In Vivo Functional Role of the *Drosophila Hyperkinetic* β Subunit in Gating and Inactivation of *Shaker* K^+ Channels

Jing W. Wang and Chun-Fang Wu
Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242 USA

ABSTRACT The physiological roles of the β, or auxiliary, subunits of voltage-gated ion channels, including Na^+, Ca^{2+}, and K^+ channels, have not been demonstrated directly in vivo. *Drosophila Hyperkinetic* (*Hk*) mutations alter a gene encoding a homolog of the mammalian K^+ channel β subunit, providing a unique opportunity to delineate the in vivo function of auxiliary subunits in K^+ channels. We found that the *Hk* β subunit modulates a wide range of the *Shaker* (*Sh*) K^+ current properties, including its amplitude, activation and inactivation, temperature dependence, and drug sensitivity. Characterizations of the existing mutants in identified muscle cells enabled an analysis of potential mechanisms of subunit interactions and their functional consequences. The results are consistent with the idea that via hydrophobic interaction, *Hk* β subunits modulate *Sh* channel conformation in the cytoplasmic pore region. The modulatory effects of the *Hk* β subunit appeared to be specific to the *Sh* α subunit because other voltage- and Ca^{2+}-activated K^+ currents were not affected by *Hk* mutations. The mutant effects were especially pronounced near the voltage threshold of I_A activation, which can disrupt the maintenance of the quiescent state and lead to the striking neuromuscular and behavioral hyperexcitability previously reported.

INTRODUCTION

K^+ channels regulate membrane repolarization, and hence the excitability of nerve and muscle cells (Rudy, 1988; Hille, 1992). Isolation of leg-shaking behavioral mutants in *Drosophila* has identified the gene *Hyperkinetic* (*Hk*), along with *Shaker* (*Sh*), *ether a go-go* (*eag*), and *slowpoke* (*slo*) (Kaplan and Trout, 1969; Elkins et al., 1986), which encode separate α subunits of K^+ channels (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988; Atkinson et al., 1991; Warmke et al., 1991). Subsequent studies in heterologous expression systems have led to the identification of molecular domains important to K^+ channel mechanisms (Jan and Jan, 1992; Sigworth, 1993; Hoshi and Zagotta, 1993). Moreover, the mutant phenotypes have provided insights into the functional role of individual K^+ channel subunits (Jan et al., 1977; Salkoff and Wyman, 1981; Wu et al., 1983; Tanouye and Ferrus, 1985; Elkins et al., 1986; Zhong and Wu, 1991; Wu and Ganetzky, 1992).

The assembly of different channel subunits generates structural variation that might enhance functional diversity. The importance of the β, or auxiliary, subunits in the proper functioning of Na^+ and Ca^{2+} channels has been well documented in heterologous expression systems (Catterall, 1991, 1992). A mammalian K^+ channel β subunit (Kvβ1) was isolated by copurification with the α subunit (Parcej and Dolly, 1989; Scott et al., 1994). Subsequent DNA sequence analysis showed remarkable conservation between the *Drosophila Hk* (Chouinard et al., 1995) and the mammalian β subunit Kvβ1 (Scott et al., 1994). Additional β subunits Kvβ2 and Kvβ3 homologous to Kvβ1 were also shown to be expressed in mammalian cardiac and brain tissue (Scott et al., 1994; England et al., 1995; Majumder et al., 1995; McCormack et al., 1995; Morales et al., 1995; Rhodes et al., 1995). Coexpression with α subunits of the *Sh* family demonstrated the influence of β subunits on the function of voltage-gated K^+ channels in heterologous expression systems. Each β subunit can modify either the kinetics of channel inactivation (Rettig et al., 1994; Chouinard et al., 1995; England et al., 1995; Majumder et al., 1995; McCormack et al., 1995; Morales et al., 1995; Sewing et al., 1996; Yu et al., 1996), or the turnover of expressed α subunits (Shi et al., 1996). However, the exact regulatory mechanisms of the β subunit in vivo and their functional consequence have not been studied in any species. Thus *Hk* mutations provide unique opportunities to identify the specific type and properties of the K^+ channels that are regulated by the β subunit.

The body-wall muscles of *Drosophila* larvae are each identifiable and accessible to two-electrode voltage clamp. In this preparation, K^+ currents can be physiologically separated into four distinct components, including two voltage-gated (the transient I_A and the delayed I_K) and two Ca^{2+}-activated (the fast I_{CF} and the slow I_{CS}) K^+ currents (Singh and Wu, 1989). It has been shown that *Sh* mutations specifically alter the voltage-gated transient K^+ current I_A (Wu and Haugland, 1985; Singh and Wu, 1989), and *slo* mutations specifically affect the fast Ca^{2+}-activated K^+ currents I_{CF} (Komatsu et al., 1990), consistent with observations in adult flight muscles (Salkoff, 1983; Elkins et al., 1986). The currents per unit membrane area among larvae are highly reproducible, allowing quantitative analysis of mutant effects (Singh and Wu, 1989; Haugland and Wu, 1990; Zhong and Wu, 1991, 1993a,b; Chopra and Singh, 1994). The role of the *Hk* subunit could be delineated in such a system in which individual K^+ channel types can be

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Address reprint requests to Dr. Chun-Fang Wu, Department of Biological Sciences, University of Iowa, Iowa City, IA 52242. Tel.: 319-335-1091; Fax: 319-335-1103; E-mail: cfwu@vaxa.weeg.uiowa.edu.

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examined. Here we report wide-ranging effects of *Hk* mutations, specifically on *Sh* channels that mediate I_A. Disruptions of the *Hk* subunit thus lead to hyperexcitability in the nerve firing pattern (Ikeda and Kaplan, 1970; Yao and Wu, 1995), enhanced neuromuscular transmission (Stern and Ganetzky, 1989), and hyperkinetic behavior (Kaplan and Trout, 1969). Part of the results here have appeared in abstract form (Wang and Wu, 1995).

MATERIALS AND METHODS

Fly stocks

All fly stocks were raised at 20–22°C on standard *Drosophila* media. The wild-type strain Canton S was used as the control in all experiments. Hk^1 was from Dr. Seymour Benzer's collection, and Hk^{1E18} flies were provided by Dr. Barry Ganetzky.

