Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10044–10048, October 1994
Neurobiology

Calcium/calmodulin-dependent protein kinase II and potassium channel subunit Eag similarly affect plasticity in *Drosophila*

(learning/memory/synaptic physiology/protein phosphorylation)

LESLIE C. GRIFFITH*, JING WANG†, YI ZHONG†‡, CHUN-FANG WU†, AND RALPH J. GREENSPAN§

*Department of Biology and Center for Complex Systems, Brandeis University, Waltham, MA 02254; †Department of Biological Sciences, University of Iowa, Iowa City, IA 52242; and §Department of Biology and Center for Neural Science, 1009 Main Building, New York University, New York, NY 10003

Communicated by Howard Nash, June 8, 1994

ABSTRACT **Similar defects in both synaptic transmission and associative learning are produced in *Drosophila melanogaster* by inhibition of calcium/calmodulin-dependent protein kinase II and mutations in the potassium channel subunit gene *eag*. These behavioral and synaptic defects are not simply additive in animals carrying both an *eag* mutation and a transgene for a protein kinase inhibitor, raising the possibility that the phenotypes share a common pathway. At the molecular level, a portion of the putative cytoplasmic domain of Eag is a substrate of calcium/calmodulin-dependent protein kinase II. These similarities in behavior and synaptic physiology, the genetic interaction, and the *in vitro* biochemical interaction of the two molecules suggest that an important component of neural and behavioral plasticity may be mediated by modulation of Eag function by calcium/calmodulin-dependent protein kinase II.**

Modulation of potassium channel activity and regulation of transmitter release at nerve terminals have emerged as key sites of regulation in synaptic plasticity (1). This has been illustrated by the physiological correlates of learning in *Aplysia* (2), where during sensitization postsynaptic potentials are increased by modulation of a potassium current. More recently, it has been demonstrated that genetic variants of *Drosophila* defective in learning have altered transmitter release (3). Calcium/calmodulin-dependent protein kinase II (CaM kinase II) has also emerged as an important mediator of plasticity. In the mammalian brain it has been shown to be required *in vitro* for induction of long-term potentiation (4), a cellular correlate of memory. A genetic "knock out" of mouse α-CaM kinase II has been shown to abolish long-term potentiation and produce defects in spatial learning (5, 6). CaM kinase II also appears to be required for plastic behaviors in *Drosophila*. Flies expressing a transgene inhibitor of CaM kinase II under control of a heat shock promoter are unable to retain the effects of conditioning in both associative and nonassociative paradigms based on courtship behavior, while locomotor activity and sensory functions remain unimpaired (7).

Mutations in the *eag* gene alter several potassium channel currents and block their modulation by various pharmacological agents affecting second messenger pathways (8–10). The gene product Eag has been shown to be a potassium channel subunit that contains a number of putative modulatory regions (8, 11) including several CaM kinase II phosphorylation sites (see *Materials and Methods*). Here we describe parallels in physiological and behavioral phenotypes caused by *eag* mutations and CaM kinase II inhibition. Furthermore, we investigate interactions of Eag and CaM kinase II at the behavioral, physiological, genetic, and biochemical levels in *Drosophila melanogaster*.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was purchased from NEN, calmodulin-Sepharose was from Pharmacia, and other reagents were of the best available commercial quality.

Behavior. Behavioral tests and rearing were conducted essentially as in ref. 7. Males were collected 5 days before testing; mated females were fertilized the day before testing. Individual males were placed in a chamber with a mated female for 1 hr, and their courtship index (CI is the number of minutes in a 10-min period spent directing courtship toward the female; 0 = no courtship, 10 = courtship for the full 10-min observation period) was measured during the first 10 min and during the final 10 min. The percentage of final CI to initial CI ($CI_{final}/CI_{initial}$) was computed for each male and averaged for each genotype (n = the number in parentheses). Data from individuals with $CI_{initial} < 1$ were excluded from analysis since learning cannot occur in the absence of some level of initial courtship; it is the temporal pairing of courtship and the aversive stimulus of the mated female that results in conditioning (12). +sib control males and mated females were from the same *C(1)DX, ywf* strain used as genetic background (7) for the *ala* and *eag* lines. These C(1) lines produce patroclinous males and females that contain a Y chromosome.

Recording at the Larval Neuromuscular Junction. Excitatory junctional potentials (EJPs) were recorded intracellularly from muscle of abdominal segments 3–5 in third instar larvae at 16°C in low Ca^{2+} (0.4 mM $CaCl_2$/4 mM $MgCl_2$) standard saline (13–15). A suction pipette with a tip opening of ≈10 μm was used to stimulate the segmental nerve (stimulation duration = 0.1 ms, strength = 10–15 V, rate = 5 Hz). The stocks were reared at 20–23°C and heat shock was carried out at 37.5°C for 30–60 min. Larvae were prepared for recording 15–60 hr after heat shock.

