A Voltage-Clamp Analysis of Gene-Dosage Effects of the *Shaker* Locus on Larval Muscle Potassium Currents in *Drosophila*

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Mutations of the *Shaker* (*Sh*) locus of *Drosophila* reduce, eliminate, or otherwise alter a transient potassium current, I_A , in muscle. Recent molecular studies indicate that the *Sh* locus produces several proteins by alternative splicing, but the relationships of the variety of *Sh* gene products to I_A channels in the various excitable membranes still remain to be determined. In *Drosophila*, many enzymes have been shown to exhibit gene-dosage effects; their amounts vary in direct proportion to the number of structural genes present. We describe a physiological isolation of I_A in larval muscle which allowed precise quantification of gene-dosage effects on I_A in *Sh* heterozygotes and aneuploids. We found that doubling the number of *Sh* genes in aneuploids increased I_A to twice that of normal, consistent with the notion that the *Sh* locus encodes the entire I_A channel in larval muscle. We further examined heterozygous combinations of different *Sh* mutations for evidence of interactions among *Sh* gene products within the I_A channel, which may yield clues to the possible subunit composition of the channel. Combinations among 5 *Sh* mutations plus their normal counterpart followed a simple gene-dosage effect; in each case the resulting I_A was about the average of the homozygous currents, compatible with the notion of additive contributions from 2 independent populations of I_A channels. Two additional *Sh* mutations caused pronounced departures from the simple dosage effect; the amplitude of I_A in heterozygotes was significantly smaller than that expected from gene dosage, a strong dominant effect attributable to interactions among protein subunits. These contrasting observations may be accounted for by certain hetero- or homo-multimeric arrangements of *Sh* products in the I_A channel.

Defective ion channels that cause altered membrane excitability have been detected in a number of *Drosophila* behavioral mutants (Ganetzky and Wu, 1986; Salkoff and Tanouye, 1986; Tanouye et al., 1986; Papazian et al., 1988). The *Shaker* (*Sh*) mutants, originally isolated on the basis of a vigorous leg-shaking behavior under ether anesthesia (Kaplan and Trout, 1969),

have been subject to the most intensive investigations. The neuromuscular junction of *Sh* larvae was found to exhibit repetitive firing of motor axons coupled with prolonged neurotransmitter release (Jan et al., 1977; Ganetzky and Wu, 1982, 1983). Subsequent intracellular recordings from adult cervical giant axons revealed abnormally prolonged action potentials (Tanouye et al., 1981; Tanouye and Ferrus, 1985). The application of voltage-clamp technique has demonstrated alterations in the transient K^+ current, I_A , in adult (Salkoff and Wyman, 1981; Salkoff, 1983), larval (Wu et al., 1983; Wu and Haugland, 1985), and embryonic (Zagotta et al., 1988) muscle.

This study was originally inspired by the suggestion that the *Sh* locus consists of a gene complex of closely linked functions (Tanouye et al., 1981; Tanouye and Ferrus, 1985). Experiments were designed to detect interactions among different *Sh* gene products in heterozygous individuals. Since the completion of our experiments (Haugland and Wu, 1986; Haugland, 1987), molecular data have become available indicating that the *Sh* locus contains a complex transcription unit producing, by alternative splicing, a family of related proteins (Baumann et al., 1987; Kamb et al., 1987, 1988; Papazian et al., 1987; Pongs et al., 1988; Schwarz et al., 1988). To date, no additional putative channel genes in the *Sh* region have been identified by molecular techniques. Nevertheless, the physiological data described in this paper indeed suggest interactions among *Sh* proteins and could therefore be used to assess the possible involvement of different *Sh* splicing variants within the I_A channel.

The *Sh* messages have been expressed in *Xenopus* oocytes to generate active K^+ channels, providing a powerful approach to the structure-function analysis of individual *Sh* splicing variants (Iverson et al., 1988; Timpe et al., 1988a, b). However, for some channels with heteromultimeric subunit composition *in situ*, a single subunit species can form functional homomeric channels in the oocyte expression system (Boulter et al., 1987; Auld et al., 1988; Blair et al., 1988). In addition, it is known that the *Sh* channels expressed in oocytes exhibit abnormal properties (MacKinnon et al., 1988). Thus, several questions regarding the functional role of the *Sh* products in the native I_A channels still remain to be resolved by using *in situ* preparations with a combination of molecular and electrophysiological techniques: How many distinct functional components within I_A channels are encoded by the *Sh* locus? For the different *Sh* gene products, how is their expression regulated in various excitable membranes and what is their stoichiometric representation in the assembled I_A channel? Are there different types of I_A channels that consist of different combinations of the *Sh* gene products?

Many mutations of the *Sh* locus are now available and their effects on I_A range from complete elimination or simple reduc-

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Table 1. Active membrane properties of larval muscles in *Sh* homozygotes and heterozygotes

	<i>Sh</i> ⁺	<i>Sh</i> ^s	<i>Sh</i> ^{K0120}	<i>Sh</i> ^{E62}
	4.78 ± 0.16 (15)	3.24 ± 0.23 (10)	3.61 ± 0.10 (12) ^b	2.58 ± 0.15 (10)
		3.54 ± 0.11 (28)	4.25 ± 0.14 (23)	3.02 ± 0.09 (27)
		2.29 ± 0.15 (13)	2.68 ± 0.16 (8)	2.01 ± 0.19 (9)
			3.00 ± 0.14 (21)	1.78 ± 0.08 (25)
<i>Sh</i> ⁺	0.75 ± 0.044 (15)		3.71 ± 0.26 (8)	2.38 ± 0.14 (13)
				2.49 ± 0.13 (20)
<i>Sh</i> ^s	0.39 ± 0.044 (10)	0.29 ± 0.023 (13)		1.26 ± 0.08 (12)
	0.52 ± 0.025 (28)			
<i>Sh</i> ^{K0120}	0.51 ± 0.025 (12) ^a	0.37 ± 0.027 (8)	0.53 ± 0.067 (8)	
	0.64 ± 0.038 (23)	0.41 ± 0.032 (21)		
<i>Sh</i> ^{E62}	0.37 ± 0.029 (10) ^a	0.24 ± 0.046 (9)	0.33 ± 0.023 (13)	0.20 ± 0.015 (12)
	0.48 ± 0.024 (27)	0.25 ± 0.014 (25)	0.37 ± 0.031 (20)	
<i>Sh</i> ^M	0.35 ± 0.033 (10)	0.15 ± 0.016 (6)	0.25 ± 0.027 (11)	0.10 ± 0.011 (4)
	0.38 ± 0.027 (20)	0.15 ± 0.014 (18)	0.27 ± 0.037 (13)	0.10 ± 0.010 (17)
<i>Sh</i> ^{K82a}	0.35 ± 0.037 (7)	0.12 ± 0.026 (4)	0.29 ± 0.013 (4)	0.11 ± 0.008 (10)
	0.38 ± 0.030 (16)	0.15 ± 0.016 (14)	0.27 ± 0.045 (9)	0.10 ± 0.011 (13)
<i>Sh</i> ^{KS133}	0.06 ± 0.006 (10) ^b	0.07 ± 0.010 (5) ^b	0.05 ± 0.009 (8) ^b	0.04 ± 0.004 (9) ^a
	0.38 ± 0.027 (20)	0.15 ± 0.014 (18)	0.27 ± 0.037 (13)	0.10 ± 0.010 (17)
<i>Sh</i> ¹⁰²	0.28 ± 0.021 (14) ^a	0.07 ± 0.007 (10) ^b	0.15 ± 0.015 (11) ^a	0.03 ± 0.004 (10) ^b
	0.38 ± 0.027 (20)	0.15 ± 0.014 (18)	0.27 ± 0.037 (13)	0.10 ± 0.010 (17)
	<i>Sh</i> ⁺	<i>Sh</i> ^s	<i>Sh</i> ^{K0120}	<i>Sh</i> ^{E62}

Upper right, Peak I_A density of +10 mV in $\mu A/\mu F$. Each cell of the half-matrix on the upper right represents data from one homozygous or heterozygous genotype whose parental genotypes are shown at the borders of the half-matrix. When the cell contains homozygous data, the observed I_A (mean $\pm$ SEM) is shown. When the cell contains heterozygous data, the observed I_A (upper value; mean $\pm$ SEM) is compared with the I_A predicted from homozygous data on the basis of gene dosage (lower value; mean $\pm$ SEM). The number of fibers used for each measurement or prediction is shown in parentheses. Lower left, Ratio of peak I_A to steady-state I_K at

tion in amplitude, to altered biophysical properties. The above questions may be explored *in situ* by combining these *Sh* mutations in a heterozygous individual. *Drosophila* is a diploid organism in which both copies of a gene contribute equal amounts of gene products (see review by Stewart and Merriam, 1980). Thus, in flies heterozygous for *Sh* alleles, a departure from the simple additive effect of gene dosage may indicate interaction between gene products. Moreover, it is also possible to study the effects of extra copies of genes in aneuploids (Lindsley and Grell, 1968; Stewart and Merriam, 1980).

