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21. Transplants were done as described (*14*). At P8, host pups were anesthetized (3.5% chloral hydrate, 10 mg/100 g of body mass) and perfused transcardially with 0.9% buffered saline, followed by 4% paraformaldehyde and 2% glutaraldehyde in 0.2 M cacodylic acid buffered to pH 7.3. Brains were removed and cortices were flattened for tangential sectioning at 40 or 50 μm on a Vibratome. Every other section was assayed for AChE (*16*). We processed the remaining sections for the binding of PNA to reveal the distribution of glycoconjugated molecules in host and transplanted tissue (*20*). Before these procedures, we examined selected sections and photographed them under ultraviolet illumination to identify the bisbenzimide-marked transplant.
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A Distinct Potassium Channel Polypeptide Encoded by the *Drosophila eag* Locus

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Many of the signaling properties of neurons and other electrically excitable cells are determined by a diverse family of potassium channels. A number of genes that encode potassium channel polypeptides have been cloned from various organisms on the basis of their sequence similarity to the *Drosophila Shaker* (*Sh*) locus. As an alternative strategy, a molecular analysis of other *Drosophila* genes that were defined by mutations that perturb potassium channel function was undertaken. Sequence analysis of complementary DNA from the *ether à go-go* (*eag*) locus revealed that it encodes a structural component of potassium channels that is related to but is distinct from all identified potassium channel polypeptides.

THE *DROSOPHILA SH* LOCUS WAS THE first K^+ channel structural gene to be isolated (*1*). Since then, a number of additional K^+ channel genes have been cloned from *Drosophila* and other organisms (*2*). The primary strategy researchers used to clone most of these genes was cross-hybridization with DNA probes derived from *Sh*. However, other K^+ channel genes less closely related to *Sh* may not have been identified by this approach. As an alternative route to isolate genes affecting K^+ channel structure and function, we made no assumptions about sequence conservation and focused on mutations that disrupt K^+ channel activity in *Drosophila* (*3*).

One of these genes is the X-linked *eag* locus, which was originally identified in *Drosophila* on the basis of mutations that cause a leg-shaking phenotype (*4*). Electrophysiological studies revealed that *eag* mutations cause spontaneous repetitive firing in motor axons and elevated transmitter release at the larval neuromuscular junction (*3, 5*). In *eag Sh* double mutants, transmitter release at the larval neuromuscular junction persists for at least an order of magnitude longer than in either single mutant, resulting in large plateau-shaped synaptic potentials. These prolonged synaptic potentials are correlated with long trains of action potentials in motor axons. Whereas *Sh* mutations eliminate I_A, a fast, transient, voltage-gated K^+ current (*6*), voltage-clamp experiments indicated that I_K, a slow non-inactivating, voltage-gated K^+ current, is reduced in *eag* mutants (*7*). Furthermore, some *eag* mutations diminish I_A, as well as I_{CF} and I_{CS}, which are fast and slow Ca^{2+}-activated K^+ currents, respectively (*7, 8*). However, it was unclear whether *eag* encoded a structural component of K^+ channels or affected the activity of these channels by another mechanism.

To elucidate the molecular basis of the *eag* phenotype, we cloned this locus by chromosome jumping and walking and identified cDNAs that correspond to the *eag* transcript (*9, 10*). These cDNAs span over 35 kb of genomic DNA and encompass the molecular lesions associated with four mutant *eag* alleles.

We determined the complete nucleotide sequence of cDNA CH20 (*10*), which is 4061 nucleotides in length. Although this cDNA is incomplete relative to the 10-kb *eag* transcript (*9*), it contains a complete open reading frame, beginning at nucleotide 464 and terminating at nucleotide 3985, thus encoding a deduced polypeptide of 1174 amino acids (Fig. 1). Translation was assumed to begin at nucleotide 464 because this is the first ATG codon of the open reading frame, and the sequence immediately preceding this codon matches the consensus translational start site for *Drosophila* (*11*). Computer analyses of the amino acid and nucleotide sequences of *eag* failed to detect any similarities with sequences of other genes in three databases (*12*). However, hydropathy analysis (Fig. 2) of the *eag* polypeptide (Eag) indicated the presence of seven potential membrane-spanning domains, reminiscent of the structure of known K^+ channel polypeptides (*1, 2*). Furthermore, comparison with a K^+ channel consensus sequence derived from the *Sh* superfamily revealed that each hydrophobic domain of Eag shared similarity with the corresponding domain of the consensus sequence (Fig. 3). The sequence similarities in two of these regions, S4 and H5, are noteworthy. A feature of most voltage-gated channels is an S4 domain, which is thought to represent the voltage sensor of these channels (*13*). This domain consists of a string of positively charged residues at every third position that are each separated by two hydrophobic residues. The fourth hydro-

