K+ currents in Drosophila muscles have been resolved into two voltage-activated currents ($I_A$ and $I_Q$) and two Ca2+-activated currents ($I_{CP}$ and $I_{CP}^M$). Mutations that affect $I_A$ (Shaker) and $I_{CP}$ (slowpoke) have helped greatly in the analysis of these currents and their role in membrane excitability. Lack of mutations that specifically affect channels for the delayed rectifier current ($I_K$) has made their genetic and functional identity difficult to elucidate. With the help of mutations in the Shab K+ channel gene, we show that this gene encodes the delayed rectifier K+ channels in Drosophila. Three mutant alleles with a temperature-sensitive paralytic phenotype were analyzed. Analysis of the ionic currents from mutant larval body wall muscles showed a specific effect on delayed rectifier K+ current ($I_K$). Two of the mutant alleles contain missense mutations, one in the amino-terminal region of the channel protein and the other in the pore region of the channel. The third allele contains two deletions in the amino-terminal region and is a null allele. These observations identify the channels that carry the delayed rectifier current and provide an in vivo physiological role for the Shab-encoded K+ channels in Drosophila. The availability of mutations that affect $I_K$ opens up possibilities for studying $I_K$ and its role in larval muscle excitability.

Voltage-activated K+ channels play a crucial role in repolarizing the membrane following action potentials, stabilizing membrane potentials and shaping firing patterns of cells (1). Many human diseases such as long QT syndrome, Jervell and Lange-Nielsen syndrome, episodic ataxia, and epilepsy are associated with mutations in these channels (2–6). Hence, it is important to understand how K+ channels function. With an excellent repertoire of available genetic tools, Drosophila provides a powerful system for such studies. The existence of distinct behavioral phenotypes that arise due to defects in neuromuscular pathways has aided in identifying mutations that affect ion channels. A functional voltage-gated K+ channel consists of four $\alpha$-subunits, each with six transmembrane domains (S1–S6) flanked by cytoplasmic amino- and carboxyl-terminal regions. A number of genes coding for K+ channel $\alpha$-subunits have been cloned. These include genes from six families, defined by six Drosophila K+ channel genes: Shaker (Kr1.1–1.7), Shab (Kr2.1–2.2), Shaw (Kr3.1–3.4), Shal (Kr4.1–4.3), ether-a-go-go (HERG) and slowpoke (maxiK) (7–10). The Shaker, ether-a-go-go (eag), and slowpoke (slo) genes were identified on the basis of behavioral mutations that helped in the cloning and extensive molecular analysis of these genes and their encoded channels (11–16). On the other hand, Shab, Shal, and Shaw were identified in homology screens using Shaker cDNA as probe (17). Expression of cRNAs of these genes results in the generation of K+ currents in Xenopus oocytes (18, 19). However, no mutations have been reported in any of these three genes, thus making it difficult to elucidate their in vivo physiological function.

We describe the identification and molecular analysis of the first behavioral mutations that disrupt the Shab gene. These mutations were initially identified as causing a temperature-induced paralytic phenotype (20). They selectively affect the delayed rectifier potassium current ($I_K$), in larval body wall muscles, without affecting other K+ currents and reveal the in vivo functional role of the Shab gene in Drosophila.

EXPERIMENTAL PROCEDURES

Isolation of 9g Mutant—A mutation (z66) with the phenotype of temperature-induced paralysis had been earlier identified to specifically affect the delayed rectifier potassium current in larval muscles. 30 male flies (3 days old) carrying the ebony (e) marker on chromosome 3 were mutagenized with 3500 rads of x-irradiation and mated in batches of three males and 10 z66 virgin female flies (3–5 days old). The males were removed from the vials after 3 days. F1 progeny from the cross were tested for temperature-induced paralysis at 39 °C for 5 min. Of the 5673 F1 progeny tested, one male fly (9g) paralyzed within 3 min at 39 °C and recovered from paralysis within 4 min of being transferred to room temperature (25 °C). The mutant was mated to virgin females carrying the third chromosome balancer TM3, Shb p+ c/TM6B, Tb H red e. F2 males and virgin females having ebony body color (with the TM3 balancer) were then mated to render the mutation homozygous. All stocks were maintained at 21 °C (21).