We show that a duplication of the *Sh* region produced twice the normal amplitude of I_A , supporting the idea that the *Sh* locus codes for the entire I_A channel. In addition, heterozygotic combinations among *Sh*⁺, *Sh*^s, *Sh*^M, *Sh*^{K82a}, *Sh*^{E62}, and *Sh*^{K0120} follow a simple gene-dosage effect, with the observed current being about the average of the homozygous currents. With different assumptions, the simple dosage effect can be explained by several possible multimeric models of *Sh* gene products within the I_A channel (see Discussion). In contrast, 2 other mutations, *Sh*^{KS133} and *Sh*¹⁰², cause decreases in I_A significantly different from that expected from simple gene-dosage effects, suggesting abnormal *Sh* products capable of interfering with other *Sh* proteins within a subunit assembly.

Comparisons with previously published data from different excitable membranes reveal considerable discrepancies in the phenotypes of certain *Sh* mutations and their heterozygotes. This indicates that I_A channel structure and function may vary in a tissue-specific manner and supports the notion that the diversity of K^+ channels arises from combinations of common as well as distinct functional components (Wu and Haugland, 1985; Wu and Ganetzky, 1986; Kamb et al., 1988; Pongs et al., 1988; Timpe et al., 1988b). Preliminary accounts of some of

the results reported here have appeared previously (Haugland and Wu, 1986).

Materials and Methods

Mutants, crosses, and nomenclature. The wild-type strain Canton-S of *Drosophila melanogaster* was used for the characterization of normal membrane currents. Normal larvae are designated *Sh*⁺. Seven different mutant *Sh* strains are characterized; 6 of these strains (*Sh*^{KS133}, *Sh*¹⁰², *Sh*^M, *Sh*^s, *Sh*^{K0120}, and *Sh*^{E62}) were obtained from the collection of Dr. S. Benzer at the California Institute of Technology. Five of these mutations (*Sh*^{KS133}, *Sh*¹⁰², *Sh*^s, *Sh*^{K0120}, and *Sh*^{E62}) have been mapped via recombination to position 57.0 of the X chromosome (see Tanouye et al., 1986, for details). Some of the *Sh*^{K0120} stocks had previously been shown to carry a second mutation, *ether α go-go* (Ganetzky and Wu, 1983), which was removed via recombination in this study. A seventh mutant strain, *Sh*^{K82a}, provided by Dr. B. Ganetzky at the University of Wisconsin, involves a chromosomal rearrangement in the *Sh* region (Schwarz et al., 1988). An additional strain, *Dp(1;3)JC153*, which possesses a duplicated copy of the *Sh*⁺ locus translocated onto the third chromosome (Tanouye et al., 1986), was used for gene-dosage experiments; this strain was provided by Dr. M. Tanouye at the California Institute of Technology. To minimize influences of unidentified loci in different genetic backgrounds, each of these *Sh* mutations was examined in a Canton-S background by replacing autosomes and, in some cases, portions of the X chromosome away from the *Sh* locus by genetic recombination. As a further precaution, additional copies of certain strains were obtained from other investigators. One strain of *Sh*^s was provided by Dr. L. Salkoff at Washington University, St. Louis, and a strain of *Sh*^{KS133} by Dr. L. Timpe at the Howard Hughes Medical Institute, San Francisco. Both strains yielded results consistent with those reported here.

Heterozygous female larvae (possessing 2 different *Sh* mutations and denoted, for example, *Sh*^{KS133/Sh}^M) were obtained by mating a single virgin mutant female with 5–10 males of a different *Sh* mutation. When multiple larvae of a given heterozygous (or aneuploid) genotype were examined (cf. Table 1), each was the result of an independent mating. For descriptive purposes, we refer to *Sh* mutations that eliminate I_A as “null” alleles as opposed to those with residual I_A as “leaky.”

Table 1. Continued

<i>Sh^M</i>	<i>Sh^{K82a}</i>	<i>Sh^{KS133}</i>	<i>Sh¹⁰²</i>	
2.49 ± 0.07 (10)	2.46 ± 0.21 (7)	0.52 ± 0.04 (10) ^b	1.75 ± 0.10 (14) ^b	<i>Sh⁺</i>
2.39 ± 0.10 (20)	2.39 ± 0.10 (16)	2.39 ± 0.10 (20)	2.39 ± 0.10 (20)	
1.08 ± 0.06 (6)	1.04 ± 0.16 (4)	0.49 ± 0.06 (5) ^b	0.51 ± 0.04 (10) ^b	<i>Sh⁵</i>
1.15 ± 0.09 (18)	1.15 ± 0.09 (14)	1.15 ± 0.09 (18)	1.15 ± 0.09 (18)	
1.74 ± 0.17 (11)	1.76 ± 0.04 (4)	0.29 ± 0.05 (8) ^b	0.89 ± 0.08 (11) ^b	<i>Sh^{KO120}</i>
1.86 ± 0.14 (13)	1.86 ± 0.14 (9)	1.86 ± 0.14 (13)	1.86 ± 0.14 (13)	
0.56 ± 0.06 (4)	0.69 ± 0.04 (10)	0.23 ± 0.02 (9) ^b	0.21 ± 0.03 (10) ^b	<i>Sh^{E62}</i>
0.63 ± 0.04 (17)	0.63 ± 0.04 (13)	0.63 ± 0.04 (17)	0.63 ± 0.04 (17)	
<0.05 (5)	<0.05 (3)	<0.05 (3)	<0.05 (4)	<i>Sh^M</i>
	<0.05 (6)	<0.05 (10)	<0.05 (6)	
	<0.05 (1)	N.D.	N.D.	<i>Sh^{K82a}</i>
<0.01 (5)		<0.05 (5)	N.D.	<i>Sh^{KS133}</i>
<0.01 (3)	<0.01 (1)		<0.05 (5)	<i>Sh¹⁰²</i>
<0.01 (6)				
<0.01 (3)	N.D.	<0.01 (5)		
<0.01 (10)				
<0.01 (4)	N.D.	N.D.	<0.01 (5)	
<0.01 (10)				
<i>Sh^M</i>	<i>Sh^{K82a}</i>	<i>Sh^{KS133}</i>	<i>Sh¹⁰²</i>	

+10 mV. Each cell of the half-matrix on the lower left contains the observed I_A/I_K ratio for the corresponding genotype; for heterozygotes the observed I_A/I_K (upper value) ratio is compared with that predicted from gene dosage (lower value). N.D., Not done.

^a $p < 0.01$.

^b $p < 0.001$.

Physiological measurements. Two-microelectrode voltage-clamp measurements of larval muscle membrane currents and potentials were made according to previously described methods (Wu and Haugland, 1985). The preparation of mature third instar larvae was identical to that described by Jan and Jan (1976). After the segmental nerves were severed, recordings were made from identified ventral longitudinal body-wall muscle fibers from the second to the sixth abdominal segment. Data were collected exclusively from fiber no. 6, 7, 12, and 13, mostly from no. 6 and 12 (see Crossley, 1978, for nomenclature).