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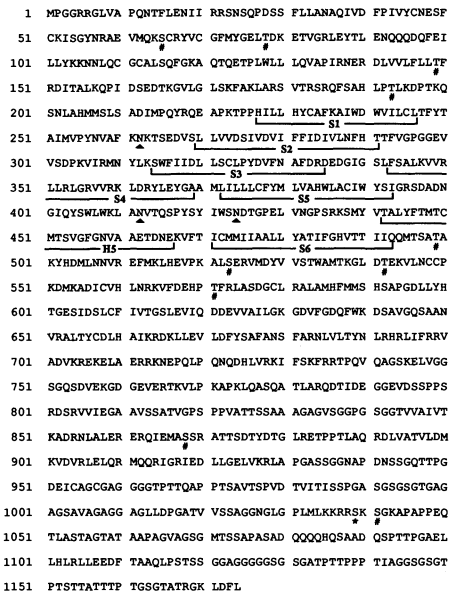

Fig. 1. Deduced amino acid sequence encoded by *eag* cDNA CH20. The seven hydrophobic domains identified by hydropathy analysis (Fig. 2) and aligned with a K^+ channel consensus sequence (Fig. 3) are underlined. The hydrophobic domains are named according to previous nomenclature (*14*). Filled triangles are marked underneath three consensus N-linked glycosylation sites (*16*) that have presumptive extracellular locations. An asterisk is marked below a single consensus site for cAMP-dependent phosphorylation (*16*) that has a presumptive cytoplasmic location. The symbol # is marked below consensus sites for PKC-dependent phosphorylation (*16*) that have presumptive cytoplasmic locations. The single letter abbreviations for amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence of cDNA CH20 has been submitted to GenBank under accession number M61157.

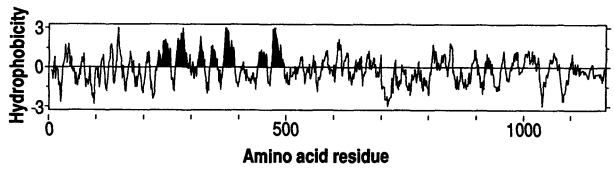

Fig. 2. Hydropathy plot for the deduced polypeptide encoded by *eag* cDNA CH20. Hydrophobicity values were calculated as described (*17*) with a window size of nine amino acids. The shaded hydrophobic peaks are named in order from left to right: S1, S2, S3, S4, S5, H5, and S6.