Electrophysiology—Body wall muscle 12 (22, 23) of mature third instar larvae of wild-type (CS) and various mutant strains was used for the two-electrode voltage clamp experiments as described previously (24). Ca2+-free recording solution contained 77.5 mM NaCl, 115 mM sucrose, 5 mM KCl, 0.5 mM EGTA, 20 mM MgCl2, 5 mM Trehalose, 2.5 mM NaHCO3, and 5 mM HEPES (pH 7.1) (25, 26). Electrodes were pulled from thin walled 1.0-mm borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) and had resistances of about 10 megohms. Voltage electrodes contained 2.5 M KCl and current electrodes contained a 3:1 mixture of 2.5 M KCl, 2 M potassium citrate (27). A Macintosh IIci computer provided the voltage clamp command pulses through a 12-bit digital-to-analog converter using the MacADIOS II/16 board (GW Instruments, Somerville, MA). Data were acquired after a 16-bit analog-to-digital conversion. Analysis was performed with a pro-

**The JOURNAL OF BIOLOGICAL CHEMISTRY**

**Vol. 274, No. 31, Issue of July 30, pp. 22109–22113, 1999**

Printed in U.S.A.

Mutational Analysis of the Shab-encoded Delayed Rectifier K+ Channels in Drosophila*  

(Received for publication, May 12, 1999)

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**This work was supported by National Science Foundation Grants IBN-9011427 and MCB-9604457 and National Institutes of Health Grant GM-50779. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF084525.

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1 M. Chopra, G.-G. Gu, and S. Singh, manuscript in preparation.

2 The abbreviations used are: CS, Canton-S; OR, Oregon-R; PCR, polymerase chain reaction.
gram written in Think-C (Symantec Corp., Cupertino, CA). Test currents were digitally sampled every 500 μs and digitally corrected for linear leakage with respect to currents obtained at −40 mV. Current densities (nanoamperes/nanofarads) were calculated as described previously (28, 29). All experiments were carried out at 4 °C.

Southern Blot Analysis—Genomic DNA was extracted from CS and 9g flies (30). The genomic DNA was digested with PstI restriction endonuclease, subjected to electrophoresis in an agarose gel, transferred to a nylon membrane, and hybridized with probe prepared from the Shab cDNA. Probe was generated using the DIG High Prime DNA labeling and chemiluminescent detection kit (Roche Molecular Biochemicals). Chemiluminescent detection was performed according to the instructions provided by the manufacturer.

Reverse Transcription-PCR Analysis—Total RNA was isolated from CS, Shab<sub>a</sub>, Shab<sub>b</sub>, and Shab<sub>b</sub> larvae and adult flies (1–2 days old) (30). 5 μg of total RNA was reverse transcribed using Superscript II (Life Technologies, Inc.) according to the instructions of the manufacturer. PCR was performed using the UITma DNA polymerase enzyme (Perkin-Elmer). RT-2 (5'-CCGCGAATTCTCCTCCTGCGGCCCGGCGAAGCTG-3'), RT-4 (5'-CGGATGACGCTTTTGCCTGTGTCGACATA-3'), and RT-6 (5'-GGATGGCGGAGCTCCTTGGG-3') were used for reverse transcription of RNA. Primers PCR-1 (5'-GAGGAGGCTATGGTGCACCGACTTGAAGGGTTGACGTGTCG-3') and RT-2 were used to amplify region 1 (base pairs 1–1231) of the cDNA templates (Fig. 5a). Primers PCR-3 (5'-GAGGAGGAGTTCCCGGAGGCCTGCTGTCG-3') and RT-6 were used to amplify region 3 (base pairs 2000–2771) of the cDNA templates. PCR amplifications were performed as described by the manufacturer (Perkin-Elmer), using the hot start PCR technique. The PCR conditions were as follows: initial denaturation (95 °C, 2 min) followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1.5 min, and extension at 72 °C for 2 min. Amplified DNA was run on a 0.8% agarose gel and extracted by centrifugal filtration using Ultrafree-MC filter (Millipore Corp.). DNA was sequenced by the Roswell Park Biopolymer Sequencing Facility.

