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Modulation of Different K^+ Currents in *Drosophila*: A Hypothetical Role for the Eag Subunit in Multimeric K^+ Channels

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We examined the role of the *ether a go-go* (*eag*) gene in modulation of K^+ currents and the possibility of its protein product Eag as a subunit in the heteromultimeric assembly of K^+ channels by voltage-clamp analysis of larval muscle membrane currents. Previous DNA sequence studies indicate that the *eag* gene codes for a polypeptide homologous to, but distinct from, the *Shaker* (*Sh*) K^+ channel subunits (Warmke et al., 1991), and electrophysiological recordings revealed allele-specific effects of *eag* on four identified K^+ currents in *Drosophila* larval muscles (Zhong and Wu, 1991). Further studies of *eag* alleles indicated that none of the *eag* mutations, including alleles producing truncated mRNA messages, eliminate any of the four K^+ currents, and that the mutational effects exhibit strong temperature dependence. We found that both W7, an antagonist of Ca^{2+}/calmodulin, and cGMP analogs modulated K^+ currents and that their actions were altered or even abolished by *eag* mutations. These results suggest a role of *eag* in modulation of K^+ currents that may subserve integration of signals at a converging site of the two independent modulatory pathways. The *Sh* locus is known to encode certain subunits of the I_A channel in larval muscle. The existence of multiple *eag* and *Sh* alleles enabled an independent test of the idea of Eag as a K^+ channel subunit by studying I_A in different double-mutant combinations. An array of allele-specific interaction between *eag* and *Sh* was observed, which reflects a close association between the *Sh* and *eag* subunits within the I_A channel. Taken together, our data strengthen the possibility that the *eag* locus provides a subunit common to different K^+ channels. The role of the *eag* subunit for modulating channels, as opposed to that of *Sh* subunits required for gating, selectivity, and conductance of the channel, suggests a combinatorial genetic framework for generating diversified K^+ channels.

[Key words: eag, cGMP, voltage clamp, potassium channels, modulation of K^+ channels]

A diverse family of K^+ channels participates in regulating membrane potentials and firing patterns in excitable cells (Rudy, 1988; Hille, 1992). A combination of genetic, molecular, and biophysical studies of the *Shaker* (*Sh*) locus in *Drosophila*, which produces multiple K^+ channel polypeptides by alternative RNA splicing (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988), has provided a physical picture of channel gating and ion selectivity mechanisms (for review, see Hoshi et al., 1991; Miller, 1991; Jan and Jan, 1992). The *Sh* polypeptides are thought to mediate voltage-gated transient K^+ current I_A in *Drosophila* muscles (Salkoff and Wyman, 1981; Wu and Haugland, 1985). Nonadditive effects on I_A have been found in heteroallelic combinations of different *Sh* mutations, suggesting oligomeric assembly of I_A channels (Haugland and Wu, 1986, 1990; Timpe and Jan, 1987). Expression of *Sh* transcripts in the *Xenopus* oocyte further indicates that multiple *Sh* subunits (Isacoff et al., 1990; McCormack et al., 1990), most likely in groups of four (MacKinnon, 1991), coassemble to form a functional channel. Although *Sh* subunits alone can form functional channels in oocytes (Iverson et al., 1988; Timpe et al., 1988), the question remains as to whether additional subunits encoded by other genes participate in assembly of native I_A channels, *in vivo*, and what functional roles the additional subunits play. It is known that vertebrate Ca^{2+} and Na^+ channels contain a number of subunits although a single subunit species, α_1 in Ca^{2+} channels or α in Na^+ channels, can form active, albeit not entirely normal, channels in oocytes (Auld et al., 1988; Mori et al., 1991). Although evidence has accumulated suggesting that these additional subunits may be involved in modulation of Na^+ or Ca^{2+} channels, the function of these subunits remains to be fully explored (Singer et al., 1991; Isom et al., 1992).

DNA sequence analysis reveals that the *ether a go-go* (*eag*) locus in *Drosophila* encodes a putative K^+ channel subunit that is homologous to, but distinct from, those of the *Sh* superfamily (Warmke et al., 1991). More interestingly, except for regions of the putative voltage sensor (S4) and the pore-forming hairpin loop (H5), other segments in the *eag* polypeptide (Eag) are more closely related to those in cyclic nucleotide–gated channels (Guy et al., 1991). Specifically, a putative cyclic nucleotide binding domain is identified in the COOH-terminal region of Eag (Guy et al., 1991). Voltage-clamp data have indicated that *eag* mutations affect four identified K^+ currents in larval muscles, including the voltage-activated transient I_A and delayed I_K, and the Ca^{2+}-activated fast I_{CF} and slow I_{CS} (Wu et al., 1983; Zhong and Wu, 1991). This raises the possibility that the *eag* locus provides a subunit common to different K^+ channels (Wu et al., 1983; Zhong and Wu, 1991). To delineate the contribution of the *eag* polypeptide to the structure and function of K^+ channels, we have analyzed K^+ currents in more than 10 *eag* alleles at different temperatures and with different pharmacological treatments.

The membrane currents in the body-wall muscles of *Drosophila* third instar larvae have been extensively characterized

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(Wu et al., 1983, 1989; Wu and Haugland, 1985; Gho and Mallart, 1986; Singh and Wu, 1989, 1990; Haugland and Wu, 1990; Zhong and Wu, 1991). Protocols based on genetic manipulations, pharmacological agents, and electrophysiological paradigms have been devised to seperate the four identified K^+ currents, I_A, I_K, I_{CF}, and I_{CS}, and the use of a small subset of identified muscle fibers has yielded highly reproducible quantitative measurements (Singh and Wu, 1989; Wu et al., 1989; Haugland and Wu, 1990; Zhong and Wu, 1991). This allows detection of relatively subtle changes in these currents caused by modulatory or mutational effects. The *eag* phenotypes were in sharp contrast to those of mutations affecting other structural genes of K^+ channels: *Sh* mutations specifically alter or eliminate I_A (Salkoff and Wyman, 1981; Wu et al., 1983; Singh and Wu, 1989) while *slowpoke* (*slo*) mutations abolish only I_{CF} (Elkins et al., 1986; Singh and Wu, 1989; Komatsu et al., 1990). In contrast, *eag* mutations affect all four identified K^+ currents in an allele-dependent manner (Wu et al., 1983; Zhong and Wu, 1991), with relatively milder effects on channel conductance as compared to *Sh* and *slo* mutations. Furthermore, the *eag* phenotype exhibited a temperature dependence; that is, the mutational effects on K^+ currents were more evident at certain temperatures (see Results). Such observations led to the speculation that the *eag* polypeptide might act as a modulatory subunit of K^+ channels. Molecular analysis of the *eag* locus has identified abundant putative phosphorylation sites for protein kinase C (PKC), cAMP-dependent protein kinase (PKA) (Warmke et al., 1991), and Ca^{2+}/calmodulin (Ca^{2+}/CaM)-dependent protein kinase (PKB) (L. Griffith and R. J. Greenspan, personal communication), in addition to the putative binding site for cyclic nucleotides (Guy et al., 1991).

In this study, we explored modulation of K^+ currents by various pharmacological agents that perturb second messenger cascades and the role of the *eag* polypeptide in such modulation. We found that an inhibitor of Ca^{2+}/CaM could reduce K^+ currents and, for the first time, demonstrated that application of cGMP and its analogs could increase K^+ currents in a voltage-dependent manner. More importantly, these modulatory responses were altered or even abolished by *eag* mutations, indicating that Eag may serve as a converging site of these two second messenger cascades.

If Eag is a channel subunit subject to modulation, it must coassemble with other subunits to form the various K^+ channels in muscles. Therefore, I_A channels should at least contain subunits derived from the *Sh* and *eag* loci. The existence of multiple *Sh* and *eag* alleles provides a unique opportunity to examine Eag-Sh interactions for indications of the physical association between these two polypeptides. Indeed, the allele-specific interactions observed in *eag Sh* double mutants support the notion of a heteromultimeric I_A channel consisting of *Sh* and *eag* subunits. Based on these observations, we discuss the possibility of a combinatorial genetic framework that could engender diverse K^+ channel subtypes with discrete functional properties.

