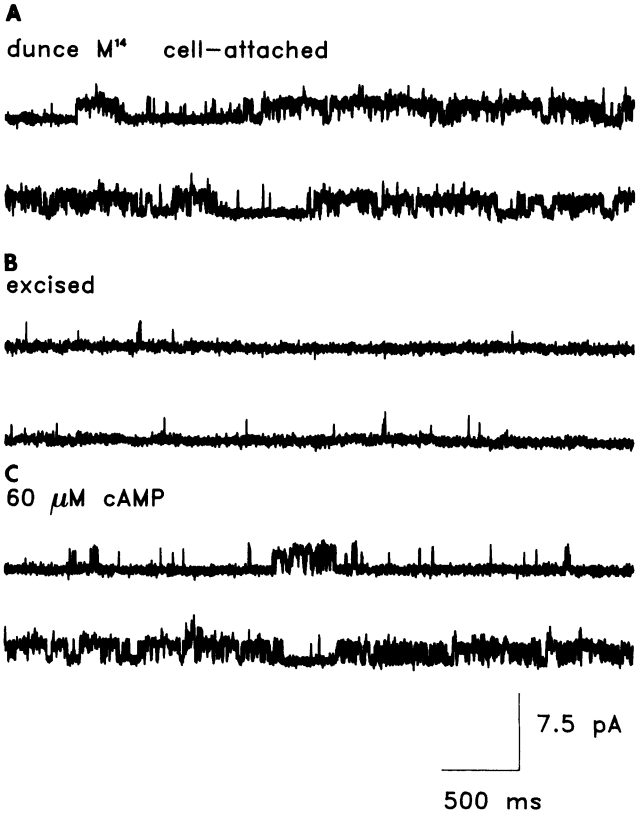
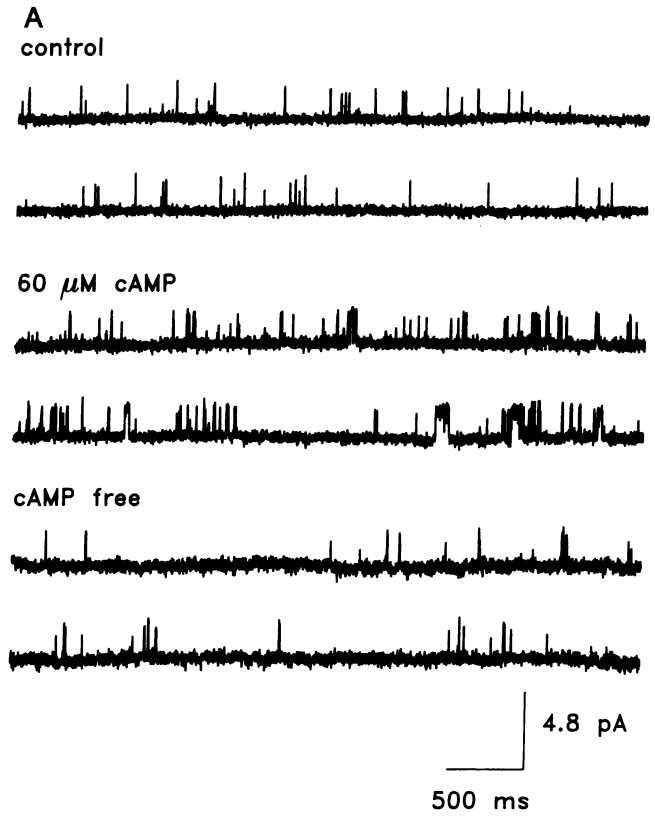