Inward Ca^{2+} currents and Ca^{2+} -dependent K^+ currents (Elkins et al., 1986; Gho and Mallart, 1986; Singh and Wu, 1989) were eliminated by the use of Ca^{2+} -free saline containing 128 mM NaCl, 2 mM KCl, 14 mM $MgCl_2$, 35 mM sucrose, and 0.5 mM EGTA. The saline was buffered with 5 mM HEPES and adjusted to pH 7.1 by the addition of about 1.5 mM NaOH.

Measurements of membrane current and voltage were stored on a digital oscilloscope (model 4094, Nicolet Instrument Corp., Madison, WI), which was interfaced with a microcomputer (Macintosh, Apple Computer, Cupertino, CA) for data analysis. Raw data were also displayed on a storage oscilloscope (model D13, Tektronix, Beaverton, OR) and photographed. All experiments were conducted at 3–5°C.

Passive membrane properties and physiological separation of outward currents. The passive membrane properties were determined under voltage-clamp conditions for each fiber before examination of the current-voltage (I - V) relation (as shown in Fig. 1). Our previous studies indicated that voltage steps within the range between –80 and –40 mV elicit only passive leakage and capacitive currents (Wu et al., 1983; Wu and Haugland, 1985). Accordingly, the average current elicited by 10 identical voltage pulses from the holding potential (V_H ; –80 mV in this study) to –40 mV was taken. Since in our experiments the voltage clamp settled within 2 msec of the voltage steps, the membrane capacity (c_m) was determined, therefore, by integrating the first 2.5 msec of the capacity surge evoked by the step. The input resistance (r_m) was determined from the difference in the holding current and the current at 45 msec after initiation of the step. The membrane time constant (τ_m) of individual fibers was estimated as the product of r_m and c_m ; fibers were discarded if the τ_m was less than 20 msec.

We took advantage of the large differences in inactivation and recovery kinetics between I_A and I_K to achieve a physiological separation of these outward currents. When the membrane potential was held at negative potentials (V_H ; –80 mV), step depolarizations elicited 2 components of active outward current (Fig. 1A). The first component was a transient outward current, I_A , which rose rapidly to a peak and then inactivated. A second, delayed component, I_K , rose more slowly to a plateau value. When a depolarizing prepulse (to –20 mV, 2 sec duration) preceded the test pulse, I_A was inactivated, unmasking I_K (Fig. 1A). Digital subtraction of the 2 traces yielded the amplitude and time course of inactivating current.

The amplitude and duration of the prepulse were chosen to maximize the fraction of I_A being inactivated while minimizing contamination from inactivation of I_K . The steady-state inactivation of I_A is essentially complete at –20 mV ($h_\infty = 0$) whereas the slow process of I_K inactivation eventually attains only a level of $h_\infty = 0.5$ at this voltage (Wu and Haugland, 1985). At 4°C, the time constant of I_A inactivation at –20 mV is on the order of 100 msec, as compared to 20 sec for I_K inactivation (Wu and Haugland, 1985, and unpublished results). The prepulse and test pulse were separated by a 10-msec repolarization to –80 mV (Fig. 1A); this brief repolarization was sufficient to close essentially all of the I_K channels activated by the prepulse but was too short to allow significant recovery of I_A from inactivation (Wu and Haugland, 1985). Because of these vast differences between I_A and I_K , contamination of peak I_A by inactivating I_K extracted by this protocol is expected to be insignificant in the voltage range examined here. As confirmed by results from the *Sh* mutants (see below), the inactivating currents extracted indeed contained very little I_K , especially near the peaking time of I_A .

This protocol is also suitable for extracting I_A in various *Sh* mutants. Previous studies have established that both kinetic and steady-state properties of I_K are not affected by *Sh* mutations (Wu and Haugland, 1985). In addition, the rates of inactivation and recovery for the residual I_A in *Sh* mutants are similar to normal values (Wu and Haugland, 1985; Haugland, 1987; see also Figs. 2 and 3).

Statistical tests of departures from gene-dosage predictions. In order to ask whether I_A in *Sh* heterozygotes followed a simple gene-dosage effect, it was necessary to obtain an expected value for heterozygous I_A .

Figure 1. Physiological separation of membrane currents in normal larval muscle at 4°C. Inward currents and Ca^{2+} -dependent outward currents were abolished by the use of Ca^{2+} -free saline in this and all subsequent figures. **A**, Two superimposed traces of total membrane currents (lower part) elicited by voltage steps (upper part) from a holding potential (V_H) of -80 mV. Upward deflections indicate outward currents or depolarizations. The depolarization elicited an early outward current, I_A , and a delayed but sustained current, I_K . When a 2-sec prepulse to -20 mV preceded the voltage step, I_A was inactivated and only I_K was observed (see Materials and Methods). **B**, Current-voltage (I - V) relation for I_K pooled from 15 fibers. After linear subtraction of leakage currents and adjustment for fiber surface area, the active outward current density (mean $\pm$ SEM) is plotted against membrane potential. All measurements were made from paradigms like that shown in **A** with the noninactivating current measured at the end of the voltage step being defined I_K . **C**, Superimposed traces of inactivating current (determined by subtraction of corresponding traces in **A**) elicited from $V_H = -80$ mV. Each trace represents the mean current density (15 fibers) elicited by voltage steps to the indicated potentials. This current is predominantly I_A in physiological isolation from I_K (see text). **D**, I - V relation for I_A as defined in **C**. Each point represents the current density (mean $\pm$ SEM) measured at the peak of I_A . Same fibers as in **B** and **C**.

The sample size used in quantifying I_A in homozygous *Sh* mutants was roughly uniform, as was the variance of the measured distributions of I_A amplitudes (see Table 1). Therefore, to obtain an expected mean and variance for I_A in *Sh* heterozygotes, the values for I_A (mean $\pm$ variance) measured in homozygous parents were simply averaged and weighing procedures for expected variances based on sample sizes were avoided. The observed and expected values were subjected to a Student's *t*-test at 0.01 and 0.001 levels of significance.

A similar test was made after normalizing the parental and offspring I_A (mean $\pm$ variance) to the amplitude of I_K . For each genotype, the mean I_A was divided by the mean I_K to give the I_A/I_K ratio (cf. Tables 1 and 2). The variance of the ratio of I_A and I_K distributions was calculated according to Fieller (1932), assuming a correlation coefficient of zero between I_A and I_K . The variance calculated with the latter assumption places an upper limit on the probable value and thus represents a conservative estimate.

Results

Normal membrane currents

Passive membrane properties. To ensure that the strains of different genotypes showed no general membrane defects, the passive membrane properties were determined in all (281) fibers in this study. The membrane capacitance, c_m , resistance, r_m , and time constant, τ_m , were determined under voltage-clamp conditions in this subset of identified fibers (see Materials and Meth-

ods). In normal female larvae the values for r_m , c_m , and τ_m were 8.12 ± 0.49 M Ω , 4.05 ± 0.28 nF, and 31.9 ± 1.99 msec (mean $\pm$ SEM, $n = 15$), respectively. Similar values were observed in male larvae (Table 2). Measurements of c_m and r_m indicated that the fibers of different genotypes were comparable in surface area. Furthermore, of the 30 mutant (homozygous and heterozygous) genotypes analyzed, 28 had τ_m that were not significantly different from normal. The differences shown in the remaining 2 genotypes (*Sh⁵/Sh^{K1020}* and *Sh^{E62}/Sh¹⁰²*), although resolved by the Student's *t*-test, were all within 37% of normal. (For details of the measurements see Haugland, 1987.)

