Voltage Clamp Analysis of Membrane Currents in Larval Muscle Fibers of *Drosophila*: Alteration of Potassium Currents in *Shaker* Mutants¹

CHUN-FANG WU² AND FRANK N. HAUGLAND

Department of Biology and Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242

Abstract

The body wall muscles in *Drosophila* larvae are suitable for voltage clamp analysis of changes in membrane excitability caused by mutations. Both inward and outward ionic currents are present in these muscle fibers. The inward current is mediated by voltage-dependent Ca^{2+} channels. In Ca^{2+} -free saline, the inward current is eliminated. The remaining outward K^{+} currents consist of two distinct components, an early transient I_A and a delayed steady I_K , which are separable by differences in the rate and voltage dependence of activation and inactivation. The steady-state and kinetic properties of the activation and inactivation processes of these two currents are analyzed. The results provide a basis for quantitative analysis of altered membrane currents in behavioral mutants of *Drosophila*.

Previous studies indicate that mutations in the *Shaker* (*Sh*) locus alter excitability in both nerve and muscle in *Drosophila*. Our results support the idea that the channels mediating I_A are molecularly distinct from those mediating I_K . All *Sh* mutations studied specifically affect I_A without changing the properties of the calcium current and I_K . In certain alleles (Sh^{KS133} , Sh^{102} , and Sh^M) I_A is eliminated, permitting detailed studies of I_K in isolation of I_A .

Studies of the alleles that do not eliminate I_A provide additional information of the channels. In one such allele, Sh^5 , voltage dependence of I_A activation is shifted to more positive potentials. This is accompanied by a less pronounced shift in the voltage dependence of inactivation. These results suggest that Sh^5 mutation affects the voltage-sensitive mechanism of both activation and inactivation processes and that these two processes are not controlled by independent parts of the channel. Furthermore, the differential effects of these alleles on different excitable membranes imply that other genes take part in the control of I_A . The effects of Sh^5 on muscle depend on developmental stage. In larval muscle, Sh^5 reduces the amplitude of I_A because of the shift in the current-voltage (I-V) relation. In

contrast, in adult Sh^5 muscles, I_A is reported to be normal in amplitude but shows abnormally rapid inactivation (Salkoff, L., and R. Wyman (1981) *Nature* 293: 228–230). A different allele, Sh^{RK0120} , causes a clear defect in nerve excitability, but analysis of I_A in Sh^{RK0120} larval muscle reveals I-V relations, inactivation, and recovery from inactivation similar to those seen in normal fibers. We suggest a possible mechanism of combinations of multiple interacting genes participating in the control of potassium channels to account for the presence of a variety of potassium channels in different excitable membranes.

Information regarding the structural and functional mechanisms of macromolecular components in excitable membranes can be inferred from the specific effects caused by mutational perturbations. In *Drosophila*, neurophysiological analyses of several behavioral mutants indicate defects in membrane excitability (Ikeda and Kaplan, 1970; Siddiqi and Benzer, 1976; Jan et al., 1977; Wu et al., 1978; Ganetzky and Wu, 1982a). In certain cases, the defects have been attributed to altered ionic currents (Jan et al., 1977; Wu and Ganetzky, 1980; Salkoff and Wyman, 1981; Wu et al., 1983a). This genetic approach is particularly useful to obtain new information regarding ionic channels that are scarce in quantity and lack high affinity ligands to facilitate biochemical analysis.

Recently, the voltage clamp technique has been satisfactorily applied to investigate the effects of mutations on the membrane currents in both adult (Salkoff and Wyman, 1981, 1983) and larval (Wu et al., 1983a; Wu and Ganetzky, 1984) muscles. In the adult flight muscles, the inward current and different components of outward currents can be separated on the basis of their differences in developmental onset and mutational alteration (Salkoff and Wyman, 1983; Salkoff, 1983a). The body wall muscles of *Drosophila* larvae are developmentally distinct from the adult muscles, which arise *de novo* following histolysis of larval muscles during pupation. Knowledge concerning whether some properties of excitable membranes are controlled by different genes at different developmental stages can be gained by comparing data from these two sets of muscles.

Muscle fibers in the larval preparation (Jan and Jan, 1976) offer certain experimental advantages. The fibers in each segment form a regular pattern and are individually identifiable (Hewitt, 1914; Crossley, 1978). Since they are directly accessible to external bathing medium, problems in membrane potential measurements due to the series resistance and equilibration time for external solution changes are minimized. These relatively short fibers are electrically isolated from one another and each is virtually isopotential (Jan and Jan, 1976). They can readily be impaled by two microelectrodes to

Received July 9, 1984; Revised March 19, 1985;
Accepted March 19, 1985

¹ We thank Drs. B. Ganetzky, P. Gettings, R. Hahin, R. Joyner, and C. Marchand for comments on the manuscript. We gratefully acknowledge the help of Dr. R. Joyner in instrumentation. This work was supported by United States Public Health Service Grants NS00675 and NS18500 and a grant from the Searle Scholars Program to C.-F. W. F. N. H. was supported by National Institute of Mental Health Training grant MH15172.

² To whom correspondence should be addressed, at Department of Biology, University of Iowa, Iowa City, IA 52242.

measure membrane currents under voltage clamp conditions (Wu et al., 1983a).

To provide a basis for quantitative analysis of altered membrane currents in behavioral mutants, we first report in detail the separation of various currents and their steady-state and kinetic properties in normal larvae. *Drosophila* larval muscles, like those of other arthropods (Fatt and Ginsborg, 1958; Werman et al., 1961; Yamamoto et al., 1981; Ashcroft and Stanfield, 1982), have voltage-dependent Ca^{2+} channels but do not have Na^{+} channels. There are at least two outward K^{+} currents: a rapid inactivating transient, similar to I_A described in molluscan neurons (Connor and Stevens, 1971b), and a delayed, sustained outward current, similar to the delayed rectifier, I_K (Hodgkin and Huxley, 1952c).

Specific effects of the X-linked *Shaker* (*Sh*) mutations on larval muscle fibers are subsequently described. *Sh* mutants display vigorous leg-shaking behavior under ether anesthesia (Kaplan and Trout, 1969). At the larval neuromuscular junction, the *Sh* mutations have been associated with antidromic repetitive firing of motor axons resulting in prolonged transmitter release (Jan et al., 1977; Ganetzky and Wu, 1982b, 1983). Intracellular recordings from adult giant axons revealed abnormal action potentials with delayed repolarization (Tanouye et al., 1981). Recent voltage clamp studies of adult (Salkoff and Wyman, 1981; Salkoff, 1983b) and larval muscles (Wu et al., 1983a; Wu and Ganetzky, 1984) confirm the previous suggestion that *Sh* mutations affect potassium currents (Jan et al., 1977).

To examine the specificity of the mutant defects, a detailed study of the kinetic and steady-state properties of I_A and I_K were conducted in an array of available *Sh* alleles. Results indicate that *Sh* mutations affect I_A without altering Ca^{2+} current and I_K . With certain *Sh* alleles, I_A is completely abolished in larval muscles. Taking advantage of these mutants, we examined the activation and inactivation processes of I_K in isolation in greater detail. In two alleles of *Sh*, I_A is altered but not eliminated. Analysis of properties of the remaining I_A suggests interrelationships among some physiological parameters and regulation of I_A function by genes besides *Sh*.

Given the complexity of molecular assembly of some ionic channels (see, for example, Catterall, 1984; Mishina et al., 1985), mutations could disrupt a variety of mechanisms that determine the function of ion channels. Ultimately, understanding of the nature of *Sh* mutations correlated with the modified properties of I_A may shed light upon the synthesis, regulation, and molecular gating mechanism of potassium channels in different excitable tissues. Preliminary accounts of some of the results reported here have appeared previously (Wu et al., 1983a; Wu and Ganetzky, 1984).

Materials and Methods

Strains. The wild-type strain, Canton-S of *Drosophila melanogaster*, was used to study the normal physiology of larval muscle fibers. Five different *Sh* alleles (Sh^{KS133} , Sh^{102} , Sh^M , Sh^5 , and Sh^{KO120}) were examined. The mutant strains were obtained from the collection of Dr. S. Benzer at The California Institute of Technology. To confirm that the observed phenotypes are specifically derived from the *Sh* mutations, the *Sh* alleles were examined with several different Canton-S and Oregon-R backgrounds. The " Sh^{KO120} stocks" used in some previous reports have been shown to carry a second mutation at the *eag* locus (Ganetzky and Wu, 1983). This second site mutation was removed by recombination from the Sh^{KO120} stock used in the present study. An additional Sh^5 stock was obtained from Dr. L. Salkoff (1983b) which yielded results consistent with data from the other Sh^5 stocks.

Preparations and salines. The ventral lateral longitudinal fibers of mature third instar larvae were used for all experiments described. The larval preparation is identical to that previously described by Jan and Jan (1976). The temperature of the preparation was controlled by a Peltier junction microscope stage (Cambion Thermionic Corp., Cambridge, MA) and monitored by a thermistor (models ICT-4 and BAT-4, Bailey Instruments, Saddle Brook, NJ) placed within 0.5 mm of the preparation in solution. The saline compositions for different experiments were modified from the standard physiological saline (Jan and Jan, 1976) containing 128 mM NaCl, 2 mM KCl, 4 mM MgCl_2 , 1.8 mM CaCl_2 , 35.5 mM sucrose, buffered at pH 7.1 with 5 mM N-2-hydroxyethyl-N'-1-ethanesulfonic acid (HEPES).

