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 Ca²⁺/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 Iₑ 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 Iₐ 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 Iₐ 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 I₅ in Drosophila melanogaster (Salkoff and Wyman, 1981; Wu and Haugland, 1985). Nonadditive effects on I₅ have been found in heteroallelic combinations of different Sh mutations, suggesting oligomeric assembly of I₅ 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 I₅ channels, in vivo, and what functional roles the additional subunits play. It is known that vertebrate Ca²⁺ and Na⁺ channels contain a number of subunits although a single subunit species, α, in Ca²⁺ 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 Ca²⁺ 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 Iₐ and delayed Iₜ, and the Ca⁺⁺-activated fast I₆ and slow I₇ (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...
Mallart, 1986; Singh and Wu, 1989, 1990; Haugland and Wu, 1990; Zhong and Wu, 1991). 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) (Warneke et al., 1991), and Ca2+calmodulin (Ca2+/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 Ca2+/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, Ic 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 Ic 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°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+/Slo. The Sh locus (mapped to 1-57;4; Kaplan and Trout, 1969; Jan et al., 1977) encodes Ic channel subunits in muscles. The Sh+/slo (designated as Sh+/1c hereafter in text) reduces the amplitude of Ic in larval muscles (Haugland and Wu, 1990).

eag+. 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; Warmke et al., 1991; Zhong and Wu, 1991).

In this study, we explored modulation of K+ currents by two microinjection of voltage-dependent ion channels for data collection and analysis. The two-microinjection technique has been described previously (Engel 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).

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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 IA and ZK. All families of current traces and voltage-current (Z-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 IA and ZK 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, IA and ZK in eag' were increased at 5°C but remained almost unchanged at 16°C (Fig. 1). Conversely, ZK in eag' was nearly normal at 5°C but decreased at 16°C (Fig. 2). A distinct temperature dependence was also observed in eagx6 (Figs. 1, 2).

Previous observations on Ica and Ics obtained at 5°C indicate that these Ca2+ activated currents are also affected by eag mutations (Zhong and Wu, 1991). However, examination of Ica and Ics at 16°C was prevented by technical difficulties due to massive muscle contractions.

Effects of a Ca2+/CaM antagonist

In addition to eag', eagx6, and eagx8, we examined eight more eag alleles, including one possible null allele, eagnull (Drysdale et al., 1991). At 5°C none of these alleles exhibited effects on Ica and Ics larger than those seen in eagx6 or eag (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, Ia and Ik 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 Ca2+/CaM (Hidaka et al., 1981), consequently inhibiting the Ca2+/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
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 \(\mu\)M W7 (Ehrlich et al., 1988). In order to avoid such nonspecific effects of W7, we used a low concentration (25 \(\mu\)M). 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, \(I_{K}\) and \(I_{A}\) in normal larvae at 16\(^\circ\)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\(^{4}\), 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\(^{PM}\) (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\(^\circ\)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 \(\mu\)M 4-aminopyridine and \(I_{K}\) by 100 \(\mu\)M quinidine (see Materials and Methods). The remaining net current was outward, the inward \(I_{K}\) 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 \(V_{m} = -80\) 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. 4C) and for the
plateau at the end of pulses (Fig. 4B) were determined. Since $I_{Cs}$ is not significantly affected in the eag alleles, the differences between normal and mutant currents represent reduction in $I_{CR}$ and $I_{CS}$ (cf. Zhong and Wu, 1991). Both $I_{CR}$ and $I_{CS}$ were greatly reduced in eag′ and eagX6 larvae while only $I_{Cs}$ was slightly decreased in eagX6 larvae (Zhong and Wu, 1991). After treatment with W7, $I_{CR}$ and $I_{CS}$ in normal fibers were reduced to the same extent as that caused by eagX6 (Fig. 4) and eag′ (Zhong and Wu, 1991) mutations. Most strikingly, these mutations blocked the action of W7 and no significant change could be detected in eagX6 (Fig. 4) and eag′ (data not shown) larvae following the same drug treatment.

**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′, and eagX6, 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.