Materials and Methods

Fly stocks. Drosophila melanogaster were reared at room temperature (20–22°C). The wild-type strain Canton-S was used for the characterization of normal membrane currents and the mutant stocks were in the Canton-S background. The molecular and/or physiological characteristics of mutants used in the experiments are described below.

Sh^5, Sh^{rKO120}. The *Sh* locus (mapped to 1-57.4; Kaplan and Trout, 1969; Jan et al., 1977) encodes I_A channel subunits in muscles. The Sh^5 allele shifts the voltage dependence and Sh^{rKO120} (designated as Sh^{120} hereafter in text) reduces the amplitude of I_A in larval muscles (Haugland and Wu, 1990).

eag^1, eag^{4PM}, eag^{X6}, eag^{24}. The locus (mapped to 1-48; Kaplan and Trout, 1969; Ganetzky and Wu, 1983) encodes a K^+ channel polypeptide. The mutations reduce different K^+ currents in larval muscles and interact synergistically with *Sh* mutations to enhance the behavioral and physiological phenotypes (Ganetzky and Wu, 1983; Wu et al., 1983; Warmke et al., 1991; Zhong and Wu, 1991).

eag^1 Sh^5, eag^1 Sh^{120}, eag^{4PM} Sh^{120}, eag^{24} Sh^5, eag^{24} Sh^{120}. All *eag Sh* double mutants exhibit vigorous abnormal leg-shaking behavior under ether anesthesia. Many of them also show a wings-down phenotype (Stern et al., 1990; Engel and Wu, 1992). More strikingly, high-frequency, spontaneous excitatory junction potentials (EJPs) of a greater amplitude are seen at larval neuromuscular junctions (Ganetzky and Wu, 1983; Budnik et al., 1990), and spontaneous discharges of EJPs or action potentials can be recorded in adult flight muscles (Engel and Wu, 1992).

Electrophysiological and pharmacological experiments. The two-microelectrode voltage-clamp setup and protocols for data collection and analysis have been previously described (Wu et al., 1989; Haugland and Wu, 1990; Zhong and Wu, 1991). Third instar larvae were dissected at room temperature in Ca^{2+}-free saline that contained NaCl (128 mM), KCl (2 mM), sucrose (35.5 mM), $MgCl_2$ (14 mM), EGTA (0.5 mM), and HEPES (5 mM, buffered at pH 7.1). After dissection, the preparation was cooled to either 16°C or 5°C by a Peltier junction. Only muscles 6 and 12 (Crossley, 1978) in abdominal segments 3, 4, and 5 were used in this study (cf. Zhong and Wu, 1991, 1993).

I_A and I_K were recorded in Ca^{2+}-free saline (containing 14 mM $MgCl_2$ and 0.5 mM EGTA). To separate I_A and I_K, a preconditioning pulse (−20 mV, 2 sec) preceded the test pulse (60 msec at 16°C, 400 msec at 5°C) with a brief repolarization (12 msec at 5°C, 6 msec at 16°C) interposed between the two pulses. This paradigm inactivated nearly all I_A (95%) but left I_K essentially intact (Haugland and Wu, 1990; Zhong and Wu, 1991). Without the prepulse, both I_A and I_K were activated by the test pulse. Thus, I_A was extracted from the difference between the currents with and without the preconditioning pulse. The density of active currents was expressed in $\mu A/\mu F$ using membrane capacitance as an indicator of surface area. This procedure has yielded measurements of I_A and I_K reproducible within 5% for larvae of a particular genotype collected years apart (Wu et al., 1989; Haugland and Wu, 1990; Zhong and Wu, 1991, 1993; present results).

To determine steady-state inactivation of I_A, the current elicited by the test pulse (+20 mV) varied with preconditioning pulses that ranged from −80 mV to −20 mV. The test pulse with a preconditioning pulse of −20 mV was assumed to elicit only I_K and leakage currents, which served as a baseline for extraction of I_A.

For recording of I_{CF} and I_{CS}, the saline contained 0.9 mM $CaCl_2$ and 4 mM $MgCl_2$ but no EGTA. Contraction of larval body-wall muscles depends on Ca^{2+} influx. At this Ca^{2+} concentration, muscle contraction is minimized while I_{CF} and I_{CS} remain at a manageable magnitude for showing modulation effects (Singh and Wu, 1989; Zhong and Wu, 1991, 1993). In addition, 100 mM 4-aminopyridine and 100 mM quinidine were added to block I_A and I_K (Haugland and Wu, 1987; Singh and Wu, 1989). The conditions for recording I_{CF} and I_{CS} were developed more recently (Singh and Wu, 1989, 1990; Zhong and Wu, 1991). Because of the technical difficulties introduced by Ca^{2+} influx and the ensuing muscle contraction, I_{CF} and I_{CS} measurements were generally more variable than those in I_A and I_K. Therefore, the more subtle changes in their amplitude or kinetics were not determined; only the stronger modifications are reported.

To examine effects of *N*-(6 aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), an antagonist of Ca^{2+}/CaM (Hidaka et al., 1981), preparation was preincubated by adding 1 mM caffeine to dissection saline and the exposure time during dissection was about 4–5 min. Afterward, the saline was replaced with recording saline containing 25 μM W7, but no caffeine. To test cGMP effects, cGMP or its analogs were added to both dissecting and recording saline, and thus preparations were preincubated (with drugs) at room temperature for about 4–5 min before recording. Treatment with cGMP and its analogs induced a slight shift of I_A inactivation to more positive voltages such that inactivation of I_A was not complete with the conditioning pulse to −20 mV (above which I_K became inactivated). Therefore, about 90% of I_A was extracted and a more complete separation of I_A from I_K was technically difficult. Such shift was observed in both normal and *eag* mutants examined.