Since quantitative conclusions of gene-dosage effects on membrane currents rely on precise determination of current density, the density was obtained by adjusting the active current amplitude to c_m , which is thought to be proportional to the membrane area under voltage clamp. This eliminated the variations due to different fiber sizes or uncertainty in estimation of membrane area by microscopic measurements (Wu and Haugland, 1985). Current density, in the units of $\mu\text{A}/\mu\text{F}$, was used in all current-voltage (I - V) relations throughout this study.

Physiological separation of outward currents. In previous studies, the analysis of I_A amplitude and kinetics in larval muscles was limited by the overlap of I_A and I_K activation over a broad

Figure 2. I_A in normal and *Sh* female larval muscle. The voltage dependence of the peak I_A for each family of traces (elicited by the same sequence of voltage steps as in Fig. 1C) is indicated in the I - V relation. In this and all subsequent figures, traces represent the mean current density from the number of fibers indicated in Tables 1 and 2 for each particular genotype. The mean and SEM of peak I_A from the same measurements are shown in the I - V relations. In *Sh^{K0120}* and *Sh^{E62}* muscle, I_A is reduced to about 78% and 28% of normal, respectively. I_A in *Sh^{K5133}* and *Sh^M* is eliminated. In *Sh⁵* muscle, the voltage dependence of I_A is shifted to more positive potentials and steepened.

range of potentials (Wu et al., 1983; Wu and Haugland, 1985). The present study circumvented these limitations by utilizing differences in inactivation properties between I_A and I_K to achieve a nearly complete separation of these currents. The fraction of inactivating I_A extracted by our pulse paradigm (Fig. 1A) was determined by comparing the normal data (with and without the prepulse) with data from *Sh* mutations (*Sh^M* and *Sh^{K5133}*) that completely eliminated I_A . These comparisons indicated that the prepulse inactivated about 86% of I_A in normal muscles.

The voltage dependence of the delayed potassium current, I_K , determined at the end of the test pulse and adjusted for linear leakage currents, is plotted in Figure 1B. This current was first detectable near -20 mV, slowly reaching a plateau after 400 msec, and became larger and rose more rapidly to a steady state in 150 msec with stronger depolarizations (cf. Wu and Haugland, 1985).

Figure 1C shows a family of I_A elicited by different depolarizing voltage steps. Both the amplitude and kinetics of I_A were voltage dependent. Normal I_A was first detectable at -30 mV and rose slowly to a peak after 25 msec. With stronger depolarizations, I_A became larger and faster; the time to peak was near 10 msec at $+40$ mV. The I - V relation at the time of peak I_A is shown in Figure 1D.

Altered I_A in homozygous *Sh* mutants

With the same protocol (Fig. 1), I_A obtained from 5 mutants is compared with normal in Figure 2. The most common phenotype was the complete elimination of I_A , as seen in *Sh^{K5133}* and *Sh^M* fibers. Two other mutations, *Sh¹⁰²* and *Sh^{K82a}*, also completely eliminated I_A (see Table 1). The residual current in

Table 2. Passive and active properties of larval muscles in normal and aneuploid *Sh* male larvae

Genotype	<i>Sh⁺/Y</i>	<i>Sh^M/Y;;Sh⁺</i>	<i>Sh^{K5133}/Y;;Sh⁺</i>	<i>Sh⁺/Y;;Sh⁺</i>
<i>n</i>	5	4	8	5
r_m (M Ω)	8.58 ± 1.11	6.12 ± 0.78	7.48 ± 0.75	7.39 ± 0.84
c_m (nF)	3.51 ± 0.19	4.40 ± 0.58	3.95 ± 0.33	3.39 ± 0.17
τ_m (msec)	29.8 ± 3.71	26.2 ± 3.73	29.8 ± 2.42	25.1 ± 1.89
I_A (μ A/ μ F)	4.17 ± 0.33	4.38 ± 0.49	1.13 ± 0.16^b	8.96 ± 1.25^b
I_K (μ A/ μ F)	6.22 ± 0.79	7.66 ± 0.44	7.75 ± 0.53	8.01 ± 0.71
I_A/I_K	0.67 ± 0.10	0.57 ± 0.07	0.14 ± 0.02^a	1.12 ± 0.19^a

The mean and SEM are shown. *n*, Number of fibers examined.

^a $p < 0.01$.

^b $p < 0.001$.

Figure 3. Comparison of the time course and voltage dependence of I_A in Sh^{K0120} and Sh^{E62} with normal. In this and all following figures (except Fig. 11B), traces represent currents elicited by voltage steps to -40 , -20 , 0 , $+20$, and $+40$ mV. Following normalization, I_A in Sh^{K0120} (upper, open circles) and Sh^{E62} (lower, open circles) muscle are superimposed on normal (traces). In these mutants, I_A is simply reduced. Same data as in Figure 2.

Sh^M and Sh^{KS133} became significant only above $+20$ mV and can be attributed to inactivating I_K extracted by the pulse protocol (less than 5% of the total I_K). These currents rose slowly with a time course and I - V relation resembling that of I_K (data not shown). Similar current was also observed in normal muscles treated with 4-aminopyridine (4-AP) at concentrations sufficient to completely block normal I_A .

In 2 other mutants, I_A was reduced. The Sh^{K0120} mutation reduces I_A to about 78% of normal (Fig. 2 and Table 1). In Sh^{E62} muscle the reduction was more striking, to only 28% of normal. The residual current in these 2 mutants was normalized with a single normalization factor (based on the I_A evoked at $+30$ mV) and superimposed on normal. As would be expected from a simple reduction in current density, the resulting amplitude and time course were nearly identical (Fig. 3). These mutations are thus hypomorphic, exhibiting a reduced, but otherwise normal, current.

Inspection of the normalized mutant data (open circles in Fig. 3) does suggest an excess of outward current at late time during the most positive voltage steps. The discrepancies at these potentials may be attributed to the disproportional magnification of residual, inactivating I_K . This interpretation is supported by the finding that the discrepancy was more pronounced in the genotype (e.g., Sh^{E62}) with the smaller residual current.

In contrast, the Sh^S mutation caused alterations that could

not be described as a simple reduction in current density. At potentials between -30 and $+20$ mV, Sh^S I_A was either absent or reduced. Depolarizations to $+40$ mV did, however, elicit a large I_A of nearly normal amplitude (see traces, Fig. 2). The I - V relation for this current was thus shifted to more positive potentials and steepened (Fig. 2). Furthermore, Sh^S I_A followed a slightly slower time course as compared to normal (see Haugland, 1987, for details). In sum, these changes in the I - V relation and kinetics of I_A in Sh^S fibers most likely reflected altered properties of the I_A channel.

As was done for normal I_A (see above), the fraction of inactivating I_A in Sh^{K0120} , Sh^{E62} , and Sh^S was determined by comparing data with those from Sh mutations that eliminated I_A . The prepulse inactivated about 78%, 90%, and 74% of I_A in Sh^{K0120} , Sh^{E62} , and Sh^S , respectively.

The delayed outward current, I_K , was analyzed using the same pulse paradigm as in Figure 1A (data not shown). The results confirmed our previous report (Wu and Haugland, 1985) that these Sh mutations do not affect the amplitude or kinetics of I_K .

Altered I_A in heterozygotes and aneuploids

Complementation tests among null alleles. The analysis of I_A in heterozygous combinations of the Sh mutations provided an opportunity to determine whether these mutations affect the same or different gene products in the I_A channel. We first examined whether heterozygous combinations of the 4 Sh mutations that eliminate I_A would result in detectable I_A . We constructed all possible combinations involving Sh^M (Sh^{KS133}/Sh^M , Sh^{102}/Sh^M , and Sh^{K82a}/Sh^M). In muscles of such genotypes I_A was not observed, indicating that these 4 mutations do not complement (Table 1, see Haugland, 1987, for details).

Sh^M and Sh^{K82a} behave as deficiencies. Although similar in phenotype, the above 4 null mutations may be categorized genetically into 2 distinct classes. One class of null mutations behaves as a simple deficiency. With an absence of gene product or production of inert, nonfunctional product, these mutations are amorphic, exhibiting no ability to affect the function of other mutations. The second class of null mutations may give rise to an abnormal gene product, which exhibits a novel, unexpected dominance when combined with other gene products in multimeric functional assembly.

