A Novel Leg-Shaking *Drosophila* Mutant Defective in a Voltage-Gated K^+ Current and Hypersensitive to Reactive Oxygen Species

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1,1′-Dimethyl-4,4′-bipyridinium dichloride (methyl viologen; paraquat), an herbicide that causes depletion of NADPH and generates excessive reactive oxygen species (ROS) *in vivo*, has been used to screen for ROS-sensitive *Drosophila* mutants. One mutant so isolated, named *quiver*[1] (*qvr*[1]), has a leg-shaking phenotype. Mutants of the *Shaker* (*Sh*), *Hyperkinetic* (*Hk*), and *ether a go-go* (*eag*) genes, which encode different K^+ channel subunits that regulate the A-type K^+ current (I_A) in different ways, exhibit leg shaking under ether anesthesia and have heightened metabolic rates and shortened life spans. We found that *Sh*, *Hk*, and *eag* mutant flies were all hypersensitive to paraquat. Double-mutant combinations among the three channel mutations and *qvr*[1] had drastically enhanced sensitivity to paraquat. Synaptic transmission at the larval neuromuscular junction was increased in the *qvr*[1] mutant to the level of *Sh* mutants. Similar to *eag Sh* double mutants, double mutants of *eag* and *qvr*[1] showed striking enhancement in synaptic transmission and a wings-down phenotype, the hallmarks of extreme hyperexcitability. Voltage-clamp experiments demonstrated that the *qvr*[1] mutation specifically disrupted the *Sh*-dependent I_A current without altering the other currents [I_K, Ca^{2+}-activated fast (I_{CF}) and slow (I_{CS}) currents, and I_{Ca}] in larval muscles. Several deficiency strains of the *qvr* locus failed to complement *qvr*[1] and confirmed that ether-induced leg shaking, reduced I_A current, and paraquat hypersensitivity map to the same locus. Our results suggest that the *qvr* gene may encode a novel K^+ channel-related polypeptide and indicate a strong link between a voltage-activated K^+ current and vulnerability to ROS.

Key words: Shaker; Hyperkinetic; ether a go-go; quiver; *potassium channel; synaptic transmission; paraquat; free radical*

A set of well studied mutations has defined a suite of phenotypes associated with defective K^+ channels in *Drosophila*. In different ways, mutations of *Shaker* (*Sh*), *ether a go-go* (*eag*), and *Hyperkinetic* (*Hk*) impair the transient A-type K^+ current (I_A) in *Drosophila* muscles (Salkoff and Wyman, 1981; Wu et al., 1983; Wu and Haugland, 1985; Zhong and Wu, 1991; Wang and Wu, 1996) and neurons (Tanouye and Ferrus, 1985; Baker and Salkoff, 1990; Saito and Wu, 1993; Zhao et al., 1995; Yao and Wu, 1999). These genes encode either the pore-forming or auxiliary subunits of *Sh*-dependent K^+ channels (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988; Warmke et al., 1991; Chouinard et al., 1995; Chen et al., 1996). These channel mutations enhance synaptic transmission at the larval neuromuscular junction (Jan et al., 1977; Ganetzky and Wu, 1983, 1985; Wu et al., 1983; Stern and Ganetzky, 1989), suggesting that the *Sh*-dependent I_A current has a functional role in terminating neurotransmitter release in the presynaptic terminal. Behavioral analysis has demonstrated that *Sh*-dependent K^+ channels are crucial for the control of the peristaltic locomotion in *Drosophila* larvae (Wang et al., 1997).

Sh, *eag*, and *Hk* mutants are well known for their leg-shaking phenotype (Kaplan and Trout, 1969). However, little attention has been given to the observations that oxygen consumption is increased by *Sh*, *eag*, and *Hk* mutations and longevity is inversely related to the enhancement of metabolic rate in these mutant flies (Trout and Kaplan, 1970). *Drosophila*, like other aerobic organisms, uses several enzymes for reactive oxygen species (ROS) homeostasis (Campell et al., 1986; Mackay and Bewley, 1989; Phillips et al., 1989; Staveley et al., 1990). The superoxide radical is catalytically reduced by superoxide dismutase (SOD) to hydrogen peroxide, which in turn is catalytically reduced to water by catalase (Fridovich, 1995). Genetic tools are available in *Drosophila* to investigate ROS homeostasis and relevant pathways (Phillips and Hilliker, 1990). 1,1′-Dimethyl-4,4′-bipyridinium dichloride (methyl viologen; paraquat) is an herbicide that generates superoxide *in vivo* at the expense of NADPH when oxygen is available. Susceptibility to millimolar concentrations of paraquat has been used successfully in screening for mutants in the ROS pathway (Phillips et al., 1989; Humphreys et al., 1993, 1996)

We demonstrated that like *quiver* (*qvr*) mutants, *Drosophila* K^+ channel mutants *Sh*, *eag*, and *Hk* were also hypersensitive to paraquat challenge. The EMS-induced *qvr*[1] mutation, along with several deficiency lines, reduced the amplitude and slowed the kinetics of I_A, like several previously isolated leg-shaking mutants. These results elucidate the physiological roles of the *qvr* polypeptide and revealed functional similarities among *qvr* and the known I_A K^+ channel mutants. *Sh*-dependent K^+ channels are known to be modulated not only by second messenger-dependent processes (Zhong and Wu, 1993b) but also by oxidoreduction (Schlief et al., 1996; Gulbis et al., 1999; J. Chen et al., 2000), which may provide a means to regulate synaptic efficacy. This study may initiate work toward a comprehensive understanding of *qvr* and K^+ channel mutants to shed light on the link between ROS and K^+ currents.