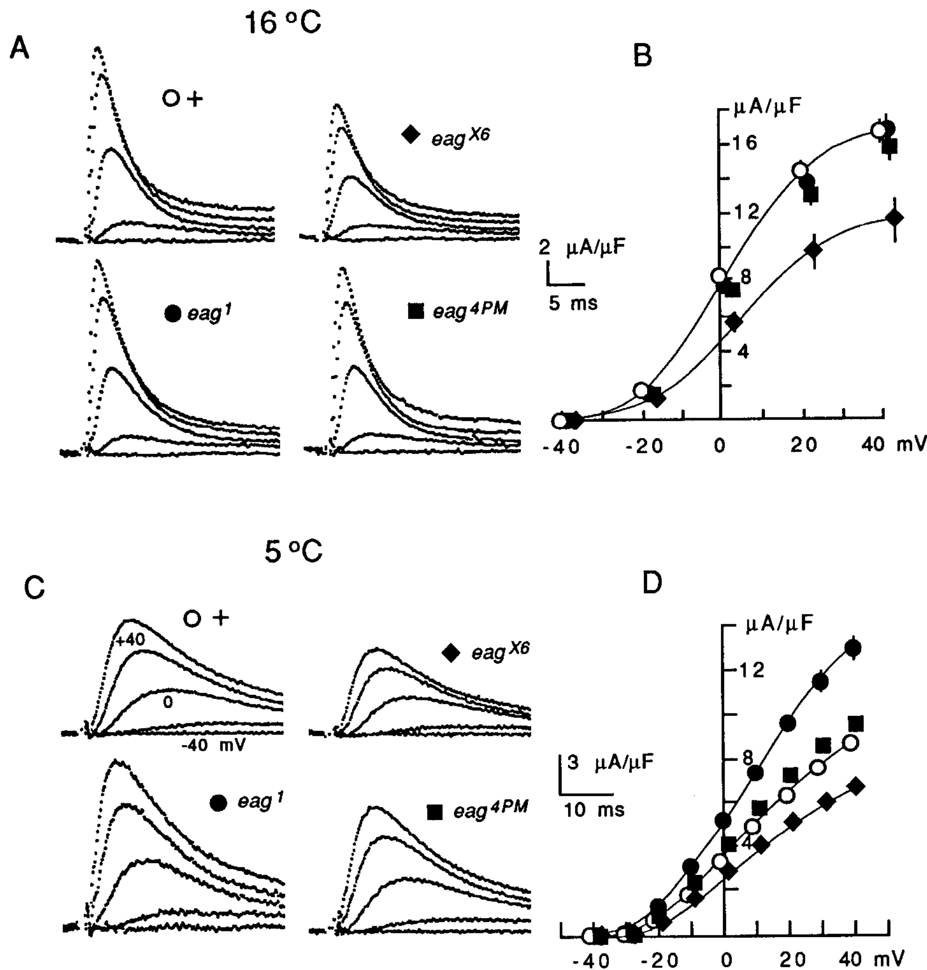

Figure 1. Temperature dependence of effects of *eag* mutations on I_A. *A*, Superimposed traces of I_A recorded at 16°C. *B*, *I–V* relations of I_A at 16°C. *C*, Superimposed traces of I_A recorded at 5°C. *D*, *I–V* relations of I_A at 5°C. The number of muscle fibers, N = 10, 9, 8, 8 (at 16°C), and N = 20, 9, 14, 11 (at 5°C), from four to nine larvae, for the alleles +, eag^1, eag^{4PM}, eag^{X6}, respectively. Note the difference in time calibration at the two different temperatures (5 msec for 16°C, 10 msec for 5°C). Also note that some *eag* mutations increased or decreased I_A depending on the temperature. For this and the following figures, each family of traces represents currents elicited by voltage steps to −40, −20, 0, +20, +40 mV from a holding potential of −80 mV, unless otherwise specified. The traces represent averaged current density in µA/µF (normalized to membrane capacitance) determined from a number of fibers as specified. In all figures, error bars indicate SEM (some masked by symbols) and the wild-type allele is indicated by +.

Results

Temperature-dependent effects of eag *mutations*

It is known that the responses of K^+ channels to modulation by protein kinases depend on temperature (Walsh and Kass, 1988), which may contribute to the temperature sensitivity observed in the gating of these channels (Hille, 1992). In contrast to other mutations affecting K^+ channels (Wu and Haugland, 1985; Singh and Wu, 1990; Y. Zhong and C.-F. Wu, unpublished observations), *eag* mutations are unique in their strong temperature-dependent phenotype. Figures 1 and 2 show the temperature dependence of *eag* effects upon I_A and I_K. All families of current traces and voltage–current (*I–V*) relations shown represent the averaged current density in which membrane currents were normalized by the corresponding membrane capacitance and averaged by the number of fibers recorded. Both I_A and I_K recorded from normal larvae at 16°C exhibited much higher amplitude as compared to those at 5°C (Figs. 1, 2, normal data). As previously reported, I_A and I_K recorded at 16°C are reduced to various extents in eag^1, eag^{4PM}, and eag^{X6} (Zhong and Wu, 1991). However, we found that the effects of these *eag* mutations were quite different at 5°C. Compared to normal larvae, I_A in eag^1 was increased at 5°C but remained almost unchanged at 16°C (Fig. 1). Conversely, I_K in eag^1 was nearly normal at 5°C but decreased at 16°C (Fig. 2). A distinct temperature dependence was also observed in eag^{4PM} and eag^{X6} (Figs. 1, 2).

Previous observations on I_{CF} and I_{CS} obtained at 5°C indicate that these Ca^{2+}-activated currents are also affected by *eag* mutations (Zhong and Wu, 1991). However, examination of I_{CF} and I_{CS} at 16°C was prevented by technical difficulties due to massive muscle contractions.

Effects of a Ca^{2+}/CaM antagonist

In addition to eag^1, eag^{4PM}, and eag^{X6}, we examined eight more *eag* alleles, including one possible null allele, eag^{sc29} (Drysdale et al., 1991). At 5°C none of these alleles exhibited effects on I_A and I_K larger than those seen in eag^{X6} or eag^1 (data not shown). Such characteristics of *eag* mutations, that is, alterations of the gene affected multiple K^+ currents in a temperature- and allele-dependent manner but eliminated none of the currents even in null alleles, led to a suspicion that the *eag* polypeptide is involved in channel modulation rather than gating and conductance in larval muscles. Therefore, we applied drugs that disrupt second messenger cascades to determine whether their actions could be affected by the *eag* mutations.

Among different drugs examined, I_A and I_K recorded at 16°C in normal fibers were suppressed severely by the application of W7 at concentrations of hundreds of micromoles. W7 is thought to be an antagonist of Ca^{2+}/CaM (Hidaka et al., 1981), consequently inhibiting the Ca^{2+}/CaM-dependent PKB. However, a high concentration of W7 is also known to inhibit PKC (Schatzman et al., 1983). It has been reported that some drugs that

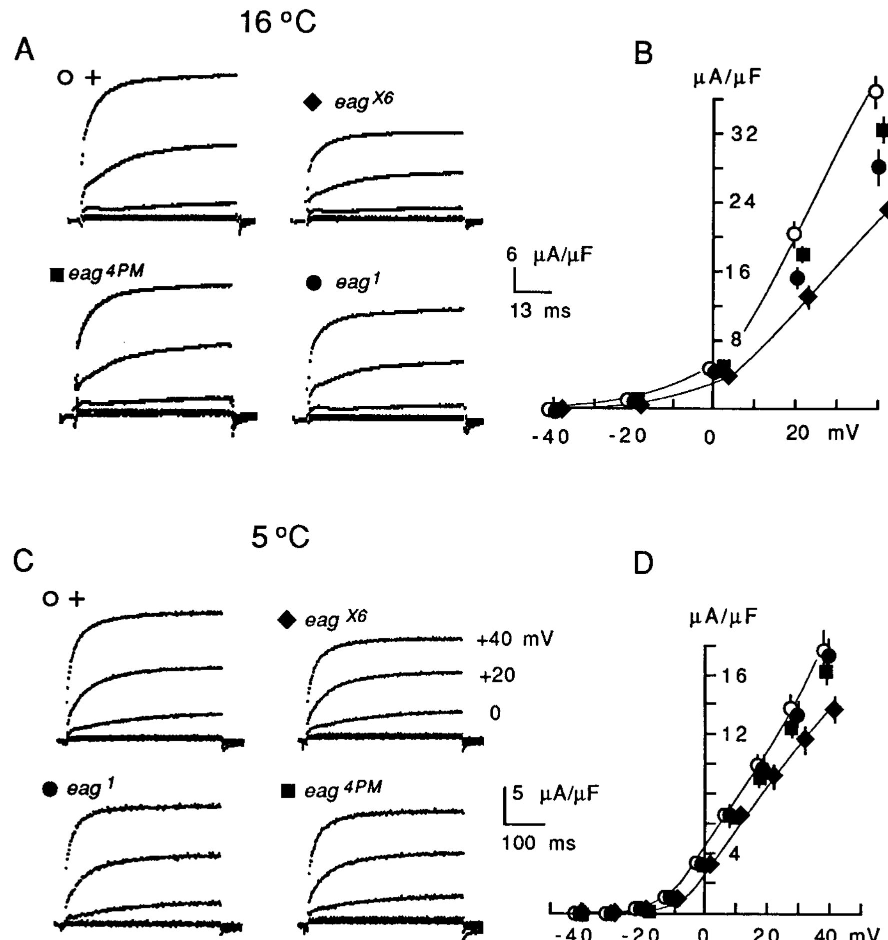

Figure 2. Temperature dependence of effects of *eag* mutations on I_K. *A*, Superimposed traces of I_K recorded at 16°C. *B*, *I–V* relations of I_K at 16°C. *C*, Traces of I_K recorded at 5°C. *D*, *I–V* relations of I_K at 5°C. Note the difference in time calibration (13 msec for 16°C, 100 msec for 5°C). Other conditions are the same as described in Figure 1.

perturb second messenger cascades also block ion channels directly in a dosage-dependent manner (Garber et al., 1990), including direct block of Ca^{2+} channels in *Paramecium* by 100 μM W7 (Ehrlich et al., 1988). In order to avoid such nonspecific effects of W7, we used a low concentration (25 μM). At this concentration, the effect of W7 was not itself evident, but could be greatly enhanced by preincubation of the preparation with 1 mM caffeine. Caffeine was present in the saline during dissection and was subsequently washed out prior to the addition of W7. Caffeine is known to exert effects on multiple cellular targets, but a major one is to trigger release of Ca^{2+} from intracellular stores (Sitsapesan and Williams, 1990). It alone appeared to reduce K^+ currents slightly in larval muscle, but its effect was diminished within 10 min after wash. Although the mechanism of the influence of caffeine on the action of W7 remains to be determined, it helped to demonstrate that the W7 effect is possibly mediated by Ca^{2+}/CaM.