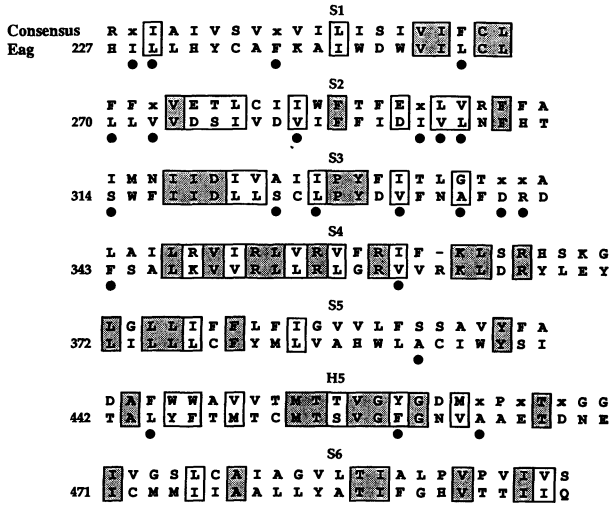

Fig. 3. Alignment of hydrophobic domains of the consensus sequences from the *Sh* family (top line) with corresponding regions of the *eag* polypeptide (bottom line). The amino acid number of the first residue of each hydrophobic domain in Eag is indicated at the left. Shaded boxes, identical amino acids; open boxes, conservative substitutions. Members of the following groups of amino acids were considered to be conserved: (M, I, L, V); (A, G); (S, T); (Q, N); (K, R); (E, D); and (F, Y, W). Filled circles mark additional positions where the amino acid present in Eag is identical at that site with at least one other member of the group of K^+ channel polypeptides that we used to define the consensus sequence. The consensus sequence was derived from alignments of the polypeptide sequences deduced from the following genes: *Sh, mbk1, bk2, rck3, rck4, drk1, Shab,* and *Shaw* (*18*). If four or more of these polypeptides shared the same amino acid at any given position, that amino acid was taken as the consensus at that position. An "x" marks those positions where no consensus existed at a particular location. A single amino acid gap, marked by a hyphen, has been introduced into the S4 sequence for maximum alignment.

phobic domain of Eag has a characteristic S4 motif, which shares nine identities and five conservative substitutions with the consensus sequence in a segment of 26 amino acids. The H5 domain, which is thought to line the channel pore, is well conserved among K^+ channels (*14*). Eag shares seven identities and six conservative substitutions with the consensus H5 segment in a span of 25 amino acids.

Overall, the membrane-spanning regions of Eag share 24% amino acid identity and 20% conservative substitution with the consensus sequence. An additional 9% of the amino acids in these regions of Eag share identity at their respective positions with at least one member of the *Sh* superfamily. This similarity, together with the observation that mutations of *eag* alter K^+ currents (*7, 8*), strongly suggests that Eag is itself a structural component of K^+ channels.

Despite the similarities between Eag and other K^+ channels, Eag has several distinctive features. The most conserved region among all polypeptides in the *Sh* superfamily is a stretch of seven amino acids near the NH_2-terminal end with the consensus sequence NEYFFDR (*2*). This sequence is absent in Eag. Furthermore, in the *Sh* superfamily, the lowest amino acid identity between the most divergent members is 35% over the conserved core of the polypeptide (*2*). In contrast, Eag shares less than 20% identity with any member of the *Sh* superfamily over the same region. Another distinct feature of Eag is its size of 1174 amino acids, about twice the size of other polypeptides in the *Sh* superfamily, which all contain about 500 to 600 amino acids, except for those encoded by *Shab* and *drk*, which have 924 and 853 amino acids, respectively (*2*).

K^+ channels in the *Sh* superfamily contain a domain that is represented four times in the principal subunits of Na^+ and Ca^{2+} channels, suggesting that K^+ channels function as tetrameric assemblies of individual subunits (*13, 15*). Because the *eag* amino acid sequence is about twice as long as that of other K^+ channel polypeptides, we examined the possibility that, unlike other K^+ channel genes, *eag* underwent an internal duplication and now specifies a polypeptide with a dimeric structure. Some hydrophobic stretches of amino acids in the COOH-terminal half of Eag are indicated in the hydropathy plot, although they appear to be too short to span the membrane (Fig. 2). In addition, several segments in this region share some similarity with the membrane-spanning domains of other K^+ channels. For example, there are eight identities and two conservative substitutions in the alignment of amino acids 1000 to 1022 of Eag with the S6 domain of the *Sh* superfamily consensus sequence. However, these segments have no marked similarity to sequences in the NH_2-terminal half of Eag, which would be expected if the *eag* gene had undergone an internal duplication. The functional significance of the long COOH-terminus of Eag is therefore still an open question.

Although *eag* is related to the *Sh* superfamily, its divergence from all known members of this family exceeds that of the distantly related members in the family. Thus, *eag* appears to encode a distinct type of K^+ channel polypeptide, which may be a component of several different types of K^+ channels (*8*). Because cloning of K^+ channel genes has been biased towards the isolation of those similar to *Sh*, the actual extent of sequence diversity among K^+ channel genes is likely to be underestimated. This possibility emphasizes the importance of genetic

approaches in identifying new K^+ channel genes that have not yet been isolated on the basis of sequence similarity. Finally, just as mammalian homologs have been found for *Drosophila* K^+ channel genes in the *Sh* superfamily, we anticipate the identification of mammalian counterparts to *eag*.