MATERIALS AND METHODS

Fly stocks. All flies were raised at room temperature (20–23°C) and fed with standard *Drosophila* medium. The parental stock *qvr*$^+$; *ry*$^{+5}$, for generating the *qvr*[1] mutant, was originally derived from the wild-type strain Oregon-R and was used in this study as the control. The Canton-S (CS) wild-type strain, used for comparison, is not significantly different from Oregon-R in many physiological aspects examined in this study. The *qvr* locus was mapped previously to 48A (Humphreys et al., 1996). *Df(2R)en-SFX31/CyO* (48A1; 48B5-7) and *w*; *Df(2R)en-B*, *b*[1] *pr*[1]*/CyO* (47E3-6; 48A4-B2) were provided by the Bloomington Stock Center (Bloomington, IN). These two deficiency lines are homozygous lethal and

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failed to complement the qvr^1 mutation in leg-shaking behavior and paraquat hypersensitivity (Humphreys et al., 1996). $qvr^{\Delta 1\text{-}1}$, $qvr^{\Delta 1\text{-}2}$, $qvr^{\Delta 1\text{-}3}$, and $qvr^{\Delta 1\text{-}4}$ are homozygous lethal deficiency lines generated by mobilization and imprecise excision of a nearby P-element P[*17en1*] (Humphreys, 1996). $qvr^{\Delta 43\text{-}1}$ is a homozygous lethal deficiency line generated by mobilization and imprecise excision of a nearby P-element P[*17en43*] (Humphreys, 1996). Except for $qvr^{\Delta 1\text{-}1}$, all P-element mutagenesis lines failed to complement the qvr^1 mutation in leg-shaking behavior in this study. P[*17en1*] and P[*17en43*] were kindly provided Dr. Judy Kassis at the Food and Drug Administration Center for Biologics Evaluation and Research.

Sh^5, Sh^M, *g sd* Sh^{rKO120} (abbreviated Sh^{120} in the text; see Table 1), Hk^1, eag^1, and nap^{ts1} were originally from the collection of Dr. Seymour Benzer at the California Institute of Technology. Sh^M is a null allele (Zhao et al., 1995) and eliminates I_A in larval muscles (Wu and Haugland, 1985). Sh^5 is a point mutation in the S4–S5 cytoplasmic linker (Gautam and Tanouye, 1990) and alters the voltage dependence of I_A (Wu and Haugland, 1985). The Hk^2 strain is the original stock described in Kaplan and Trout (1969) and is kindly provided by Dr. Rodney Williamson at the Beckman Research Institute of the City of Hope. The compound mutants eag^1 Sh^{120}, Hk^1 eag^1, and eag^1 Sh^{120} nap^{ts1} are the same stocks used in previous studies (Budnik et al., 1990). Other compound mutants were generated for this study. Compound mutants were all confirmed by scoring leg-shaking phenotype and electrophysiological phenotype in larval muscles. The semicolon for indication of mutations on separate chromosomes is omitted in the text for simplicity. nap^{ts1} is an EMS-induced mutation (Wu et al., 1978), which reduces the expression of sodium channels and is allelic with *mle* mutations (Kernan et al., 1991). Flies bearing this mutation become paralyzed at 37°C or higher because of the blocking of nerve action potentials.

Wings-down frequency. The frequency of wings-down flies was determined in male F1 noncurly flies of the following cross within 72 hr after eclosion: A/*Y;* qvr^1/*CyO* × *XX/Y;* qvr^1/*CyO*, where A represents the X chromosome carrying Hk^1, Hk^2, eag^1, *f* eag^{4pm}, Sh^5, *g sd* Sh^{120}, or Sh^M. *CyO* is a second chromosome balancer carrying a dominant marker *Cy* (curly wings), and XX indicates a compound X chromosome, which carries *y* and *f* markers. Flies with noncurly wings in the F1 generation were homozygous for qvr^1, whereas those with curly wings were heterozygous for qvr^1. Wings-down flies are flightless and sluggish in locomotion (Engel and Wu, 1992).

Paraquat feeding. The procedure for paraquat (from Sigma, St. Louis, MO) feeding was described previously (Humphreys et al., 1993, 1996). Briefly, 0- to 24-hr-old adult male flies were collected and allowed 24 hr to recover from ether anesthesia before being transferred to vials (10 flies/vial) for paraquat exposure. Flies were then exposed for 48 hr at 25°C to filter paper presoaked with paraquat dissolved in 1% sucrose solution. Flies were held in the dark during exposure, because paraquat is light sensitive. The survival rate was determined at the end of the 48 hr exposure period.

Electrophysiology. Dissection of *Drosophila* third-instar larvae was performed in Ca^{2+}-free saline to minimize muscle contraction. Excitatory junctional potentials (EJPs) were recorded intracellularly from muscles of abdominal segment 3–5 in third-instar larvae at room temperature (20–25°C) in HL3 saline (Stewart et al., 1994) containing 1 mM $CaCl_2$. For measuring excitatory junctional currents (EJCs), muscle fibers were maintained at −80 mV with two-electrode voltage clamp at 16°C (Wang et al., 1994). A suction pipette with a tip opening of ~1 μm was used to stimulate the segmental nerve to evoke synaptic transmission. Stimulus pulses of 0.1 msec duration were delivered at a low repetition rate of 0.5 Hz with a stimulator (model S88; Grass Instruments, Quincy, MA). Normally, two discrete EJC amplitudes were evoked at two different thresholds (Jan and Jan, 1976). A stimulus voltage slightly higher than the upper threshold was therefore used. Signals were low-pass filtered at 2 kHz (model 3202R; Krohn-Hite, Avon, MA). Temperature was controlled by a Peltier stage (Cambion, Cambridge, MA) when specified as different from room temperature.