With this combined treatment, I_A and I_K in normal larvae at 16°C were reduced to the extent observed in eag^{X6} (Fig. 3). The priming effect of caffeine did not depend on its simultaneous presence with W7. The suppression of currents in normal fibers by W7 was stable for at least 30 min after caffeine was washed out.

I_A and I_K in eag^1, which were less affected by this mutation than by eag^{X6} (Figs. 1, 2), responded to W7 like normal larvae and were reduced to a level similar to that in eag^{X6} (Fig. 3). Similar response was also observed in eag^{4PM} (data not shown). More intriguingly, both I_A and I_K were not further reduced by the drug treatment in the allele eag^{X6}, which produces a truncated mRNA (Drysdale et al., 1991; Warmke et al., 1991), but instead they showed a slight but reproducible increase in amplitude (Fig. 3).

Since *eag* mutations also alter I_{CF} and I_{CS} (Zhong and Wu, 1991), we extended the same drug treatment to see the response of I_{CF} and I_{CS}. The Ca^{2+}-activated I_{CF} and I_{CS} were studied at a lower temperature (5°C) and an external Ca^{2+} concentration of 0.9 mM (reduced from 1.8 mM in standard saline; see Materials and Methods). Under these conditions, muscle contraction was minimized while the two currents could still be readily measured (Fig. 4). We removed the voltage-activated I_A by addition of 100 μM 4-aminopyridine and I_K by 100 μM quinidine (see Materials and Methods). The remaining net current was outward, the inward I_{Ca} being totally masked by I_{CF} and I_{CS} (Fig. 4; also see Salkoff, 1983; Elkins et al., 1986; Singh and Wu, 1989). The inward tail currents (Fig. 4), following the depolarization-induced outward currents, were characteristic of Ca^{2+}-activated K^+ currents and distinct from I_K, which lacks inward tails at $V_H = -80$ mV (Fig. 2; see also Wu and Haugland, 1985; Singh and Wu, 1989, 1990). Further isolation of I_{CF} and I_{CS} was not conducted because, unlike the case of I_A and I_K, simple physiological separation of I_{CF} and I_{CS} is not yet feasible. However, as indicated by previous studies using genetic elimination of I_{CF} (Elkins et al., 1986; Singh and Wu, 1989, 1990), I_{CF} contributes mainly to the early outward transient whereas I_{CS} to the delayed plateau (Fig. 4).

The *I–V* relations for the transient peak (Fig. 4*C*) and for the

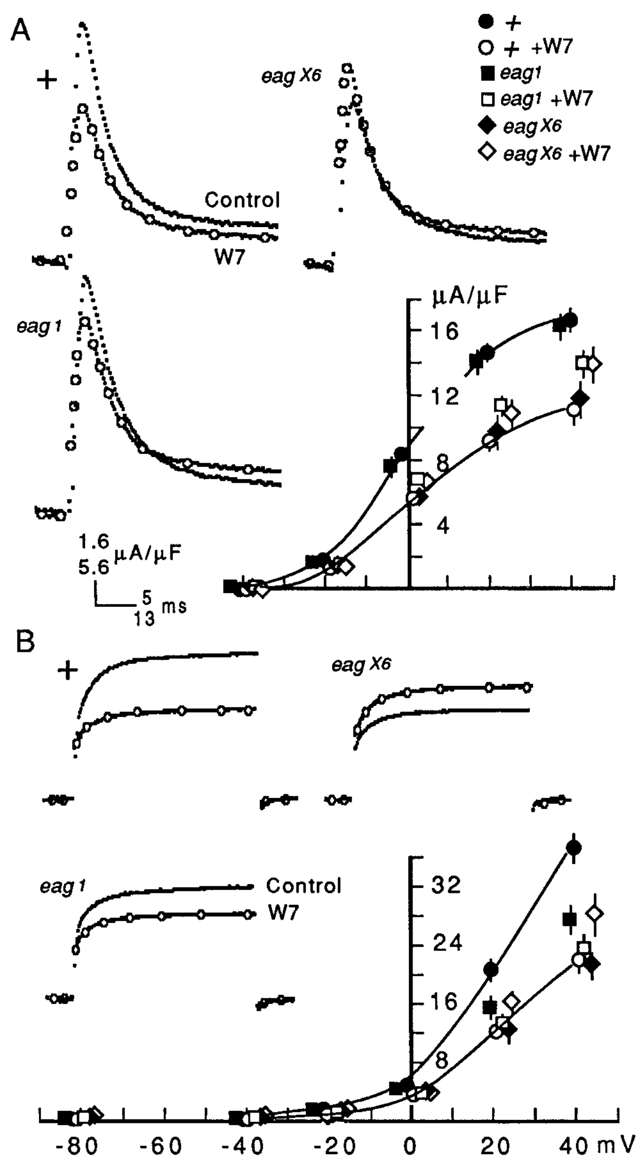

Figure 3. Effects of W7 on I_A and I_K in different *eag* alleles. I_A and I_K were elicited by voltage steps to +40 mV and recorded at 16°C in Ca^{2+}-free saline from larvae with or without treatment of 25 μM W7 (following preincubation with 1 mM caffeine; see Materials and Methods). The *traces marked with circles* represent currents following W7 treatments. *A*, I_A traces and *I–V* relations. *B*, I_K traces and *I–V* relations. For data from W7 experiments, N = 8, 7, 8 fibers from 3, 3, 4 larvae for wild type, eag^1, and eag^{X6}, respectively. The control data (unmarked traces and *solid symbols* in *I–V* relations) are identical to the corresponding sets of data shown in Figures 1 and 2.

plateau at the end of pulses (Fig. 4*B*) were determined. Since I_{Ca} is not significantly affected in the *eag* alleles, the differences between normal and mutant currents represent reduction in I_{CF} and I_{CS} (cf. Zhong and Wu, 1991). Bolth I_{CF} and I_{CS} were greatly reduced in eag^1 and eag^{X6} larvae while only I_{CS} was slightly decreased in eag^{4PM} larvae (Zhong and Wu, 1991). After treatment with W7, I_{CF} and I_{CS} in normal fibers were reduced to the same extent as that caused by eag^{X6} (Fig. 4) and eag^1 (Zhong and Wu, 1991) mutations. Most strikingly, these mutations blocked the action of W7 and no significant change could be detected in eag^{X6} (Fig. 4) and eag^1 (data not shown) larvae following the same drug treatment.

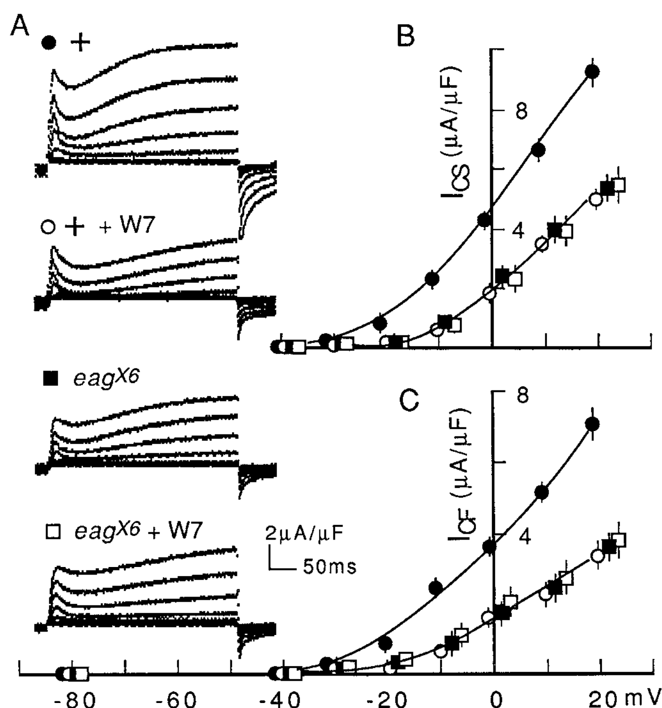

Figure 4. Effects of W7 on I_{CF} and I_{CS} in different *eag* alleles. *A*, Currents collected with or without 25 μM W7 treatment in wild-type and eag^{X6} larvae. *B*, *I–V* relations at I_{CS} plateau. *C*, *I–V* relations at peak I_{CF}. With W7 treatment, N = 7 and 8 fibers from three wild-type and four eag^{X6} larvae, respectively. For experiments without W7 treatment, N = 10 and 8 fibers from four wild-type and five eag^{X6} larvae, respectively. I_{CF} and I_{CS} were recorded at 5°C in saline containing 0.9 mM $CaCl_2$ and 4 mM $MgCl_2$, and other conditions were identical to those specified in Figure 3.

cGMP-dependent modulation of I_A *and* I_K

Although the action of W7 observed at a low concentration (Figs. 3, 4) was not likely to be a consequence of direct interaction of W7 with K^+ channels or other nonspecific effects of W7, the interpretation of the above results was complicated by the uncertainty of the exact mechanisms in the priming action of caffeine. However, the following experiments prompted by the finding of a consensus sequence of the cGMP binding site in the Eag sequence (Guy et al., 1991) provide further evidence that the *eag* mutations affect K^+ channel modulation.