For characterization of outward currents, the inward Ca^{2+} current was

eliminated by the use of saline containing 0 mM CaCl_2 , 0.5 mM EGTA. Excess MgCl_2 (14 mM) was added to stabilize the membrane (Salkoff and Wyman, 1981; Yamamoto et al., 1981). As in other species (Yamamoto et al., 1981; Hahin and Campbell, 1983), the presence of high concentration of Mg^{2+} circumvents the problems of muscle surface potential change and membrane leakage increase caused by Ca^{2+} -free solution. To block K^{+} channels, 5 to 10 mM tetraethylammonium (TEA) (Eastman, Rochester, NY) was added to the bathing solution. In the studies of the voltage-dependent channels mediating the inward currents, SrCl_2 (10 mM) was substituted for CaCl_2 to minimize muscle contractions evoked by Ca^{2+} influx. The physiological effects of the slight changes in osmolarity or ionic strength caused by the additional solutes described above were expected to be within experimental variation (Jan and Jan, 1976), and no special effort was made to adjust for these changes. In some experiments (Fig. 7), the K^{+} concentration was greatly increased (from 2 to 30 mM). This was achieved by substitution of KCl for NaCl.

Physiological recordings. The glass microelectrode used for the application of current was filled with 2 M potassium citrate and the voltage recording electrode was filled with 2 to 3 M KCl. The tip resistance of these microelectrodes usually ranged between 20 and 30 megohms, and in many experiments the resistance was reduced to a range between 10 and 15 megohms by bevelling according to methods described previously (Ogden et al., 1978). The current electrode was positioned near the middle of the muscle fiber and the voltage electrode was about 50 to 100 μm apart from it. To minimize capacity coupling between the two electrodes, both electrode assemblies were electrically shielded to expose only the last 1 cm of the microelectrodes and mounted on the micromanipulators such that the angle between the two electrodes was 60° or more.

The voltage clamp circuitry was similar to that used by previous investigators (Connor and Stevens, 1971a), except for the use of a high voltage operational amplifier (model AM-303B, Datel Systems, Inc., Canton, MA) as the source of the applied current. The membrane voltage and the signal from the virtual ground were displayed on a storage oscilloscope (Tektronix D13). Photographs of oscilloscope traces were taken and a chart recorder (Brush model 2200, Gould Inc., Cleveland, OH) was used to keep continuous records of the relatively slow I_K . Measurements requiring a high precision were obtained from the digital readout of a digital oscilloscope equipped with magnetic disks for data storage (model 4094, Nicolet Instrument Corp., Madison, WI), which was also interfaced to produce hard copies of the voltage and current traces on an X-Y plotter.

Previous analysis (Jan and Jan, 1976) showed that these relatively short (about $400 \times 80 \times 25 \mu\text{m}$) larval fibers are virtually isopotential. Calculations based on the short cable model (Weidmann, 1952) using the dimension of the fibers and the maximum active membrane conductance encountered in our experiments indicate that the deviation from isopotentiality was less than 10% throughout the fiber over the range of clamping potential used (−100 to +30 mV).

Relatively low series resistance was expected because larval muscle fibers are free from the thick envelope of connective tissue and are highly accessible to drug agents in the bath solution. The series resistance was estimated by the initial step change in membrane potential preceding the exponential charging phase caused by application of a current step. The value was 54.1 ± 24.2 kilohms or $24.6 \pm 8.9 \text{ ohm} \cdot \text{cm}^2$ (mean $\pm$ SD) for the fibers ($n = 10$) tested. No compensation for the series resistance was attempted because the total membrane current in our study was generally within ± 100 nA. Error in membrane potential measurements due to the series resistance was therefore within the range of 5.4 mV even in the extreme cases when the membrane was strongly depolarized ($V \geq +30$ mV).

Step changes in membrane potential reached the steady level and membrane capacity currents settled within 2 msec under optimal conditions and generally within 5 msec. The membrane voltage control was adequate for the membrane currents reported here as judged by the absence of "notches" in the membrane current traces and the lack of abrupt jumps in the current-voltage relations (Taylor et al., 1960) for the inward current responsible for the regenerative action potential in larval muscles.

All fibers examined had a linear current-voltage relationship over the range −35 to −70 mV. We assumed this to be a leakage conductance the value of which was independent of voltage. The stability of the leakage conductance was checked by hyperpolarizing or weak depolarizing voltage steps applied frequently throughout the course of an experiment. For stronger depolarizations the leakage current was extrapolated by using the calculated leakage conductance and subtracted from the total current to give the net active current.

Anatomical observations. The surface area of the identified fibers was estimated using scanning electron micrographs of mature third instar larvae dissected to expose the ventral longitudinal muscles. Computations of the

surface areas of fibers were estimated according to the method of Jan and Jan (1976). This method neglects membrane invaginations, which would lead to underestimation of the actual cell membrane area but does allow for standardization and comparison of current density between fibers of different sizes. Since individual muscle fibers are readily identifiable and the mature third instar larvae used in these experiments were of rather uniform size, the surface area of each identified fiber was estimated without repeated individual measurements.

Results

Normal membrane currents

Passive membrane properties. The passive membrane properties of normal larval muscle fibers were determined under voltage clamp conditions, and the results are summarized in Table I. The specific membrane resistivity (R_m) was estimated by the leakage conductance adjusted for the surface area of the muscle fiber. Among 214 normal fibers included in this report, the mean R_m was 2.03 kilohm·cm² and the maximum R_m was 5.6 kilohm·cm². The distribution was skewed to the lower values, which is attributable to the increased leakage introduced by microelectrode penetration.

To estimate the specific membrane capacitance (C_m), a triangular wave was used as the voltage command signal (Serrano, 1982). The membrane capacitance current I_c is determined by

$$I_c = c_m \frac{dV}{dt} \quad (1)$$

where C_m is the membrane capacitance and V is the membrane potential. The change in slope in the triangular waveform should induce a jump in I_c and, hence, in the total membrane current of an absolute value of $2c_m \frac{dV}{dt}$, where $\frac{dV}{dt}$ represents the slope of the rising phase of the triangular wave. The average value of C_m computed from the cell surface area and c_m thus determined was 7.1 $\mu\text{F}/\text{cm}^2$. Our estimates of C_m and R_m are in general agreement with the previously reported values determined under current clamp conditions (Jan and Jan, 1976).

Inward currents. Previous studies (Suzuki and Kano, 1977) suggest that Ca^{2+} channels mediate the inward current in *Drosophila* larval muscles. Direct measurements of Ca^{2+} currents were difficult in these supercontracting muscles (Osborne, 1967) because the Ca^{2+} influx elicited strong contractions which immediately damaged the doubly impaled muscle fibers. When Sr^{2+} (10 mM) was substituted for Ca^{2+} , this problem was minimized. Thus, as an initial characterization of the Ca^{2+} channels in *Drosophila* larval muscles, we measured the voltage dependency of Sr^{2+} current, as has been studied previously in other preparations (Hagiwara, 1973; Hagiwara and Byerly, 1981).

Step depolarization from a holding potential (V_H) of -50 mV to a level above -30 mV elicited an inward current which peaked rapidly and then decayed to a steady-state value. Both the rise and decay of the inward current became more rapid with increasing amplitude of depolarization steps. Pooled current-voltage (I-V) relations from four different fibers indicate that the net inward current reached a maximum at about -5 mV with a peak inward current density of $47.5 \pm 26 \mu\text{A}/\text{cm}^2$ (mean $\pm$ SD).

TABLE I

Passive membrane properties of normal and *Sh* larval muscle fibers

The mean and SD of the specific membrane resistance (R_m) and the specific membrane capacitance (C_m) are shown. The number of fibers used to determine each value is given in parentheses.

	R_m	C_m
	kilohm·cm ²	$\mu\text{F}/\text{cm}^2$
Normal	2.03 ± 1.1 (214)	7.1 ± 2.3 (9)
<i>Sh</i>	1.91 ± 1.1 (129)	8.8 ± 2.7 (14)

Outward currents. We have studied the outward currents in greater detail because several mutations in *Drosophila* are known to affect the outward K^+ currents in larval muscles (Wu et al, 1983a; Wu and Ganetzky, 1984). Elimination of the inward Ca^{2+} current facilitated the analysis of outward K^+ currents and could be achieved by the use of saline containing 0 mM Ca^{2+} and 0.5 mM EGTA (see "Materials and Methods"). Muscular contractions also ceased within a few seconds after substitution of physiological saline by the Ca^{2+} -free saline. In this saline, the preparation could be maintained in good condition for at least 2 hr at 5 to 15°C, as evidenced by a negligible rundown of the active currents.

The lower traces in Figure 1A represent the currents recorded in response to step depolarizations (Fig. 1A, upper traces) from a holding potential of $V_H = -50$ mV in the Ca^{2+} -free saline at 6°C. Depolarization above -30 mV elicited a transient outward current which rose to a peak and then inactivated. With stronger depolarization, a delayed component also appeared. This component rose more slowly to a plateau without evidence of inactivation for these short pulses (≤ 420 msec).

Figure 1B shows the I-V relations of the outward currents at the peak of the transient and the end of depolarizing pulse at 6°C for the same cell of Figure 1A. The I-V plot reflects the difference in the voltage dependence of the two components. The transient component was activated at less depolarized levels as compared with the delayed component. Similar I-V relations of the outward currents have been observed at all other temperatures examined (refer to Figs. 5 and 11 for data of 15°C).