Both cases were observed when we examined I_A in various combinations of null Sh mutations with other Sh mutations. In all combinations of Sh^+ , Sh^{K0120} , Sh^{E62} , and Sh^S with Sh^M , I_A appeared to follow a simple gene-dosage effect; the resulting current was always about the average of the 2 currents observed in the homozygotes (Fig. 4). Even the shifted and steepened voltage dependence of Sh^S I_A was expressed according to gene dosage in the Sh^S/Sh^M heterozygotes (Fig. 4B), as though the remaining currents were contributed solely by the Sh^S chromosome. The adherence of I_A to a simple gene-dosage effect suggests that the Sh^M allele behaves in a fashion indistinguishable from a simple deficiency (an amorph), neither enhancing nor suppressing the function of the other alleles.

A further test for an amorphic mutation is to determine whether the presence of a duplication of the normal locus restores the wild-type phenotype. We examined I_A in Sh^M aneuploid male larvae possessing an additional copy of the Sh^+ locus on the third chromosome (by using the translocation $Dp(1;3)JC153$; see Materials and Methods). In *Drosophila*, expression of sex-linked genes, such as Sh , is dosage-compensated. Male flies

Figure 4. Comparison of I_A observed in Sh^M heterozygotes with that expected from gene dosage. *A*, I_A in Sh^+/Sh^M muscle (traces) is superimposable on the average of the Sh^+ and Sh^M currents (open circles, from same data as in Fig. 2). The graph represents the I - V relation of peak I_A in normal, Sh^+/Sh^M , and Sh^M muscles. The upper continuous curve was fit by eye to the Sh^+ data while the lower continuous curve represents the peak I_A predicted from gene dosage. In Sh^+/Sh^M muscle, I_A is reduced to about 50% of normal. *B-D*, Similarly, I_A in Sh^5/Sh^M , Sh^{E62}/Sh^M , and Sh^{K0120}/Sh^M heterozygotes is near that expected from gene dosage.

possess only a single copy of the X chromosome, as opposed to 2 copies in females. Each X chromosome in females contributes equally to the synthesis of gene products while the single X chromosome in males synthesizes as much as the 2 female X chromosomes combined. Adult aneuploid male flies of this genotype were behaviorally normal and did not exhibit the leg-shaking behavior characteristic of the Sh mutants. In larval muscle fibers of these aneuploids, the density of I_K and passive membrane properties were not different from normal (Table 2).

Most significantly, as shown in Figure 5 they possessed an I_A nearly identical to that observed in the controls, Sh^+ males (see also Table 2).

We have examined another mutation, Sh^{K82a} , which gave results similar to that described for Sh^M . Heterozygote combinations of Sh^{K82a} with Sh^+ , Sh^{K0120} , Sh^{E62} , and Sh^5 also followed a simple gene-dosage effect (Table 1). Thus, the Sh^{K82a} allele also appears to be amorphic, with the current in heterozygotes reflecting the synthetic capacity of the other allele.

Figure 5. I_A in a Sh^M male larva possessing a duplication of Sh^+ on the third chromosome is near normal. The continuous curve was fit by eye to the normal data and also represents the prediction from gene dosage.

Figure 6. Comparison of I_A observed in Sh^s heterozygotes with that expected from gene dosage. *A*, I_A in Sh^+/Sh^s muscle (traces) is superimposable on the average of the Sh^+ and Sh^s currents (open circles, from same data as in Fig. 2). The graph represents the $I-V$ relation of peak I_A in normal (filled circles), Sh^+/Sh^s (open squares), and Sh^s (filled squares) muscle. The upper and lower continuous curves were fit by eye to the normal and Sh^s data while the middle curve represents the average of the 2 curves and is peak I_A predicted from gene dosage. In Sh^+/Sh^s muscle, I_A exhibits a voltage dependence intermediate between that of normal and Sh^s . *B* and *C*, Similarly, I_A in Sh^rK0120/Sh^s , Sh^E62/Sh^s heterozygotes is near that expected from gene dosage.

Complementation tests among leaky alleles. A different set of experiments examined I_A in all possible heterozygotic combinations among Sh^+ , Sh^s , Sh^rK0120 , and Sh^E62 . The I_A observed in Sh^+/Sh^s heterozygotes (traces in Fig. 6*A*) is compared with that expected from gene dosage (open circles in Fig. 6*A*). The peak I_A in Sh^+/Sh^s muscle exhibited an $I-V$ relation (Fig. 6*A*) inter-

Figure 7. I_A in heterozygotic combinations of Sh^+ , Sh^rK0120 , and Sh^E62 is compared with that expected from gene dosage (cf. Fig. 6 legend for details regarding the $I-V$ relations).

mediate between those of Sh^+ and Sh^s homozygous muscles. This is consistent with the prediction that the I_A channels in the heterozygotes consist of 2 equal populations of normal and Sh^s channels.

Also shown are 2 other parallel experiments that compared the I_A observed in Sh^rK0120/Sh^s and Sh^E62/Sh^s muscles with that expected from gene dosage (Fig. 6, *B*, *C*). In both examples, the observed current was intermediate from that observed in the homozygous muscles, in close agreement with the prediction that the total I_A is composed of 50% of the Sh^s type and 50% of the other type. In neither case was the observed current significantly different from that predicted (Table 1).

Figure 8. I_A in Sh^{KS133} heterozygotes. The traces represent I_A in the indicated Sh^{KS133} heterozygotes evoked by voltage steps from -80 mV to -40 , -20 , 0 , $+20$, and $+40$ mV. The open circles indicate the I_A at $+40$ mV predicted from gene dosage. The graph represents the I - V relation of peak I_A in Sh^+/Sh^{KS133} (filled circles), Sh^{K0120}/Sh^{KS133} (filled squares), Sh^{E62}/Sh^{KS133} (filled diamonds), and Sh^5/Sh^{KS133} (open squares) muscles. In these muscles, I_A is much less than expected from gene dosage (see also Table 1). Nevertheless, the voltage dependence of the peak I_A in Sh^5/Sh^{KS133} is characteristic of that in Sh^5 whereas peak I_A in the other heterozygotes is simply reduced but otherwise not different from normal.

Figure 7 shows experiments of the remaining 3 combinations, Sh^+/Sh^{K0120} , Sh^+/Sh^{E62} , and Sh^{K0120}/Sh^{E62} . In Sh^{K0120}/Sh^{E62} the observed I_A was nearly identical to the expected value (Fig. 7C). In Sh^+/Sh^{K0120} and Sh^+/Sh^{E62} fibers (Fig. 7, A, B), I_A showed a small shortage from the expected value at more positive potentials (see also Table 1); in Sh^+/Sh^{K0120} the difference was statistically significant (Table 1). Since the latter discrepancy is small (within 15% of the expected value), it probably does not indicate a violation of the gene-dosage effect but, rather, reflects a fluctuation due to small sample size. In sum, adherence of I_A to a simple gene-dosage effect in heterozygotic combinations among Sh^5 , Sh^{K0120} , Sh^{E62} , Sh^M , and Sh^{K82a} suggests that these mutations are allelic, affecting a transcript coding for a structural component of the I_A channel.