The two-electrode voltage-clamp technique for measuring muscle K^+ currents (I_A, I_K, I_{CF}, and I_{CS}) and Ba^{2+} currents has been described previously (Singh and Wu, 1989; Haugland and Wu, 1990; Wang and Wu, 1996). In brief, the voltage-gated I_A and I_K were recorded in Ca^{2+}-free standard saline containing 128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 35 mM sucrose, 5 mM EGTA, and 5 mM HEPES, pH 7.1. The Ca^{2+}-activated I_{CF} and I_{CS} were measured in the Ca^{2+}-free standard saline plus 20 mM $CaCl_2$ (Singh and Wu, 1989). This saline was made hypertonic with addition of 353 mM sucrose to prevent muscle contraction, and 1 mM 4-aminopyridine (4-AP) and 100 μM quinidine were added to block I_A and I_K (Zhong and Wu, 1991). For experiments measuring the voltage-gated Ca^{2+} channel, Ba^{2+} was used as the charge carrier to assess the Ca^{2+} conductance without activating I_{CF} and I_{CS} (Gielow et al., 1995). Ca^{2+} channel-mediated Ba^{2+} currents were examined in the Ca^{2+}-free HL3 saline, with 1 mM 4-AP, 50 μM quinidine, and 20 mM tetraethylammonium (TEA) to block K^+ currents and with 4 mM $BaCl_2$. A Master-8 programmable stimulator (A.M.P.I., Jerusalem, Israel) and an IBM-compatible computer equipped with PClamp 5.0 (Axon Instruments, Foster City, CA) were used for voltage-pulse generation and data collection. Data analysis was performed off-line on Macintosh computers with AxoGraph 2.0 (Axon Instruments).

Table 1. Paraquat sensitivity of *Drosophila* channel mutants and *qvr*

	0 mM Paraquat		10 mM Paraquat	
Genotype	% Survival	*n*	% Survival	*n*
qvr^+	99	259	97	340
qvr^1	99	238	42	445
eag^1	98	207	48	269
Hk^1	100	219	48	328
Sh^5	100	30	34	70
Sh^{120}	97	30	32	60
nap^{ts1}	80	20	28	50
eag^1 Sh^{120}	80	20	0	50
eag^1 Sh^{120}; nap^{ts1}	100	30	12	60
eag^1 Hk^1	Not done		0	30
eag^1; qvr^1	82	33	0	59
Sh^5; qvr^1	90	20	0	50
Hk^1; qvr^1	93	40	2	90

RESULTS

Increased paraquat sensitivity and excitability in double mutants

Vigorous leg shaking when ether-anesthetized, a phenotype similar to that of the previously identified K^+ channel mutants *Sh*, *Hk*, and *eag*, was observed in qvr^1 mutant flies (Humphreys et al., 1996). This phenotypic similarity led us to perform a comprehensive test of paraquat sensitivity in those molecularly characterized leg-shaking mutants. As seen in Table 1, when exposed to 10 mM paraquat for 48 hr, Sh^5, Sh^{120}, Hk^1, and eag^1 mutant flies had 32–48% survival rates, similar to that seen in qvr^1 (42%) but much lower than that of wild-type controls (97%). These numbers for controls and mutants are consistent with those reported previously for some of these alleles (Humphreys et al., 1996).

Double mutants of *eag* and *Sh* are even more hyperexcitable than are *eag* or *Sh* single mutants in synaptic transmission at the larval neuromuscular junction (Ganetzky and Wu, 1983, 1985; Zhong and Wu, 1993a) and in the adult flight muscle system (Engel and Wu, 1992). To see whether ROS sensitivity was similarly enhanced in double-mutant combinations, we extended the paraquat-feeding study to include various double combinations among qvr^1 and mutations of the three K^+ channel genes. A survival rate of 0% was observed in eag^1 Sh^{120} double-mutant flies fed with 10 mM paraquat, which was much more extreme than that of any single mutant (Table 1; 48% for eag^1; 32% for Sh^{120}). Sh^5 qvr^1, Hk^1 qvr^1, and eag^1 qvr^1 double-mutant flies showed 0, 2, and 0% survival rates after exposure to paraquat, lower than that of each single mutant. nap^{ts1}, a mutation reducing the expression of a Na^+ channel and suppressing the hyperexcitability in eag^1 Sh^{120} nap^{ts1} triple mutants (Wu and Ganetzky, 1992), lowered the paraquat-induced mortality in eag^1 Sh^{120} nap^{ts1} mutants, despite the fact that the nap^{ts1} mutant flies showed a significant lower survival rate compared with that of the wild-type controls (Table 1). These results indicate that hyperexcitability is closely correlated with paraquat hypersensitivity.

The dosage dependence of survival rate after exposure to paraquat is shown in Figure 1. A noticeable number of double-mutants flies died even without exposure to paraquat. For example, the survival rates for eag^1 qvr^1 and Hk^1 qvr^1 in 0 mM paraquat were 82 and 93%, respectively. This could be attributed to the shorter life span of hyperactive flies (Trout and Kaplan, 1970).