Since the transient current decayed rapidly during depolarizing pulses, we examined the currents in the same cell with $V_H = -25$ mV and obtained results strikingly different from those with $V_H = -50$ mV. The transient phase was virtually completely inactivated (Fig. 1C).

Thus, there appear to be two distinct outward currents which exhibit different voltage sensitivities and kinetic properties. The characteristics of these two components are similar to the K^+ currents I_A and I_K described in molluscan neurons (Connor and Stevens, 1971a, b) as well as in adult *Drosophila* flight muscles (Salkoff and Wyman, 1983). Indeed, both of these components were sensitive to the K^+ channel blocker TEA. Within seconds after adding 10 mM TEA to the bath, the transient component declined to 33% and the delayed component declined to 30% of control values. Hereafter we will refer to the transient outward current as I_A and the delayed outward current as I_K .

The I-V relations of outward currents with $V_H = -25$ mV (Fig. 1D) confirms that the remaining active currents have properties expected for I_K . The steady-state currents are similar to those with $V_H = -50$ mV (cf. solid symbols in Fig. 1, B and D), whereas at the time that peak I_A is observed with $V_H = -50$ mV, only the rise of I_K is detected (cf. open symbols in Fig. 1, B and D). Therefore, I_A and I_K are indeed separable components. (The amplitude of the I_K plateau in Fig. 1D is smaller due to a partial inactivation of I_K at $V_H = -25$ mV; see Fig. 10.)

Activation and inactivation of I_A . It has been suggested that changes in voltage dependence of I_A inactivation occur in certain *eag* (Wu and Ganetzky, 1984) and *Sh* (see below) mutants. We thus characterized the normal steady-state inactivation of I_A over a wide range of holding potentials, V_H .

Transient outward current (I_A) was evoked at various holding potentials (V_H) by test pulses to either -10 or +10 mV (cf. Fig. 12A for the experimental paradigm). The peak I_A was normalized and plotted as a function of V_H . Figure 2 represents the pooled data from several fibers of different larvae illustrating the steady-state inactivation at 10 to 15°C. It can be seen that, irrespective of the amplitude of test pulses (to -10 mV, Fig. 2, open symbols, or to +10 mV, Fig. 2, solid symbols), the data can be described by the empirical function (continuous curve in Fig. 2):

$$h_A = 1 / \left[1 + \exp\left(\frac{V + 40}{4.5}\right) \right] \quad (2)$$

Figure 1. Membrane currents recorded from a normal fiber at 6°C. Inward currents were eliminated by using Ca^{2+} -free saline in experiments shown in this and all subsequent figures. *A*, Superimposed traces of total membrane currents (lower part) elicited by various voltage steps (upper part) from a holding potential (V_H) of -50 mV. Upward deflections indicate depolarizations or outward currents. Note that depolarizing steps positive to -30 mV elicited a transient outward current (I_A) which inactivated rapidly. Depolarizing steps to more positive levels activated a delayed but sustained component (I_K) in addition to the transient I_A . See the text for the nomenclature. *B*, Current-voltage (I - V) relation at $V_H = -50$ mV. After correction for linear leakage currents, the amplitude of active currents at the peak of I_A (○) and the end of the depolarizing pulse (●) are plotted against the membrane potential. *C*, Superimposed traces of total membrane currents and voltage steps from $V_H = -25$ mV. Note that the transient I_A is absent. *D*, I - V relation for the remaining active currents at $V_H = -25$ mV. At 20 msec after the onset of depolarizing pulses (near the peak of I_A in *A*), only the rise of I_K was detected (○). The measurements at the end of pulses (●) represent steady-state I_K .

where h_A represents the fraction of the unactivated I_A and V is the potential (in millivolts) at which the membrane is held. This equation is of a form similar to that used by Hodgkin and Huxley (1952b) for their description of Na inactivation, which has been subsequently used to quantify inactivation of I_A in other preparations (Neher, 1971; Hagiwara et al., 1981). Equation 2 specifies the 50% level of I_A inactivation, i.e., -40 mV, and the steepness of its voltage dependence about this midpoint, e.g., a positive or negative deviation of 4.5 mV from -40 mV will change the level of inactivation to $1/(1 + e)$ or $1/(1 + e^{-1})$, respectively. It should be noted that the larval muscle fibers normally have a resting potential of about -50 mV (Jan and Jan, 1976; C.-F. Wu, unpublished data). This would correspond to a resting inactivation of I_A of approximately 15%.

To characterize the kinetic properties of I_A , we examined the time course of I_A activation and inactivation following step depolarizations. From the appearance of the traces shown in Figure 1*A*, the qualitative conclusion may be drawn that both activation and inactivation (the rising phase and falling phase) of I_A occur more rapidly with stronger depolarizations. At 6°C the time to peak I_A was 35 msec at -15 mV, 25 msec at $+5$ mV, and 18 msec at $+25$ mV.

We limited our analysis of the kinetics of I_A inactivation to depolarizations below the level where the rise of I_K significantly overlaps the decay of I_A . Traces similar to those with depolarizing pulses < -5 mV shown in Figure 1*A* were analyzed in semilogarithmic plots.

The initial phase of I_A decay can be approximated by first-order kinetics, but the later phase deviates from a first-order decay. The deviation became more pronounced with stronger depolarizations. This slower component may be accounted for by the slow rise of a small but detectable I_K at these depolarized levels (cf. Figs. 1 and 6).

The time constant of the initial decay, τ_A , obtained by this method is shown as a function of voltage in Figure 3*A*, which allows the quantitative comparison with mutant data (cf. Fig. 3*B*). It is evident that I_A decays more rapidly with stronger depolarizations. Data from three different temperature ranges indicate that τ_A becomes faster at higher temperature with a Q_{10} of about 2 over the range of 5 to 15°C (Fig. 3*A*).

Time course of I_A recovery from inactivation. To gain knowledge about the reversibility of I_A inactivation, we investigated the time course of I_A recovery at $V = -50$ mV, the normal resting potential, from inactivation induced by a conditioning pulse to -10 mV. The membrane was first depolarized to -10 mV and then repolarized to -50 mV for various intervals before a brief test pulse (to -10 mV) was applied. The inset of Figure 4 shows results obtained from two such experiments at 15°C. It can be seen that the I_A was rapidly inactivated by the conditioning pulses and that I_A recovery (at -50 mV) is a much slower process.

Figure 4 represents pooled data at 15°C from a number of cells.

Note that the peak I_A was normalized to allow comparison. The recovery time course could not be fitted by a simple first-order kinetics. The continuous lines in Figure 4 were drawn according to the sum of two exponentials

$$Y = A(1 - e^{-t/\tau_1}) + B(1 - e^{-t/\tau_2}) \quad (3)$$

where $\tau_1 = 75$ msec and $\tau_2 = 900$ msec and A and B are proportionality constants, which vary with the duration of conditioning pulses. Thus, the observed time course may reflect two different

Figure 2. Effect of V_H on the amplitude of I_A in normal fibers. Pooled data are shown from four fibers with test pulses to -10 mV (open symbols) and five fibers to $+10$ mV (solid symbols), 11.5 to 15°C (cf. Fig. 12A for examples of experimental records). Peak amplitudes of I_A are normalized and plotted as a function of V_H . The continuous curve is calculated from equation 2 in the text.

steps in the reversible process of inactivation. The recovery from inactivation was more rapid with shorter conditioning pulses. The proportionality of the two components was $A = 0.80$ and $B = 0.20$ for 50 msec conditioning duration and $A = 0.45$ and $B = 0.55$ for 350 msec duration.

Isolation of I_K in *Sh* mutants

Passive membrane properties in *Sh* fibers. The passive membrane properties of larval muscle fibers of the *Sh* mutants were studied by the voltage clamp methods described above. Results obtained from the five different *Sh* alleles (see "Materials and Methods") showed no significant differences and data were combined and summarized in Table I. The average value of specific membrane resistance, R_m , derived from the leakage conductance was 1.91 kilohm $\cdot$ cm^2 . As in normal fibers, the distribution was skewed toward lower values and the apparent upper limit was 4.9 kilohm $\cdot$ cm^2 . The specific membrane capacitance, C_m , was 8.8 $\mu\text{F}/\text{cm}^2$. These values are in close agreement with those of normal fibers, indicating no abnormality in the passive properties of *Sh* muscle fibers.

Inward current in *Sh* muscle fibers. It is important to determine whether *Sh* mutations specifically affect the outward current and leave the inward current intact. As in normal larvae, the inward current in *Sh* larvae was measured in Ca^{2+} -free saline containing 10 mM Sr^{2+} . Results similar to those of normal fibers were obtained. The I - V relation of the inward current and its time course appeared normal. The voltage at which inward currents were first detected (above -30 mV) and peak amplitude of the inward Sr^{2+} current (46.5 ± 25 $\mu\text{A}/\text{cm}^2$, mean $\pm$ SD, at -5 mV, $n = 4$) were not significantly different from normal (47.5 ± 26 $\mu\text{A}/\text{cm}^2$).