Electrophysiology

The two-electrode voltage-clamp technique for measuring K^+ currents in larval muscle has previously been described (Wu and Haugland, 1985; Haugland and Wu, 1990). In brief, third instar larvae were dissected, and I_A and I_K were recorded in Ca^{2+}-free standard saline containing 128 mM NaCl, 2 mM KCl, 14 mM $MgCl_2$, 35 mM sucrose, 5 mM EGTA, and 5 mM HEPES (pH 7.1). I_{CF} and I_{CS} were measured in standard saline containing 20 mM $CaCl_2$ and 4 mM $MgCl_2$ (Singh and Wu, 1989). In addition, 353 mM sucrose was used to increase the tonicity for preventing muscle contraction, and 1 mM 4-aminopyridine (4-AP) and 100 μM quinidine were used to block I_A and I_K (Zhong and Wu, 1991). Ca^{2+} channels were examined in the HL3 saline (Stewart et al., 1994), with the same concentrations of 4-AP and quinidine used to block I_A and I_K. Ba^{2+} was used as the charge carrier (4 mM $BaCl_2$), which allows assessment of current through Ca^{2+} channel without activating I_{CF} and I_{CS} (Gielow et al., 1995).

Muscle 6 of abdominal segments 3 to 5 was used exclusively. For separation of I_A and I_K, a 2-s preconditioning pulse from a holding potential of −80 mV to −20 mV was used to inactivate I_A while leaving I_K intact. Subtraction of the voltage-activated current with a prepulse from the one without gives I_A (Haugland and Wu, 1990; Zhong and Wu, 1991). Current signals were filtered at 2 kHz (4-pole Bessel; Krohn-Hite, Avon, MA), digitized, and analyzed with pCLAMP and AxoGraph software (Axon Instruments, Foster City, CA). The preparation temperature was controlled by a Peltier stage (Cambion, Cambridge, MA).

Analysis of inactivation kinetics

The decay of I_A was fit with a single exponential (decay time constant τ and the extrapolated amplitude I_o at $t = 0$) and a small residual constant (I_s). The kinetics scheme for inactivation can be expressed as (Hoshi et al., 1991)

$$C_1 \rightleftharpoons O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I_1 \rightleftharpoons I_2, \quad (1)$$

where C_1 and I_1 are the closed and inactivated states, respectively, immediately associated with the open state, O. The forward and backward rate constants k_1 and k_{-1} for the transition between O and I_1 are indicated. The transition between I_1 and I_2 is significant only if prolonged depolarization steps are considered (Hoshi et al., 1991). The activation process from C_1 to O affects the rise and early decay phase of I_A and contributes relatively little to the exponential decay phase (see below). Thus, $d[O]/dt = -k_1[O] + k_{-1}[I]$. At steady state, $d[O]/dt = 0$. Therefore, $k_1 + k_{-1} = k = 1/\tau$ and $k_{-1}/(k_1 + k_{-1}) = I_s/I_o$. Because I_s/I_o is small, k_{-1} is negligible and $1/\tau = k \approx k_1$.

The inactivation rate constant k was used to deduce E_a and A according to the Arrhenius equation $k = A \exp(-E_a/RT)$, where R is the ideal gas constant, T is the absolute temperature, E_a is the energy of activation in Joules per mole, and A is the preexponential constant in s^{-1}. The values of E_a and A shown in Table 1 were derived from the slope and intercept (at $1/T = 0$) of regression lines in the log-transformed Arrhenius plot using Systat software (Systat, Evanston, IL).

RESULTS

Macroscopic conductance and kinetics of I_A

In the majority of experiments, Ca^{2+}-free saline was used to eliminate Ca^{2+} and Ca^{2+}-dependent K^+ currents, and only the voltage-activated transient I_A and delayed I_K remained. I_A and I_K can be further separated physiologically, because of differences in their inactivating kinetics. A depolarizing preconditioning pulse can be applied to inactivate I_A without affecting I_K. Thus subtraction of the voltage-activated current with a prepulse from the one without gives I_A (Wu and Haugland, 1985; Haugland and Wu, 1990).

Striking defects in I_A were seen in different *Hk* mutant alleles, including HK^{1E18}, a null mutation, and Hk^1, a point mutation (Schlimgen, 1991). As shown in Fig. 1, the extracted I_A was evidently reduced in *Hk* muscles, whereas I_K remained unaltered. There was no indication of a change in ion selectivity because the reversal potential of I_A was normal (−60 mV; cf. Haugland, 1987) in *Hk* mutants. Conversion of current density to membrane conductance

TABLE 1 Effects of a *Hk* null mutation on the inactivation kinetics of I_A at different membrane potentials

	E_a (J mol^{-1} × 10^4)		ln A		A (s^{-1})		I_s/I_o (%)	
V_m (mV)	Normal	Hk^{1E18}	Normal	Hk^{1E18}	Normal	Hk^{1E18}	Normal	Hk^{1E18}
20	10.0 ± 0.3	9.6 ± 0.4	47 ± 1	45 ± 2	2.6×10^{20}	3.5×10^{19}	3.9 ± 0.7	5.4 ± 1.0
10	9.8 ± 0.4	9.5 ± 0.4	46 ± 2	45 ± 2	1.5×10^{20}	2.6×10^{19}	3.2 ± 0.7	5.2 ± 0.7
0	9.7 ± 0.4	8.7 ± 0.4	46 ± 2	41 ± 2	9.5×10^{19}	6.4×10^{17}	2.3 ± 0.5	6.5 ± 0.8
−10	9.7 ± 0.3	7.4 ± 0.6	42 ± 1	35 ± 3	4.4×10^{19}	1.9×10^{15}	4.0 ± 1.8	9.9 ± 1.6
−20	8.6 ± 0.5	3.5 ± 1.0	40 ± 2	18 ± 4	2.4×10^{17}	6.6×10^{7}	ND*	ND*

Regression of the transformed Arrhenius equation, $\ln k = \ln A - E_a/RT$, provided E_a(J mol^{-1}), the energy of activation, and A (s^{-1}), a preexponential constant at different membrane potentials (V_m). The same data were also plotted in Fig. 3 *B*. I_o is the current amplitude of I_A at $t = 0$ ms from exponential fitting, and I_s the residual I_A at steady state at 11°C.

*Because of the small amplitude of I_A (I_s) at this mark point, the value of I_s/I_o cannot be determined accurately. The uncertainty of the subtraction process for I_A isolation (I_s has a gain of <5% of I_K, the inactivating component; Haugland, 1987) affects I_s, and the noise level affects I_o. The upper limit of I_s/I_o is estimated to be 43 ± 4% for the mutant, and 8.6 ± 3% for normal fibers.