Purification of *Drosophila* CaM Kinase. Purified kinase was obtained by expressing the R3 *Drosophila* CaM kinase II cDNA clone in COS cells (16). COS cell supernatant was applied to an S-Sepharose column in 150 mM Tris, pH 7.2/1 mM EGTA/1 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/20 mM benzamidine/1 μg of leupeptin per ml/1 μg of pepstatin per ml/1 μg of aprotinin per ml. Activity was eluted stepwise with 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M NaCl in the same buffer. Active fractions were brought to 1 mM $CaCl_2$, pooled, and applied to a calmodulin-Sepharose column. The CaM-Sepharose column was washed with 25 mM Tris, pH 7.0/10% glycerol/0.5 M NaCl/1 mM $CaCl_2$/1 mM phenylmethylsulfonyl fluoride and eluted in 25 mM Tris, pH 7.0/10% glycerol/0.2 M NaCl/1 mM EGTA. Protein was

Abbreviations: CaM kinase II, calcium/calmodulin-dependent protein kinase II; EJP, excitatory junctional potential; CI, courtship index.

‡Present address: Beckman Center for Neuroscience, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

measured using Bio-Rad protein assay reagent with bovine gamma globulin as a standard.

Phosphorylation Assays. Four micrograms of purified fusion protein was phosphorylated in a 100-μl reaction mixture [50 mM Pipes, pH 7.0/1 mM $CaCl_2$/15 mM $MgCl_2$/10 μg of bovine calmodulin/50 μM [γ-^{32}P]ATP (specific activity, 1 Ci/mmol; 1 Ci = 37 GBq)/10 μl of purified cloned *Drosophila* CaM kinase II]. The reaction was allowed to proceed for 1 min at 30°C and then stopped by boiling for 3 min with 50 μl of 9% sodium lauryl sulfate/25% glycerol/0.186 M Tris, pH 8.9/trace amounts of bromophenol blue.

Kinetic constants for Eag phosphorylation were determined by phosphorylating various concentrations (10 nM–25 μM) of fusion protein as described above and analyzing the SDS/polyacrylamide gels using a Molecular Dynamics PhosphorImager system. All reactions were done in duplicate. A nonlinear curve was fit to the data using Kaleidagraph. Data are given in text as value ± SE of parameter estimate.

Fusions were generated by cloning portions of the *eag* cDNA (8) into a vector containing the coding region of the *Escherichia coli* maltose binding protein and were a generous gift of B. K. Talbot and B. Ganetzky. The sequence of the fusion proteins is as follows: *N-terminal fusion* (amino acids 78–212): [MBP] **Thr** Asp Lys Glu Thr Val Gly Arg Leu Glu Tyr Thr Leu Glu Asn Gln Gln Gln Asp Gln Phe Glu Ile Leu Leu Tyr Lys Lys Asn Asn Leu Gln Cys Gly Cys Ala Leu Ser Gln Phe Gly Lys Ala Gln Thr Gln Glu Thr Pro Leu Trp Leu Leu Leu Gln Val Ala Pro Ile Arg Asn Glu Arg Asp Leu Val Val Leu Phe Leu Leu **Thr** Phe Arg Asp Ile Thr Ala Leu Lys Gln Pro Ile Asp Ser Glu Asp Thr Lys Gly Val Leu Gly Leu Ser Lys Phe Ala Lys Leu Ala Arg Ser Val Thr Arg Ser Arg Gln Phe Ser Ala His Leu Pro **Thr** Leu Lys Asp Pro Thr Lys Gln Ser Asn Leu Ala His Met Met Ser Leu Ser Ala Asp. *C-terminal fusion* (amino acids 628–800): [MBP] Leu Gly Lys Gly Asp Val Phe Gly Asp Gln Phe Trp Lys Asp Ser Ala Val Gly Gln Ser Ala Ala Asn Val Arg Ala Leu Thr Tyr Cys Asp Leu His Ala Ile Lys Arg Asp Lys Leu Leu Glu Val Leu Asp Phe Tyr Ser Ala Phe Ala Asn Ser Phe Ala Arg Asn Leu Val Leu Thr Tyr Asn Leu Arg His Arg Leu Ile Phe Arg Arg Val Ala Asp Val Lys Arg Glu Lys Glu Leu Ala Glu Arg Arg Lys Asn Glu Pro Gln Leu Pro Gln Asn Gln Asp His Leu Val Arg Lys Ile Phe Ser Lys Phe Arg Arg Thr Pro Gln Val Gln Ala Gly Ser Lys Glu Leu Val Gly Gly Ser Gly Gln Ser Asp Val Glu Lys Gly Asp Gly Glu Val Glu Arg Thr Lys Val Leu Pro Lys Ala Pro Lys Leu Gln Ala Ser Gln Ala Thr Leu Ala Arg Glu Asp Thr Ile Asp Glu Gly Gly Glu Val Asp Ser Ser Pro Pro Ser. Potential C kinase (boldface) and CaM kinase II (underlined) phosphorylation sites are marked; "[MBP]" indicates amino acids of the maltose binding protein.