**Figure 4.** Effects of W7 on $I_{CS}$ and $I_{CS}$ in different eag alleles. A, Currents collected with or without 25 µM W7 treatment in wild-type and eagX6 larvae. B, $I-V$ relations at $I_{CS}$ plateau. C, $I-V$ relations at peak $I_{CR}$. With W7 treatment, $N = 7$ and 8 fibers from three wild-type and four eagX6 larvae, respectively. For experiments without W7 treatment, $N = 10$ and 8 fibers from four wild-type and five eagX6 larvae, respectively. $I_{CR}$ and $I_{CS}$ were recorded at 5°C in saline containing 0.9 mM CaCl₂ and 4 mM MgCl₂, 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
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_K 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', 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'' (Fig. 5). Unlike eag' and eag'', eag'' 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'' 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 \textit{eag} and \textit{Sh} mutations on \textit{I}_A and their interactions in double mutants. \textit{A}. Each family of traces represents averaged density of \textit{I}_A elicited by steps to \(-40, -20, 0, +20, +40\) mV in different \textit{eag}--\textit{Sh} combinations at 5°C. In each column, current traces illustrate the phenotype of a specific \textit{eag} allele (wild-type allele \textit{eag}+, and mutant alleles \textit{eag} and \textit{eag}*, from left to right) when combined with different \textit{Sh} alleles (\textit{Sh}+, \textit{Sh}5, or \textit{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 \textit{I}_A elicited at 0 and +40 mV. The effect of \textit{eag} on the \textit{I}_A time course depends on the \textit{Sh} allele in the background. The rise and decay of \textit{I}_A became faster in \textit{eag} Sh+ and \textit{eag} Sh120 but \textit{eag} did not alter the time course of \textit{Sh}s \textit{I}_A in \textit{eag} Sh5 double mutants. \textit{B}. \textit{I}--\textit{V} curves. The effects of \textit{eag} mutations on \textit{I}_A amplitude vary with the \textit{Sh} background. The \textit{eag} and \textit{eag}* mutations increased \textit{I}_A, against the \textit{Sh} background but reduced \textit{I}_A when combined with \textit{Sh}5, and had no effect on \textit{I}_A amplitude when combined with \textit{Sh}120. \textit{N} = 20, 9, 12, 9, 10, 9, 7, 9, 8, 15 fibers from four to nine larvae, for wild type, \textit{eag}, \textit{eag}*, \textit{Sh}5, \textit{eag} Sh5, \textit{eag}*, \textit{Sh}120, \textit{eag} Sh120, \textit{eag}* Sh120, respectively.

\textbf{Allele-specific interaction between \textit{eag} and \textit{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 \textit{I}_A, \textit{I}_C, and \textit{I}_K could be altered by \textit{eag} mutations in an allele-dependent fashion. If both the \textit{eag} and \textit{Sh} subunits participate in forming \textit{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, \textit{eag} \textit{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 \textit{I}_A kinetics among different genotypes, all data were recorded at 5°C, at which the rise and decay phases of \textit{I}_A are slowed down (Wu et al., 1989). The \textit{I}--\textit{V} relations and averaged current traces based on a large number of fibers are shown in Figure 7, summarizing the allele-dependent \textit{eag}--\textit{Sh} interaction observed, which displays the effect of \textit{eag} mutations upon \textit{I}_A amplitude and kinetics against different \textit{Sh} backgrounds. The first column in Figure 7A illustrates the background effects conferred by different \textit{Sh} alleles without altering the \textit{eag} gene. As compared to normal larvae (\textit{Sh}+), \textit{Sh}120 reduced \textit{I}_A amplitude, while \textit{Sh}5 decreased \textit{I}_A only at lower voltages but not at higher voltages (+40 mV), indicating a shift in voltage dependence of \textit{I}_A (cf. Haugland and Wu, 1990). When \textit{eag} (second column) and \textit{eag}* (third column) were combined with these different \textit{Sh} alleles, it was evident that the \textit{eag} effect on \textit{I}_A varied with the \textit{Sh} background. For example, against the \textit{Sh}+ background, both the \textit{eag} and \textit{eag}* mutations increased \textit{I}_A.
amplitude (Fig. 7B). The weaker effect of eagg was statistically significant at +20 mV or above (0.05 level, t test). In contrast, both mutations showed an opposite effect on Ia against the ShI background (no statistically significant change in eagg but significant reduction in eagg). When combined with Shl20, the two eagg mutations did not exert any detectable effect on the Ia amplitude (Fig. 7). To compare the kinetics of Ia, the currents of each Sh allele at two voltages (0 and +40 mV) shown in the first column of Figure 7A are normalized (circles) and superimposed on the corresponding Ia traces in the second and third columns. Notably, the eagg mutation appeared to make the rise and decay phases of Ia faster (Fig. 7A) against the ShI+ and Shl20 background, but did not alter the kinetics of Ia against the ShI background.