A novel phenotype arose from double mutants of eag^1 and qvr^1, similar to the synergistic effects seen in *eag Sh* double mutants. Wings of the double mutants pointed downward instead of extending horizontally as in normal flies. This "wings-down" phenotype has been studied previously in *eag Sh* double mutants (Engel and Wu, 1992). It is a hallmark of hyperexcitable mutants (Ganetzky

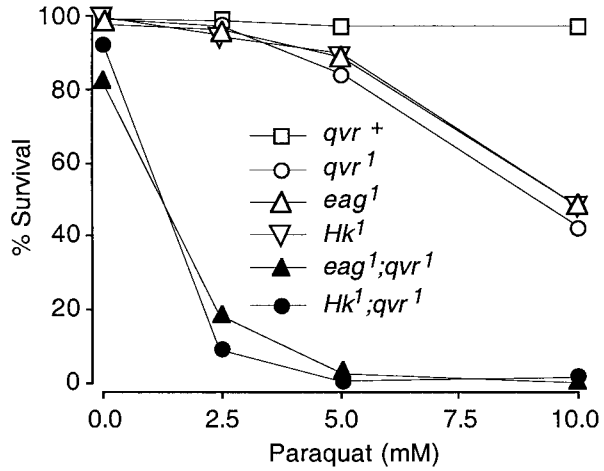

Figure 1. Paraquat hypersensitivity. Adult flies (24–48 hr old) were fed with paraquat for 48 hr at 25°C in darkness, and the survival rate was determined at the end of the 48 hr period. K^+ current mutants eag^1 and Hk^1 were as sensitive to paraquat as was the qvr^1 mutant. The double mutants $eag^1\ qvr^1$ and $Hk^1\ qvr^1$ were more sensitive to paraquat than were any of the single mutants, indicating synergistic interactions between the qvr^1 mutation and the K^+ current mutations. For qvr^+, n = 259, 259, 293, and 340 (from 0 to 10 mM paraquat); for qvr^1, n = 238, 257, 268, and 445; for Hk^1, n = 219, 189, 190, and 328; for eag^1; n = 207, 250, 250, and 269; for $eag^1\ qvr^1$, n = 33, 44, 37, and 97; for $Hk^1\ qvr^1$, n = 40, 47, 49, and 140.

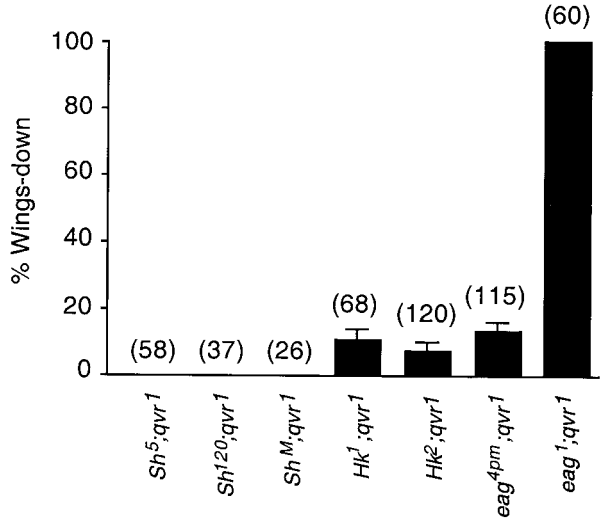

Figure 2. Wings-down frequency in hyperactive mutants. Nearly 100% of the double-mutant $eag^1\ qvr^1$ flies were wings-down. See Materials and Methods for the determination of wings-down frequency. Error bars represent SD. SD = $\sqrt{(p(1-p)/n)}$, where p is the wings-down frequency and n is the number of flies. The number of flies examined is indicated *above* each *bar* in *parentheses*.

and Wu, 1985) and has been used in mutant screening (Stern and Ganetzky, 1992).

As can be seen in Figure 2, nearly 100% of $eag^1\ qvr^1$ double-mutant flies showed the wings-down phenotype. $Hk^1\ qvr^1$ and $eag^{4pm}\ qvr^1$ double mutants had 10 and 13% of the flies, respectively, exhibiting the wings-down phenotype. No wings-down flies were observed in $Sh^5\ qvr^1$, $Sh^M\ qvr^1$, or $Sh^{120}\ qvr^1$, although leg shaking was more vigorous in these double mutants than in *Sh* or qvr^1 alone. Furthermore, no wings-down flies were seen in $Hk^1\ Sh^5$ and $Hk^1\ Sh^{120}$ double mutations, in contrast to the 10% wings-down frequency seen in the $Hk^1\ qvr$ stock. The sequence of potency for causing the wings-down phenotype is $eag^1 > eag^{4pm} > Hk^1 > Hk^2 > Sh^M$ (Fig. 2).

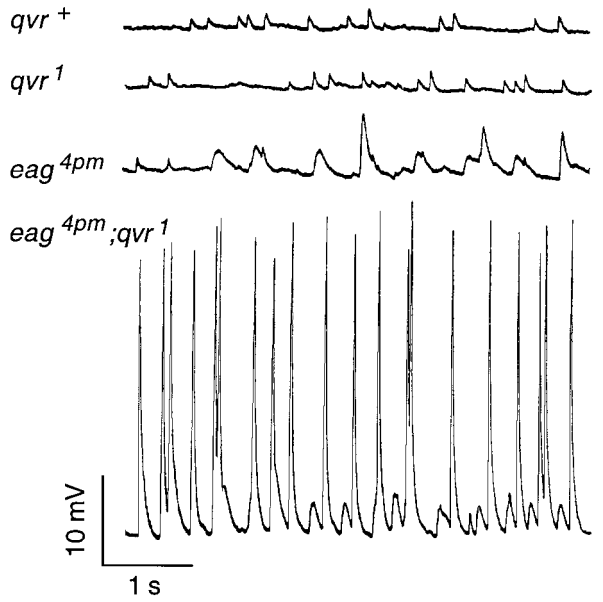

Figure 3. Enhancement of nerve activity by the qvr^1 mutation. In wild-type neuromuscular junctions of third-instar larvae, MEJPs were observed without nerve stimulation (*top trace*) as a result of spontaneous quantal release. The qvr^1 mutant displayed MEJPs similar to that of the wild-type control (*second trace* from *top*). However, the amplitude and frequency of the spontaneous EJPs (recognized by amplitudes larger than quantal size) seen in eag^{4pm} mutants were both drastically increased by the qvr^1 mutation in the $eag^{4pm}\ qvr^1$ double mutant (*bottom two traces*). Experiments were done at room temperature (23°C) in HL3 saline containing 1.0 mM $CaCl_2$ and 20 mM $MgCl_2$.