Sh^{KS133} and Sh^{102} are dominant null Sh mutations. In contrast to Sh^M and Sh^{K82a} , the other 2 null mutations, Sh^{KS133} and Sh^{102} , produced dramatic departures from the gene-dosage effect in heterozygotes (see Table 1). Figure 8 shows the results obtained in various combinations of Sh^+ , Sh^{K0120} , Sh^{E62} , and Sh^5 with Sh^{KS133} . In all cases the observed current (traces) was far below that predicted from a simple gene-dosage dependence (open circles, which show the expected current at $+40$ mV). The traces in Figure 9 show the I_A measured in all combinations of Sh^+ , Sh^{K0120} , Sh^{E62} , and Sh^5 with Sh^{102} . The observed currents were consistently less than expected (Fig. 9, open circles, which show the predicted values at $+40$ mV). The degree of departure from gene dosage varied in an allele-specific fashion, with Sh^+/Sh^{102} I_A being 37% of normal while Sh^{E62}/Sh^{102} I_A was only 17% of that in Sh^{E62} homozygotes (Table 1, comparisons at $+10$ mV). In contrast to the amorphic phenotypes of Sh^M and Sh^{K82a} , the

Sh^{102} and Sh^{KS133} mutant gene products appeared to interfere or interact with the product altered by the rest of the Sh mutations (see Discussion).

The dominant phenotype of Sh^{KS133} was further demonstrated in male Sh^{KS133} flies possessing one duplicated copy of the Sh^+ locus ($Dp(1;3)JC153$). These flies do shake under ether anesthesia (Tanouye et al., 1981), but the tremor is less severe. The larval muscles of these aneuploids exhibited an I_A that was only 25% of that measured in a normal male (Fig. 10 and Table 2), in marked contrast to the 100% restoration of normal I_A observed in Sh^M males with the same duplication (Fig. 5).

Sh gene products but not other factors limit I_A density

To explore the possibility that the I_A channel in larval muscle consists of only the Sh products, we examined Sh^+ male larvae possessing an additional copy of the Sh^+ locus (the same duplication as in Figs. 5 and 10). As shown in Figure 11, the I_A observed in muscles of these aneuploids was about twice that seen in normal male larvae, whereas the size of I_K remained normal. Consequently, there was a striking increase in the ratio I_A/I_K in fibers of these aneuploids (Table 2).

The result implies that the limiting factor determining I_A density is the amount of Sh products but not other limited gene products or resources, consistent with the idea that the Sh locus codes for the entire I_A channel. This is exactly what is expected from gene-dosage compensation experiments in male *Drosophila*; 1 copy and 2 copies of X-linked structural genes produce 100% and 200% of the gene products, respectively (see review by Stewart and Merriam, 1980).

Figure 9. I_A in Sh^{102} heterozygotes. The traces represent I_A in the indicated Sh^{102} heterozygotes evoked by voltage steps from -80 mV to -40 , -20 , 0 , $+20$, and $+40$ mV. The open circles indicate the I_A at $+40$ mV predicted from gene dosage. The graph represents the I - V relation of peak I_A in Sh^+/Sh^{102} (filled circles), Sh^{K0120}/Sh^{102} (filled squares), Sh^{E62}/Sh^{102} (filled diamonds), and Sh^5/Sh^{102} (open squares) muscles. In these muscles, I_A is less than expected from gene dosage (see also Table 1). Nevertheless, the voltage dependence of the peak I_A in Sh^5/Sh^{102} is characteristic of that in Sh^5 whereas peak I_A in the other heterozygotes is simply reduced but otherwise not different from normal.

Discussion

This study demonstrated that the gene-dosage dependence of membrane channel proteins in *Drosophila* can be determined with precision by the voltage-clamp technique. A method of physiological separation of I_A was used to quantify its amplitude and kinetics over a large range of membrane potentials. The results obtained from various heterozygous and aneuploid combinations of *Sh* mutants provide some information about the nature of the *Sh* mutations examined and the tissue-specific expression pattern of the *Sh* products. The interpretation of the data is also relevant to the questions of whether the I_A channel contains multiple *Sh* proteins and of whether a homomeric or heteromeric arrangement is involved if the larval muscle channel consists of a subunit aggregate.

Does the *Sh* locus encode the entire I_A channel?

The aneuploid males possessing an extra *Sh* locus ($Sh^+/Y;;Sh^+$) produce an I_A that is 2-fold of that in normal males (Fig. 11).

Thus, the small translocated chromosome segment (cytological location 16E2-4;17A-B, Tanouye et al., 1981) containing the duplicated *Sh* locus appears to be fully capable of producing the normal amount of I_A channels, consistent with the idea that the *Sh* locus codes for the entire structure of the I_A channel.

This observation differs from the previous reports of the lack of gene-dosage effect in adult muscle fibers carrying duplicated copies of the *Sh* locus (Salkoff, 1983; Timpe and Jan, 1987; see below for details). Our result ensures that the number of functional I_A channels is limited only by the amount of the *Sh* products but not by other resources, such as limited membrane sites or other channel subunits. Therefore, it is still possible that the I_A channel contains a different subunit(s) that is produced in excess by a gene located in a region outside of the *Sh* locus, including the small flanking regions within the *Dp(1;3)JC 153* segment.

Another conclusion that could be drawn from the results is that regulation of I_K appears to be independent of the amount of I_A present. Duplication of the *Sh* locus affects only the channel

Figure 10. I_A in Sh^{K5133} male larvae possessing a duplication of Sh^+ is near 25% of normal. The continuous line was fit by eye to the normal data and also represents the I_A expected from gene dosage (contrast with Fig. 5).

Figure 11. *I*_A in *Sh*⁺ male larvae possessing a duplication of *Sh*⁺ is near twice that of normal but *I*_K is near normal. **A**, The families of peak *I*_A are shown and plotted in the *I*-*V* relations. The lower continuous curve was drawn by eye to fit the normal data while the upper continuous curve represents the peak *I*_A predicted from gene dosage. **B**, Two superimposed traces of membrane currents elicited by a voltage step to +20 mV with or without a prepulse (see Fig. 1A for the pulse paradigm). The *Sh*⁺ duplication only increases the amplitude of *I*_A whereas *I*_K remains normal. The noninactivating *I*_K measured at the end of the voltage step is plotted in the *I*-*V* relations. A continuous curve is fit to the normal data and represents the expected *I*_K.

density of *I*_A, but not *I*_K. Furthermore, in all homozygous, heterozygous, and aneuploid forms of *Sh* mutants examined, the density of *I*_K was not up-regulated when *I*_A was reduced or eliminated (Tables 1 and 2).

The nature of the *Sh* mutations

The functional alteration in *Sh*^s mutants is most likely related to altered structure of the *I*_A channel. This is supported by our recent experiments in *Sh*^s larvae which revealed a striking increase in the sensitivity of *I*_A to blockade by 4-AP concomitant with a change in channel gating properties (Haugland, 1987; Haugland and Wu, 1987).

The 2 amorphic alleles, *Sh*^M and *Sh*^{K82a}, have been characterized molecularly. Both mutations affect the common regions that may interfere with multiple transcripts; *Sh*^{K82a} causes an inversion (Papazian et al., 1987) and *Sh*^M involves an insertion (Kamb et al., 1987). An altered production of *Sh* transcripts was reported in *Sh*^{K82a} flies (Schwarz et al., 1988). However, *Sh*^M may still produce a nonfunctional but inert translation product since polyclonal antibodies specific to a common region of the putative *Sh* proteins identify a protein species in immunoblots of *Sh*^M as well as *Sh*⁺, *Sh*^s, and *Sh*^{KS133}, but not of *Sh* deficiencies (Barbas et al., 1989).

The other 2 null mutations, *Sh*^{KS133} and *Sh*¹⁰², appear antimorphic, being dominant over their normal allele. These may be missense or nonsense mutations resulting in a gene product that interferes with other products in heterozygotes. Recent DNA sequence analysis and transformation experiments of *Sh*¹⁰² indicated truncated *Sh* products which interact with their normal

counterpart in transformed flies carrying a normal dose of the wild-type *Sh* gene (Gisselmann et al., 1989).

The *Sh*^{rK0120} and *Sh*^{E62} mutations reduce the amplitude of *I*_A without causing a detectable change in properties. This phenotype could be explained by a simple reduction in the number of functional *I*_A channels, although a decreased channel conductance cannot be ruled out. Previous genetic experiments suggest that the *Sh*^{E62} and *Sh*^{rK0120} mutations may affect sites relatively distant (Tanouye et al., 1981) from the regions where *Sh*^s, *Sh*^{KS133}, and *Sh*¹⁰² reside (Kamb et al., 1987) and might be associated with different (e.g., regulatory) functions.