RESULTS

Previously we had examined the effects of a CaM kinase II inhibitory peptide transgene on the ability of male flies expressing the transgene (*ala1*, hemizygous for a first chromosome insert and *ala2* heterozygous for a second chromosome insert) to learn and remember in an associative courtship conditioning paradigm (7, 17, 18). The courtship conditioning behavior can be divided into two parts: an acquisition phase (or learning), where the male is trained with a mated female and responds to her presence and multiple chemical cues (12) by decreasing his courtship toward her, and a retention phase (or memory), where the same male is subsequently presented with a virgin female and fails to court her. In previous experiments at 25°C, *ala* males showed normal acquisition of training in this paradigm but were defective in retention (7). The memory defects produced were dose dependent; the strain expressing a higher level of the peptide (*ala2*) was more severely affected than the one expressing a lower level (*ala1*). The differences in expression level are presumably due to differences in the basal activity of the *hsp70* promoter due to the different insertion sites of the transgenes. The memory defect of *ala1* could be made more severe by induction of the transgene with heat shock (7). Since the more severely affected strain, *ala2*, was incapable of retaining the effects of training even without heat shock, we first asked whether there would be further, perhaps qualitatively different, consequences, such as defects in acquisition, of producing an even higher level of inhibitor by heat shocking this strain or increasing the transgene dosage.

To examine the effects of higher levels of inhibitor, we tested *ala1* and *ala2* males carrying either one or two doses of the inhibitor gene with mild heat shock. Fig. 1 demonstrates that at higher levels of inhibitor, males now failed to be conditioned by the mated female. Acquisition of training—i.e., learning—was affected. Fig. 1 shows the percentage of the ratio of final CI (CI_{final}, CI after 50 min of exposure to the mated female) to initial CI ($CI_{initial}$, CI at the beginning of the training period) for heat-shocked *ala1* and *ala2* flies. A percentage ≥100 indicates no learning, while ratios of <100 are a quantitative measure of learning, with smaller numbers indicating more effective conditioning. A progressive decrease in learning with increased dosage of inhibitory peptide (achieved both genetically and by inducing the transgene promoter) in the *ala* lines is shown. As previously reported (7), there is no effect on the *ala1* strain, which expresses a lower level of inhibitory peptide.

Modification of potassium channels has been implicated in cellular mechanisms of learning and memory in *Aplysia* and *Drosophila* (19–21). Because the Eag gene product has been hypothesized to be a potassium channel regulatory subunit (8–10), we tested the ability of flies carrying the mutant alleles eag^1 and eag^{4PM} to be conditioned by mated females (Fig. 2). These flies showed the same complete failure to learn as the most severely affected line in Fig. 1, the heat-shocked *ala2* homozygotes. eag^1 males show an average increase in their courtship of the mated female (mean % $CI_{final}/CI_{initial}$) of 130.8% ± 57.1% and eag^{4PM} males show an increase to 153.2% ± 32.3% ($P < 0.01$ and 0.001, respectively, when compared to +sib controls by Wilcoxon nonparametric rank sums test).

As shown in Table 1, the mean $CI_{initial}$ for all strains and treatments did not vary significantly and had a range of

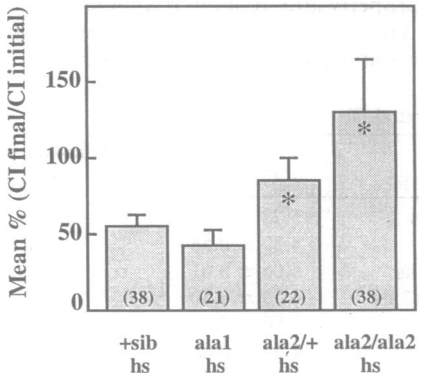

FIG. 1. Courtship conditioning of heat-shocked male *ala1*, *ala2*, and +sib controls by mated females. A progressive decrease in learning in males with increasing amounts of CaM kinase II inhibition is demonstrated. Males carrying a heat-shock-inducible transgene for a CaM kinase II inhibitory peptide (at independent insertion sites in the *ala1* and *ala2* lines) were tested in varying gene dosage after a 30 min, 37°C heat shock (hs). Data from individuals with $CI_{initial}$ <1 were excluded from analysis since learning cannot occur in the absence of some level of initial courtship (12). Error bars represent SEM. Asterisks denote scores that were significantly different from the +sib control by a nonparametric Wilcoxon test ($P < 0.05$). *n* for each genotype is indicated in parentheses.