Synaptic transmission at the larval neuromuscular junction

A unique property of *eag* mutants is that they display spontaneous EJPs, caused by spontaneous firing in the hyperexcitable motor axons (Ganetzky and Wu, 1982), which are different in amplitude and frequency from miniature EJPs (MEJPs). The frequency of spontaneous EJPs is higher in eag^1 than in eag^{4pm} (Ganetzky and Wu, 1983). This is correlated to the degree in hyperexcitability conferred by different *eag* mutations, with eag^1 affecting K^+ currents in larval muscles more than eag^{4pm} (Zhong and Wu, 1991). The frequency and amplitude of the spontaneous EJPs were drastically increased by the qvr^1 mutation in $eag^{4pm}\ qvr^1$ double mutants (Fig. 3). However, the qvr^1 mutation itself did not cause any noticeable alteration in the amplitude, time course, or frequency of MEJPs. Similar synergistic interaction has been observed between *Sh* and *eag* in double-mutant combinations (Ganetzky and Wu, 1983). This suggests that a presynaptic rather than a postsynaptic alteration conferred by the qvr^1 mutation is responsible for the enhancement of the spontaneous EJP phenotype of *eag*.

EJCs serve as a quantitative measurement of synaptic transmission, because muscles are held at a constant membrane potential by the voltage-clamp technique to provide a constant driving force and thus avoid nonlinear summation of multiple quantal release in EJP recordings. As described in other species, the amplitude of EJCs follows a fourth-power relationship with external Ca^{2+} concentration in the *Drosophila* larval neuromuscular system (Zhong and Wu, 1991; Stewart et al., 1994; Wang et al., 1994). At an external Ca^{2+} concentration of 0.4 mM, the increase in EJC amplitude caused by the qvr^1 mutation [27.2 ± 7.4 nA (EJC ± SD); n = 6] was no greater than that by a null mutation, Sh^M (32.2 ± 9.1; n = 5), and was not further enhanced in $qvr^1\ Sh^M$ double mutants (34.6 ± 3.4; n = 5), suggesting that *qvr* and *Sh* share the same pathway in the regulation of synaptic transmission. The difference between qvr^1 and qvr^+ control is proportionally greater at 0.5 than at 1.0 mM $[Ca^{2+}]_o$ (Fig. 4). At these Ca^{2+} concentrations, the EJC amplitudes of qvr^1 and $qvr^1/qvr^{\Delta 43\text{-}1}$ mutants did not follow the fourth-power relationship, indicating that defective K^+ currents in

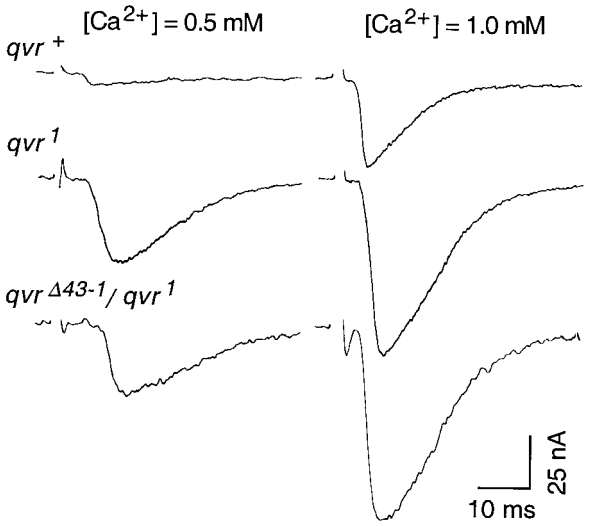

Figure 4. Enhanced EJCs in *qvr* mutations. Muscle membrane potential was voltage-clamped at −80 mV. Experiments were done at 16°C in HL3 saline containing the indicated $CaCl_2$ concentrations and 20 mM $MgCl_2$.

these mutants could weaken membrane repolarization and cause transmitter release approaching the saturation level at relative lower concentrations. This could be caused by approaching saturation of the glutamate receptors on the postsynaptic membrane at the higher $[Ca^{2+}]_o$, which sets the ceiling of EJCs.

qvr mutations specifically affect the I_A current

Outward K^+ currents in *Drosophila* larval muscles can be separated into at least four different components: two voltage-dependent currents, a transient (I_A) and a delayed rectifier (I_K) current, and two Ca^{2+}-activated currents, a fast (I_{CF}) and a slow (I_{CS}) current (Singh and Wu, 1989). Invertebrate muscles generally do not express Na^+ channels, and their inward currents are mediated by Ca^{2+} channels (Schwartz and Stühmer, 1984), which is also true for *Drosophila* muscles. We first examined Ca^{2+} channels for possible defects in *qvr*^{1} because of their important role in neurotransmitter release. Quinidine, 4-AP, and TEA were used to block I_A and I_K (Gielow et al., 1995). Ba^{2+} ions, which pass through Ca^{2+} channels with high permeability, were used here as the charge carrier to avoid activating the Ca^{2+}-activated K^+ currents I_{CF} and I_{CS}. Figure 5 shows that the Ca^{2+} current in larval muscle was not affected by the *qvr*^{1} mutation.