I_K recorded in normal larvae at 5°C showed clear responses to bath application of 500 μM 8-bromo-cGMP (8-Br-cGMP), a membrane-permeable cGMP analog (Figs. 5, 6). Following the treatment, there was a drastic change in I_K in normal larvae that indicated a voltage-dependent alteration in conductance (Fig. 5, top panel). I_K was suppressed if the membrane was clamped at lower voltages but greatly enhanced at higher voltages (see the normal I_K traces and *I–V* curve in Fig. 5). If the *I–V* curve of normal I_K is normalized (open squares) to that with 8-Br-cGMP treatment (solid circles, top panel, Fig. 5), it is clear that I_K activation was shifted toward more positive membrane voltages by cGMP-dependent modulation. Therefore, 8-Br-cGMP treatments altered both I_K amplitude and its voltage dependence of activation. However, the kinetics of I_K remained largely unaltered (Fig. 5). The normal I_K traces without 8-Br-cGMP treatment, when normalized (circles, Fig. 5), extensively overlap with those following the drug treatment.

Similar changes could be observed at lower concentrations of

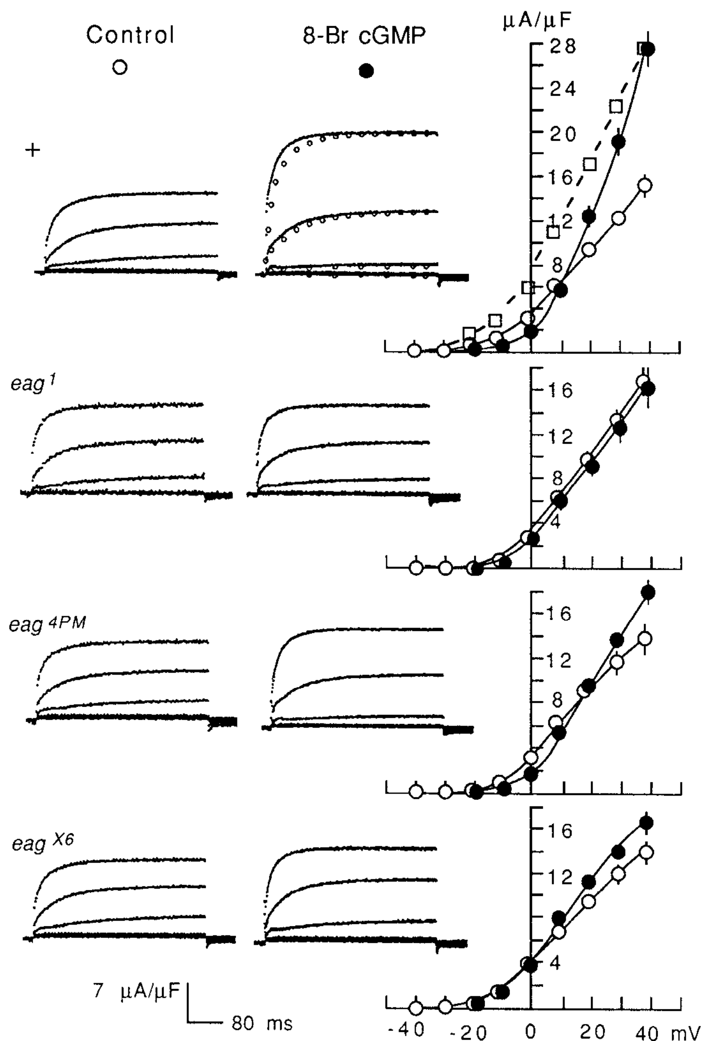

Figure 5. Alteration of cGMP-dependent modulation of I_K by *eag* mutations. The traces and *I–V* relations compare I_K recorded at 5°C without (*left column*) and with (*right column*) 500 μM 8-Br-cGMP in saline. 8-Br-cGMP altered I_K amplitude and voltage dependence of activation with relatively little effect on its kinetics. Note the change in the voltage dependence of normal I_K, indicated by a reduction in current amplitude at lower voltages but a drastic increase at higher voltages. Such a shift in the voltage dependence is more evident when the *I–V* curve of normal I_K is normalized (*squares*) to that following drug treatment (*solid circles*). However, changes in the kinetics are not evident as control currents in wild type show a time course similar to the drug-treated traces after normalization (*circles superimposed on corresponding traces*). These effects of 8-Br-cGMP are altered by *eag* mutations. N = 10, 8, 10, 8 from six to eight larvae for wild type, eag^1, eag^{4PM}, eag^{X6}.

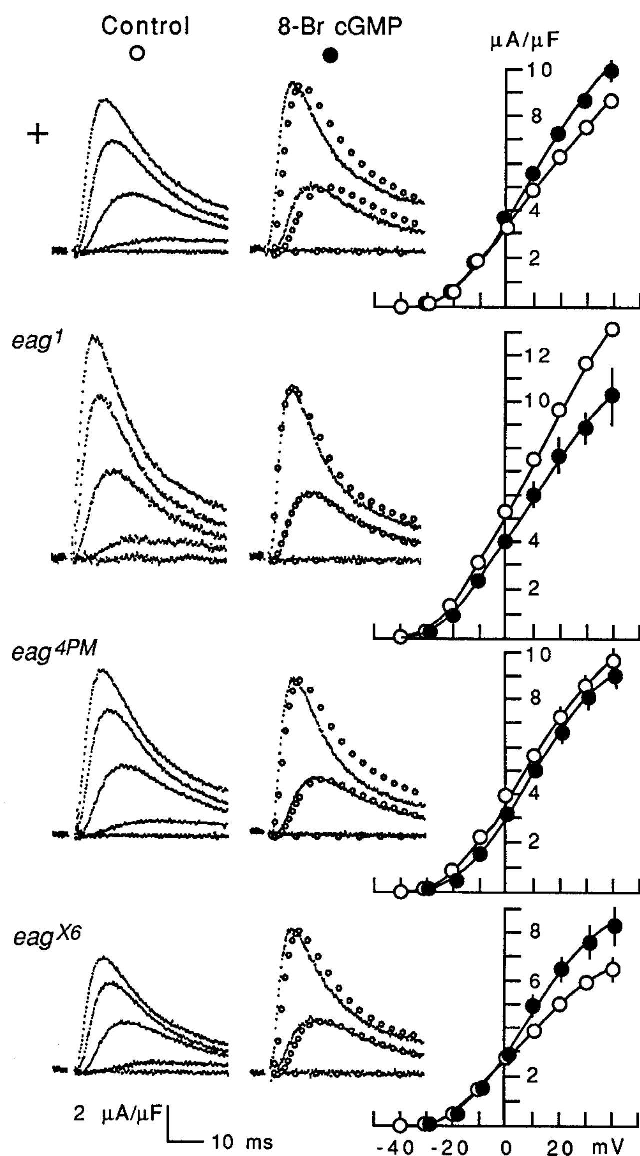

Figure 6. Alteration of cGMP-dependent modulation of I_A by *eag* mutations. The arrangement of current traces is similar to that in Figure 5. *Circles* represent control currents normalized to and superimposed on the corresponding drug-treated traces at −40, 0, +40 mV. The I_A amplitude after drug treatment is underestimated because of incomplete separation from I_K (see Materials and Methods). Same experimental conditions and number of fibers as in Figure 5.

8-Br-cGMP (e.g., 250 μM; data not shown) and could also be induced by application of either dibutyryl-cGMP at 500 μM or cGMP at a higher concentration, for example, 2 mM (data not shown), possibly due to low membrane permeability to cGMP.