Allele-specific interactions were further observed in steady-state inactivation of Ia (Fig. 8). When the membrane was held at different preconditioning voltages, Ia was inactivated to different degrees. Figure 8 illustrates the fraction of remaining Ia (vertical axis) activated by a fixed test pulse with varying preconditioning voltages (horizontal axis). Among a number of eagg Sh double mutants examined, eagg Shl20 and eagg Shl20 showed a clear indication for allele-specific interaction in the voltage dependence of channel inactivation. The eagg and eagg Shl20 mutations alone did not significantly alter the inactivation of Ia. The Shl20 mutation shifted the voltage dependence of Ia inactivation toward the positive direction (cf. Wu and Haugland, 1985). This defect became more extreme in eagg Shl20 while it was restored to nearly normal in eagg Shl20 (Fig. 8). The difference observed between Shl20 and eagg Shl20 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 (Ia/Io) at -35 mV (Fig. 8) indicates that the shift is significant (F[4, 50] = 18.95, p < 0.01). Furthermore, such interaction between eagg and Shl20 has also been observed in aneuploid backgrounds, in which an additional copy of the normal Sh gene was introduced into Shl20 and eagg Shl20 flies. Even with increased Ia, due to the contribution from the duplicated Sh locus (Haugland and Wu, 1990), the inactivation of Ia observed in aneuploid eagg Shl20 larvae occurred at more positive voltages than that in aneuploid Shl20 larvae (not shown).

**Discussion**

**Involvement of Eag polypeptide in modulation of multiple K+ currents**

We have demonstrated in a previous paper that eagg 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 eagg mutations on Ia and Io were qualitatively dependent on temperature (Figs. 1, 2), which provides a clue that the effects are due to 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 Io and W7/caffeine-dependent modulation on Io, G, and Icx.

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 Ca2+ and Na+ currents in neurons (Paupardin-Tritsch et al., 1986; Levitan

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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 Ia 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; Doemer 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 Ia 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 eagg or eagg mutations (Zhong and Wu, unpublished observations). These data implicate cGMP as a biological second messenger that modulates Ia and Icx. Interestingly, it has been reported that glutamate, a major excitatory transmitter at neuromuscular junctions in Drosophila, increases GMP synthesis in a vesicle preparation of larval muscles (Robinson et al., 1982). It is not known whether this leads to a functional regulation of Ia and Icx. 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, eagg muta-
eliminate a fast Ca\(^{2+}\)-activated K\(^+\) current ZcF (Elkins et al., Wu et al., 1983; Haugland and Wu, 1990) while slo mutations specifically affect IA (Jan et al., 1977; Salkoff and Wyman, 1981; Elkins et al., 1986; Singh and Wu, 1989; Komatsu et al., 1990). Only additive effects, restricted to their individual defects in IA and ICF, 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 IA 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, 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).

Participation of the eag subunit in the assembly of I, 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 IA (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 ICF (Elkins et al., 1986; Singh and Wu, 1989; Komatsu et al., 1990). Only additive effects, restricted to their individual defects in IA and ICF, 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 IA 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, 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.

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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 Ia and Ic 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 Ia and Ic channels (Singh and Wu, 1989). In contrast, eag mutations affect multiple K+ currents and alter their modulation. Thus, the eag polypeptide and its possible homologues, yet to be identified, represent type II subunits.

References


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