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Alteration of Four Identified K^+ Currents in *Drosophila* Muscle by Mutations in *eag*

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Voltage-clamp analysis of *Drosophila* larval muscle revealed that *ether à go-go (eag)* mutations affected all identified potassium currents, including those specifically eliminated by mutations in the *Shaker* or *slowpoke* gene. Together with DNA sequence analysis, the results suggest that the *eag* locus encodes a subunit common to different potassium channels. Thus, combinatorial assembly of polypeptides from different genes may contribute to potassium channel diversity.

A VARIETY OF K^+ CHANNELS PARTICIPATE in many cellular functions and contribute to the signaling capacity of excitable cells (*1*). However, the genetic basis that underlies this diversity still remains to be fully explored (*1–3*). Studies have shown that several distinct K^+ currents in *Drosophila* muscle are selectively affected by mutations in different genes (*4–6*). We investigated the effect of mutations in the *eag* gene (*7*) on all identified K^+ currents in *Drosophila* larval muscle. These include the transient (I_A) and delayed (I_K) voltage-activated K^+ currents and the fast (I_{CF}) and slow (I_{CS}) Ca^{2+}-activated K^+ currents (*8–10*).

A voltage-clamp analysis with two microelectrodes was performed on the body-wall muscle fibers in *Drosophila* third instar larvae (*10–12*). We measured the voltage-activated I_A and I_K in Ca^{2+}-free saline, which eliminated the inward Ca^{2+} current (I_{Ca}), as well as the Ca^{2+}-activated I_{CF} and I_{CS} (*8–10*). I_A and I_K could be separated by their kinetic differences in inactivation and recovery (*11*). A previous inactivating pulse to −20 mV from the holding potential (V_H = −80 mV) inactivated most I_A but left I_K virtually intact (Fig. 1A). The difference between corresponding traces with and without the inactivating pulse was I_A (*11*).

Current-voltage (*I-V*) relations were determined for the peak I_A and the maximum I_K at different voltages for several *eag* alleles (Fig. 1). I_K was reduced in eag^{X6} and, to a lesser extent, in eag^{4PM} and eag^1 (Fig. 1C). I_A decreased in eag^{X6} even though it was not significantly affected in eag^{4PM} and eag^1 (*13*). The extreme phenotype of eag^{X6} is consistent with molecular data that indicate that the *eag* transcript is truncated in this mutant. Therefore, eag^{X6} is likely to exhibit the null phenotype (*14*).

Measurements of Ca^{2+}-dependent currents requires the presence of Ca^{2+} in saline. As in other invertebrate species, the inward current in *Drosophila* muscle fibers is carried by Ca^{2+}, which is required for contraction (*15*). Although it is desirable to measure isolated I_{Ca} with pharmacological elimination of all K^+ currents, such experiments are prevented by the resulting contraction of all muscle fibers in the larval preparation. We instead determined the current mediated by Ca^{2+} channels by replacing Ca^{2+} with Ba^{2+}, which permeates Ca^{2+} channels and blocks K^+ channels but does not initiate muscle contraction (*9, 16*). These experiments indicated that the I_{Ba}'s in eag^{4PM} and eag^{X6} larvae were not different from that found in normal larvae (*17*).

The Ca^{2+}-activated I_{CF} and I_{CS} were studied at a lower temperature, and the external Ca^{2+} concentration was reduced to 0.9 mM from the standard 1.8 mM (*8, 11*). Under these conditions, muscle contraction was minimized, but the two currents could still be measured (Fig. 2A). We removed the voltage-activated I_A by addition of 4-aminopyridine (*18*) and the voltage-activated I_K by addition of quinidine (*10*). The remaining net current was outward; the inward I_{Ca} was masked by the I_{CF} and I_{CS}. The inward tail currents (Fig. 2A) that followed the depolarization-induced outward currents were characteristic of Ca^{2+}-activated K^+ currents and distinct from I_K, which lacks inward tails at a V_H of −80 mV (Fig. 1C).

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