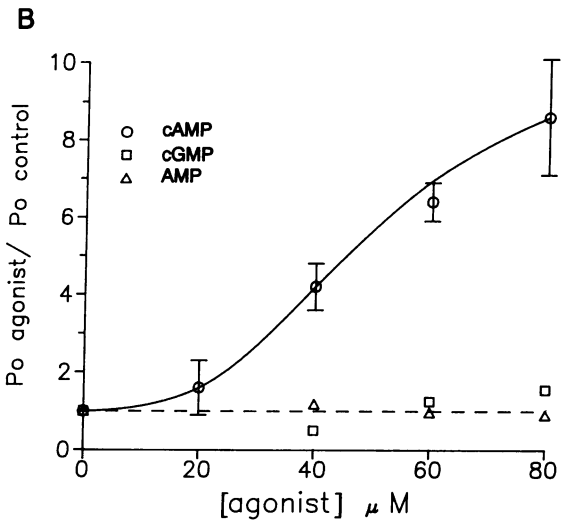
FIG. 2. Potassium channel activity in male larval muscle from dunce^{M14} cultures. (*A*) Channel current records in a cell-attached patch from dorsal longitudinal larval muscle from dunce^{M14} culture showing a high level of activity. Openings shown as upward deflections. $V_m = 10$ mV. (*B*) Channel current fluctuations recorded after excising the patch shown in *A*. $V_m = 10$ mV. (*C*) Channel current fluctuations after exposing the patch shown in *B* to a pseudointracellular buffer containing 60 μM cAMP. Other conditions were identical to those in Fig. 1. $V_m = 10$ mV.

external solution to a final concentration of 100 μM (Fig. 1*C*, open squares). Fig. 1*D* shows that the channel displays a very low, voltage-independent probability of opening (see also Table 1).

Muscle from dunce^{M14} male larvae obtained from dunce^{M14} cultures contains a channel with an identical current–voltage relationship (Fig. 1*C*, triangles) but with a much higher probability of opening (Fig. 2 *A* and *B*). Excising the patch from the cell leads to a decrease in the probability of opening to levels similar to those found in muscle from wild-type larvae (Fig. 2*B*). In view of these observations, and the fact that dunce^{M14} has been shown to display abnormally high intracellular levels of cAMP (15, 16), we investigated whether or not this K^+ channel is directly activated by cAMP. Fig. 2*C* shows that exposure of the same excised patch shown in Fig. 2*B* to 60 μM cAMP results in an increase in P_o to levels found in the dunce^{M14} cell-attached patch shown in Fig. 2*A* ($P_o \approx 0.2$; see Table 1).

We used other dunce alleles to test whether the dunce cAMP elevation correlates with the channel opening phenotype. Table 1 shows that all the dunce mutants tested had a much higher channel activity than the wild type. Considering all the male larvae used in the patch clamp studies, 60% (5/8), 50% (3/6), and 38% (15/39) of dunce^2, dunce^{M11}, and dunce^{M14} male larvae, respectively, show a high level of channel activity.