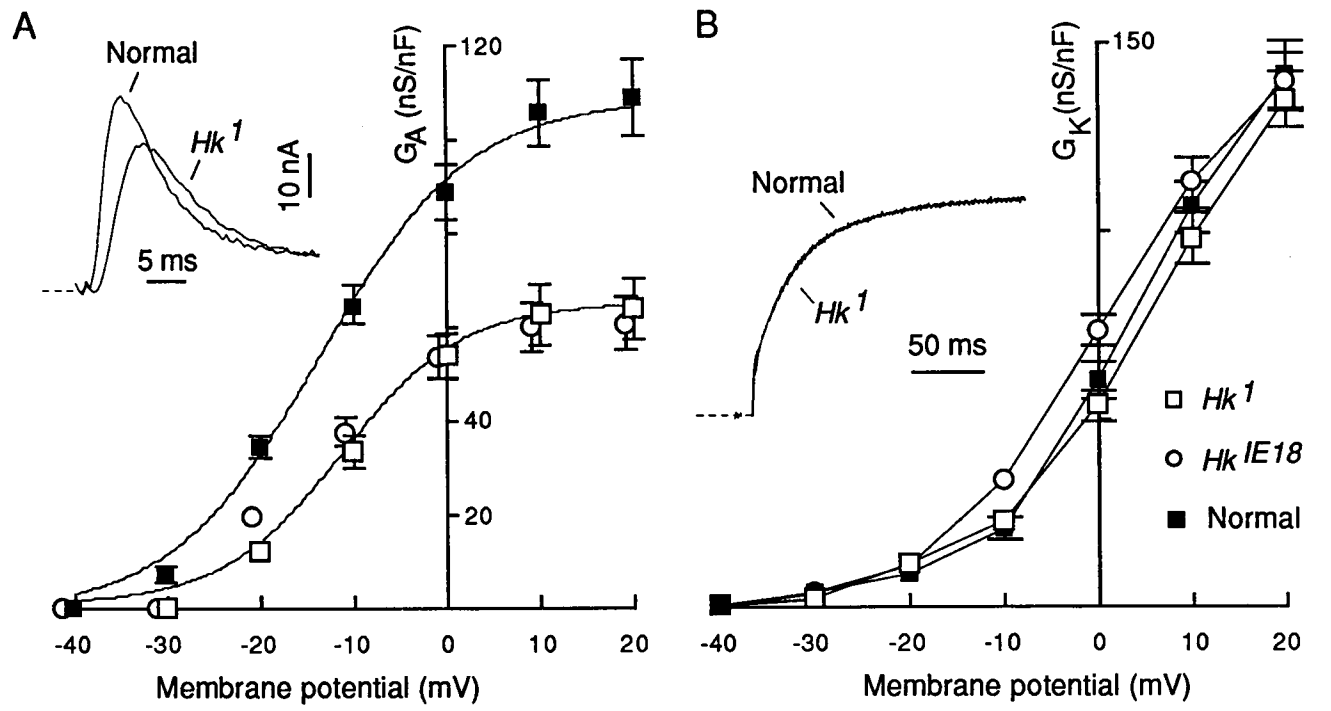

FIGURE 1 Effects of *Hk* mutations on the transient K^+ current I_A in body-wall muscles of *Drosophila* larvae. (*A*) *G-V* relationship of I_A for Hk^1, Hk^{IE18}, and normal fibers. The membrane conductance G was obtained by the relationship $G = I/(V - V_r)$, where I is the current density in nA/nF and V_r is the reversal potential of I_A (−60 mV; cf. Haugland, 1987, which was not altered in *Hk* mutants; data not shown). Conductance data were fit to the relationship $G = G_0/(1 + \exp((V_{m1/2} - V)/V_{mslope}))$. The maximum conductance G_0 was significantly reduced in the mutants. The half-activation voltage ($V_{m1/2}$) and the limiting slope (V_{mslope}) were −13.5 mV and 8.0 mV/*e*-fold for normal fibers, and −11.0 mV and 6.5 mV/*e*-fold for mutant fibers. (*B*) *G-V* relationship of the delayed rectifier I_K exhibiting no significant differences between mutant and normal fibers. The insets show typical traces of I_A and I_K activated by depolarization to 10 mV for normal and Hk^1 larvae. The peak I_A and maximum I_K were measured at 11°C from 10 fibers in five to six larvae for each genotype. For this and the following figures, each data point represents mean ± SEM (■, normal; □, Hk^1; ○, Hk^{IE18}). SEM is not shown if smaller than the symbol.

based on the reversal potential allows an assessment of the voltage dependence of channel conductance. We found that voltage dependence of I_A activation in *Hk* mutants was similar to that of normal fibers with a half-activation voltage of −11 mV, steepness of voltage dependence of 6.5 mV/*e*-fold, and a saturation voltage above +20 mV (Fig. 1). However, the maximum conductance of I_A, attained at +20 mV (Fig. 1 *A*), was reduced to about 60% of normal in both Hk^1 and Hk^{IE18}. This could indicate a reduction in either the single-channel conductance or the number of functional channels.

The rise and decay of I_A were markedly slower in mutant fibers. The time (t_p) to peak I_A from the onset of depolarization was measured (Fig. 2), and the decay phase during I_A inactivation was approximated with a single exponential decay plus a small residual amplitude constant (see Materials and Methods). The kinetic processes were examined at several temperatures for indications of changes in thermal stability or energetic requirements of the mutant subunits. We found that I_A activation was slower, as indicated by a longer t_p in the two mutant alleles at all temperatures examined (Fig. 2). Readily reproducible changes in the kinetics of I_A decay were seen in the mutants. The decay time constants (τ) from Hk^{IE18} at 4°C and 16°C are compared with a normal control in Fig. 3; similar results were obtained in Hk^1 (data not shown). Notably, the differences in t_p and τ between normal and mutant fibers were more pronounced at lower membrane potentials (Figs. 2 and 3). It is conceivable that the presence of the β subunit could facilitate these kinetic processes at low levels of depolarization, but is less crucial when the energy requirement for activation and inactivation is reduced by larger depolarizations.