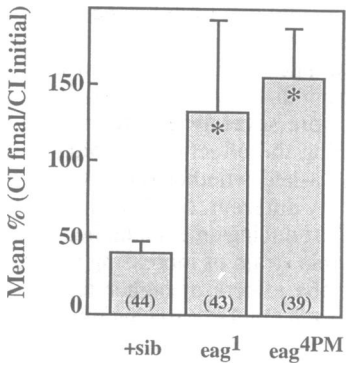

FIG. 2. Courtship conditioning of male eag^1, eag^{4PM}, and +sib controls by mated females. Males mutant at the *eag* locus and +sib controls were tested at 25°C. Data from individuals with $CI_{initial} < 1$ were excluded from analysis since learning cannot occur in the absence of some level of initial courtship (12). Error bars represent SEM. Asterisks denote scores that were significantly different from the +sib control by a nonparametric Wilcoxon test ($P < 0.05$). *n* for each genotype is indicated in parentheses.

4.32–6.54. This indicates that fitness of the strains and heat shock had no significant effect on the baseline ability of the flies to carry out courtship.

Having observed behavioral similarities between *ala2* and the *eag* mutants, we compared their synaptic physiology at the larval neuromuscular junction and observed a drastic defect in the response to repetitive stimulation, a parameter relevant to activity-dependent plasticity. Stimulation of segmental nerve that contains the motor axons normally produces one-for-one EJPs in the body-wall muscle fibers (15). We found that after a few seconds of repetitive stimulation at 5 Hz, normal flies continue to exhibit one-for-one following, which is unaffected by heat shock (Fig. 3). In contrast, without heat shock, both eag^1 and *ala2* flies show supernumerary synaptic discharges, which produce EJPs after several seconds of such stimulation. Spontaneous discharges occurred at a frequency as high as 25 Hz and lasted up to tens of seconds after the cessation of stimulation, in contrast to controls in which unevoked discharges were never seen. These results suggest a failure of the nerve terminal to repolarize properly after repetitive stimulation and imply that both CaM kinase II activity and a normal Eag potassium channel subunit are required for repolarization.

Table 1. Initial CI

Genotype	$CI_{initial}$	Wilcoxon vs. +sibs *P* value
+sibs hs	5.78 ± 0.55	
ala1 hs	4.32 ± 0.58	>0.13
ala2/+ hs	5.06 ± 0.61	>0.35
ala2/*ala2* hs	6.50 ± 0.50	>0.27
+sibs	5.90 ± 0.46	
eag^1	6.54 ± 0.48	>0.26
eag^{4PM}	4.33 ± 0.53	>0.1

Mean CI ± SEM is indicated for the initial 10 min of exposure to a mated female of males of the indicated genotypes and treatments shown in Figs. 1 and 2. Males carrying a heat-shock-inducible transgene for a CaM kinase II inhibitory peptide (at independent insertion sites in the *ala1* and *ala2* lines) were tested in varying gene dosage after a 30-min, 37°C heat shock (hs). Males mutant at the *eag* locus and +sib controls were tested at 25°C. Data from individuals with $CI_{initial} < 1$ were excluded from analysis since learning cannot occur in the absence of some level of initial courtship (12). Experimental CIs were compared to the appropriate +sib control using a Wilcoxon nonparametric test. *n* for each genotype is the same as in Figs. 1 and 2.