K^+ channels are thought to terminate synaptic transmission by a rapid membrane repolarization (Hille, 1992). The paraquat hypersensitivity and the enhanced synaptic transmitter release seen in both *qvr* mutants and K^+ channel mutants suggest that the *qvr*

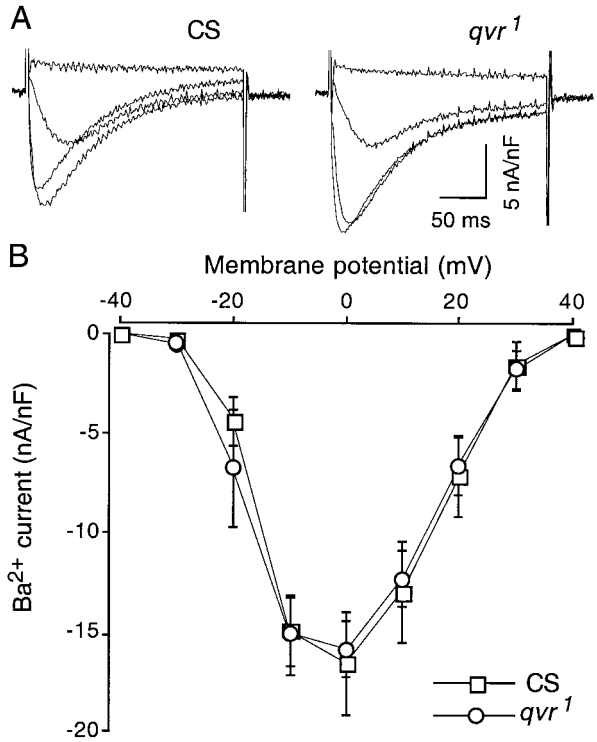

Figure 5. Ca^{2+} currents in muscle cells were not altered by the *qvr*^{1} mutation. *A*, Representative *traces* of inward currents mediated by Ca^{2+} channels at membrane potentials from −30 to 0 mV in 10 mV increment. *B*, *I–V* curves for *qvr*^{1} and CS larvae. The holding potential was −80 mV. Ba^{2+} (4 mM $BaCl_2$) replaced Ca^{2+} in the standard saline as the charge carrier to assess the Ca^{2+} conductance without activating I_{CF} and I_{CS}. Other K^+ currents were blocked by 1 mM 4-AP, 20 mM TEA, and 50 μM quinidine. Data are the mean ± SEM measured at 11°C.

polypeptide might have a functional role in the modulation of K^+ channels. All four K^+ currents mentioned above were examined in *qvr*^{1} mutant larvae (Figs. 6, 7). The Ca^{2+}-activated outward K^+ currents I_{CF} and I_{CS} were examined in the presence of 20 mM Ca^{2+}, and the saline contained 1 mM 4-AP and 100 μM quinidine to block the voltage-activated I_A and I_K. Under these conditions there were no significant differences in the amplitude or kinetics of the outward currents I_{CF} and I_{CS} induced by membrane depolarization (Fig. 6).

When Ca^{2+}-free saline is used, only I_A and I_K are activated by a step of depolarizing voltage. I_A and I_K can be separated physiologically by their different responses to a 2 sec conditioning prepulse from a holding potential of −80 to −20 mV, which inactivates

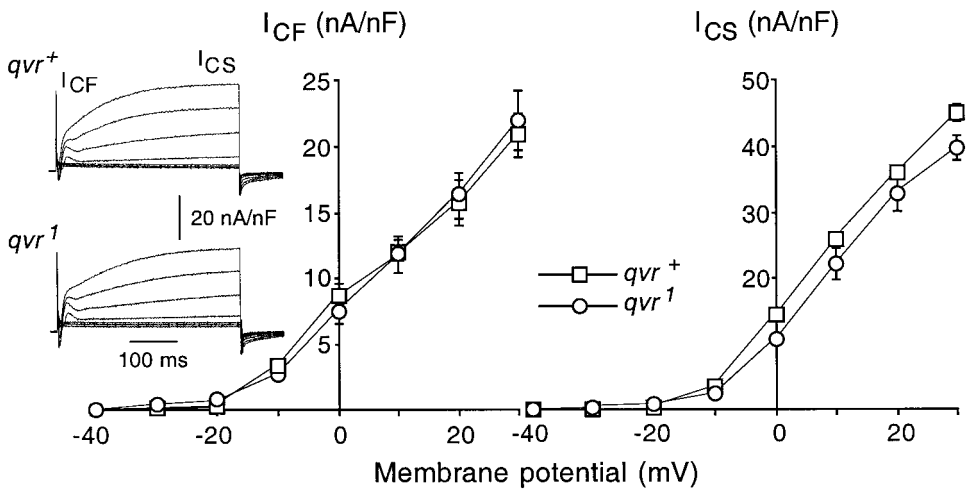

Figure 6. Ca^{2+}-activated K^+ currents were not altered by the *qvr*^{1} mutation. *Traces* (*left*) represent outward currents generated by membrane depolarization to different voltages ranging from −40 to 30 mV at an increment of 10 mV from a holding potential of −80 mV. Standard saline contained 20 mM $CaCl_2$ and 4 mM $MgCl_2$. Voltage-gated K^+ currents were blocked by 1 mM 4-AP and 100 μM quinidine. Tonicity of the saline was increased by adding 353 mM sucrose to reduce muscle contraction. Data are the mean ± SEM measured at 11°C.