Specificity of *Hk* mutant effects

At each temperature examined, there were no detectable differences in the kinetics and conductance of I_K between mutant and normal muscles (see Fig. 1 for data at 11°C; data not shown for 4 and 16°C). Other properties of I_K, including steady-state inactivation (see below, with 5–10 s depolarization), appeared normal (data not shown). In addition, no obvious alterations by *Hk* mutations were indicated in Ca^{2+} channels or in Ca^{2+}-activated K^+ channels (data not shown, see Materials and Methods). This differential effect of *Hk* mutations on I_A suggests that the *Hk* β subunit interacts specifically with the *Sh* α subunits.

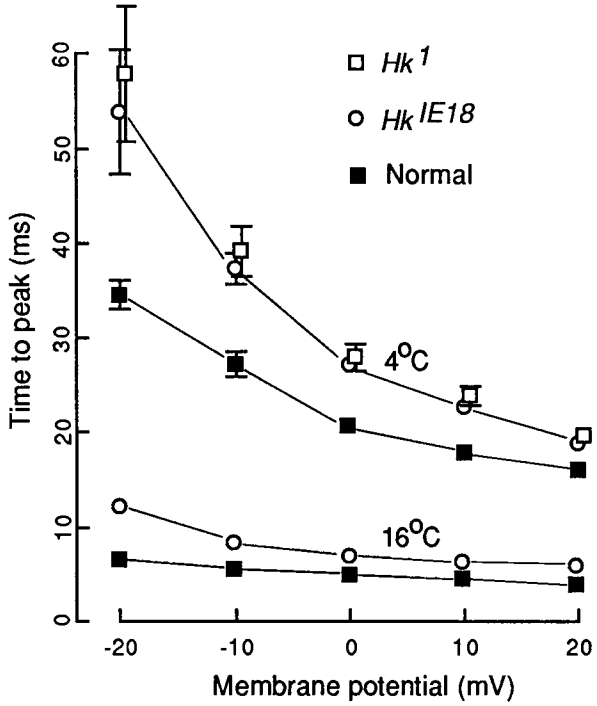

FIGURE 2 Activation of I_A. Time to peak of I_A from the onset of voltage steps (t_p) was lengthened in *Hk* mutants. Data were pooled from eight mutant fibers in four larvae and 12 normal fibers in five larvae at 4°C, six mutant fibers in four larvae and four normal fibers in three larvae at 16°C.

Temperature dependence of I_A kinetics

In contrast to the clear temperature-dependent alterations in I_A kinetics (Figs. 2 and 3), the reduction (about 60–70%) in conductance (Fig. 1) in mutant fibers appeared to be independent of temperature (4, 11, and 16°C). This is consistent with a change in properties of the channel, but a possibility of reduction in number of channels cannot be ruled out.

The rise of I_A in larval muscle is relatively rapid compared to the decay. It is technically difficult to analyze quantitatively the activation process in this preparation, because the rising phase could be distorted by the slow settling time in the two-microelectrode voltage clamp system. In contrast, the decay of I_A in larval muscle is slower and follows a simpler time course. The decay phase could be approximated by an exponential process plus a small residual steady-state component (Wu and Haugland, 1985; Haugland, 1987). The time constant τ of the exponential decay of I_A traces recorded from normal and *Hk* mutant muscle fibers was determined (see Fig. 3 *A*, *inset*).

If the open state (O) and its immediately associated closed (C_1) and inactivated (I_1 and I_2) states are modeled as in the following scheme

$$C_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O \rightleftharpoons I_1 \rightleftharpoons I_2, \qquad (2)$$

the time constant τ derived from the late phase of I_A decay can be used to deduce the rate constants of the rate-limiting step that governs the I_A decay process. Because the activation of I_A is not instantaneous, the peak and the early decay phase of I_A are determined by the convolution of the activation and inactivation processes (Zagotta et al., 1989). Because the activation of I_A is relatively rapid as reflected by the rapid rise of I_A, the late phase of I_A decay would be largely determined by the inactivation process with much less contribution from the activation process. The fact that the time course of I_A decay, except for the first few milliseconds after the peak, could be satisfactorily fit by a single time constant supports the above assumption (see Discussion for futher details). In other words, the transition between C_1 and O is rapid, and the fitting reflects the process predominantly determined by the transition between O and I_1 with rate constants k_1 and k_{-1}. The time constant τ can be used to deduce the combined rate constant k, where $k = k_1 + k_{-1}$. Because the steady-state I_A (I_s) is no more than 5% of the extrapolated I_A at the initial decay in normal fibers (Haugland, 1987, and Table 1), the time constant τ would be largely determined by the forward rate constant k_1 ($\tau = 1/k = 1/(k_1 + k_{-1}) \approx 1/k_1$; see Materials and Methods). Additional states (I_2, etc.) of inactivation for I_A channels have been proposed (cf. N- and C-type inactivation; Hoshi et al., 1991), but they do not contribute significantly to the inactivation process during the relative short depolarization considered here. The second inactivation state was revealed by the recovery from prolonged inactivation, which requires two time constants to fit (see Fig. 5 and below).

Fig. 3 *B* shows the temperature dependence of k at different voltages in normal and Hk^{IE18} fibers, presented in an Arrhenius plot. Studies of the temperature dependence of kinetic processes have provided insights into the energetic requirements for transition between conformational states of K^+ channels (Hille, 1992; Murrel-Lagnado and Aldrich, 1993). It is evident that differences between mutant and normal fibers were proportionally more pronounced at 16°C than at 4°C. This observation suggests that the normal functioning of *Hk* subunits may be promoted by thermal energy, and their influence on I_A thus appears to be more important at higher than at lower temperatures. In addition, the proportional difference in k for a given temperature between normal and mutant fibers is greater at lower voltages, implying that the physiological function of *Hk* subunits is more significant at lower membrane potentials, especially near the voltage threshold of I_A activation (see Fig. 3 and Discussion).