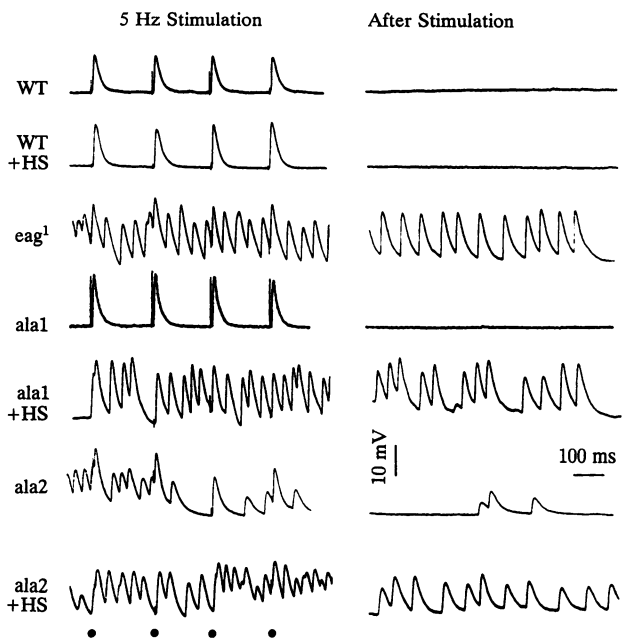

FIG. 3. EJPs recorded from larval neuromuscular junctions of eag^1, *ala1* with or without heat shock (HS), *ala2* homozygotes with or without heat shock, and Canton-S controls (wild-type, WT). Stimulus artifacts are indicated by filled circles. (*Left*) High-frequency supernumerary discharges seen with 5-Hz stimulation in *ala* and eag^1 flies. Supernumerary discharges were never observed in controls and were not observed in all heat-shocked *ala1* larvae. (*Right*) Continued activity after stimulation has ceased. This activity could last up to tens of seconds after cessation of stimulation.

In contrast to eag^1 and *ala2*, *ala1*, the line with a lower level of inhibitory peptide, showed normal one-for-one following and no spontaneous discharges without heat shock. Only after heat shock were supernumerary synaptic discharges evident. Heat shock also increased the severity of *ala2*'s physiological phenotype (Fig. 3). This gradient of severity of synaptic abnormalities parallels both the level of peptide in the transgenic lines and the severity of courtship behavior defects (7), suggesting that inhibition of CaM kinase II is involved in both synaptic transmission and courtship conditioning.

The analysis of double mutants in *Drosophila* has been used fruitfully to investigate the direct molecular and indirect physiological relationships between synaptic phenomena (13, 22). If two processes are closely related physiologically, the phenotype of the double mutant will often differ from a simple addition of the single mutant phenotypes. Interactions that produce nonadditive phenotypes can be either direct or indirect. To investigate if the phenotypes of *eag* mutants and *ala* flies were the result of disruption of a common process, we constructed an eag^1;*ala2* line. When asked to perform in the courtship conditioning assay, this line showed a high rate of failure to court in the initial observation period with the mated female (Table 2). This high failure rate was also observed in more severe (i.e., null) *eag* alleles such as eag^{sc29} and eag^{X-6} (23).

The courtship failure rate (percentage of flies with a $CI_{initial} < 1$) for the four *eag* alleles tested (Table 2) paralleled the severity of the defects previously reported (9) in potassium conductance, $eag^{X-6} = eag^{sc29} > eag^1 > eag^{4PM}$. Comparatively, eag^1 and *ala2* are midway in severity. Addition of a transgene containing a CaM kinase II inhibitor to eag^1 changes this genotype's behavioral deficits so that they resemble the null alleles eag^{X-6} and eag^{sc29}. This is not simply an additive effect of the *ala2* and eag^1 phenotypes, but a qualitative change associated with complete absence of *eag*

Table 2. Courtship failure

Genotype	$CI_{initial}$ <1.0	%	*P* value: χ^2 vs. +sibs	*P* value: χ^2 vs. *eag*^{1};*ala2*
+sibs hs	8/46	17.4	—	<0.005
ala1 hs	6/27	22.2	>0.1	<0.005
ala2/+ hs	2/24	8.3	>0.1	<0.005
ala2/*ala2*	5/26	19.2	>0.5	<0.001
ala2/*ala2* hs	6/44	13.6	>0.1	<0.005
eag^{4PM}	5/44	11.3	>0.1	<0.005
eag^1	21/64	32.8	<0.005	<0.005
eag^{X-6}	29/39	74.4	<0.005	>0.1
eag^{sc29}	40/49	81.6	<0.005	>0.1
eag^1;*ala2*	30/37	81.1	<0.005	—

The number of flies with CI <1.0 for the initial 10 min of exposure to a mated female out of the total number of males assayed is shown. Males are of the indicated genotypes and treatments shown in Figs. 1, 2, and 4. Males carrying a heat-shock-inducible transgene for a CaM kinase II inhibitory peptide (at independent insertion sites in the *ala1* and *ala2* lines) were tested in varying gene dosage after a 30-min, 37°C heat shock (hs) or without heat shock at 25°C. Males mutant at the *eag* locus, *eag*^{1};*ala2*, and +sib controls were tested without heat shock at 25°C. χ^2 was computed for each line compared to +sibs and to *eag*^{1};*ala2*. *eag*^{1} is also significantly different from *ala2*/*ala2* hs ($P < 0.005$) and from *eag*$^{X-6}$ ($P < 0.005$) and *eag*^{sc29} ($P < 0.005$).

function. The courtship failure rate of *ala2* was not significantly different from +sibs, yet in combination with *eag*^{1} the failure rate resembles that of an *eag* null (Table 2).

