Mutation of the Axonal Transport Motor Kinesin Enhances *paralytic* and Suppresses *Shaker* in Drosophila

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ABSTRACT

To investigate the possibility that kinesin transports vesicles bearing proteins essential for ion channel activity, the effects of kinesin (*Khc*) and ion channel mutations were compared in Drosophila using established tests. Our results show that *Khc* mutations produce defects and genetic interactions characteristic of *paralytic* (*para*) and *maleless* (*mle*) mutations that cause reduced expression or function of the alpha-subunit of voltage-gated sodium channels. Like *para* and *mle* mutations, *Khc* mutations cause temperature-sensitive (TS) paralysis. When combined with *para* or *mle* mutations, *Khc* mutations cause synthetic lethality and a synergistic enhancement of TS-paralysis. Furthermore, *Khc* mutations suppress *Shaker* and *ether-a-go-go* mutations that disrupt potassium channel activity. In light of previous physiological tests that show that *Khc* mutations inhibit compound action potential propagation in segmental nerves, these data indicate that kinesin activity is required for normal inward sodium currents during neuronal action potentials. Tests for phenotypic similarities and genetic interactions between kinesin and sodium/potassium ATPase mutations suggest that impaired kinesin function does not affect the driving force on sodium ions. We hypothesize that a loss of kinesin function inhibits the anterograde axonal transport of vesicles bearing sodium channels.

DUE to their extended morphology, neurons provide an excellent system for the study of cytoplasmic transport processes. Most organelles and proteins that are required in axons and terminals are synthesized exclusively in the cell body and require active anterograde transport to reach their destinations. The majority of fast anterograde axonal transport requires microtubules and motor proteins (reviewed by BRADY 1991; VALLEE and BLOOM 1991; HIROKAWA 1993; HOYT 1994; MERCER *et al.* 1994). It is thought that the motor proteins bind to organelles or other cargo and use axonal microtubules as directional tracks for generating processive movement. While substantial advances have been made in understanding the mechanochemistry of microtubule-based movement (for example, see HACKNEY 1994; SVOBODA and BLOCK 1994; BERLINER *et al.* 1995; GILBERT *et al.* 1995), the identity of the specific cargoes carried by most microtubule motors remains unclear.

Kinesin was the first anterograde microtubule-based motor to be discovered and characterized (VALE *et al.* 1985). It is a heterotetramer of two identical heavy chains and two light chains. The kinesin heavy chain (KHC) consists of two globular regions separated by an extended alpha-helical stalk. The amino-terminal globular region, or motor domain, hydrolyzes ATP and generates movement directed toward the plus ends of microtubules (reviewed by GOLDSTEIN 1993). The predicted sequence of the Drosophila KHC motor domain served as a founder for the identification of the kinesin superfamily of microtubule-based motor proteins (reviewed by GOLDSTEIN 1993; GOODSON *et al.* 1994). At least three different members of the kinesin superfamily are expressed in Drosophila neural tissue (STEWART *et al.* 1991), and at least five are expressed in mouse neural tissue (AIZAWA *et al.* 1992). The motor domains of these proteins show extensive sequence similarity, but the stalk and carboxyl-terminal globular regions, which are thought to bind cargo, show little or no sequence similarity (GOLDSTEIN 1993).

The divergence of the cargo-binding regions suggests that different neuronal motors carry different cargoes. A comparison of data from studies of three members of the kinesin superfamily supports this hypothesis. An ultrastructural study revealed that axon terminals in *Caenorhabditis elegans unc-104* mutants possessed low concentrations of synaptic vesicles but appeared to have a normal complement of mitochondria (HALL and HEDGECOCK 1991). Recent biochemical studies have shown that the murine homologue of UNC-104 can associate with anterograde axonal vesicles that bear several protein components of synaptic vesicles (OKADA *et al.* 1995). These data support the hypothesis of HALL and HEDGECOCK (1991) that UNC-104 is a motor for the anterograde transport of synaptic vesicles or their

This article is dedicated to the memory of William Laughner (1943–1994).

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precursors. Another kinesin superfamily member, KIF1b, can associate with mitochondria and can move them *in vitro* toward the plus ends of microtubules (NANGAKU *et al.* 1995). Thus KIF1b may be a motor for the anterograde axonal transport of mitochondria.

The third kinesin superfamily member whose function has been studied in neurons is kinesin itself. Studies in Drosophila have shown that axon terminals in kinesin mutants are reduced in size (D. HURD and W. SAXTON, unpublished data) but contain normal concentrations of synaptic vesicles and mitochondria (GHO *et al.* 1992). Thus, the cargoes transported by kinesin are likely to be somewhat different than those transported by UNC-104 and KIF1b. A variety of other studies suggest that kinesin may carry anterograde transport vesicles (reviewed by BLOOM and ENDOW 1994; HOYT 1994). Two classes of anterograde transport vesicles have been distinguished by electron microscopy: "dense-core vesicles" with osmiophilic neuropeptides in their lumens and "clear vesicles" whose lumens are not osmiophilic (GRAFSTEIN and FORMAN 1980; DE BIASI and RUSTIONI 1988; ATWOOD *et al.* 1993; WELDON *et al.* 1993). Additional diversity within each of these two classes has not been clearly demonstrated, but it probably exists (OKADA *et al.* 1995). Therefore, determining the specific cargoes transported by kinesin and by other putative vesicle motors will require identification of the specific vesicle-associated proteins that each transports.