The Arrhenius plot shows differences in both the slope (related to the activation energy E_a) and intercept (the preexponential constant A) with the equation

$$k = A \exp(-E_a/RT), \qquad (3)$$

where R is the gas constant and T is the absolute temperature. Regression of the transformed Arrhenius equation, $\ln k = \ln A - E_a/RT$, shows that both E_a and A were reduced in the null mutant Hk^{IE18} (Fig. 3 *B* and Table 1) as well as Hk^1 (data not shown). Therefore, the β subunit influences

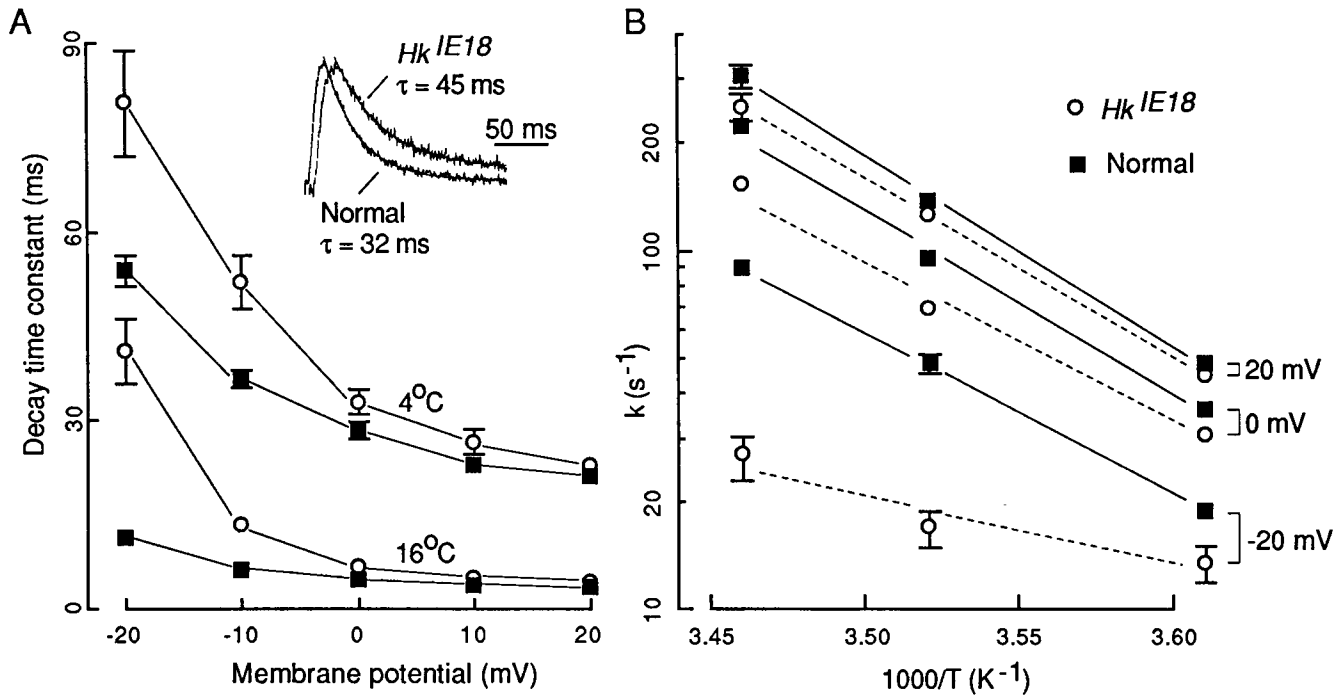

FIGURE 3 Inactivation kinetics of I_A at different voltages and temperatures. (*A*) The decay of I_A was fit with a single time constant τ and a small residual amplitude constant (*inset*). (*B*) Arrhenius plot of the rate constant k ($k = 1/\tau$; see text). Decay time constants were measured at three different temperatures, 4, 11, and 16°C, and data from three different depolarizing potentials −20, 0, and 20 mV are plotted. The inactivation rate constant k was fit with the equation $k = A \exp(-E_a/RT)$, where A is the preexponential constant, and E_a is the activation energy (see Experimental Procedures). E_a and A are extracted from the slope and intercept (at $1/T = 0$) of regression lines (– – –, *Hk*; ——, normal). At −20 mV, $A = 2.4 \times 10^{17}$ s^{-1}, $E_a = 8.6 \times 10^4$ J mol^{-1} for normal fibers; $A = 6.6 \times 10^7$ s^{-1}, $E_a = 3.5 \times 10^4$ J mol^{-1} for Hk^{IE18} (see Table 1). The cells from Fig. 2 were used.

the potential energy barrier (E_a) that prevents the molecular switch to an intermediate state prerequisite for inactivation, and shapes the intrinsic conformation properties and other entropy factors (related to A; cf. Johnson et al., 1974) that determine the structural match needed to realize channel inactivation after the switch (see Discussion). It should be noted that lacking the β subunit in Hk^{IE18} muscles in fact leads to a slightly lower E_a but a reduction of several orders of magnitude in A, especially at lower voltages (e.g., at −20 mV, $E_a = (8.6 \pm 0.5) \times 10^4$ and $(3.5 \pm 1.0) \times 10^4$ J mol^{-1}, and $A = 2.4 \times 10^{17}$ and 6.6×10^7 s^{-1}, for normal and Hk^{IE18} fibers, respectively; see Table 1). This line of reasoning leads to the hypothesis that the β subunit increases the inactivation rate primarily by promoting proper conformational fit rather than lowering the energy barrier.

Steady-state inactivation and recovery from inactivation

After investigation of the influence of the β subunit on the kinetics of I_A decay, the proportion of channels that are inactivated at steady state was also determined. The experimental data indicate that after a prolonged depolarization the fraction of I_A channels becoming inactivated was less in mutant than in normal fibers (Fig. 4). The half-inactivation potential ($V_{h1/2}$) in the Boltzmann equation used to fit the data reveals a shift of about 6 mV to a more positive direction (−39 mV to −33 mV) in the mutants Hk^1 and Hk^{IE18}. It is worth noting that a voltage shift of a similar magnitude in the same direction was also seen in the voltage dependence of inactivation kinetics (Fig. 3 *A*), in spite of the fact that the voltage ranges in which the kinetic processes (> −20 mV) and the steady-state properties (< −20 mV) were determined do not overlap (Figs. 3 *A* and 4).

Significantly, recovery of I_A from inactivation was again slower in the mutants (Fig. 5). This further weakens the repolarization efficacy of I_A and may contribute to the *Hk* phenotype. The process of recovery can be fit by two exponentials (Wu and Haugland, 1985) with time constants of 50 and 500 ms for normal versus 160 and 1200 ms for mutant fibers. These two time constants are thought to reflect two processes analogous to the N- and C-type inactivation described for *Sh* channels characterized in heterologous expression systems (Hoshi et al., 1991). Therefore, the mutant channels appear sluggish; not only are they slower in inactivation, but once inactivated, they require a much longer time to recover.