The *eag*^{1};*ala2* line also exhibits a complex nonadditive synaptic phenotype. *ala* flies show no spontaneous activity (Fig. 4), whereas alleles of *eag*, including *eag*^{4PM} and *eag*^{1}, cause low-frequency spontaneous firing (5–10 Hz) at the neuromuscular junction (13, 14). The severity of this phenotype is allele-dependent in the *eag* mutants. The frequency of spontaneous EJPs parallels the defects in potassium currents (9). Addition of the *ala* transgene completely suppresses *eag*^{1}-dependent spontaneous activity at the neuromuscular junction (Fig. 4 *Left*).

A second *eag* synaptic phenotype, generation of high-frequency supernumerary discharges induced by repetitive stimulation, is not noticeably affected in the *eag* flies with an *ala* transgene. The afterdischarges in the *eag*^{1};*ala2* flies are similar to that of *eag*^{1} in this regard (Fig. 4 *Center* and *Right*). The severity of this phenotype in *eag* mutants is also allele-dependent but does not parallel the potassium current defects. *eag*^{1} is the most severely affected, with supernumerary discharges seen in every animal tested, and *eag*^{X6} the least affected, with supernumerary discharges seen in a quarter of animals tested (Fig. 4). For *ala* larvae, the occurrence of supernumerary discharge paralleled peptide dosage; it was never seen in *ala1* without heat shock, seen in *ala1* with heat shock in about one-third of animals tested, and always seen with *ala2* regardless of heat shock (Fig. 3).

One way to account for the similarity in behavioral and physiological phenotypes between *ala* flies and *eag* mutants is that function of Eag is regulated by CaM kinase II. A deficit in the channel's activity or modifiability would then produce a phenotype similar to that produced by a decrease in kinase activity. In support of this idea, a recent study indicates that W7, a calmodulin antagonist, reduces voltage-activated I_A and I_K and calcium-activated I_{CF} and I_{CS} potassium currents in wild-type larval muscle, but not in *eag* mutants (10).

To address this possibility, we asked whether the Eag protein could serve as a substrate for *Drosophila* CaM kinase II. Two separate fusion proteins corresponding to N-terminal amino acid residues 78–212 and to C-terminal residues 628–800 were tested as potential CaM kinase II substrates. Both portions are presumed to be on the cytoplasmic side of the membrane (8) and both have consensus sequences for CaM kinase II (see *Materials and Methods*). As shown in Fig. 5, only the C-terminal cytoplasmic domain is actually phosphorylated by purified *Drosophila* CaM kinase II, consistent with the putative sites suggested by DNA sequence analysis. The V_{max} for this reaction is 3.7 ± 0.2 μmol/min per mg, and the K_m is 1.7 ± 0.4 μM for this fusion protein, indicating that it is a very good substrate for CaM kinase II. In comparison, the V_{max} for synapsin I phosphorylation by the rat brain CaM kinase II has been reported to be 2.9 μmol/min per mg (24).