RESULTS

Identification of Mutations Affecting I<sub>K</sub>—Several ethylmethylsulfonate-induced mutations had been previously isolated in our laboratory by using the conditional phenotype of temperature-induced paralysis (20). These mutations were identified with the use of compound chromosomes (31–34). Compound chromosomes have homologous copies of a chromosomal arm attached at the centromere. The use of compound chromosomes facilitates the isolation of mutants, because this allows recombination. They enable identification of recessive autosomal mutations without setting up individual fly lines.

Initial characterization showed that the delayed rectifier current, I<sub>K</sub>, was reduced in two of the temperature-sensitive paralytic mutants, z66 and z4 (Fig. 1a). Genetic analysis demonstrated that the mutations do not complement each other and therefore reside in the same gene. The effect of the z66 mutation on various other currents expressed in larval muscles and therefore reside in the same gene. The effect of the z4 mutation on various other currents expressed in larval muscles was investigated. No significant change was seen in the fast transient calcium-activated K<sup>+</sup> current, I<sub>Ca<sup>2+</sup></sub>; the fast transient calcium-activated K<sup>+</sup> current, I<sub>Ca<sup>2+</sup></sub>; the slow sustained calcium-activated K<sup>+</sup> current, I<sub>Ca<sup>2+</sup></sub>; and the total Ca<sup>2+</sup> current, thus suggesting that the mutation selectively affects I<sub>K</sub> (20).<sup>1</sup> As shown in Table I, I<sub>K</sub> was reduced by 46.0 ± 3.0% in z66 mutants. The z4 mutations showed a 43.6 ± 3.4% reduction in I<sub>K</sub>. To obtain additional alleles of the gene affected by the z66 and z4 mutations, including some with chromosomal aberrations that would aid in identifying the gene affected by the mutations, we performed X-ray mutagenesis (35). Male ebony flies were subjected to X-ray mutagenesis and mated with virgin z66 females (see “Experimental Procedures”). Screening of 5673 F<sub>1</sub> progeny for temperature-induced paralysis at 39 °C led to the identification of a new mutant, 9g.

In paralysis tests performed on 9g/z66 flies, the two mutations did not complement each other and hence are alleles of the same gene. As in z66, I<sub>K</sub> was affected in 9g mutants (Fig. 1a). There was a 61.2 ± 2.7% reduction of I<sub>K</sub> from CS-derived strains. As shown in Fig. 2, a comparison between the PstI-digested CS and 9g DNA revealed a restriction fragment length polymorphism between mutant and wild type DNAs, indicating that the 9g mutation disrupts

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**Table I**

| Genotype | Current amplitude nA/nF | n | Reduction in I<sub>K</sub> % | Reduction in Shab component % |
|----------|-------------------------|---|-----------------------------|-----------------------------|
| CS       | 16.5 ± 0.4              | 10| 0                           | 0                           |
| Shab<sup>a</sup> | 9.3 ± 0.4              | 9 | 43.6 ± 3.4                 | 71.2 ± 7.0                  |
| Shab<sup>b</sup> | 8.9 ± 0.3              | 12| 46.0 ± 3.0                 | 75.2 ± 6.6                  |
| Shab<sup>c</sup> | 6.4 ± 0.2              | 10| 61.2 ± 2.7                 | 100                         |

<sup>a</sup> Nanoamperes/nanofarads.

The 9g Mutation Maps to the Shab Locus—Recombination and deletion mapping localized the z66 and z4 mutations to the left arm of chromosome 3 at 63A1-B6 (20).<sup>1</sup> Deletion analysis of the z66 mutation disrupts a gene that is homologous to the Shab gene. Shab, a K<sup>+</sup> channel gene cloned by homology to Shaker lies on chromosome 3 at position 63A17 (20). By using deletions in this region (63A1-B9), Tsunoda and Salkoff (36) showed that I<sub>K</sub> is reduced in embryonic neurons and myotubes of these deletion strains and suggested that Shab may code for a delayed rectifier potassium channel.