Pharmacological inhibition of I_A

Pharmacological agents have been routinely used as a tool to probe the molecular conformation of ion channels. Binding affinity and the mode of action often depend on the conformational state of the channel (Rudy, 1988; Hille,

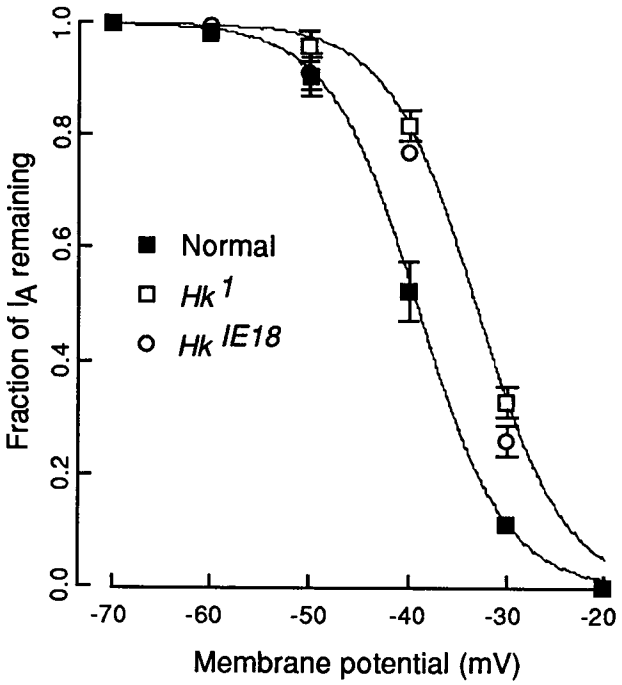

FIGURE 4 The effects of *Hk* mutations on steady-state inactivation. The steady-state inactivation h, indicated by the fraction of attainable I_A remaining at different holding potentials, was shifted to more positive potentials in *Hk* mutant muscles. The curves represent $h = 1/(1 + \exp((V - V_{h1/2})/V_{hslope}))$. $V_{h1/2}$, the half-inactivation voltage, and V_{hslope}, the limiting slope of the inactivation curve, were −39 mV and 4.5 mV/*e*-fold for normal fibers ($n = 15$), and −33 mV and 4.5 mV/*e*-fold for mutant fibers ($n = 11$ for Hk^{IE18} and 21 for Hk^1). Depolarizing potential, 0 mV.

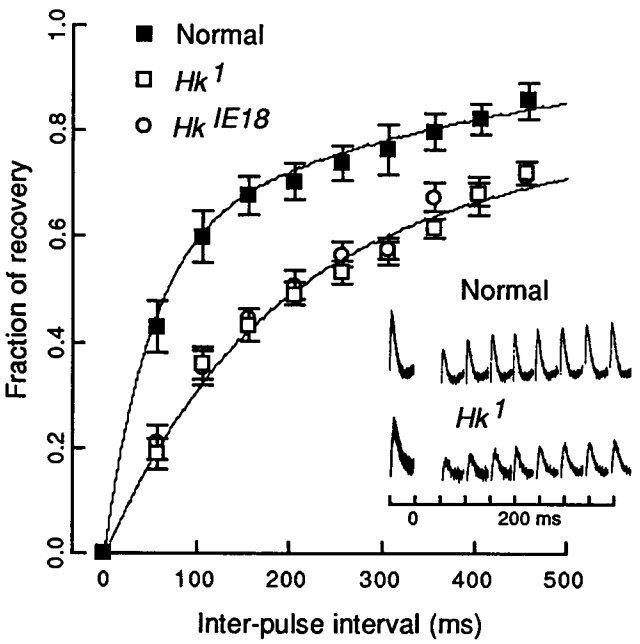

FIGURE 5 Slower recovery of I_A from inactivation in *Hk* mutants. Two identical pulses to 0 mV from holding potential −80 mV for a duration of 50 ms with varying interpulse intervals were used to test recovery from inactivation, r, which could be fit with two time constants τ_a and τ_b in the equation $r = 1 - 0.6\exp(-t/\tau_a) - 0.4\exp(-t/\tau_b)$. For normal fibers ($n = 6$), τ_a and τ_b were 50 and 500 ms, and for mutant fibers ($n = 8$ for Hk^1, and 5 for Hk^{IE18}), they were 160 and 1200 ms. Temperature, 11°C.

1992). As in other preparations, I_A, but not I_K, is sensitive to 4-AP at micromolar concentrations in *Drosophila* larval muscles (Haugland, 1987). We found that I_A was blocked by 4-AP less completely in *Hk* muscles than in normal muscles at all concentrations examined, even after prolonged incubation (>20 min). After this equilibration procedure, no further reduction in I_A could be induced by repeated depolarizing pulses, indicating that the differential effects of 4-AP on the two genotypes are not use-dependent. The half-inhibition concentration (IC_{50}) was 17.3 μM for *Hk* and 6.6 μM in normal fibers (Fig. 6). It is generally agreed that 4-AP binds to the cytoplasmic pore region of *Sh* channels (McCormack et al., 1991; Kirsch et al., 1993; Yao and Tseng, 1994). The lower sensitivity of mutant fibers suggests that the β subunit modifies channel conformation to affect the cytoplasmic recognition site(s) for 4-AP. Therefore, *Hk* subunits may either be associated with the inner mouth directly or influence the mouth conformation through second-site interaction.

DISCUSSION

We have examined the muscle membrane currents in a point and a null mutant of the *Hk* gene and found that these mutations specifically alter many aspects of the transient K^+ current I_A, suggesting an interaction between the *Hk* β and the *Sh* α subunits. Conformational modulation of the *Sh* channel by the *Hk* β subunit is further supported by pharmacological evidence that mutant I_A was less sensitive to 4-AP, which binds to the cytoplasmic pore region of *Sh* channels (McCormack et al., 1991; Kirsch et al., 1993; Yao and Tseng, 1994).

Our results help delineate the functional role of the *Hk* β subunit in vivo. The observation that *Hk* subunits modulate I_A properties, especially near the voltage threshold of activation, is consistent with a special role for β subunits in regulating the quiescent state of excitable cells. Defects in the *Hk* β subunit thus lead to hyperexcitability seen in mutant neurons (Ikeda and Kaplan, 1970; Stern and Ganetzky, 1989; Yao and Wu, 1995) and hyperkinetic behavior in mutant flies (Kaplan and Trout, 1969).