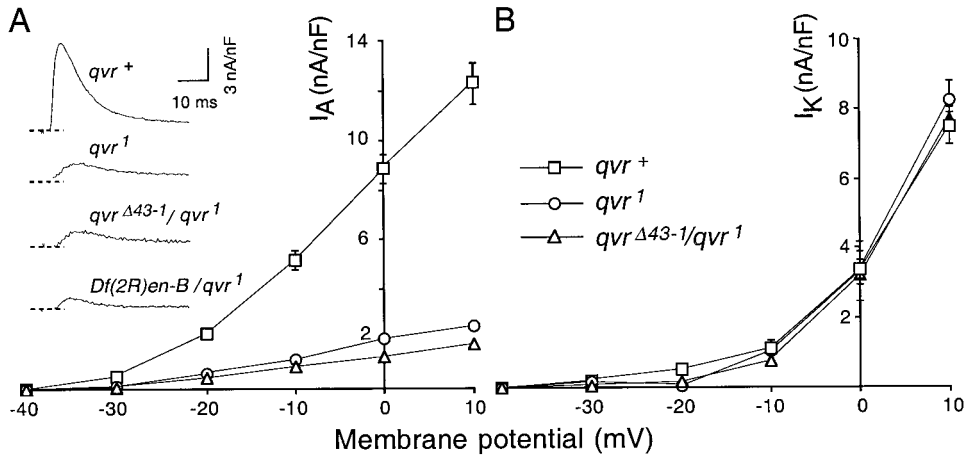

Figure 7. The transient I_A and delayed I_K currents in *qvr* mutant muscles. Larval preparations were dissected and recorded in Ca^{2+}-free standard saline containing 14 mM $MgCl_2$ and 5 mM EGTA. The membrane potential was held at −80 mV. *A*, *Right*, The amplitude of I_A was drastically reduced by *qvr* mutations. *Left*, The activation kinetics of I_A was slower in *qvr* mutants as shown in the representative current *traces* generated by membrane depolarization to +10 mV (see also Table 2, Time to peak). Recordings during the first 3 msec show a capacitive transient and have been omitted for clarity. *B*, I_K was not altered by *qvr* mutations. *I–V* curves show I_K measured at the end of the depolarization pulse (between 190 and 200 msec after the onset of depolarization) when a plateau was reached. Data are the mean ± SEM.

I_A but leaves I_K intact when they are assessed by a test pulse delivered 10 msec later (Haugland and Wu, 1990). Figure 7 shows that only the I_A current was affected by the mutation. The I_A current in qvr^1 mutants appeared to have a very unstable component that inactivated easily and recovered slowly and incompletely (J. W. Wang and C.-F. Wu, unpublished observations). For simplicity, only the stable and fast-recovery component is presented here. The amplitude of the transient I_A was greatly reduced at various membrane potentials as seen in the *I–V* curve, and the kinetics of I_A was slower in the *qvr* mutations as the time to peak I_A was lengthened (Table 2; Fig. 7, representative *traces*). When larval muscles were depolarized to +10 mV from a holding potential of −80 mV, the average amplitude of I_A for the qvr^1 mutant larvae was 2.5 ± 0.3 nA/nF, only 20% of the wild-type I_A current (12.3 ± 0.8 nA/nF) in qvr^+ larvae.

The EMS mutagenesis of the second chromosome yielded only one paraquat-hypersensitive leg-shaking allele, qvr^1. To attribute the observed physiological phenotype to the *qvr* locus defined on the basis of paraquat hypersensitivity, we examined two deficiencies, *Df(2R)en-B* and *Df(2R)en-SFX31*, that cover a chromosome region that contains qvr^1. In addition, we generated five new deficiency lines from the mobilization of two P-elements that map near the *qvr* locus. All of these deficiency lines are homozygous lethal. Four deficiency lines designated $qvr^{\Delta 1\text{-}1}$, $qvr^{\Delta 1\text{-}2}$, $qvr^{\Delta 1\text{-}3}$, and $qvr^{\Delta 1\text{-}4}$ were obtained by mobilizing the P-element in P[*17en1*]. The $qvr^{\Delta 43\text{-}1}$ mutation was obtained by mobilizing the P-element in P[*17en43*]. All of these deficiency lines except $qvr^{\Delta 1\text{-}1}$ failed to complement the qvr^1 mutation in the leg-shaking behavioral test. As shown in Table 2, heterozygotes between these deficiencies and qvr^1 showed a reduction in I_A amplitude and slower I_A kinetics as indicated by the time to peak I_A. These heterozygotes, except $qvr^{\Delta 1\text{-}1}/qvr^1$, did not show a significantly different amplitude of I_A from that of the qvr^1 mutation. These results establish that the physiological phenotype comaps with the leg-shaking and paraquat hypersensitivity to the *qvr* locus.

Table 2. Alteration of I_A in *qvr* deficiency lines

Genotype	I_A (nA/nF)	Time to peak (msec)	*n*
qvr^+	12.3	7.2	8
qvr^1	2.5	11.9	5
$qvr^{\Delta 1\text{-}1}/qvr^1$	9.3	8.0	3
$qvr^{\Delta 1\text{-}2}/qvr^1$	1.2	10.8	2
$qvr^{\Delta 1\text{-}3}/qvr^1$	1.6	10.6	2
$qvr^{1\text{-}4}/qvr^1$	1.3	11.3	2
$qvr^{\Delta 43\text{-}1}/qvr^1$	1.8	11.6	5
$Df(2R)en\text{-}B/qvr^1$	1.8	10.4	2
$Df(2R)en\text{-}SFX31/qvr^1$	1.9	11.7	2