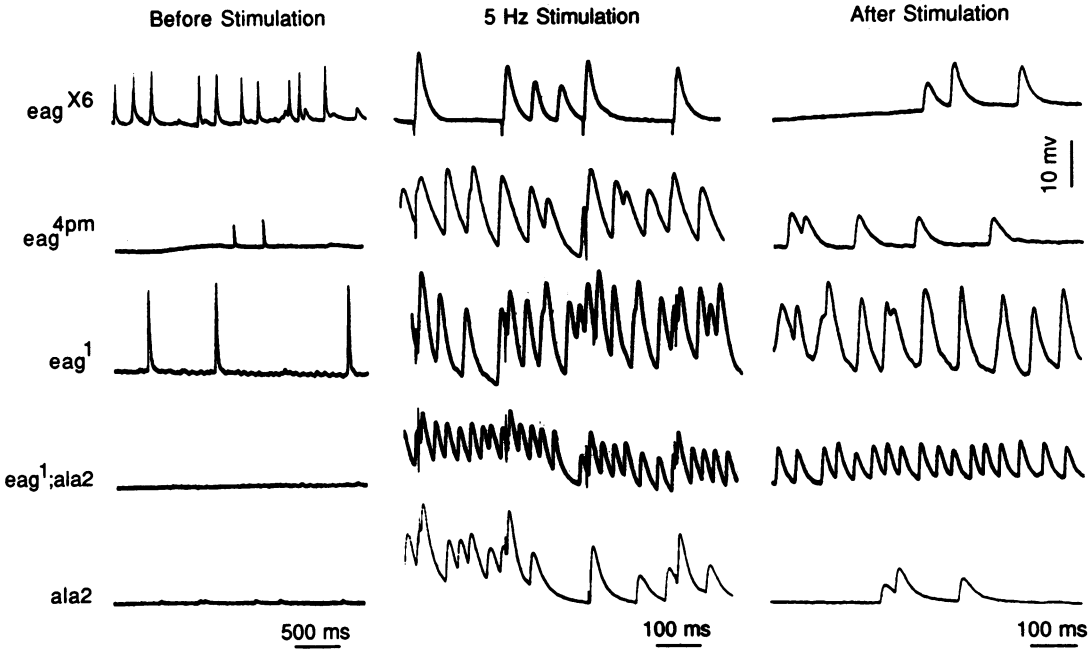

FIG. 4. EJPs recorded from larval neuromuscular junctions of *eag*$^{X-6}$, *eag*^{4PM}, *eag*^{1}, *eag*^{1};*ala2*, and *ala2*. Animals were not heat shocked. (*Left*) Low-frequency spontaneous discharges recorded from unstimulated muscle. Note the difference in time scale. Spontaneous activity was seen in every *eag*$^{X-6}$, *eag*^{4PM}, and *eag*^{1} preparation but was never seen in *eag*^{1};*ala2* animals. (*Right*) Continued activity after stimulation has ceased.

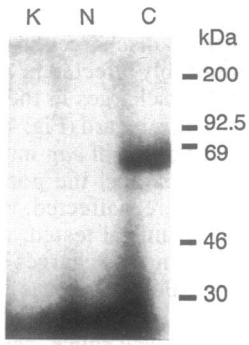

FIG. 5. Phosphorylation of Eag fusion proteins by *Drosophila* CaM kinase II. Lanes: K, kinase alone; N, kinase plus N-terminal fusion protein; C, kinase plus C-terminal fusion protein. Four micrograms of purified fusion protein was phosphorylated by purified cloned *Drosophila* CaM kinase II in the presence of calcium and calmodulin. The reaction was allowed to proceed for 1 min at 30°C. Proteins were separated on a 9% SDS/polyacrylamide gel. Autoradiography was carried out with an intensifying screen at −80°C for 12 hr.

DISCUSSION

In this paper we present four different lines of evidence—behavioral, physiological, genetic, and biochemical—for a functional relationship between CaM kinase II and the potassium channel subunit Eag. The biochemical interaction, between a kinase and a substrate, has both physiological and behavioral consequences. One of the CaM kinase II consensus phosphorylation sites (Thr-655) in the Eag C-terminal fusion protein lies in the middle of the Eag cyclic nucleotide-binding site (25). This site is contained in the fusion protein shown to be a substrate. Both W7, a calmodulin antagonist, and cGMP have been shown to modulate multiple potassium currents in *Drosophila* larval muscles and this modulation is blocked by *eag* mutations (10). Phosphorylation of Thr-655 by CaM kinase II could potentially alter the ability of cyclic nucleotides to regulate potassium currents.

In *eag* mutants containing an *ala* transgene, we see evidence for a complex genetic interaction. Behaviorally, the eag^1;*ala2* flies recapitulate the phenotype of *eag* null mutants, a failure to court. This parallel change in the character of the defect raises the possibility that the two genes are affecting the same pathway. Synergistic effects, however, do not prove a direct interaction since separate convergent pathways could be involved. The strong biochemical interaction between the two molecules, however, makes direct interaction a likely possibility.

The physiological results are complex, with two separate synaptic phenomena, spontaneous activity and supernumerary discharge (22), affected in different manners. The suppression of *eag* spontaneous activity by the *ala* transgene shows that for this synaptic process, CaM kinase and Eag are likely to be in the same pathway. Supernumerary discharges are seen in both *ala* and *eag* flies, and the phenotype is relatively unaffected in the eag^1;*ala2* animals, which resemble eag^1. Epistasis for this effect and for behavior is not demonstrated by this experiment, and this may prove difficult to do if CaM kinase affects multiple pathways involved in these phenomena. In light of the multifunctional nature of CaM kinase II, it would be expected to affect many processes. The association of the physiological result (continued hyperexcitability) with an increase in the failure rate for the behavior is probably a reflection of the nonlinear nature of the behavioral switch.