Outward currents in *Sh* muscle fibers. The outward currents in *Sh* muscle fibers were studied in the same types of experiments described above for normal fibers. Among the five alleles examined, *Sh*^{K5133}, *Sh*^M, and *Sh*¹⁰² apparently caused a loss of the transient outward current I_A in the larval muscle membrane (Fig. 5). In contrast, I_A was not completely abolished in the larval muscle of the other two alleles, *Sh*⁵ and *Sh*^{K0120}, suggesting a difference in the nature of the

Figure 3. Kinetics of I_A inactivation. A, Normal fibers. The time constant τ_A of the decay phase of I_A elicited by voltage steps to various potentials from $V_H = -50$ mV was determined (see the text). The plot shows τ_A as a function of voltage. Pooled data from different cells (represented by individual symbols) at different temperature ranges were used: 6 to 8°C (crosses), 10 to 12°C (solid symbols), and 14 to 15°C (open symbols). B, Pooled data from five *Sh*^{K0120} fibers at 15°C . Results are similar to those of normal fibers at this temperature.

Figure 4. Time course of I_A recovery from inactivation in normal fibers. Inactivation of I_A was induced by a conditioning pulse depolarizing the membrane from $V_H = -50$ mV to -10 mV for the durations of 350 msec (open symbols) and 50 msec (solid symbols). To monitor the recovery of I_A upon repolarization, a test pulse to -10 mV was applied at variable time intervals following the end of the conditioning pulse. Examples of superimposed active current traces are shown in the inset. The graphs illustrate the fraction of I_A recovery from inactivation as a function of duration of repolarization to V_H . Pooled data from different fibers at 15°C are represented by individual symbols. Continuous curves are derived from summation of two exponentials with time constants of 75 and 900 msec of different proportions. See equation 3 in the text.

mutations. A more detailed analysis of these two alleles will be presented later (see "Altered I_A properties in *Sh* alleles").

The traces in Figure 5 represent total membrane currents recorded from Sh^M , Sh^{102} , and Sh^{KS133} and normal fibers elicited by step depolarizations from a V_H of -50 mV. Variation in the amplitude of total membrane currents at the plateau of I_K reflects differences in fiber sizes (cf. Figs. 5 and 11). After adjustments for leakage currents and fiber surface area (see "Materials and Methods"), the density of the remaining active currents measured from a number of cells of the various *Sh* alleles is plotted as a function of voltage (Fig. 6). In the aforementioned three alleles, I_A was entirely missing whereas the steady-state amplitude of the delayed outward current I_K was not significantly different from normal (Fig. 6A).

We have studied the remaining I_K in greater detail for two reasons. First, normality of other properties of the I_K in various *Sh* mutants provides a stringent proof for the hypothesis that I_A and I_K channels are genetically distinct and separable in larval muscles. Second, if the remaining I_K is normal, then the *Sh* larval muscle fibers will be a favorable preparation for quantitatively describing normal I_K in isolation from I_A . Such a description provides a basis for comparison with other mutations reported to result in defective I_K (Wu et al, 1983a; Wu and Ganetzky, 1984). The above considerations prompted us to examine I_K further in the *Sh* larval muscle, comparing their kinetic and steady-state properties with those obtained in normal fibers.

Instantaneous I-V relation and reversal potential of I_K . It is important to test whether the reversal potential for I_K is consistent with the hypothesis that K^+ is the major charge carrier through the channels. It is also of interest to examine whether voltage-dependent conductance change mediating the current has a linear instantaneous I-V relationship (Hodgkin and Huxley, 1952a).

Figure 7A shows the current tails obtained from a normal and a *Sh* fiber upon repolarizations to various potentials following a 200-msec conditioning depolarization to $+10$ mV in saline containing 2

mm KCl. The current tails decayed more rapidly with stronger repolarizations and became inward at certain levels of hyperpolarizations. The reversal potential and the instantaneous I-V relationships can be estimated from a family of I_K tail currents. The speed of our voltage clamp system allowed measurements of tail currents 4 msec after the initiation of the repolarizing pulse. Estimation of an instantaneous I-V relation relied on extrapolation of the tail current. This procedure probably introduced little error because the time constant of the tail currents was generally much greater than 4 msec (cf. Fig. 9) and the extrapolated values at 0 msec were not substantially different from those at 4 msec. The instantaneous I-V relations for a normal fiber and a Sh^{102} fiber in saline containing 2 mm KCl are compared in Figure 7B (solid symbols). Results from these cells and from 32 normal and 30 *Sh* cells indicate that I_K channels exhibit rectification. For hyperpolarizations, inward current was much less than would have been predicted from a linear relationship.

The reversal potential V_K was determined in two ways, as the repolarization voltage at which the I_K tail current reversed direction, and as the voltage at which the I-V relation and the instantaneous I-V relation intersected. We found that the two methods gave the same value for V_K . For all normal cells examined, $V_K = -58.6 \pm 9.0$ mV (mean $\pm$ SD, $n = 33$) in the Ca^{2+} -free saline containing 2 mm KCl. Among all *Sh* alleles, including Sh^{KS133} , Sh^M , and Sh^{102} in which I_A was completely absent, the reversal potential $V_K = -56.8 \pm 11.5$ mV (mean $\pm$ SD, $n = 31$ fibers) closely resembled that determined in normal fibers. Thus the $[\text{K}^+]$ gradient for *Sh* fibers appears to be normal.

The above reversal potential measurements lend support to the idea that K^+ is the principle charge carrier for I_K . This notion is further confirmed by the observation of shifts in the reversal potential caused by changes in external $[\text{K}^+]$. As shown in Figure 7B (open symbols), an increase in $[\text{K}^+]$ from 2 to 30 mm by substitution of external K^+ for Na^+ shifted the V_K to a more positive value (in 10 fibers, -30.0 ± 3.2 mV, mean $\pm$ SD). However, the magnitude of the shift is

Figure 5. Total membrane currents recorded from normal, Sh^{KS133} , Sh^M , and Sh^{102} muscle fibers. $V_H = -50$ mV, 15°C . Note that, in these *Shaker* mutants, the transient I_A is not observed.

smaller than that which would be predicted by the Nernst equation for a perfect K^+ electrode (a predicted shift of 66 mV in contrast to the observed value of about 28 mV at 10°C). This suggests that, as in other preparations (Hille, 1973; Yamamoto et al., 1981), I_K channels in *Drosophila* larval muscles are also slightly permeable to other ionic species.

The instantaneous I-V relation determined in saline containing higher $[\text{K}^+]$ (30 mM) remains nonlinear (Fig. 7B, open symbols). Therefore, the observed rectification property of I_K channels cannot be fully accounted for by a steep concentration gradient for the charge carrier, K^+ .

Voltage dependency of conductance associated with I_K . Once V_K is determined, the "steady-state" conductance (g_K) associated with I_K at various voltages can be defined according to the equation

$$I_{K_{\max}} = g_K(V - V_K) \quad (4)$$

where $I_{K_{\max}}$ is the maximum or "steady-state" current density driven by the potential gradient ($V - V_K$) at the membrane potential, V .

Strictly speaking, this is an operational definition for g_K , which represents the conversion factor between $I_{K_{\max}}$ and $(V - V_K)$ measurements. Converting these measurements into permeability (Dodge and Frankenhaeuser, 1959) was not pursued because, without additional assumptions, the present definition of g_K provides a convenient means for comparison among fibers from different mu-

tants. Furthermore, this definition of g_K is also appropriate for our purpose because the instantaneous I-V relation for I_K is essentially linear over the voltage region of interest (-30 to $+30$ mV; cf. Fig. 7B).

Results from a number of normal fibers examined at different V_H and temperatures were first analyzed. Calculated values of g_K were normalized and plotted as a function of the membrane potential V in the semilogarithmic graph shown in Figure 8A. The continuous line in the figure represents the initial slope of e-fold increase in g_K per 6 mV, similar to the value found in squid axons (Hodgkin and Huxley, 1952a). It is evident that, within the range tested, the conductance approaches a maximum value for strong depolarizations (≥ 30 mV), and that the initial slope is not markedly altered by changes in temperature (6 to 21°C) or in holding potential (-30 to -50 mV) (Fig. 8A).

We subsequently analyzed the voltage dependency of g_K for each of the alleles of *Sh*. In Figure 8B, representative data from various alleles are normalized and compared. As in normal fibers, g_K increases with the strength of depolarization and approaches a maximum value for depolarizations exceeding $+30$ mV. For direct comparisons between alleles, most fibers were examined at a temperature of 13 to 15°C . No significant difference among fibers of all five mutants and the normal muscle could be detected. The line shown in Figure 8B represents an initial slope (e-fold increase in g_K per 6 mV) identical to that drawn to fit the data for normal (Fig. 8A).

Figure 6. Pooled I-V relations for normal (●, 26 fibers), Sh^{KS133} (○, 12 fibers), Sh^M (■, 5 fibers), Sh^5 (□, 9 fibers), and Sh^{K0120} (△, 18 fibers) at $V_H = -50$ mV, 14 to 15°C. Each data point represents the current density (mean $\pm$ SEM) after adjustment for leakage current and surface area. A, Active outward currents measured at the steady-state of I_k . B, Active outward currents measured at the time of peak of I_A . I_A is absent in Sh^{KS133} , Sh^M , and Sh^{102} fibers, and only small active currents are present at more depolarized levels due to the rapid rise of I_k (only Sh^{KS133} is represented in this I-V plot). In Sh^{K0120} cells, the current density of I_A is not decreased. In Sh^5 , I_A is reduced and is activated only at more depolarized levels.

To illustrate the effect of temperature, data from one fiber (Sh^{KS133}), at 6°C, have been included as open circles in Figure 8B. The voltage dependency was not markedly altered or shifted by the reduced temperature. Neither was it affected by changing the holding potential to $V_H = -30$ mV (Fig. 8B, solid squares). Although the absolute value of g_k was reduced at this holding potential due to inactivation (cf. Fig. 10), following normalization, the general form of the voltage dependency was not obviously altered in a number of normal and mutant cells examined.