Kinetic and thermodynamic considerations

The results clearly demonstrated that the *Hk* β subunit is an important factor that modulates both the activation (Figs. 1 and 2) and inactivation (Figs. 3–5) processes of I_A. The simple exponential decay of I_A enabled a straightforward analysis of the possible kinetic schemes and the energetic requirements for the rate-limiting step in the process. Single-channel analysis of *Sh* currents evoked by depolarizing steps has shown that for certain channel subtypes, activation

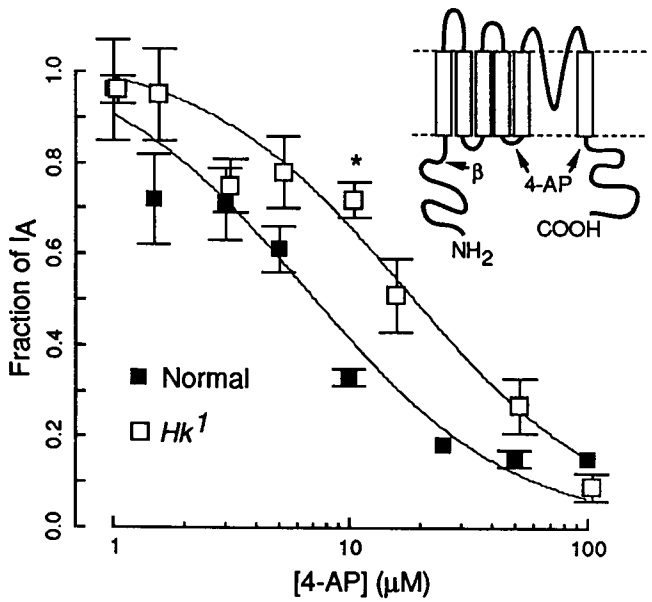

FIGURE 6 Inhibition of I_A by the channel blocker 4-aminopyridine (4-AP). The sensitivity of I_A to 4-AP was reduced in Hk^1 mutant fibers with IC_{50} = 17.3 μM in contrast to 6.6 μM in normal fibers. IC_{50} was determined by a nonlinear regression with the following equation assuming the Hill coefficient of unity: $I_d/I_o = 1/(1 + [4 - AP]/IC_{50})$, where I_d is the I_A amplitude after and I_o the amplitude before drug treatment, when membrane was depolarized to 10 mV from a holding potential of −80 mV. At least 20 min of incubation time was allowed for 4-AP to have a stable measurement of I_d. The fraction of I_A remaining in Hk^1 fibers was significantly greater than normal at 10 μM (n = 10, t-test, $p < 0.0001$, indicated by *, n ranging from 3 to 7 for other concentrations). The inset displays the sites on the *Sh* α subunit believed to be involved in 4-AP binding and the location for association between the α and β subunits.

is relatively slow in the lower voltage range, as indicated by the first latency distribution of channel opening. One can argue for the possibility that the slower decay of I_A in *Hk* mutants observed here is due to a slower activation, rather than inactivation, process. Single-channel studies of *Sh* channels suggest that the processes of activation and inactivation are coupled (Zagotta et al., 1989), so that the *Hk* mutations may affect certain transitions between closed states to change both the time to peak and the rate of decay. However, the following observations suggest that this alternative possibility cannot explain all of the observed kinetic changes in our experiments. In addition to the clear alterations in the activation process (Figs. 1 and 2), certain major defects could be attributed to the inactivation process. First, recovery from inactivation was significantly slower in *Hk* mutants (Fig. 5). It is clear that the process of recovery from inactivation is more closely related to inactivation than to activation. Second, there was a clear shift in voltage dependence of steady-state inactivation (Fig. 4). Third, in *Drosophila* myotubes (Zagotta et al., 1989), the first latency of single-channel currents induced by step depolarizations in the voltage range studied here is relatively short (less than 10 ms at 0 mV) and does not overlap with the decay phase considered here. Fourth, the decay phase of I_A in *Hk* mutants still followed a simple exponential process with no obvious indication of additional exponential components. Therefore, in the Arrhenius plot, the rate constant k, which is derived from the time constant τ of the simple exponential decay of I_A, is most likely related to the inactivation kinetics. Furthermore, I_A decays to a small residual level, indicating that the decay is largely determined by the forward rate constant k_1 of the transition from O to I_1 in Eq. 1 ($\tau = 1/k = 1/(k_1 + k_{-1}) \approx 1/k_1$; see Results).

Regression of the transformed Arrhenius equation, $\ln k = \ln A - E_a/RT$, gives the slope E_a and the intercept $\ln A$, as shown in Fig. 3 *B*. The preexponential constant A allows for adjustment of the effective orientations of colliding molecules in chemical reactions (Johnson et al., 1974), and in our case is roughly equivalent to the collective, averaged conformation states of channels effective for inactivation. In simple bimolecular chemical reactions, factors contributing to the constant A can be categorized into those affecting collision rate and those related to entropy factors (Johnson et al., 1974), including the favorable orientations of the colliding molecules and the environment in which the reaction takes place (e.g., hydration, viscosity, etc.).

It is surprising that *Hk* mutations alter the preexponential constant A more profoundly than the activation energy E_a. This implies that *Hk* β subunits influence the conformational match between molecular domains involved in channel inactivation more than the energy barrier for the conformational change prerequisite for inactivation. For example, it is thought that inactivation in *Sh* channels is the consequence of association between the N-terminal "ball" and the acceptor domain in the cytoplasmic pore region (Hoshi et al., 1990; Zagotta et al., 1990; Isacoff et al., 1991; cf. Armstrong and Bezzanilla, 1977). In this scheme, E_a would be the barrier to be overcome by thermal energy and electric potential before switching, and A could reflect the probability of the actual match allowing inactivation after a collision between the structural domains (Johnson et al., 1974). It is of interest to note that similar observations on the relationships of the two parameters A and E_a to the inactivation process have been reported for *Sh* channels expressed in *Xenopus* oocytes (Murrell-Lagnado and Aldrich, 1993). The free "ball" peptide in saline can cause the inactivation of the noninactivating *Sh* channel (with the N-terminal "ball" domain deleted), and this process is facilitated by certain charge modifications of the peptide. An analysis of temperature dependence of the association rate demonstrates that the facilitated inactivation is coincident with a change of more than 2 orders of magnitude in the preexponential constant A, whereas the activation energy E_a is not significantly changed. These findings by Murrell-Lagnado and Aldrich represent an interesting parallel to our hypothesis that the *Hk* β subunit facilitates I_A channel inactivation by influencing the conformational match rather than lowering the activation energy.