Relationship between *Sh* gene products and structure of *I*_A channels in larval muscle

It is now known that the *Sh* locus contains a complex transcription unit that generates multiple messages by alternative exon splicing (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988). DNA sequence analysis indicates that some of the transcripts share a common central region flanked by variable 3' and 5' ends and may code for proteins resembling 1 of the 4 internally similar domains of Na⁺ and Ca²⁺ channels (Baumann et al., 1987; Tempel et al., 1987; Kamb et al., 1988; Schwarz et al., 1988). Thus, the different *Sh* products may be separate *I*_A channel subunits. Indeed, several species of *Sh* transcripts are each capable of generating *I*_A-like currents in *Xenopus* oocytes, suggesting a homomultimeric structure (Iverson et al., 1988; Timpe et al., 1988a, b). However, it is still not known how these different *Sh* products are related to the *I*_A channels in different nerve and muscle membranes and whether other

transcripts from the *Sh* region still remain to be identified. Moreover, the channels expressed in oocytes may not reflect the complete structure in the natural setting.

For a number of ligand-gated channels that are known to be aggregates of different subunits, a single subunit species can form homomeric channels with apparently normal properties when expressed in oocytes or cell lines (Boulter et al., 1987; Blair et al., 1988; and other cases cited in Pritchett et al., 1988). On the other hand, expression of the rat brain Na⁺ channel α -subunit in oocytes requires additional RNA species to attain normal gating properties (Auld et al., 1988). Similarly, a fraction of rat brain mRNA has been used to express I_A in *Xenopus* oocytes, but the full expression of channel kinetics and pharmacology depends on additional mRNA species (Rudy et al., 1988). Most significantly, some homomeric *Sh* channels expressed in oocytes are sensitive to charybdotoxin whereas the native I_A channels are not (MacKinnon et al., 1988). Therefore, it is important to examine I_A channels *in situ* to determine whether they contain heteromeric subunits with a combination of the different *Sh* products.

Functional assays in heterozygous combinations can be used to probe for nonlinear interactions between structurally dissimilar subunits generated by different mutant alterations. Leg shaking under ether anesthesia, excitatory junctional potentials (e.j.p.s) at neuromuscular junctions (unpublished data), and axonal action potentials (Tanouye et al., 1981) in heterozygotes show varying degrees of abnormality that cannot be readily quantified. The difficulty could be circumvented by direct voltage-clamp measurements of the current, which is proportional to the density and conductance of the channels.

Strict gene-dosage effects in heterozygous combinations among leaky and amorphic alleles. The *Sh*^s mutation, which produces I_A with clearly altered properties, is especially useful for detecting departures from simple gene-dosage dependence within heterozygous channels. In heterozygotes of *Sh*^s with amorphic mutations *Sh*^M and *Sh*^{K82a}, we found no evidence of wild-type I_A but 50% of *Sh*^s I_A. Moreover, in all possible combinations among *Sh*⁺, *Sh*^s, *Sh*^{K0120}, *Sh*^{E62}, *Sh*^M, and *Sh*^{K82a}, the currents in heterozygotes follow closely the simple average of the parental I_A (Figs. 4, 6, 7, and Table 1), as though only 2 populations of I_A channels are formed, each like the parental homozygous channels, and no hybrid channels are present.

The simplest model to explain the above results is that each channel contains only one *Sh* protein which is affected by the 5 amorphic and leaky mutations. However, this monomeric model is not favored by the current molecular view of the I_A channel being a multimer of *Sh* products (see above). On the other hand, the simple average effect can also be explained by a heteromultimeric channel in which a single copy of a *Sh* subunit subject to modification by the above 5 alleles is present along with the products of some other splicing variants not affected by these mutations. Both models do not give rise to hybrid channels in these *Sh* heterozygotes and would lead to a simple average effect.

Under different assumptions, a homomeric channel assembly could also account for the observed simple average effect: (1) Like products preferentially associate with each other such that hybrid channels do not form; or (2) hybrid channels of combinations between the 5 alleles do form but the ensemble behavior fortuitously resembles the simple average of the parental phenotypes. [In addition to *Sh*^s, *Sh*^{K0120}, *Sh*^{E62}, and *Sh*^M may also produce altered *Sh* proteins, as indicated by immunochem-

ical studies (Barbas et al., 1989) described above.] These possibilities could most definitively be resolved by single-channel measurements. Deviations in conductance and gating properties would be readily detectable because hybrid channels should constitute a majority in the population (e.g., 1/2 for homodimeric and 3/4 for homotrimeric form).

*Departures from the simple gene-dosage effect in *Sh*^{KS133} and *Sh*¹⁰² heterozygotes.* The dominant effects of the *Sh*^{KS133} and *Sh*¹⁰² mutations observed in complementation and gene duplication experiments (Figs. 8–10), especially the strong allele-specific effect of *Sh*¹⁰² (Fig. 10), suggest interactions among *Sh* gene products. This is also consistent with the recent observations on transgenic flies carrying a heat-inducible *Sh*¹⁰² gene (Gisselmann et al., 1989). In both *Sh*^{KS133}/*Sh*⁺ and *Sh*^{KS133}/*Y*; *Sh*⁺ larvae I_A appears normal in activation, inactivation, and kinetic properties but is reduced to about 1/8 of the wild-type amplitude (Tables 1, 2, and Figs. 8, 10). The reduction in I_A found in *Sh*^s/*Sh*^{KS133}, *Sh*^{K0120}/*Sh*^{KS133}, and *Sh*^{E62}/*Sh*^{KS133} varies between 1/8 and 1/4 of the homozygous current (Table 1). In contrast, the dominant effect of *Sh*¹⁰² varies more clearly in an allele-specific fashion; the departure from the simple gene-dosage prediction is much greater in *Sh*^{E62}/*Sh*¹⁰² muscles and is least in *Sh*⁺/*Sh*¹⁰² (Fig. 9 and Table 1).

The exact nature of the dominant effects associated with these 2 mutations is not known. Opportunities for interactions among gene products exist during the synthesis, modification, assembly, transport, and localization of channel proteins; and the affected gene product may be altered in its functional conformation, association affinity, or production level (see Haugland, 1987, for a detailed discussion). However, the most straightforward explanation for the strong reduction of I_A in *Sh*^{KS133}/*+* heterozygotes and aneuploids is that the channel consists of multiple subunits. For example, a channel containing more than 3 subunits subject to *Sh*^{KS133} modification could account for the probability of less than 1/8 aggregates being completely normal after random association among normal and mutant subunits in *Sh*^{KS133}/*+* heterozygotes to produce less than 1/8 normal I_A (less than 1/4 of the expected averaging effect, see Table 1). Along this line of argument, the *Sh*¹⁰² mutation could affect the same subunit species in question if the truncated subunits (Gisselmann et al., 1989) are produced at a significantly lower amount as compared to the *Sh*^{KS133} product. In multimeric aggregation, this would cause I_A in *Sh*⁺/*Sh*¹⁰² to be only moderately reduced as a larger number of channels would not receive a defective *Sh*¹⁰² subunit (cf. Fig. 9 and Table 1). The greater reduction observed in *Sh*^{K0120}/*Sh*¹⁰² and *Sh*^{E62}/*Sh*¹⁰² heterozygotes could then be explained if the *Sh*^{K0120} and *Sh*^{E62} mutants produce decreased amounts of normal products.