Potassium channels determine the initiation and recurrence of action potentials and thus play an important role in influencing the calcium influx necessary for transmitter release. The S-channel of *Aplysia* sensory neurons, which is a target of phosphorylation by protein kinase A during sensitization, acquires a reduced probability of being open, thus increasing postsynaptic potentials (2). Reduction in CaM kinase II activity has been shown to affect long-term potentiation in the mammalian hippocampus (4, 5), an important model for plasticity in learning, and genetic manipulation of CaM kinase II has demonstrated its importance for learning and memory in the mouse (6) and in *Drosophila* (7).

The phenotype we demonstrate here, produced by both kinase inhibition and channel mutation, suggests that under conditions of repetitive stimulation, there is normally a regulated compensatory mechanism initiated to ensure proper repolarization of the nerve terminal and regulation of neurotransmitter release. We postulate that the relevant synapses in the brain are modulated similarly to the neuromuscular junction synapses. In line with this idea, Eag has been suggested to be a regulatory subunit of a number of potassium channels. Voltage clamp studies have demonstrated that *eag* mutations reduce several types of potassium currents in larval muscles (9) and abolish the ability of calmodulin antagonists and cGMP to regulate potassium channel activity (10). The results presented here support the hypothesis that Eag is a potassium channel regulatory subunit and identify the Eag protein as a possible down-stream element in CaM kinase II-mediated modulation of neuronal plasticity.

We thank Nanci Kane and Sue Broughton for help with behavioral data collection and Becky Talbot and Barry Ganetzky for the generous gift of Eag fusion proteins. These studies were supported by National Institutes of Health Grants GM33205 to L.C.G. and NS18500 and NS26528 to C.-F.W., a grant from the Esther A. and Joseph Klingenstein Fund to R.J.G., and a grant to New York University (R.J.G.) from the W. M. Keck Foundation.

1. Jessel, T. M. & Kandel, E. R. (1993) *Neuron* **10,** 1–30.
2. Schwartz, J. H. & Greenberg, S. M. (1987) *Annu. Rev. Neurosci.* **10,** 459–476.
3. Zhong, Y. & Wu, C.-F. (1991) *Science* **251,** 198–201.
4. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245,** 862–866.
5. Silva, A. J., Stevens, C. F., Tonegawa, S. & Wang, Y. (1992) *Science* **257,** 201–206.
6. Silva, A. J., Paylor, R., Wehner, J. M. & Tonegawa, S. (1992) *Science* **257,** 206–212.
7. Griffith, L. C., Verselis, L. M., Aitken, K. M., Kyriacou, C. P., Danho, W. & Greenspan, R. J. (1993) *Neuron* **10,** 501–509.
8. Warmke, J., Drysdale, R. & Ganetzky, B. (1991) *Science* **252,** 1560–1562.
9. Zhong, Y. & Wu, C.-F. (1991) *Science* **252,** 1562–1564.
10. Zhong, Y. & Wu, C.-F. (1993) *J. Neurosci.* **13,** 4669–4679.
11. Bruggemann, A., Pardo, L., Stuhmer, W. & Pongs, O. (1993) *Nature (London)* **365,** 445–448.
12. Tompkins, L., Siegel, R. W., Gailey, D. A. & Hall, J. C. (1983) *Behav. Genet.* **13,** 565–578.
13. Ganetzky, B. & Wu, C.-F. (1983) *J. Neurogenet.* **1,** 17–28.
14. Wu, C.-F., Ganetzky, B., Liu, A.-X. & Haugland, F. (1983) *Science* **220,** 1076–1078.
15. Jan, L. Y. & Jan, Y. N. (1976) *J. Physiol. (London)* **262,** 189–214.
16. Griffith, L. C. & Greenspan, R. J. (1993) *J. Neurochem.* **61,** 1534–1537.
17. Siegel, R. W. & Hall, J. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3430–3434.
18. Ackerman, S. L. & Siegel, R. W. (1986) *J. Neurogenet.* **3,** 111–123.
19. Shuster, M. J., Camardo, J. S., Siegelbaum, S. A. & Kandel, E. R. (1985) *Nature (London)* **313,** 392–395.
20. Cowan, T. M. & Siegel, R. W. (1984) *J. Neurogenet.* **1,** 333–344.
21. Cowan, T. M. & Siegel, R. W. (1986) *J. Neurogenet.* **3,** 187–201.
22. Ganetzky, B. & Wu, C.-F. (1982) *J. Neurophysiol.* **47,** 501–514.
23. Drysdale, F., Warmke, J., Kreber, R. & Ganetzky, B. (1991) *Genetics* **127,** 497–505.
24. Bennett, M. K., Erondu, N. E. & Kennedy, M. B. (1983) *J. Biol. Chem.* **258,** 12735–12744.
25. Guy, H. R., Durell, S. R., Warmke, J., Drysdale, R. & Ganetzky, B. (1991) *Science* **254,** 730.