To determine if the 9g mutation lies in the Shab gene, Southern blot analysis was performed on genomic DNA from wild type (CS) and 9g flies using the entire Shab cDNA as probe. CS was used in the analysis because the mutants were obtained from CS-derived strains. As shown in Fig. 2, a comparison between the PstI-digested CS and 9g DNA revealed a restriction fragment length polymorphism between mutant and wild type DNAs, indicating that the 9g mutation disrupts...
the Shab gene. The 9g mutation and its two noncomplementing alleles, z66 and z4, contain the first mutations to be identified in the Shab gene. The mutant alleles in z66, z4, and 9g will be referred to hereafter as Shab1, Shab2, and Shab3 respectively.

Molecular Characterization of Shab Mutations—To investigate the underlying molecular defects in the mutants, Shab cDNA was prepared by reverse transcription-PCR from Shab1, Shab2, and Shab3, and wild type (CS) flies (Fig. 3a). PCR products were sequenced, and results were compared with the published sequence of Shab derived from the OR strain (17). Comparison of the sequences revealed that the CS sequence has an insertion of a single base (G) at position 2708 of the published OR sequence. This produces a shift in the reading frame, resulting in the addition of 60 amino acids at the C terminus of the Shab channel in CS. In addition to the insertion, we found other nucleotide changes, seven of which alter the encoded amino acid sequence. These changes from OR to CS are as follows (the number system is as previously defined for the Shab (OR) gene; GenBank accession no. M32659): 1) a T to A transition at nucleotide 92 that changes amino acid 31 from a leucine to a glutamine; 2 and 3) two G to A transitions at nucleotides 658 and 1084 that change amino acids 220 and 362 from glycine to serine; 4) a T to G transversion at nucleotide position 1483 that changes amino acid 495 from serine to alanine; 5) a G to T transversion at nucleotide 2481 that changes amino acid 827 from an aspartic acid to a glutamic acid; 6) a G to C transversion at nucleotide 2482 that changes amino acid 828 from a glutamic acid to a glutamine, and 7) a G to C transversion at nucleotide position 2630 that changes amino acid 877 from glycine to alanine. Butler et al. (17) reported the presence of a 90-nucleotide coding region (nucleotides 2151–2240) in the longest correctly spliced Shab cDNA (Shab11) (GenBank accession no. M32659) not found in other Shab cDNAs. In our experiments, the CS cDNA did not show the presence of these 90 nucleotides. The Shab gene sequence from CS has been entered in GenBank.

Sequence comparison between CS and Shab1 cDNAs revealed a single G to A transition at nucleotide position 1304, which changes an arginine at amino acid 435 to a glutamine (Fig. 3b). This arginine at position 435 is thought to be the last residue before the protein enters into the membrane as the first transmembrane segment (S1) (Fig. 4) and is an arginine or a lysine in most members of voltage-gated potassium channels (Fig. 5). In Shaker potassium channels, the NH2-terminal region and the S1 segment are essential for subunit interactions as well as expression of functional channels at the cell membrane (37–39). The position of the Shab1 mutation is interesting and may suggest an important role for this region in the formation of a functional Shab channel.

Analysis of the Shab2 cDNA revealed a T to A transversion at nucleotide position 1823 (Fig. 3c). This missense mutation changes a valine to an aspartic acid at amino acid 608. This residue is found in the pore region of the Shab1 channel (Fig. 4). The pore region consists of a turret, pore helix, and selectivity filter (40). While the pore helix and the selectivity filter

![Fig. 2. Southern blot analysis of 9g genomic DNA.](https://example.com)

**a**. PCR products were analyzed by agarose gel electrophoresis, transferred to nylon, and probed with the entire sequence of the Shab cDNA. Analysis of the blot shows the presence of a restriction fragment length polymorphism (arrow) in the 9g genomic DNA.