A weaker response to the 8-Br-cGMP treatment was observed in I_A. As shown in the top panel of Figure 6, the I_A rise and decay phases appeared to be faster after the drug treatment and the amplitude of I_A in normal larvae was increased. The increase in I_A amplitude was, in fact, underestimated because of incomplete separation of I_A from I_K due to a shift in I_A inactivation (see Materials & Methods). Because of this uncertainty and small increase in the amplitude, further investigation is required to clarify the modulatory effect of cGMP on I_A.

Alterations of the cGMP responses by eag *mutations*

The response of I_K to 8-Br-cGMP was completely abolished in eag^1, in which the amplitude and voltage dependence of I_K were essentially the same before and after the drug treatment (Fig. 5). This blockade of the modulatory effect on I_K response was also evident at a high concentration of cGMP (2 mM; data not shown). Similarly, the 8-Br-cGMP effect on I_K was also nearly eliminated in eag^{X6} (Fig. 5). Unlike eag^1 and eag^{X6}, eag^{4PM} exhibited a characteristic but diminished cGMP-dependent modulation. After the 8-Br-cGMP treatment, a change in voltage dependence of I_K was still evident in eag^{4PM} larvae, that is, suppression at lower voltages and enhancement at higher voltages, as in normal larvae (Fig. 5).

There were no detectable effects of 8-Br-cGMP on the ampli-

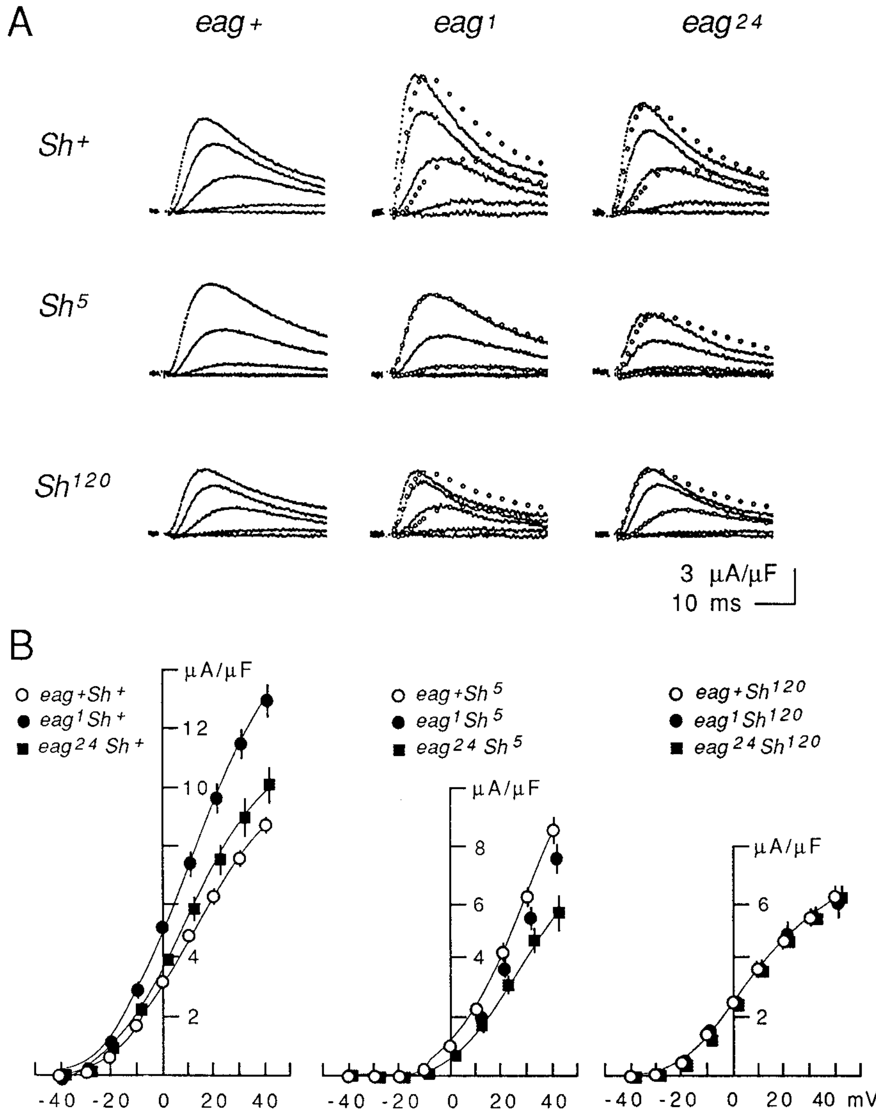

Figure 7. Effects of *eag* and *Sh* mutations on I_A and their interactions in double mutants. *A*, Each family of traces represents averaged density of I_A elicited by steps to −40, −20, 0, +20, +40 mV in different *eag–Sh* combinations at 5°C. In each column, current traces illustrate the phenotype of a specific *eag* allele (wild-type allele eag^+, and mutant alleles eag^1 and eag^{24}, from left to right) when combined with different *Sh* alleles (Sh^+, Sh^5, or Sh^{120}, from top to bottom). *Circles* represent currents from the first column normalized and superimposed on the corresponding traces to compare the time course of I_A elicited at 0 and +40 mV. The effect of eag^1 on the I_A time course depends on the *Sh* allele in the background. The rise and decay of I_A became faster in eag^1 Sh^+ and eag^1 Sh^{120} but eag^1 did not alter the time course of Sh^5 I_A in eag^1 Sh^5 double mutants. *B*, *I–V* curves. The effects of *eag* mutations on I_A amplitude vary with the *Sh* background. The eag^1 and eag^{24} mutations increased I_A, against the Sh^+ background but reduced I_A when combined with Sh^5, and had no effect on I_A amplitude when combined with Sh^{120}. N = 20, 9, 12, 9, 10, 9, 7, 9, 8, 15 fibers from four to nine larvae, for wild type, eag^1, eag^{24}, Sh^5, eag^1 Sh^5, eag^{24} Sh^5, Sh^{120}, eag^1 Sh^{120}, eag^{24} Sh^{120}, respectively.

tude of I_A in eag^{4PM}. The amplitude of I_A was increased slightly in eag^{X6} and reduced in eag^1, although the kinetics of I_A remained unchanged. However, as mentioned above for the case of normal I_A, the exact magnitude and the significance of such changes in the 8-Br-cGMP effects on I_A need to be further examined by using other techniques.

Allele-specific interaction between eag *and* Sh *mutations*

DNA sequence analysis has revealed that Eag is a polypeptide displaying features characteristic of K^+ channel subunits (Warmke et al., 1991), and the above results indicate that modulation of I_A, I_K, I_{CF}, and I_{CS} could be altered by *eag* mutations in an allele-dependent fashion. If both the *eag* and *Sh* subunits participate in forming I_A channels, these two polypeptides may have a close association such that mutations in one subunit may also influence function of the other through direct physical contact and conformational interaction. In that case, *eag Sh* double mutants may express novel phenotypes depending on specific allele combinations. Such genetic approaches using double-mutant interactions have been successful in demonstrating relations among subunit components in multimeric enzymes or in cytoskeletal assemblies (Huffaker et al., 1987; Fyrberg et al., 1990; Beall and Fyrberg, 1991).