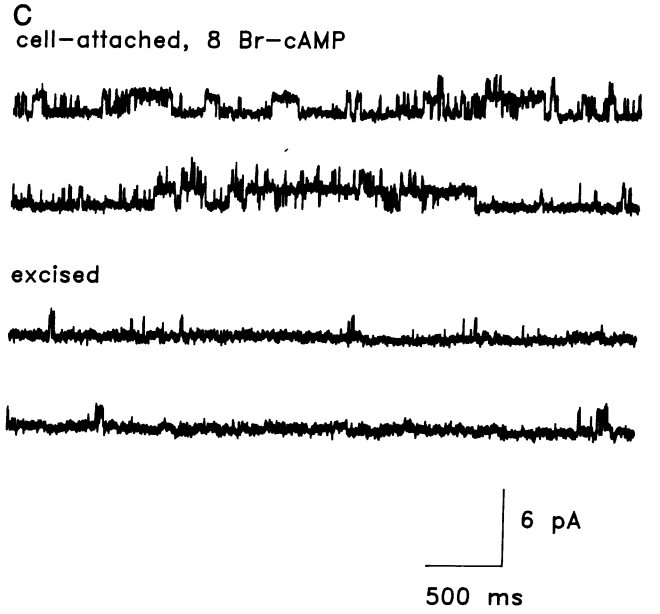


FIG. 3. Channel activation by cAMP in excised patches. (*A*) Channel activity in an excised patch before cAMP exposure (upper records). Channel activity in the presence of 60 μM cAMP (middle records). Channel open probability increased in this particular experiment from 0.01 in the absence to ≈0.1 in the presence of cAMP. Channel activity after perfusion with cAMP-free pseudointracellular buffer (lower records). Openings shown as upward deflections. V_m = 10 mV in all records. (*B*) Probability of channel opening as a function of cAMP, cGMP, and AMP concentration. Solid line is a fit to the experimental data obtained in the presence of cAMP (open circles) by using the equation $P_o/P_c = 1 + (P_m/P_c)([cAMP]^N/(K^N + [cAMP]^N)$, where N is the Hill coefficient, K is the apparent dissociation constant, P_o is the fraction of time in the open state in the presence of cAMP, P_c is the fraction of time in the open state in the absence of cAMP, and P_m is the fraction of time in the open state at very high [cAMP]. The best fit using a nonlinear least-squares fitting procedure (22) gives N = 2.9, K = 50.8 μM, and P_m = 9.6. The experimental points represent the average values of the ratio P_o/P_c ± SEM obtained from three different patches over the concentration range shown. Squares and triangles represent the values of P_o/P_c ratios in the presence of cGMP and AMP, respectively. (*C*) Effect of 8-BrcAMP on channel activity in cell-attached patches from wild-type larval muscle. The larval muscles were treated for 20 min with SSB containing 200 μM 8-BrcAMP. After this treatment, a cell-attached patch was established and channel activity was measured (upper records). Lower records were taken after excising the patch in the presence of pseudointracellular buffer. c, Baseline. V_m = 0 mV in all records.

Thus, the percentage of trials in which the channel is active in male larvae from dunce correlates well with the fraction of dunce adult males in the population (see *Materials and Methods*). In dunce[1] we found that 80% (4/5) of the larvae show a high level of activity. This higher frequency of appearance of cAMP-activated channels in dunce[1] compared to the other dunce is to be expected, given the fact that all males in the population are dunce. Table 1 shows only the results obtained in active patches. In a series of experiments, we identified dunce males in the dunce^{M14} culture by the color of the malpighian tubules; as expected, in all cases the channel was active. In all mutants, channel conductance remains as in the wild type (≈50 pS at 40 mV; see Fig. 1*C*) and the channels were activated by cAMP after excising the membrane patch from the muscle cell. Channel activity appears to be related with intracellular cAMP concentration. Thus, dunce[1] and dunce[2] with the lower intracellular cAMP content [≈2-fold larger than the wild type (15)] show on the average lower P_o values than dunce^{M14}. In this mutant, the cAMP level is ≈6-fold larger than the wild type (16). Table 1 also shows that the main effect of this single-gene mutation is on the channel open probability rather than the number of channels per patch. At the macroscopic level, this persistent activation of the K^+ channel in dunce muscle correlates with resting potential values that are 4–7 mV more hyperpolarized than in wild-type muscle.

In a series of experiments carried out in inside-out patches from wild-type muscle, we proceeded to study channel activation by cAMP. Fig. 3*A* (middle records) shows that exposure of the cytoplasmic side of the patch to micromolar concentrations of cAMP results in activation of the channel

to levels similar to those found in cell-attached patches in dunce^{M14}. Furthermore, as illustrated in Fig. 3*A* (lower records), the effect of cAMP is reversed upon perfusing the chamber with cAMP-free buffer. Fig. 3*B* documents that, at micromolar concentrations, cAMP, but not cGMP or AMP, is effective in activating the channel and that cAMP activation is dose dependent. In addition, inositol trisphosphate (50 μM), ATP (1 mM), and Ca^{2+} (100 μM) failed to increase the probability of channel opening in cell-free patches.