Structural considerations

Our findings of altered 4-AP sensitivity and inactivation properties are consistent with the notion that the inner mouth region on the α subunit contains the potential sites that are regulated by the β subunit in the modulation of I_A. As shown previously, the sites important to both 4-AP binding (McCormack et al., 1991; Kirsch et al., 1993; Yao and Tseng, 1994) and reception for the inactivation ball (Isacoff et al., 1991) are located in this region. So far, site-directed mutagenesis has not been performed within this region of the α subunit to examine the changes in the outcome of interaction with the β subunit.

Unlike the α subunit, an integral membrane protein, the *Hk* β subunit, lacks putative transmembrane domains (Chouinard et al., 1995) and may behave as a peripheral membrane protein, as recently shown for the mammalian β1 subunit (Scott et al., 1994). Site-directed mutagenesis experiments in heterologous expression systems suggest that the association between α and β subunits involves an N-terminal region on the α subunit (Yu et al., 1996; Sewing et al., 1996). This appears to depend on hydrophobic interaction, because replacing certain hydrophobic amino acid residues severely affects the modulation by β subunits. Our results indicate that similar hydrophobic interactions may be present in vivo, because of the temperature dependence of manifestations of interaction between the α and β subunits. The kinetic differences between normal and *Hk* muscle fibers are diminished when the temperature is lowered, indicating that the normal function of β subunits is hampered at low temperatures (Fig. 3 *B*). This is consistent with a mechanism of hydrophobic interaction, which is usually cold-sensitive (Balwin, 1986; Privalov and Gill, 1988; Chen et al., 1993).

The *Hk* polypeptide is a member of the aldo-keto reductase superfamily, of which aldose reductase is a representative molecule containing a characteristic eight-strand parallel β/α barrel structure (Rondeau et al., 1992; Wilson et al., 1992). High conservation between *Hk* and aldose reductase polypeptides lies in the β sheet present in the $(\beta/\alpha)_8$ barrel structure, and the highly hydrophobic area for the binding of NADPH (Chouinard et al., 1995). It will be important to determine whether the characteristic $(\beta/\alpha)_8$ structural motif is preserved in the K^+ channel β subunits and how this motif interacts with specific domains in the α subunit. To our knowledge, extensive structural analysis of the functional domain in the β subunit by site-directed mutagenesis has not been accomplished. It should be noted that there were no substantial differences between the point mutation Hk^1 and the null mutation Hk^{IE18} in all aspects of I_A examined. The Hk^1 mutation might therefore define an important site in the molecular region of the β subunit crucial for in vivo interaction.

In vivo functions

The *Shaker* family of K^+ channel α subunits in *Drosophila* consists of the splicing variants of the *Sh* gene and the *Sh* homologs *Shab*, *Shaw*, and *Shal* (corresponding to mammalian Kv2α, Kv3α, and Kv4α, respectively, Salkoff et al., 1992). Heterologous expression and immunohistochemical staining studies have demonstrated that the mammalian β subunits associate with members of the *Sh* subfamily (Kv1α), but not with other *Sh* homologs in the *Shaker* family. Moreover, individual isoforms of Kvβ subunits interact with specific members within the *Sh* subfamily (Nakahira et al., 1996; Yu et al., 1996). It is known that *Drosophila* muscle I_A channels contain the *Sh* α subunit, whereas I_K channels contain *Shab* (Salkoff and Wyman, 1981; Wu and Haugland, 1985; Tsunoda and Salkoff, 1995). Our in vivo observations that *Hk* mutations specifically affect I_A without altering I_K and other K^+ currents are consistent with the above findings that the β subunits interact with only specific members of the *Sh* subfamily.

Except for certain distinct differences (see below), our in vivo data in large part agree with the observations from coexpression experiments in *Xenopus* oocytes (Rettig et al., 1994; Chouinard et al., 1995). The β subunits can speed up the activation and inactivation processes and shift their voltage dependence toward more negative potentials. Furthermore, with the presence of the β subunits, the density of *Sh* currents is substantially increased. It is not known whether this is due to an increase in single-channel conductance or simply reflects a higher channel density resulting from a better expression efficiency or channel stability (Shi et al., 1996). Resolution of this question requires single-channel recording from heterologous expression systems or in vivo preparations.

Despite the consistency mentioned above, there can be significant differences in experimental results and hence physiological implications between in vivo and heterologous systems. For example, our results demonstrate that the normal function of the *Hk* β subunit is to accelerate the recovery of the I_A channel from inactivation in vivo (Fig. 5). The slower recovery from inactivation in *Hk* mutants can be one of the factors causing hyperexcitability phenotypes in neurons. However, coexpression of the *Hk* β subunit with the *Sh* α subunit slows I_A recovery from inactivation in the oocyte expression system (Chouinard et al., 1995). This difference might be due to uncertainty in the stoichiometry of α and β subunits and other undefined cytoplasmic factors in heterologous systems. Moreover, K^+ channels expressed in a heterologous system are less sensitive to pharmacological agents such as 4-AP and tetraethylammonium (TEA) compared to native channels (Haugland, 1987; Iverson and Rudy, 1990; Zhao et al., 1995), suggesting that channel structure in a non-native environment can differ from the native one. Thus the functional consequences of channel modulation conferred by auxiliary subunits may be illustrated best in native excitable cells.

Results presented here demonstrate that the *Hk* β subunit modulates all aspects of the *Sh* channels in vivo, including kinetics, voltage dependence, pharmacology, and temperature dependence. Thus these results 1) define the range of the in vivo functioning of the β subunit; 2) provide in vivo

evidence for the modulation of *Sh* channels by association with a new class of subunit; 3) extend the previous studies in *Xenopus* oocytes to show that the β subunit confers a conformational change in *Sh* channels, particularly the cytoplasmic pore region; 4) demonstrate that such modulation is most important to *Sh* channel operation near the threshold range, revealing a special role for the β subunit in regulating the quiescent state of excitable cells; 5) explain the hyperexcitability seen in mutant neurons and the hyperkinetic behavior of the mutant flies.

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