We examined a number of double-mutant combinations for indication of allele-specific interactions. In order to facilitate the comparison of I_A kinetics among different genotypes, all data were recorded at 5°C, at which the rise and decay phases of I_A are slowed down (Wu et al., 1989). The *I–V* relations and averaged current traces based on a large number of fibers are shown in Figure 7, summarizing the allele-dependent *eag-Sh* interaction observed, which displays the effect of *eag* mutations upon I_A amplitude and kinetics against different *Sh* backgrounds. The first column in Figure 7*A* illustrates the background effects conferred by different *Sh* alleles without altering the *eag* gene. As compared to normal larvae (Sh^+), Sh^{120} reduced I_A amplitude, while Sh^5 decreased I_A only at lower voltages but not at higher voltages (+40 mV), indicating a shift in voltage dependence of I_A (cf. Haugland and Wu, 1990). When eag^1 (second column) and eag^{24} (third column) were combined with these different *Sh* alleles, it was evident that the *eag* effect on I_A varied with the *Sh* background. For example, against the Sh^+ background, both the eag^1 and eag^{24} mutations increased I_A

amplitude (Fig. 7*B*). The weaker effect of *eag*[24] was statistically significant at +20 mV or above (0.05 level, *t* test). In contrast, both mutations showed an opposite effect on I_A against the *Sh*[5] background (no statistically significant change in *eag*[1] but significant reduction in *eag*[24]). When combined with *Sh*[120], the two *eag* mutations did not exert any detectable effect on the I_A amplitude (Fig. 7). To compare the kinetics of I_A, the currents of each *Sh* allele at two voltages (0 and +40 mV) shown in the first column of Figure 7*A* are normalized (circles) and superimposed on the corresponding I_A traces in the second and third columns. Notably, the *eag*[1] mutation appeared to make the rise and decay phases of I_A faster (Fig. 7*A*) against the *Sh*+ and *Sh*[120] background, but did not alter the kinetics of I_A against the *Sh*[5] background.

Allele-specific interactions were further observed in steady-state inactivation of I_A (Fig. 8). When the membrane was held at different preconditioning voltages, I_A was inactivated to different degrees. Figure 8 illustrates the fraction of remaining I_A (vertical axis) activated by a fixed test pulse with varying preconditioning voltages (horizontal axis). Among a number of *eag Sh* double mutants examined, *eag*[1] *Sh*[120] and *eag*[4PM] *Sh*[120] showed a clear indication for allele-specific interaction in the voltage dependence of channel inactivation. The *eag*[1] and *eag*[4PM] mutations alone did not significantly alter the inactivation of I_A. The *Sh*[120] mutation shifted the voltage dependence of I_A inactivation toward the positive direction (cf. Wu and Haugland, 1985). This defect became more extreme in *eag*[4PM] *Sh*[120] while it was restored to nearly normal in *eag*[1] *Sh*[120] (Fig. 8). The difference observed between *Sh*[120] and *eag*[4PM] *Sh*[120] was highly reproducible among the larvae examined and was highly significant in paired *t* tests (see Fig. 8 caption). In addition, one-way ANOVA of the current ratio ($I : I_o$) at −35 mV (Fig. 8) indicates that the shift is significant ($F[4, 50] = 18.95$; $p < 0.01$). Furthermore, such interaction between *eag*[4PM] and *Sh*[120] has also been observed in aneuploid animals, in which an additional copy of the normal *Sh* gene was introduced into *Sh*[120] and *eag*[4PM] *Sh*[120] flies. Even with increased I_A due to the contribution from the duplicated *Sh* locus (Haugland and Wu, 1990), the inactivation of I_A observed in aneuploid *eag*[4PM] *Sh*[120] larvae occurred at more positive voltages than that in aneuploid *Sh*[120] larvae (not shown).

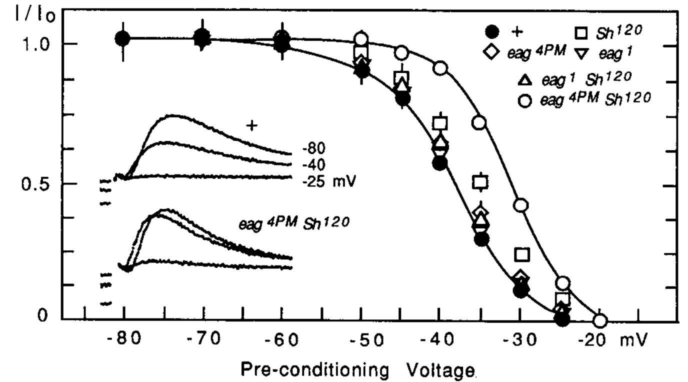

Figure 8. Effects of *eag-Sh* interactions on steady-state inactivation of I_A at 5°C. I_A elicited by a test pulse to +10 mV was inactivated to varying degrees (see *inset*) by preconditioning depolarization at different voltages (*horizontal axis*). The *vertical axis* indicates the ratio ($I : I_o$) of the remaining I_A to the maximally attainable I_A preconditioned at −80 mV. In paired *t* tests, $I : I_o$ in *Sh*[120] is significantly higher than that in normal larvae at −35 mV ($p < 0.01$) while $I : I_o$ in *eag*[4PM] *Sh*[120] is significant larger than both normal ($p < 0.001$) and *Sh*[120] larvae ($p < 0.005$). In contrast, the *eag*[1] *Sh*[120] double mutant showed no significant difference from normal larvae, but a marginal reduction from *Sh*[120] ($p < 0.05$). N = 13, 11, 9, 7, 9, 7 fibers from three to six larvae, for wild type, *eag*[1], *eag*[4PM], *Sh*[120], *eag*[1] *Sh*[120], *eag*[4PM] *Sh*[120], respectively. The curves are fit by eye to data from wild type and *eag*[4PM] *Sh*[120] fibers.

Discussion

Involvement of Eag polypeptide in modulation of multiple K+ currents

We have demonstrated in a previous paper that *eag* mutations are capable of attenuating the amplitude of four distinct K+ currents, but eliminating none of them (Zhong and Wu, 1991). The different lines of evidence presented in this article suggest that the Eag polypeptide plays an essential role in modulation of these K+ currents. It was first shown that the effects of *eag* mutations on I_A and I_K were qualitatively dependent on temperature (Figs. 1, 2), which provides a clue that the effects are of modulatory nature. More direct evidence came from the pharmacological studies. In contrast to the milder effects on the amplitude of K+ currents, certain mutations could abolish the cGMP-dependent modulation on I_K and W7/caffeine-dependent modulation on I_A, I_K, I_{CF}, and, I_{CS}.

To our knowledge this is the first report of K+ currents modulated by cGMP. Modulation mediated by cGMP-induced kinase activity has previously been reported on Ca^{2+} and Na^+ currents in neurons (Paupardin-Tritsch et al., 1986; Levitan and Levitan, 1988; Hemmings et al., 1989; Ichinose and McAdoo, 1989) and on cation channels in epithelial apical membrane (Light et al., 1990). Direct cGMP binding to channels for modulation of light- and ordorant-sensitive cation flux has been shown in visual and olfactory receptor cells (Yau and Baylor, 1989; Goy, 1991). It is interesting to note that, in addition to the putative cGMP binding site, general sequence homology between cGMP-gated channels and the Eag polypeptide has been reported (Guy et al., 1991). The exact mechanisms of cGMP-dependent modulation of I_K in *Drosophila* must await further experimentation.

It may be thought that the cGMP effect actually reflects physiological responses caused by cAMP. In principle, cGMP can exert its effect indirectly by regulating the activity of certain phosphodiesterases specific for cAMP degradation (Hartzell and Fischmeister, 1986; Doerner and Alger, 1988). In addition, some cAMP-gated channels can also be activated by cGMP (Goy, 1991), and, in particular, a cAMP-gated single-channel K+ current has been identified in *Drosophila* larval muscles (Delgado et al., 1991). However, these cAMP-gated K+ channels are cAMP specific and voltage independent (Delgado et al., 1991). Moreover, the effect of cAMP treatment on macroscopic I_K, detected by voltage clamp (Zhong, 1991, Zhong and Wu, 1993), is different from that of cGMP reported here and such cAMP effects were not reduced or abolished by *eag*[1] or *eag*[X6] mutations (Zhong and Wu, unpublished observations). These data implicate cGMP as a biological second messenger that modulates I_A and I_K. Interestingly, it has been reported that glutamate, a major excitatory transmitter at neuromuscular junctions in *Drosophila*, increases cGMP synthesis in a vesicle preparation of larval muscles (Robinson et al., 1982). It is not known whether this leads to a functional regulation of I_A and I_K. Hormonal regulation of cGMP levels during insect molting has also been reported to modulate firing patterns of central neurons, perhaps via modifications of membrane currents (Truman et al., 1979).