Kinetics of I_k tail currents. The above results indicate that the steady-state properties of I_k activation in *Sh* are normal. The relaxation process of I_k activation was studied by analyzing the tail current kinetics of I_k in both *Sh* and normal fibers. Under the assumption that I_k is mediated by a homogeneous species of channels, any substantial change in the time course of the tail current decay reflects an alteration in the rates of conversion between open and closed states of I_k channels.

The same families of tail currents obtained with the experimental paradigm used to estimate V_k (e.g., Fig. 7A) were analyzed. Repolarizing steps to a voltage above V_k from a depolarization of about +10 mV produced current tails which were outward and declined over tens of milliseconds. Upon hyperpolarization to a level more negative than V_k , the tail current turned inward but became smaller, due to the apparent rectification, and its decay followed a more rapid time course.

The time course of current tails in semilogarithmic plots were nonlinear, composed of a rapid initial decay and a slower second phase. However, the slow phase constituted the major part (>70%) of the total tail current and could be approximated by exponential decay with a single time constant.

The time constant (τ_s) chosen to fit the slow phase is plotted as a function of voltage. Pooled data of τ_s from a number of normal and *Sh* cells at three different temperatures are summarized in Figure 9. The τ_s shows a strong voltage dependence, decreasing at more negative membrane potentials. Figure 9 also illustrates that τ_s increased as the temperature was decreased. For example, at -15 mV, τ_s was about 45 msec at 15°C and about 120 msec at 6°C. This represents a Q_{10} of about 3 over this temperature range.

Because of its more rapid kinetics and smaller amplitude, the time course of the initial fast decay could be reliably resolved only at low temperatures. Analysis of this component at 6°C revealed a process about 4 times faster than the slower major component in the tail current.

The families of data obtained from normal and *Sh* fibers are comparable in all aspects. The above results and the measurements of instantaneous I-V relations (Fig. 7) also imply that the tail currents in normal fibers reported here represent the true relaxation process of I_k . At the plateau of I_k , I_A is inactivated and does not produce a detectable tail current upon abrupt membrane repolarization.

Inactivation of I_k . As described above, I_k , unlike I_A , did not appear to inactivate during short depolarizing pulses. However, for prolonged depolarizations above -10 mV, a slow decay of I_k over many seconds could be observed. Brief hyperpolarizing pulses of less than a second completely reversed the effect of prolonged depolarization.

Inactivation of I_k also occurs at potentials between -10 and -50 mV, where I_k activation is minimal. Inactivation over this range may be demonstrated indirectly by an increase in current induced by a test pulse following a hyperpolarizing conditioning pulse (Fig. 10A). For various holding potentials, a brief (200 msec) test pulse was first applied to measure the attainable I_k . Interposed between this and a second identical test pulse was a period of hyperpolarization with an amplitude and duration sufficient to remove inactivation completely. After adjustment for leakage currents, the increment in I_k evoked by the second test pulse was assumed to be I_k recovering from inactivation. The ratio, h_k , between the I_k evoked by the first and the second test pulse represents the fraction of I_k which was not inactivated at the particular holding potential, V_H .

In a large number of experiments, h_k was determined as a function of holding potentials in both normal and *Sh* muscle fibers (Fig. 10B). The results show that the fraction of I_k attainable, or h_k , varied with the holding potential, ranging from 1.0 at -50 mV (no inactivation) to 0.2 at -10 mV (80% inactivation). Figure 10B suggests that the steady-state I_k inactivation in both *Sh* and normal larval muscles shows a similar voltage dependency. The continuous curve is given by the equation

$$h_k = 1 / \left[1 + \exp \left(\frac{V + 20}{8} \right) \right] \quad (5)$$

where V is the potential in mV at which the membrane is held. This equation is similar to the one used to fit h_A for I_A inactivation (equation 2), with the difference that I_A inactivation occurs at more hyperpolarized levels (-40 instead of -20 mV for 50% inactivation) and shows a steeper voltage dependency.

Figure 7. Reversal potential and instantaneous I-V relations of I_K in normal and *Sh* fibers. A, Examples of current tails associated with abrupt repolarization to different potentials after 200 msec depolarization to +10 mV in standard saline (2 mM K^+). $V_H = -50$ mV, 10°C. The superimposed voltage records of the normal fiber are shown to indicate the pulse paradigm. B, Instantaneous I-V relations in standard saline and in high K^+ saline (30 mM). Data from normal (●), *Sh*¹⁰² (■), and *Sh*^{K5133} (○) fibers are plotted after subtraction of the leakage currents. Note that the nonlinearity in the instantaneous I-V relation is apparent at both $[K^+]$. The reversal potential (V_K) for each case is indicated by an arrow.

The data of Figure 10B also indicate that the voltage dependence of the steady-state I_K inactivation did not vary significantly within the temperature range used (9 to 21°C). Although the kinetics of inactivation slowed down at lower temperatures, similar steady-state levels of inactivation were reached.

The time course of inactivation of I_K at various V_H was determined. Upon returning to a V_H in question after a duration of hyperpolarization to remove all I_K inactivation, a train of brief test pulses was applied. The currents elicited by the successive test pulses declined to a steady-state value. The time course of inactivation followed a simple first-order kinetics. Normal and *Sh* muscle fibers were analyzed in this fashion and both yielded similar results. Inactivation of I_K above -10 mV was directly monitored by the decay of I_K during prolonged depolarization. The inactivation of I_K is voltage dependent, exhibiting faster kinetics at more depolarized levels. The time constant of inactivation at 15°C decreased from roughly 30 to 5 sec when the membrane depolarization increased from -40 to +10 mV.

The kinetics of recovery from inactivation were measured by two identical test pulses separated by a variable duration of hyperpolarization (cf. Fig. 10A). I_K recovered more rapidly with stronger hyperpolarizations. The recovery process can be approximated by first-order kinetics with time constants of 100, 325, and 700 msec at hyperpolarizing potentials of -95 mV, -62 mV, and -55 mV, respectively.

Altered I_A properties in *Sh* alleles

Analysis of *Sh*^{K0120}. As mentioned above, I_A in larval muscles is completely abolished in three different alleles, *Sh*^{K5133}, *Sh*^M, and *Sh*¹⁰², but not in two other alleles, *Sh*⁵ and *Sh*^{K0120}. It is of interest to examine the residual I_A in *Sh*⁵ and *Sh*^{K0120} for possible alteration in the function of I_A channels.

At the larval neuromuscular junction, *Sh*⁵ exhibits less enhanced synaptic transmission than do *Sh*^{K5133} (Jan et al., 1977), *Sh*^M, and *Sh*¹⁰² (C.-F. Wu and B. Ganetzky, unpublished observation). Neuromuscular transmission in *Sh*^{K0120} larvae is, in contrast, elevated to a degree similar to that found in *Sh*^{K5133} (Ganetzky and Wu, 1983). Recombination of either of these two alleles with alleles of *eag*, a second gene found to affect potassium currents (Wu et al., 1983a), results in the same extreme phenotype of prolonged transmitter release lasting for hundreds of milliseconds (see "Materials and Methods" and Ganetzky and Wu, 1983, for a review of *Sh*^{K0120} stocks). Therefore, it is reasonable to expect *Sh*^{K0120} larvae to have a defect in I_A more severe than in *Sh*⁵ larvae (see below) and nearly as extreme as that in *Sh*^{K5133} larvae. However, we consistently observed that I_A in *Sh*^{K0120} was large and qualitatively similar to that in normal larval muscles.

The outward currents recorded from a *Sh*^{K0120} fiber are shown in Figure 11A. Results from 18 fibers examined are summarized in

Figure 8. Voltage dependence of the conductance mediating I_K . After determination of V_K for each cell, maximum conductances at different voltages are calculated according to equation 4 in the text and plotted in relative scale. A, Normal fibers. Data are from different fibers at $V_H = -50$ mV (open symbols) or -30 mV (solid symbols) at temperature ranges of 6 to 8°C (circles), 15°C (squares and triangles), and 21°C (diamonds). The continuous line represents an e-fold increase in g_K per 6 mV. B, *Sh* fibers. Data are from *Sh*^{K0133} at 6°C (open circles), *Sh*^M at 15°C (squares and diamonds), *Sh*^{K0120} at 15°C (open triangles), and *Sh*⁵ at 13 to 15°C (crosses). $V_H = -50$ mV for all fibers except one *Sh*^M fiber (solid squares) where $V_H = -30$ mV. The continuous line (e-fold increase in g_K per 6 mV) is identical to that shown in A.

Figure 6 and are compared with data obtained from fibers of other *Sh* alleles and normal larvae. Activation of I_A in *Sh*^{K0120} occurred at normal potential levels (more negative than I_K). The I-V relations are not significantly different from normal at the plateau of I_K (Fig. 6A) and the peak of I_A (Fig. 6B). Furthermore, no obvious change in the activation kinetics of I_A was detected. The time to peak I_A upon step depolarization to -15 mV was about 9 msec at 15°C, as compared to about 9 msec in normal fibers. Thus, it is important to inspect more closely other possible differences in the properties of I_A in *Sh*^{K0120} larvae. Experiments similar to those described above for normal I_A were performed on *Sh*^{K0120} fibers.