Additional experiments were performed to determine the effects of increasing the intracellular cAMP concentration on P_o in cell-attached patches of wild-type *Drosophila* larval muscle. As shown in Fig. 3*C* (upper records) and Table 1, levels of channel activity similar to those obtained in cell-attached patches of dunce^{M14} muscles can be recorded when wild-type muscles are incubated in Ringer's solution containing 200 μM 8-BrcAMP. Moreover, as found in the mutant, excising the patch causes a decrease in channel activity to levels measured in the untreated wild-type muscles (Fig. 3*C*, lower records).

DISCUSSION

Some second messengers, such as Ca^{2+} and cGMP, exert their actions by directly interacting with binding site(s) in the channel protein (23–25). On the other hand, channel modulation by cAMP is usually mediated via cAMP-dependent kinases whose activation leads to a covalent modification (26, 27). We have found that cAMP is able to activate directly a K^+ channel present in *Drosophila* larval muscle. The possibility that cAMP exerts its effect indirectly by activating a kinase is unlikely since channel activation occurs in cell-free patches in the absence of ATP. Furthermore, no agent other than cAMP seems to be required to achieve channel activation; the effect is obtained rapidly after exposing the patch to this cyclic nucleotide, and it is readily reversed when the chamber is perfused with a cAMP-free buffer. Thus, the cAMP site(s) appears to be specific and the K^+ channel described here has a Hill coefficient of 2.9 and an apparent dissociation constant of 50.8 μM (Fig. 3*B*, solid line), similar to the values reported for the cyclic nucleotide-gated conductances in vertebrate photoreceptors (24) and olfactory neurons (28).

We showed that muscle exposure to 8-BrcAMP activated the channel in cell-attached patches. Attempts to increase the intracellular cAMP levels through the activation of the adenyl cyclase were made by adding forskolin to the bath to a final concentration of 50 μM. However, we found that this compound greatly increases the patch noise and produces muscle contraction, apparently due to an increase in transmitter release from nerve terminals. Indeed, in the presence of forskolin, intracellular recordings reveal a higher frequency and larger amplitude of spontaneous postsynaptic potentials (data not shown).

Patch clamp studies in cultured embryonic myotubes derived from *Drosophila* indicate the presence of at least four different types of K^+ channels (2–4). Three of these channels (A_1, K_D, and K_O) display voltage-dependent kinetics. A fourth type (K_{ST}) is voltage independent and is activated by membrane stretch. K_O has a conductance similar to the cAMP-activated channel described here. However, in contrast with the cAMP-activated channel recorded from ventral longitudinal larval muscle, K_O is voltage dependent. K_{ST}, which is voltage independent, has a larger conductance and is stretch activated. We found no signs of activation by stretch of the cAMP-activated channel.

Previous studies have demonstrated that dunce mutants display poor retention in a classical conditioning test (13, 14), lack a form of phosphodiesterase, and have cAMP levels 2- to 6-fold higher than in the wild type (15, 16). We show here that *Drosophila* larval muscles possess a conductance directly activated by cAMP, and we present evidence that this channel is persistently activated in a series of dunce mutants, indicating that dunce cAMP elevation segregates with the channel opening phenotype. It remains to be established whether this conductance is present in adult muscle or in other excitable tissues in *Drosophila*. The physiological importance of this channel is yet unclear, but the evidence presented in Table 1 indicates that it contributes to the total resting conductance in dunce, making the resting potential $\approx$7 mV larger in the case of dunce^{M14}. Therefore, the results reported here render feasible the hypothesis that abnormal regulation of a cAMP-gated K^+ channel might underlie the behavioral defects in dunce mutants.

We thank Drs. J. G. Nicholls, R. W. Aldrich, and T. Tully for comments and criticisms on an early version of this manuscript. We are indebted to Drs. L. Hall and T. Tully for providing us with the dunce mutants in which preliminary studies were done. We are much indebted to Dr. R. Davis for his generosity in providing the mutants and for comments on the manuscript. This work was supported by National Institutes of Health Grant GM-35981, Fondecyt 1167/88, and by a grant from the Tinker Foundation. R.L. is a recipient of a John Guggenheim Fellowship. He also wishes to thank the Dreyfus Bank (Switzerland) for generous support from a private foundation that they made available to him.

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