In addition to the cGMP-dependent modulation, *eag* muta-

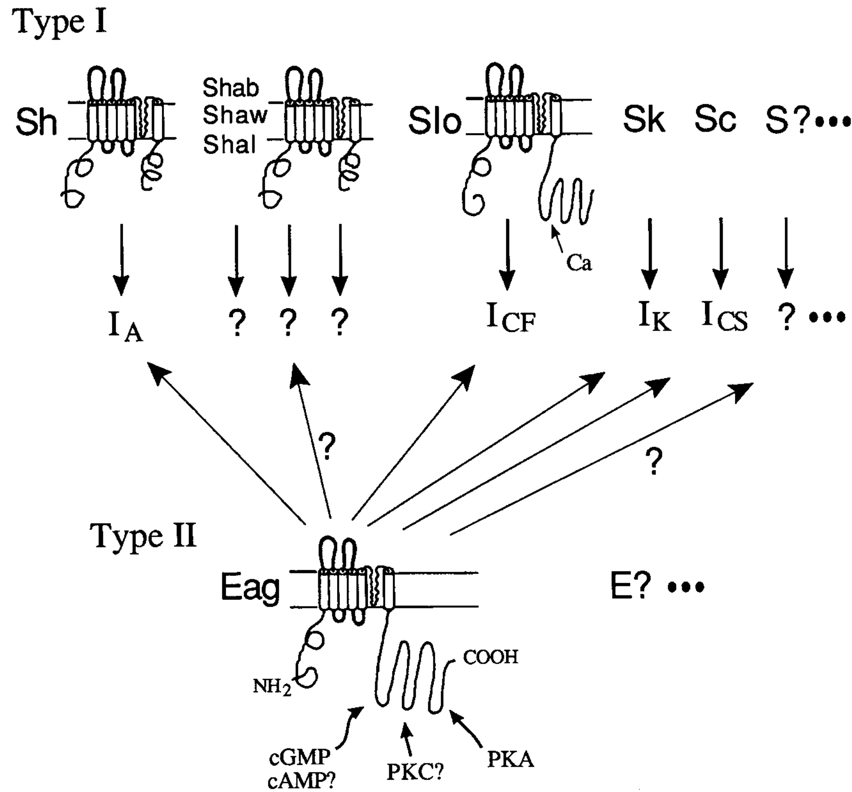

Figure 9. A hypothetical genetic framework for generating a diverse family of K^+ channels. The properties of the I_A, I_K, I_{CF}, and I_{CS} channels have been characterized in *Drosophila* muscles. Sh, Shab, Shaw, Shal, Slo, and Eag are K^+ channel subunits encoded by different genes that have been cloned and sequenced. *Sk* and *Sc* represent subunits, not yet identified molecularly, for the I_K and I_{CS} channels. *Shab, Shaw,* and *Shal* have been expressed in the *Xenopus* oocyte, but their functions have not yet been demonstrated in *Drosophila* muscles. *S*? and *E*? represent undiscovered subunits for K^+ channels. *PKC,* protein kinase C. *PKA,* cAMP-dependent protein kinase. The K^+ channel subunits in *Drosophila* may be classified into two types. Type I subunits, including Sh, determine the channel gating and conductance. Subunits in this group have not been observed to coassemble intergenically in a functional channel. Type II subunits, represented by Eag, so far the only identified member, are assumed to coassemble with different type I subunits to mediate channel modulation.

tions also diminished the W7/caffeine-dependent modulation on I_A, I_K, I_{CF}, and I_{CS}. W7, an antagonist of Ca^{2+}/CaM complex, could show a strong suppression on the muscle K^+ currents, but at a concentration range that raises concerns of its nonspecific interactions with other protein kinases (Schatzman et al., 1983) and direct interaction with the channels (Ehrlich et al., 1988). Therefore, we have also examined the effects of W7 at a relatively low concentration (25 μM), at which W7 alone did not affect K^+ currents appreciably, but significantly reduced these currents with preincubation of the preparation in the saline containing caffeine (Figs. 3, 4). As previously mentioned, caffeine is capable of triggering release of Ca^{2+} from intracellular stores and inhibiting phosphodiesterase, among a large range of effects (Sitsapesan and Williams, 1990). It cannot be certain which of these mechanisms is responsible for potentiating the W7 effect. However, it should be noted that among the abundant putative phosphorylation sites in Eag, several consensus sequences of PKB sites have been identified (Griffith and Greenspan, personal communication).

These results indicate that modulation of multiple K^+ currents through different second messenger pathways can be altered by the same *eag* mutations. Thus, the Eag polypeptide may serve as a convergent site of the cGMP and PKB-related mechanisms to integrate modulatory effects on K^+ currents.

Participation of the eag *subunit in the assembly of* I_A *channels*

At least three different K^+ channel genes, *Sh, eag,* and *slowpoke* (*slo*), have been identified by their mutational effects on membrane currents and by the structural homology of their protein products deduced from DNA sequence data. *Sh* mutations specifically affect I_A (Jan et al., 1977; Salkoff and Wyman, 1981; Wu et al., 1983; Haugland and Wu, 1990) while *slo* mutations eliminate a fast Ca^{2+}-activated K^+ current I_{CF} (Elkins et al., 1986; Singh and Wu, 1989; Komatsu et al., 1990). Only additive effects, restricted to their individual defects in I_A and I_{CF}, are observed when *Sh* and *slo* mutations are combined in double mutants (Singh and Wu, 1989; Komatsu et al., 1990). In contrast, when *eag* and *Sh* mutations were combined, allele-specific, novel phenotypes of I_A were observed. As explained in Results, such observations lend strong support to the idea that these two polypeptides may have close spatial relations and interact through physical contact.

We cannot rule out the possibility that the *eag* polypeptide might function as an enzyme or as a cytoskeleton component that contacts and modulates the *Sh* subunits. However, *eag* mutations interrupting modulatory responses of K^+ currents to two independent mechanisms suggest that the *eag* polypeptide may not be a specific protein kinase. In addition, the deduced structure of the *eag* polypeptide has a resemblance closer to a K^+ channel subunit than to other classes of proteins. Hence, we favor the interpretation that the allele-specific interaction between *eag* and *Sh* indicates the coexistence of *Sh* and *eag* polypeptides in the I_A channel complex. A similar arrangement may be proposed for other K^+ channels affected by *eag* mutations, although double-mutant analysis in those cases is hindered by the limited number of mutations in other genes.

It has to be pointed out that our results based on the four identified K^+ currents in larval muscles cannot exclude the possibility that Eag alone can form other types of K^+ channels in other cell types or in an expression system such as *Xenopus* oocytes. It is well established that a single subunit species of a heteromultimeric channel, either ligand gated or voltage gated, can form functional, although not entirely normal, channels in different expression systems (Boulter et al., 1987; Auld et al., 1988; Blair et al., 1988; Mori et al., 1991; Singer et al., 1991; Isom et al., 1992).

A hypothetical genetic framework for K^+ channel diversity

In addition to sequence homology, the Eag polypeptide as a K^+ channel subunit is supported by evidence from electrophysiological studies of the mutational effects *in vivo.* The allele-dependent differential effects of individual *eag* mutations on separate sets of K^+ currents (Zhong and Wu, 1991) and the remarkable functional role of the Eag polypeptide in mediating modulation of multiple K^+ currents by different biochemical cascades in *Drosophila* muscle prompted a hypothesis, which may serve to stimulate further studies on the important issues of channel assembly and modulation, as detailed below.

The role of the Eag subunit in modulation of different K^+ channels contrasts with those of the other K^+ channel polypeptides so far identified, Sh and Slo, which determine the gating and conductance of I_A and I_{CF} channels, respectively. As schematically presented in Figure 9, such distinctions lead to a plausible genetic framework for the diverse K^+ channel families based on two categories of K^+ channel polypeptides. Type I includes those members that are individually associated with a specific K^+ channel and determine the channel gating and conductance. Type II subunits coassemble with different type I subunits in multiple K^+ channels to mediate modulation of these channels.

Type I subunits may be represented by members in the *Sh* family and by the *slo* polypeptide (Schwarz et al., 1988; Butler et al., 1989; Atkinson et al., 1991). Oocyte coexpression experiments have indicated that subunits derived from different members in the *Sh* family, that is, *Sh, Shal, Shab,* and *Shaw,* are not able to coassemble with each other to form K^+ channels (Covarrubias et al., 1991). Specific effects of *Sh* and *slo* mutations suggest that Sh and Slo do not coexist in the I_A and I_{CF} channels (Singh and Wu, 1989). In contrast, *eag* mutations affect multiple K^+ currents and alter their modulation. Thus, the *eag* polypeptide and its possible homologs, yet to be identified, represent type II subunits.

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