The steady-state level of inactivation was determined by the amplitude of I_A activated upon step depolarization to $+10$ mV from various holding potentials (Fig. 12A). In Figure 12B, the peak I_A normalized to the maximum attainable value in *Sh*^{K0120} fibers (Fig. 12B, open triangles) is plotted against the holding potential and

compared to the normal data (Fig. 12B, solid circles). The continuous curve in Figure 12B is drawn according to equation 2, which was previously used to fit the normal data (Fig. 2). A slight but statistically significant change in the level of inactivation is indicated (Fig. 12B).

The time course of I_A decay from its peak value at different depolarization levels was analyzed by using semilogarithmic plots. As in normal fibers, the decay is composed of an initial major component, which can be fitted by first-order kinetics, and a slower but smaller component, which is attributable to the slight activation of I_K . The time constants of the major component determined at various potentials in a number of fibers at 15°C are shown in Figure 3B. The values exhibit a voltage dependence resembling that found in normal fibers (cf. 15°C data of Fig. 3A).

Figure 13 shows the time course of recovery of I_A (at -50 mV) from inactivation induced by a 50-msec depolarizing pulse to -10 mV. The data can be satisfactorily described by the same kinetic function (continuous curve in Fig. 13) used to fit normal data (Fig. 4, equation 3).

Except for a slight change in the voltage dependence of I_A inactivation (Fig. 12B), our present investigation failed to reveal any clear qualitative change in the properties of I_A in *Sh*^{K0120} muscle fibers despite the fact that the abnormal neuromuscular transmission in this mutant is as extreme as that found in *Sh*^{K0133} (see "Discussion").

Analysis of *Sh*⁵. The *Sh*⁵ larvae exhibit only a mild increase in neuromuscular transmission. Ether-anesthetized *Sh*⁵ adults display a characteristic scissoring of the wings (Kaplan and Trout, 1969) in addition to the leg-shaking behavior associated with all *Sh* alleles. It has been reported that in developing adult flight muscles of *Sh*⁵, activation of I_A is normal but inactivation of I_A follows abnormally rapid kinetics (Salkoff, 1983b).

Surprisingly, contrasting results were obtained from *Sh*⁵ larval muscles demonstrating that I_A is reduced in amplitude and altered in voltage sensitivity. Among cells from different larvae, or even within the same larva, the extent of decrease in I_A was variable. Its appearance varied from barely detectable shoulders (Fig. 11B) to more defined peaks (Fig. 12A) riding on the rising phase of I_K .

The diminished I_A in *Sh*⁵ muscles is a result of a shift in the voltage dependence of activation to more positive levels but not a consequence of an altered reversal potential or excessive inactivation.

Figure 9. Voltage and temperature dependence of the time constant τ_s of the major component of I_K tails. See the text for details. Time constants at 6°C (*Sh*^{K0133}: $\circ$; normal: $\square$), 9 to 11°C (*Sh*⁵: $\blacktriangle$; normal: $\blacksquare$, $\blacklozenge$), and 15°C (*Sh*^M: $\triangle$; normal: $\diamond$) are plotted as a function of membrane potential.

Figure 10. Voltage dependence of the steady-state inactivation of I_K . A, Total membrane currents elicited from $V_H = -25$ mV by test pulses to 20 mV before and after a hyperpolarizing interval sufficient to remove inactivation. Sh^{rKO120} , 15°C. B, Pooled data from normal fibers at 9 to 11°C (■, ●), 15°C (▲), and 21°C (◆), Sh^M at 15°C (□, ○); Sh^{KS133} at 15°C (△); Sh^{rKO120} at 15°C (◇); and Sh^5 at 10°C (+) and 15°C (x). Maximum amplitudes of I_K are normalized and plotted as a function of V_H . The continuous curve is calculated from equation 5. See the text.

Figure 11. Total membrane currents recorded from Sh^{rKO120} , Sh^5 , and normal fibers at 15°C. A, Sh^{rKO120} , $V_H = -50$ mV. Note the presence of the transient I_A and its amplitude relative to I_K (cf. Figs. 5A and 6). B, Sh^5 , $V_H = -50$ mV. Note that I_A is reduced in amplitude and activated only at the same depolarization levels as I_K (cf. Fig. 6). C, Sh^5 , $V_H = -30$ mV. Insufficient inactivation of I_A is evident in this fiber with larger residual I_A (see the text). D, Normal, $V_H = -30$ mV. I_A is nearly completely inactivated at this V_H (cf. Fig. 2). This fiber was smaller in size than the fiber used in C and had less leakage and active currents. Total membrane currents are shown in A and B; active currents (after adjustment for leakage) are presented in C and D.

Figure 12. Voltage dependence of steady-state inactivation of I_A in Sh^{rKO120} and Sh^5 as compared to normal at 15°C. *A*, Membrane currents elicited by voltage steps to +10 mV from the different holding potentials (V_H) indicated. Note that the rise of I_A delineates the base line. The superimposed voltage records of the normal fiber are shown to indicate the pulse paradigm. *B*, Pooled data from 11 Sh^{rKO120} (Δ) and 11 Sh^5 ($\square$) fibers are compared to data from 14 normal fibers ($\circ$). Peak amplitudes of I_A are normalized and the mean and SEM are presented. The continuous curve (equation 2) fit to normal data is identical to that shown in Figure 2. All data were obtained by using the procedure shown in *A*.

Figure 13. Time course of I_A recovery from inactivation in Sh^{rKO120} . The membrane was depolarized from $V_H = -50$ mV by a 50-msec conditioning pulse to -10 mV and then repolarized to V_H . The recovery of I_A was tested by a second identical test pulse after various time intervals following the repolarization. An example of superimposed current traces is shown in the inset. Each symbol represents data from one of four fibers at 15°C. The continuous curve is identical to the one used to fit normal data in Figure 4.

Examination of the tail currents of I_A in Sh^5 did not reveal a substantial change in the reversal potential (about -50 mV in normal fibers) to account for the reduced amplitude of I_A . Changing the holding potential ($V_H = -50$ mV) to more negative voltages ($V_H = -60$ and -70 mV) did not significantly increase the amplitude of I_A . Nevertheless, in all 47 Sh^5 cells studied, I_A was no longer activated at more negative potentials but was only detectable at the same depolarizing potentials which activated I_K (Fig. 11, B and C).

Statistics from Sh^5 cells at $V_H = -50$ mV are compared with those of normal and other Sh alleles in the I-V relations shown in Figure 6. It can be seen that there is no significant difference in the I-V relations at the plateau of I_K (Fig. 6A), whereas the I-V relations at the peak of I_A is clearly abnormal in Sh^5 (Fig. 6B). Alteration in the voltage dependence of I_A activation in Sh^5 becomes even more evident when the I-V relations at the peak of I_A are corrected for the contributions from I_K activation at this time (see data of Sh^{KS133} in Fig. 6B).

In view of the change in the voltage dependence of I_A activation, it is of importance to investigate I_A inactivation in Sh^5 cells for clues of a relationship between these two voltage-sensitive processes. The voltage dependence of I_A inactivation is in fact also shifted to more positive levels, as indicated by the persistence of I_A at more depolarized holding potentials. Insufficient inactivation of I_A was evident at $V_H = -30$ mV in Sh^5 cells with larger residual I_A (Fig. 11C), whereas I_A in normal cells (Fig. 11D) was virtually completely inactivated at this V_H .

The steady-state inactivation of I_A in Sh^5 was determined by depolarizing pulses to $+10$ mV from various holding potentials (V_H). The I_A evoked in Sh^5 was smaller than normal but readily quantifiable in cells with larger I_A (Fig. 12A). Results from a number of Sh^5 cells are summarized and compared to those of normal and Sh^{KS120} fibers in Figure 12B. Compared with normal fibers, the steady-state inactivation of I_A in Sh^5 cells shows a less steep voltage dependence with a shift (about 5 mV) to more positive potentials. The difference at more positive levels (-20 mV) may be underrepresented because the small residual I_A in Sh^5 at these potentials was often masked by noise, leading to an overestimate of inactivation.

Measurements of kinetic properties of the remaining I_A in Sh^5 larvae were difficult, because it is not possible to separate it from I_K in any voltage range. However, estimations of the rise time (about 7 msec to peak $+10$ mV, 15°C) and the half-time of decay (about 8 msec at $+10$ mV, 15°C) of the residual I_A were not substantially different from normal.

Discussion

Membrane currents in larval muscles. Analysis of *Drosophila* larval muscles has revealed several physiologically distinct membrane currents similar to those found in other insect muscles (Yamamoto and Washio, 1981; Yamamoto et al., 1981; Ashcroft and Stanfield, 1982). Voltage-dependent Ca^{2+} channels are responsible for the inward current but Na^+ channels are not detectable in the larval muscles, consistent with a previous current clamp study (Suzuki and Kano, 1977). The outward currents can be effectively isolated by using Ca^{2+} -free saline which completely abolishes the inward current, revealing two different components of outward K^+ currents, I_A and I_K .

We have not systematically searched for a Ca^{2+} -dependent K^+ conductance (Meech, 1978) in the larval muscle. A fast transient of Ca^{2+} -dependent outward current has been reported in the larval muscle of *Tenebrio* (Yamamoto and Washio, 1981) and in the adult flight muscles of *Drosophila* (Salkoff, 1983a). The present voltage clamp study can be extended, by using saline containing Ca^{2+} , to determine whether a Ca^{2+} -dependent K^+ conductance is also present in the larval muscles of *Drosophila*.