It is important to note that *Sh*^s/*Sh*^{KS133} (and *Sh*^s/*Sh*¹⁰²) larvae exhibit only a small, *Sh*^s-like I_A with no indication of normal I_A (see *I*–*V* relations in Figs. 8 and 9). With different assumptions, several multimeric models can be proposed to explain this observation. One example is a heteromultimeric channel that contains a single copy of an *Sh* splicing variant that could be affected by *Sh*^s and all other alleles (including *Sh*^{KS133}) plus multiple copies of a different splicing variant that is subject to modification by *Sh*^{KS133} but not by *Sh*^s. Association among these subunits would produce no normal I_A, but only drastically reduced *Sh*^s I_A in *Sh*^s/*Sh*^{KS133} heterozygotes (see Haugland, 1987, for detail). Conversely, if the condition of preferential association of like products [see assumption (1) in the previous section] is relaxed, a homomeric model, in which *Sh*^s and *Sh*^{KS133} poly-

peptides (of the same splicing variant) are able to associate, could also produce the observed effect. It will be important to obtain additional information about the molecular lesion in *Sh^s* polypeptides. In contrast to the truncated product in *Sh¹⁰²*, the *Sh^s* mutation confers intriguing alterations in channel gating and drug sensitivity (Haugland and Wu, 1987) and interesting subunit interactions with products of other alleles.

Comparisons with pupal flight muscle and sources of variation

Our data are consistent with some of the findings in pupae and adults. In adult cervical giant fibers, addition of 2 extra copies of *Sh⁺* to one copy of *Sh^{KSI33}* in females does not restore normal action potentials (Tanouye et al., 1981; Tanouye and Ferrus, 1985), likewise indicating that the *Sh^{KSI33}* allele produces an abnormal gene product. Experiments in pupal flight muscle (Salkoff, 1983; Timpe and Jan, 1987) showed that all null *Sh* mutations, including the 4 examined here, fail to complement, yielding no detectable I_A in heterozygous combinations. Timpe and Jan (1987) also suggested interactions among *Sh* gene products based on the absence of I_A in *Sh^{E62}/Sh¹⁰²* heterozygotes.

Surprisingly, there are gross differences between the pupal and larval observations. Salkoff's (1983) voltage-clamp measurements in pupal flight muscles show that I_A in aneuploid *Sh^{KSI33}* males possessing a duplication of *Sh⁺* (*Y, B^s*) is only 1/2 of the expected (normal) value (compared to 1/3 in larvae) but I_A in *Sh⁺/Sh^{KSI33}* heterozygous females is reportedly also 1/2 of normal. Similarly, I_A in *Sh⁺/Sh¹⁰²* heterozygous pupae was reported to be 52% of normal, indicating no dominant effect, whereas in *Sh⁺/Sh^{K82a}* pupae, I_A was 83% (Timpe and Jan, 1987). In comparison, about 37% and 50% I_A were seen in larvae of these genotypes, respectively (Table 1). While the residual I_A measurements in homozygous *Sh^{E62}* and *Sh^{K0120}* pupae (27% and 63%, respectively) agree with larval data (about 26% and 75%, respectively), the results from *Sh^{E62}/Sh⁺* and *Sh^{K0120}/Sh⁺* differ markedly, being 84% and 136%, respectively, of normal I_A in pupae vs. about 54% and 75% in larvae (compare Table 1 with Timpe and Jan, 1987).

The sources of these variations are not known. However, at least part of these discrepancies may be artifactual. Measurements of gene-dosage effects and allelic interactions are quantitative; the amplitude of a membrane current depends on the density of ion channels and on sarcolemmal surface area. Unfortunately, the pupal muscle measurements cited above (Salkoff, 1983; Timpe and Jan, 1987) were not adjusted for fiber surface area; thus variation in animal and fiber size would increase the variation in these data. In addition, the developing pupal flight muscle may not be a suitable preparation for determinations of gene-dosage effects. Although it allows isolation of I_A from I_K , the amplitude of I_A is rapidly developing from 55 to 72 hr after puparium formation. Near the end of this interval, I_K appears (Salkoff and Wyman, 1981), but it is not known whether I_A has reached its full development. Thus differences in developmental rates of I_A in different strains combined with uncertainties in staging the pupal development would also increase the variation in measurements.

Nevertheless, at least part of the discrepancies may reflect true differences in the genetic control of I_A between larval and pupal muscles. In one set of experiments with different doses of *Sh⁺*, Timpe and Jan (1987) have adjusted measurements using membrane capacitance. For one copy of *Sh⁺* in females (with an *Sh* deficiency), a reduction to 67% of normal I_A was seen while no significant increase in I_A was detected in pupae

with 3 (wild-type females with a single duplication) and the equivalent of 4 (wild-type males with a single duplication) copies of *Sh⁺*; as though the expression of *Sh⁺* products in pupal muscle depends on a limited supply of other gene products. This is contrary to the larval data (Figs. 5, 11) where I_A is proportional to the *Sh⁺* dose up to the equivalent of 4 copies (*Sh⁺/Y⁺;Sh⁺* males). We cannot exclude the possibility of the presence of additional species of subunits within larval I_A channels, for instance, a *Sh* product not affected by the mutations examined or a nonlimiting product of a gene outside of the *Sh* locus (see above).

Genetic basis for the diversity of K⁺ channels

Since the various *Sh* products differ in their distribution in different body parts (Schwarz et al., 1988), they may not be present in a fixed stoichiometry in all tissues and may contribute to different subtypes of I_A channels or even different types of K^+ channels. The difference in dosage dependence on *Sh⁺* could indicate that the number of I_A channels is limited only by the amount of *Sh* products in larval muscle, whereas it is restricted by the products of other loci in pupal muscle. There are also intriguing differences in the effects of certain *Sh* mutations on the properties of I_A in these 2 sets of muscles. In *Sh^s* pupal muscle, I_A was reported to show normal peak amplitude but abnormally rapid kinetics of inactivation and recovery (Salkoff, 1983). In striking contrast, the *Sh^s* mutation greatly alters the voltage dependence of I_A in larval muscle (Fig. 2), actually slowing its kinetics of activation and inactivation (Haugland, 1987). Furthermore, as previously pointed out (Wu and Haugland, 1985; Timpe and Jan, 1987), the *Sh^{K0120}* mutation exerts markedly different effects on nerve and muscle membranes. Although it causes in larval nerve terminals aberrations nearly as extreme as *Sh^{KSI33}* (Ganetzky and Wu, 1983), voltage-clamp studies on larval (Wu and Haugland, 1985, and Fig. 2) and pupal (Timpe and Jan, 1987) muscles indicate only mild reductions in I_A amplitude. [There has been some confusion about *Sh^{K0120}* strains. The one that was reported to show no I_A in pupal muscle (Salkoff and Wyman, 1981) has been renamed *Sh^{K0120}* (Salkoff, 1983; Timpe and Jan, 1987) and should not be confused with the present discussion.] The picture is further complicated by recent patch-clamp studies which showed that certain inactivating current resembling I_A in cultured neurons remains intact in the presence of either the *Sh^{KSI33}* mutation (Solc et al., 1987) or a deletion of the *Sh* locus (Baker and Salkoff, 1988).

The above discrepancies suggest that I_A channels in different excitable membranes may be regulated by other genes outside of the *Sh* locus as well (see Butler et al., 1989). In fact, mutations of 2 different loci, *eag* (*ether a go-go*) (Wu et al., 1983; Ganetzky and Wu, 1986; Y. Zhong and Wu, unpublished data) and *Hk* (*Hyperkinetic*) (Wu and Ganetzky, 1986; Wu, 1988; Stern and Ganetzky, 1989) have been suggested to alter I_A and also other K^+ currents. Taken together, these results suggest that, as previously proposed in a combinatorial hypothesis (Wu and Haugland, 1985; Wu and Ganetzky, 1986), diversity of K^+ channel types may arise from combinations of common, as well as distinct, structural or regulatory components. In this respect, the possibility that different *Sh* proteins may be involved in generating I_A channel subtypes and even contribute to other types of K^+ channels (Iverson et al., 1988; Pongs et al., 1988; Timpe et al., 1988b) is especially intriguing. Considering the large number of *Sh* products so far identified, combinations of different

Sh products and those of other genes could give rise to a great diversity of channel subtypes.

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