**b**. Sequence analysis of Shab1, Shab2, and Shab3 reverse transcribed cDNA. a, a schematic diagram of the Shab cDNA. Oligonucleotide primers used for reverse transcription of total RNA isolated from adult CS, Shab1, Shab2, and Shab3 flies are represented as left arrows. Primers (sense strand) used to PCR amplify the three regions of reverse transcribed Shab cDNA are shown as right arrows. The entire Shab coding region was sequenced from all four strains. The CS Shab sequence has been placed in the GenBank data base, b-d, comparison of mutant and wild type DNA sequences. The R435Q mutation in Shab1 (b) and the V608D mutation in Shab2 (c) are shown in boldface type. The Shab2 deletions are represented in d by dashed lines.

**c**. Pore-helix sequence comparison between CS and Shab1 cDNA revealing a single G to A transition at nucleotide position 1304, which changes an arginine at amino acid 435 to a glutamine (Fig. 3b). This arginine at position 435 is thought to be the last residue before the protein enters into the membrane as the first transmembrane segment (S1) (Fig. 4) and is an arginine or a lysine in most members of voltage-gated potassium channels (Fig. 5). In Shaker potassium channels, the NH2-terminal region and the S1 segment are essential for subunit interactions as well as expression of functional channels at the cell membrane (37–39). The position of the Shab1 mutation is interesting and may suggest an important role for this region in the formation of a functional Shab channel.

**d**. Analysis of the Shab2 cDNA revealed a T to A transversion at nucleotide position 1823 (Fig. 3c). This missense mutation changes a valine to an aspartic acid at amino acid 608. This residue is found in the pore region of the Shab1 channel (Fig. 4). The pore region consists of a turret, pore helix, and selectivity filter (40). While the pore helix and the selectivity filter
FIG. 4. A schematic representation of the Shab potassium channel. The Shab<sup>1</sup> deletions are in the cytoplasmic amino terminus and result in a null allele. The R435Q missense mutation in Shab<sup>1</sup> occurs at the last amino acid before the protein enters into the membrane as S1. The V608D mutation in Shab<sup>2</sup> is in the entryway to the pore.

![Diagram of Shab potassium channel]

FIG. 5. Conservation of a positively charged amino acid immediately before S1. A sequence alignment of K<sup>+</sup> channels in the region of S1 shows conservation of basic residues (arginines and lysines) at the position immediately preceding the S1 transmembrane segment. The sequences are as follows: Shab, Drosophila melanogaster (Swiss-Prot P17970); Kv2.2 (CDRK), Rattus norvegicus (Swiss-Prot Q63099); Kv2.1 (DRK1), R. norvegicus (Swiss-Prot P15387); Kv1.5, Homo sapiens (Swiss-Prot P22460); Kv3.4, H. sapiens (Swiss-Prot Q03721); RCK1, R. norvegicus (Swiss-Prot P10499).

are highly conserved in their amino acid sequence, the turret, which lies at the external mouth of the pore, is less conserved. The amino acids in this region form high affinity binding sites for peptide inhibitors from scorpion venom (41). The V608D mutation falls at the junction of the turret and the pore helix in the toxin binding site of the pore region.

The sequences of Shab<sup>1</sup> and Shab<sup>2</sup>, both of which were isolated in the same mutagenesis, corroborate the sequence of the Shab gene and provide evidence that the mutations observed are not due to polymorphic differences between the parent strain used for the isolation of the Shab gene and the original CS strain. Two alleles, Shab<sup>1</sup> and Shab<sup>2</sup>, contain missense mutations. The third allele, Shab<sup>3</sup>, contains two deletions in the cytoplasmic amino-terminal region of the protein and is a null allele (Fig. 4). It is significant that the delayed rectifier current, I<sub>K</sub>, is only reduced by 61.2 ± 2.7% and not completely abolished in Shab<sup>3</sup> (Fig. 1; Table I). If Shab channels carried all of the I<sub>K</sub>, then a null mutation in the Shab gene would be expected to lack delayed rectifier current. Thus, our results indicate that I<sub>K</sub> consists of more than one component, with a large fraction of the current being carried by Shab channels. Detailed pharmacological and physiological studies performed on Shab<sup>3</sup> mutants support this hypothesis. Electrophysiological studies performed using genetic aberrations that delete a large region of chromosome containing the Shab locus in Drosophila show a 77% reduction of I<sub>K</sub> in embryonic myotubes (36). The gene that codes for the remaining current seen in the Shab<sup>3</sup> mutants remains to be identified. One possible candidate is the Shab gene that codes for a noninactivating K<sup>+</sup> channel (18). The availability of mutations that affect the residual current in the Shab<sup>3</sup> mutants will be helpful in studying this current and the gene coding for the channels.