Properties of I_K . Comparison between the properties of I_K in normal and Sh larval muscles does not reveal any essential difference (cf. Figs. 6 to 10). These results strongly suggest that Sh mutations do

not affect I_K and that, in normal larvae, at the time I_K reaches a plateau value, I_A is inactivated and does not contribute to the behavior of the outward current (e.g., tail currents).

The delayed outward current I_K in *Drosophila* larval muscles resembles the delayed rectifier reported in other excitable tissues. The voltage dependency of activation of conductance g_K has an initial slope of about e-fold/6 mV (e-fold increase per 6-mV depolarization) and reaches saturation at about $+30$ mV as compared to the corresponding values of e-fold/6.5 mV and $+30$ mV in the squid giant axon (Hodgkin and Huxley, 1952a) and about e-fold/10 mV and $+112$ mV in the larval mealworm muscle (Yamamoto and Washio, 1981).

In *Drosophila* larval muscles, I_K inactivates slowly, resembling the observations reported in a number of different tissues including frog skeletal muscles (Adrian et al., 1970) and adult stick insect muscles (Ashcroft and Stanfield, 1982). Depletion of intracellular K^+ or accumulation of external K^+ in the vicinity of the membrane surface may also lead to decline of I_K . However, these factors are not likely to interfere with our measurements of I_K inactivation to a significant extent for the following reasons. First, I_K inactivation reaches a level of 50% at $V_H = -20$ mV, a potential at which I_K is not even activated (Figs. 6A and 10). Second, in saline containing a high concentration (30 mM) of K^+ , the same steady-state level of inactivation is reached ($h_A = 0.5$ at $V_H = -20$ mV), indicating that the measurements are independent of external K^+ concentrations (unpublished observations). Third, the actual depletion or accumulation of K^+ is probably not serious after all, because prolonged activation of I_K (at $V_H = +10$ mV for seconds) does not shift the reversal potential V_K (data not shown).

The instantaneous I-V relation of I_K displays a pronounced outward rectification. This tail current behavior cannot be entirely attributed to the steep concentration gradient for the charge carrier because the apparent rectification persists in saline containing higher concentrations of K^+ (Fig. 7B). However, we cannot rule out the possibility of a very rapid ($\ll 5$ msec) decay of inward tail currents not resolvable by the present voltage clamp system.

The time course of I_K tail currents deviates from that of first-order kinetics. This can be accounted for by a heterogeneous population of I_K channels or a sequential scheme of the I_K channel kinetics with a number of closed and open states (Conti and Neher, 1980). The possibility of distortion of channel kinetics by accumulation of K^+ in restricted extracellular space can be excluded as described above. In fact, tail currents observed in high K^+ saline (30 mM) displayed the same kinetics (data not shown).

Comparison with adult muscles. Our observations are in general agreement with the findings in the adult flight muscles of *Drosophila* (Salkoff and Wyman, 1983). However, there are certain important differences between these two sets of muscle fibers in the properties of I_A and I_K . These two currents in the larval muscle can be physiologically separated. Because I_K is activated at a more positive level, depolarization to the range between -35 and -20 mV from a holding potential, $V_H < -50$ mV evokes mainly I_A . Conversely, at more positive holding potentials ($V_H > -30$ mV), I_A is inactivated and only I_K remains. In contrast, I_A and I_K in the adult flight muscle show similar I-V relations and are both activated at about -40 mV. Moreover, the behavior of tail currents indicates that the channels mediating I_K are different in rectification properties in these two sets of muscles. In larval muscles, the current is strongly rectified in the outward direction (Fig. 7), whereas large inward tail currents of I_K can be elicited in the adult muscles (Salkoff and Wyman, 1983). However, a more quantitative comparison between I_K in these two sets of muscles of different developmental stages is not yet possible because analyses of the instantaneous I-V relation, the reversal potential, and the kinetics of tail currents of I_K in adult muscles have not been reported. In addition to the above discrepancies, there appear to be intriguing differences in the effects of certain Sh mutations on the properties of I_A in the larval and adult muscles, as discussed in detail below.

Nature of *Sh* mutations and properties of I_A . Alterations in the functioning of ionic channels may be derived from changes in a number of molecular and cellular mechanisms, including primary protein structures, post-transcriptional or post-translational modifications, and assembly of subunits. At present, it is not possible to pinpoint the mechanism responsible for the altered I_A in *Sh* mutants solely by electrophysiological means. Nevertheless, useful information concerning I_A channels can be gained by comparing the present findings with previous observations on various *Sh* alleles.

Evaluation of the effects of the same mutation on different excitable tissues at various developmental stages is of particular interest. In terms of severity, consistency is found in the defects of *Sh*^{K^{S133}}, *Sh*^M, and *Sh*¹⁰², each causing delayed repolarization of action potentials in adult "giant axons" (Tanouye et al., 1981), prolonged transmission in larval neuromuscular junctions (Jan et al., 1977; Ganetzky and Wu, 1983; B. Ganetzky and C. -F. Wu, unpublished data), and complete elimination of I_A in both pupal (Salkoff, 1983b; *Sh*^M not tested) and larval muscles. In contrast, *Sh*^{K^{O120}} causes abnormal larval neuromuscular transmission to an extent indistinguishable from that in *Sh*^{K^{S133}} but leads to only mild defects in the adult "giant axon" action potentials and no obvious change in properties of I_A in larval muscles. (There has been a report (Salkoff and Wyman, 1981) on the complete elimination of I_A in adult *Sh*^{K^{O120}} muscles. It was later found to be due to a different strain which has since been renamed *Sh*^{K^{O120}} (Salkoff, 1983a, b; L. Salkoff, personal communication).)

The defects caused by *Sh*⁵ have been found to be consistently less extreme than those in *Sh*^{K^{S133}} in all tissues examined (larval neuromuscular junctions: Jan et al., 1977; C. -F. Wu and B. Ganetzky, unpublished observations; adult "giant axons": Tanouye et al., 1981; adult muscles: Salkoff and Wyman, 1981; larval muscles: this report). However, voltage clamp studies of the residual I_A in *Sh*⁵ revealed important differences in the manner in which adult and larval muscles are affected. The I_A in adult flight muscles retains a normal peak amplitude but exhibits abnormally fast decay (Salkoff and Wyman, 1981) due to a change in the voltage dependence of inactivation kinetics (Salkoff, 1983b). In contrast, the I_A in *Sh*⁵ larval muscles shows a reduced amplitude and a shift in the voltage dependency of both activation and inactivation, indicating that these two processes are not controlled by two independent, discrete parts of the channel. The fact that the same mutation alters both activation and inactivation in larvae but only inactivation in adults points up a differential expression of a common mechanism underlying these two processes in the two sets of muscles.

A comparative study of nerve and muscle membranes of different *Sh* alleles can reveal additional interrelationships among various functional components of potassium channels and provide a more complete view of the roles of the *Sh* gene product (or products) in different excitable membranes. More incisive analysis of mutant potassium channels can be achieved by patch recording from cultured *Drosophila* neurons (Wu et al., 1983b; Sun and Wu, 1984).

Molecular identities of potassium channels. By physiological and pharmacological criteria, a variety of potassium channels have been identified, which vary in distribution among different cellular regions and cell types displaying a spectrum of signaling capability (Adams et al., 1980; Kandel and Schwartz, 1982; Serrano, 1982; Alkon, 1983). It should be possible to modify and separate various potassium currents by genetic means. This approach will ultimately lead to the molecular identities of potassium channels by isolating the gene products of interest with recombinant DNA techniques (Jan et al., 1983; Ganetzky and Wu, 1984; Kamb et al., 1984). Evidence supporting the hypothesis that the *Sh* locus contains the structural gene for a potassium channel has been reviewed by Jan et al. (1983). However, caution must be exercised in implicating a simple one-to-one correspondence between a specific gene product and a particular potassium channel. The physiological properties and pharmacological sensitivities of I_A and I_K often show a varying degree of overlap in different excitable tissues, and sometimes an absolute

distinction is difficult (Adams et al., 1980; Serrano, 1982). Furthermore, differences between the properties of I_A in the adult and larval muscles and the differential effects of *Sh*^{K^{O120}} and *Sh*⁵ on various types of excitable membranes suggest that the genetic control of I_A channels involves other genes in addition to *Sh*. In fact, mutations of a second gene, *eag*, have been reported to alter potassium currents in both larval nerve and muscle membranes (Ganetzky and Wu, 1983; Wu et al., 1983a, Wu and Ganetzky, 1984).

Taking the above findings together, the hypothesis can be put forward that particular types of potassium channels are composed of specific functional components (e.g., subunits) and may share some components with other types of potassium channels. In consequence, a single-gene mutation can affect different potassium channels and a particular channel type can be affected by mutations of different genes. Thus, combination of a relatively small number of interacting gene products can yield a rich variety of potassium channels subserving an array of physiological activities in different excitable membranes. It should be possible to verify this notion by analysis of double-mutant combinations of the relevant genes. The interrelationships among subunits will be indicated by altered functional parameters of channels which may serve as a guide for molecular genetic analysis of potassium channels.