DISCUSSION

In this study we present a genetic and physiological characterization of a novel leg-shaking mutation, qvr^1. The observed paraquat hypersensitivity in *Sh*, *Hk*, and *eag* mutant flies may be related to the shorter life span and increased metabolic rate in these hyperactive mutants (Trout and Kaplan, 1970), which could increase ROS production and thus confers paraquat hypersensitivity. The measurement of survival rate in double mutants suggests that the hypersensitivity to paraquat is closely related to membrane hyperexcitability. It should be noted that the Cu/Zn superoxide dismutase mutation $cSOD^{n108}$ or exposure of wild-type flies to 1 mM paraquat did not alter the conductance or kinetics of the I_A current in larval muscles (Wang and Wu, unpublished observations) and that the enzymatic levels of catalase or cSOD are normal in qvr^1 mutant flies (Humphreys, 1996). These results suggest that general disturbance in ROS homeostasis per se does not alter I_A currents. Similar to the *eag Sh* double mutants, double mutants of *eag* and qvr^1 showed a wings-down phenotype, the hallmark of extreme hyperexcitability. However, the mutation $cSOD^{n108}$, when combined with Sh^5, Hk^1, or eag^1, did not generate any wings-down double-mutant flies (J. M. Humphreys, A. J. Hilliker, and J. P. Phillips, unpublished observations), suggesting that the wings-down phenotype may be caused by hyperexcitability instead of an increase in the ROS level. These observations raise an interesting possibility that a defect in I_A K^+ channels can disrupt K^+ ion homeostasis and in turn results in excessive ROS. This could be confirmed in the future by measuring the ROS level in all of these K^+ channel mutants.

Null mutations of the *Sh* gene eliminate the I_A current (Wu et al., 1983), whereas the major component of I_K in *Drosophila* muscles is abolished by a deficiency in the *Shab* locus (Tsunoda and Salkoff, 1995; Singh and Singh, 1999). Deletion of the *slowpoke* gene removes I_{CF} current (Elkins et al., 1986; Komatsu et al., 1990). In contrast to these mutations of K^+ channel α subunits, null mutations of the β subunit modify but do not abolish I_A (Wang and Wu, 1996; Yao and Wu, 1999). Furthermore, the specific effect of *qvr* mutations on I_A current instead of a more global effect on K^+ currents parallels the phenotype of *Hk* mutations. Mutation of *qvr* disrupted the modulation of but did not eliminate I_A. The phenotypic similarities of physiological hyperexcitability and leg-shaking behavior between *qvr* and the other K^+ channel mutants *Sh*, *Hk*, and *eag* suggest that the *qvr* gene might encode a novel K^+ channel-related polypeptide. Heterozygotes between several deficiencies and qvr^1 showed phenotypes similar to that of the qvr^1 homozygote in the amplitudes of I_A and EJC, suggesting that qvr^1 may be a null mutation.

The molecular cloning and physiological characterization of the *Sh*, *eag*, and *Hk* genes have served to point out the complex molecular machinery required for the proper functioning of K^+ channels. On the basis of the reduction of all four muscle K^+

currents, I_A, I_K, I_{CF}, and I_{CS}, in *eag* mutations (Zhong and Wu, 1991) and a multiplicity of modulation sites by protein kinases and cyclic nucleotides on the *eag* polypeptide (Warmke et al., 1991; Griffith et al., 1994), it has been hypothesized that the *eag* polypeptide interacts with other K^+ α channel subunits of the *Sh* family to confer channel modulation (Zhong and Wu, 1993a). Interacting channel aggregates or heteromultimeric channel assemblies can therefore increase the functional diversity of K^+ currents. Coexpression of *eag* and *Sh* in the *Xenopus* oocyte has subsequently confirmed an interaction between gene products of *eag* and *Sh* (Chen et al., 1996; M. L. Chen et al., 2000). The intricacy of K^+ channel function is further increased by the β subunit encoded by the *Hk* gene (Chouinard et al., 1995), which modulates the properties of the I_A channel in conductance and kinetics (Wang and Wu, 1996; Yao and Wu, 1999). The rich modulation seen in the I_A channel appears to be reasonable for its important role in regulating the delay in initiation and frequency coding of action potentials (Connor and Stevens, 1971; Zhao and Wu, 1997; Yao and Wu, 1999). A comprehensive study of the *qvr* gene by mutational analysis will lead to a more complete picture of the intricate molecular mechanism underlying the wide-ranging function of K^+ channels. Apparently, the lack of proper *qvr* function could lead to unstable *Sh* channels. In *qvr* mutants, the amplitude of *Sh* I_A was highly use dependent. It had a component that was very easily inactivated and recovered very slowly after being inactivated. Therefore, I_A in *qvr* muscle declined quickly to a steady-state level during repeated activation (see Results; Fig. 7). This property may have important functional implications that can only be elucidated in further physiological experiments using *in vivo* preparations. It is not likely that the expression of *Sh* channels is affected by the *qvr* mutation because the *Sh* current after full recovery displayed nearly normal amplitude. With further information from molecular cloning and the availability of highly specific *Sh* antibodies, some of the above issues may be resolved in a more definitive manner.

Recent studies have demonstrated that oxidation of amino residues in K^+ channels can modify their kinetic and gating properties. In particular, several cloned channels of the *Sh* family have been shown to be regulated by oxidation (Schlief et al., 1996; J. Chen et al., 2000). Future experiments to sequence the *qvr* gene may elucidate the molecular mechanism of the *qvr* gene. The relevant biochemical or physiological pathways will be important in understanding the link between neuronal excitability and ROS homeostasis and the pathology of diseases that have been correlated to abnormal levels of ROS (Rosen et al., 1993; Busciglio and Yankner, 1995; Youdim and Riederer, 1997).

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