In Shab<sup>1</sup>, an R435Q mutation exhibits a 46.0 ± 3.0% reduction in I<sub>K</sub> (Fig. 1). Since Shab channels carry 61.2% of the delayed rectifier current, Shab<sup>1</sup> mutants show a 75.2 ± 6.6% reduction in the Shab component of I<sub>K</sub> (Table I). The Shab<sup>3</sup> mutation changes a highly conserved arginine to a glutamine (R435Q) at the last amino acid before the protein enters the membrane as the first transmembrane segment (S1). Most transmembrane proteins contain signal/anchor sequences prior to the first transmembrane segments. These sequences include positively charged arginines or lysines, which are essential for determining membrane topology (42–44). Substituting the arginines or lysines with neutral or negatively charged residues results in a significant fraction of membrane-spanning proteins anchoring in reverse topological orientation in the membrane (42, 43). Voltage-gated K<sup>+</sup> channels show similarly conserved arginines and lysines just before S1 (Fig. 5). It will be interesting to examine if the R435Q mutation in Shab<sup>1</sup> results in a topological inversion.

Mutations in the pore region affect various properties such as gating, channel activation, ion selectivity, and permeability (45–47). The V608D missense mutation in Shab<sup>2</sup> is in the pore region of the channel. This region is subdivided into the turret, pore helix, and selectivity filter (40). The turret lies at the extracellular entryway of the pore and forms a high affinity binding site for various scorpion venom toxins such as charybdotoxin (41) and agitoxin (48). The amino acid composition in the turret is not highly conserved, but the overall structure of this region appears to be conserved (49). Using agitoxin foot-

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3 Singh, A., and Singh, S. (1999) J. Neurosci., in press.
printing on mutant Shaker channels, Gross and MacKinnon (48) determined the spatial location of amino acids in the pore entryway. According to their studies, Lys427 in Shaker channels, which corresponds to Val508 in Shab channels, lies away from the center of the pore but makes direct contact with agiotxin. Mutating Lys427 to a negatively charged glutamic acid does not alter single channel conductance in Shaker channels when expressed in Xenopus oocytes (41). However, in Shab2 mutants, I(K) is reduced in amplitude by 43.6% (a 71.2 ± 7.0% reduction in the Shab-encoded I(K)). Further functional studies will have to be performed to understand the mechanism for the reduction of I(K) in Shab2.

**Shab Mutations Will Facilitate Further Analysis of I-K**—In vivo mutations have been used extensively to characterize genes and their protein products. Due to a large repertoire of genetic mutations and the ease of performing electrophysiological analysis, *Drosophila* has provided a very useful system for ion channel study. Two voltage-activated K+ currents (I(A) and I(C)) and two Ca2+-activated K+ currents (I(CP) and I(CG)) have been reported in the larval muscles of *Drosophila* (27, 50, 51). Mutations that affect I(A) (Shaker) and I(CP) (slowpoke) have helped greatly in the analysis of these currents and their role in membrane excitability. Lack of mutations that specifically affect the delayed rectifier channels had made their genetic and functional identity difficult to elucidate. Availability of the *Shab* mutations that affect I(K) opens up many possibilities for studying this current and its role in larval muscle excitability.

**Acknowledgments**—We thank Scott Chouinard and Barry Ganetzky for the generous gift of the *Shab* cDNA clone. We thank Maninder Chopra for identifying the *Shab*1 and *Shab*2 mutants and performing the initial characterization.

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