References

- Adams, D. J., J. S. Stephen, and S. H. Thompson (1980) Ionic currents in molluscan soma. *Annu. Rev. Neurosci.* 3: 141-167.
- Adrian, R. H., W. K. Chandler, and A. L. Hodgkin (1970) Voltage clamp experiments in striated muscle fibers. *J. Physiol. (Lond.)* 208: 607-644.
- Alkon, D. L. (1983) Learning in a marine snail. *Sci. Am.* 249: 70-85.
- Ashcroft, F. M., and P. R. Stanfield (1982) Calcium and potassium currents in muscle fibers of an insect (*Carausius morosus*). *J. Physiol. (Lond.)* 323: 93-115.
- Catterall, W. (1984) The molecular basis of neuronal excitability. *Science* 223: 653-661.
- Connor, J. A., and C. F. Stevens (1971a) Inward and delayed outward membrane currents in isolated neural somata under voltage clamp. *J. Physiol. (Lond.)* 213: 1-19.
- Connor, J. A., and C. F. Stevens (1971b) Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol. (Lond.)* 213: 21-30.
- Conti, F., and E. Neher (1980) Single channel recording of K⁺ currents in squid axons. *Nature* 285: 140-143.
- Crossley, A. C. (1978). The morphology and development of the *Drosophila* muscular system. In *Genetics and Biology of Drosophila*, M. Ashburner and T. R. F. Wright, eds., Vol. 2b, pp. 499-559, Academic Press, Inc., New York.
- Dodge, F. A., and B. Frankenhaeuser (1959) Sodium currents in the myelinated nerve fiber of *Xenopus laevis* investigated with the voltage clamp technique. *J. Physiol. (Lond.)* 148: 188-200.
- Fatt, P., and B. L. Ginsborg (1958). The ionic requirements for the production of action potentials in crustacean muscle fibers. *J. Physiol. (Lond.)* 142: 516-543.
- Ganetzky, B., and C. -F. Wu (1982a) Indirect suppression involving behavioral mutants with altered nerve excitability in *Drosophila melanogaster*. *Genetics* 100: 597-614.
- Ganetzky, B., and C. -F. Wu (1982b) *Drosophila* mutants with opposing effects on nerve excitability: Genetic and spatial interactions in repetitive firing. *J. Neurophysiol.* 47: 501-514.
- Ganetzky, B., and C. -F. Wu (1983) Neurogenetic analysis of potassium currents in *Drosophila*: Synergistic effects on neuromuscular transmission in double mutants. *J. Neurogenet.* 1: 17-28.
- Ganetzky, B., and C. -F. Wu (1984) Mutations of a gene affecting potassium currents induced by transposable elements in *Drosophila*. *Soc. Neurosci. Abstr.* 10: 1090.
- Hagiwara, S. (1973) Ca spike. *Adv. Biophys.* 4: 71-102.
- Hagiwara, S., and L. Byerly (1981) Calcium channel. *Annu. Rev. Neurosci.* 4: 69-125.
- Hagiwara, S., S. Yoshida, and M. Yoshii (1981) Transient and delayed potassium currents in the egg cell membrane of the coelenterate, *Renilla koellikeri*. *J. Physiol. (Lond.)* 318: 123-141.
- Hahn, R., and D. Campbell (1983) Simple shifts in the voltage dependence

- of sodium channel gating caused by divalent cations. *J. Gen. Physiol.* 82: 785–805.
- Hewitt, C. G. (1914) *The House Fly*, Cambridge University Press, Cambridge, England.
- Hille, B. (1973) Potassium channels in myelinated nerve. Selective permeability to small cations. *J. Gen. Physiol.* 61: 669–686.
- Hodgkin, A. L., and A. F. Huxley (1952a) The components of membrane conductance in the giant axon of *Loligo*. *J. Physiol. (Lond.)* 116: 473–496.
- Hodgkin, A. L., and A. F. Huxley (1952b) The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol. (Lond.)* 116: 497–506.
- Hodgkin, A. L., and A. F. Huxley (1952c) A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.)* 117: 500–544.
- Ikeda, K., and W. D. Kaplan (1970) Patterned neural activity of a mutant *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 66: 765–772.
- Jan, L. Y., and Y. N. Jan (1976) Properties of the larval neuromuscular junction in *Drosophila melanogaster*. *J. Physiol. (Lond.)* 262: 215–236.
- Jan, L. Y., S. Barbel, L. Timpe, C. Laffer, L. Salkoff, P. O'Farrell, and Y. N. Jan (1983) Mutating a gene for a potassium channel by hybrid dysgenesis: An approach to cloning of the *Shaker* locus in *Drosophila*. *Cold Spring Harbor Symp. Quant. Biol.* 48: 233–245.
- Jan, Y. N., L. Y. Jan, and M. J. Dennis (1977) Two mutations of synaptic transmission in *Drosophila*. *Proc. R. Soc. Lond. (Biol.)* 198: 87–108.
- Kamb, A., L. Iverson, and M. Tanouye (1984) Molecular analysis of the *Shaker* (*Sh*) gene complex in *Drosophila melanogaster*. *Soc. Neurosci. Abstr.* 10: 1089.
- Kandel, E. R., and J. H. Schwartz (1982) Molecular biology of learning: Modulation of transmitter release. *Science* 218: 433–443.
- Kaplan, W. D., and W. E. Trout, III (1969) The behavior of four neurological mutants of *Drosophila*. *Genetics* 61: 399–409.
- Meech, R. W. (1978) Calcium-dependent potassium activation in nervous tissues. *Annu. Rev. Biophys. Bioeng.* 7: 1–18.
- Mishina, M., T. Tobimatsu, K. Imoto, K.-i. Tanaka, Y. Fujita, K. Fukuda, M. Kurasaki, H. Takahashi, Y. Morimoto, T. Hirose, S. Inayama, T. Takahashi, M. Kuno, and S. Numa (1985) Location of functional regions of acetylcholine receptor α -subunit by site-directed mutagenesis. *Nature* 313: 364–369.
- Neher, E. (1971) Two fast transient current components during voltage clamp on snail neurons. *J. Gen. Physiol.* 58: 36–53.
- Ogden, T. E., M. C. Citron, and R. Pierantoni (1978) The jet stream microbeveler: An inexpensive way to bevel ultrafine glass micropipettes. *Science* 201: 469–470.
- Osborne, M. P. (1967) Supercontraction in the muscles of the blowfly larva: An ultrastructural study. *J. Insect Physiol.* 13: 1471–1482.
- Salkoff, L. (1983a) *Drosophila* mutants reveal two components of fast outward current. *Nature* 302: 249–251.
- Salkoff, L. (1983b) Genetic and voltage clamp analysis of a *Drosophila* potassium channel. *Cold Spring Harbor Symp. Quant. Biol.* 48: 221–231.
- Salkoff, L., and R. Wyman (1981) Genetic modification of potassium channels in *Drosophila Shaker* mutants. *Nature* 293: 228–230.
- Salkoff, L., and R. Wyman (1983) Ion currents in *Drosophila* flight muscles. *J. Physiol. (Lond.)* 337: 687–708.
- Serrano, E. E. (1982) Variability in molluscan neuron soma currents. Ph.D. thesis, Stanford University, Palo Alto, CA.
- Siddiqi, O., and S. Benzer (1976) Neurophysiological defects in temperature-sensitive paralytic mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 73: 3253–3257.
- Sun, Y. A., and C.-F. Wu (1984) Voltage-dependent single-channel currents in dissociated CNS neurons of *Drosophila*. *Soc. Neurosci. Abstr.* 10: 1090.
- Suzuki, N., and M. Kano (1977) Development of action potential in larval muscle fibers in *Drosophila melanogaster*. *J. Cell. Physiol.* 93: 383–388.
- Tanouye, M., A. Ferrus, and S. C. Fujita (1981) Abnormal action potentials associated with *Shaker* locus of *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 78: 6548–6552.
- Taylor, R. E., J. W. Moore, and K. S. Cole (1960) Analysis of certain errors in squid axon voltage clamp measurements. *Biophys. J.* 1: 161–202.
- Weidmann, S. (1952) The electrical constants of Purkinje fibers. *J. Physiol. (Lond.)* 118: 348–360.
- Werman, R., F. V. McCann, and H. Grundfest (1961) Graded and all-or-none electrogenesis in arthropod muscle. I. The effect of alkali-earth cations on neuromuscular system of *Romalea microptera*. *J. Gen. Physiol.* 44: 979–995.
- Wu, C.-F., and B. Ganetzky (1980) Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of *Drosophila melanogaster*. *Nature* 286: 814–816.
- Wu, C.-F., and B. Ganetzky (1984) Properties of potassium channels altered by mutations of two genes in *Drosophila*. *Biophys. J.* 45: 77–78.
- Wu, C.-F., B. Ganetzky, L. Y. Jan, Y.-N. Jan, and S. Benzer (1978) A *Drosophila* mutant with a temperature-sensitive block in nerve conduction. *Proc. Natl. Acad. Sci. U. S. A.* 75: 4047–4051.
- Wu, C.-F., B. Ganetzky, F. Haugland, and A.-X. Liu (1983a) Potassium currents in *Drosophila*: Different components affected by mutations of two genes. *Science* 220: 1076–1078.
- Wu, C.-F., N. Suzuki, and M.-m. Poo (1983b) Dissociated neurons from normal and mutant *Drosophila* larval central nervous system in cell culture. *J. Neurosci.* 3: 1888–1889.
- Yamamoto, D., and H. Washio (1981) Voltage clamp studies on insect skeletal muscle. II. The outward currents. *J. Exp. Biol.* 90: 13–22.
- Yamamoto, D., J.-I. Fukami, and H. Washio (1981) Voltage clamp studies on insect skeletal muscle. I. The inward current. *J. Exp. Biol.* 90: 1–12.