To identify the cargoes transported by kinesin and to determine the role that such transport has in a complex organism, we have taken a genetic approach using Drosophila. Previous work has shown that the kinesin heavy chain (*Khc*) gene is essential and that it is expressed maternally (SAXTON *et al.* 1991). Severe mutations cause paralysis and death in the second larval instar. Moderate mutations cause paralysis of the posterior region of third instar larvae and death during the pupal stages. Mild mutations can show slight posterior paralysis in larvae but allow the development of fertile adults. Physiological studies of the effects of moderate mutations on segmental nerves revealed that compound action potential propagation was impaired (GHO *et al.* 1992). Although the interpretation of that observation was complicated by the fact that the recordings measured the aggregate function of many individual axons, the results suggested that kinesin's cargo might include vesicles that carry ion channels and associated proteins to the axonal membrane (GHO *et al.* 1992).

In the present study, we have tested this hypothesis by using established behavioral and genetic tests to compare the effects of *Khc* mutations with those of mutations that reduce the activity of ion channels in Drosophila. The rationale behind this approach is that if kinesin is involved in the anterograde transport of vesicles bearing ion channels or proteins that regulate ion channel activity, then kinesin mutations should lower the dosage of those proteins in the axonal membrane and thereby generate phenotypes characteristic of reduced ion channel activity. Our results show that the effects of kinesin mutations are remarkably similar to those of mutations that reduce the activity of voltage-gated sodium channels.

MATERIALS AND METHODS

Drosophila culture and mutant alleles: Flies were cultured at 22° with a 12-hr light and 12-hr dark cycle on a standard soft medium (0.5% agar, 7% molasses, 6% cornmeal, and 0.8% killed yeast) seeded with live yeast. General descriptions of most of the mutations used in this study can be found in LINDSLEY and ZIMM (1992). For more detailed information about *paralytic*^{ts1}, *maleless*$^{nap\text{-}ts1}$ (formerly known as *no-action-potential*^{ts1}), *Shaker*^{KS133} and *ether-a-go-go*^{1} see WU and GANETZKY (1992). The *Atpα*^{2206} allele of the gene that encodes the alpha-subunit of the sodium/potassium ATPase is described by SCHUBIGER *et al.* (1994). Except for *Khc*^{BD}, detailed descriptions of the genetics of the *Khc* mutations used can be found in SAXTON *et al.* (1991). *Khc*^{BD} was generated using ethyl methanesulfonate and isolated in a screen for recessive suppressers of *Shaker*^{KS133} on the second chromosome as described by STERN and GANETZKY (1992). Chromosomes carrying the channel mutations and the *Khc* mutations were rendered isogenic by descent before use in the tests described here.

Temperature-sensitive paralysis assay: For tests of single mutants, adults of the genotypes *mle*$^{nap\text{-}ts1}$, *para*^{ts1}, *y w*; *Khc*^{BD}/*Khc*^{6}, *y w*; *Khc*^{BD}/*Khc*^{6}; P{*Khc*$^{+}$ *w*$^{+}$} and *Khc*^{4}/*Khc*^{5} were collected after eclosion, then aged 2–4 days. The different classes of flies were placed in 1-cm diameter glass vials with a drop of 2.5% sucrose and a cotton pad at the bottom of each. Vials were first placed in a circulating water bath at ambient temperature (~22°) and agitated to get the flies to climb the vial walls. They were then transferred to a circulating water bath heated to a given test temperature for 3 min and subsequently transferred back to the ambient temperature bath. Data shown in Figures 1 and 4 were collected after 2 min at the indicated test temperatures. Temperatures were monitored with thermometers in the water baths and a type T thermocouple thermometer probe (Cole-Parmer) suspended in one of the test vials. The rates of cooling and heating recorded inside the tubes were ~2°/sec. Behavior was recorded with a video camera as described previously (SAXTON *et al.* 1991) or at 15-sec intervals with a 35-mm camera and a macro lens. To test for interactions between *Khc* and sodium channel mutations, progeny from the matings detailed in the following paragraph were used.

Lethal interactions of *Khc* and ion channel mutations: The relative viability of flies with double mutant and various control genotypes was determined by counting the number of progeny in each genotypic class produced from the following crosses: 1) *w*; *mle*$^{nap\text{-}ts1}$ *Khc*^{6}/CyO × *w*; *mle*$^{nap\text{-}ts1}$ *Khc*^{BD}/CyO; P{*Khc*$^{+}$ *w*$^{+}$}/+ (total progeny counted n = 571), 2) *w*; *mle*$^{nap\text{-}ts1}$ *Khc*^{BD}/CyO; P{*Khc*$^{+}$ *w*$^{+}$}/+ × *w*; *mle*$^{nap\text{-}ts1}$ *Khc*^{6}/CyO; P{*mle*$^{+}$}/TM3 (n = 1680), 3) *para*^{ts1}; *Khc*^{BD}/CyO × *para*^{ts1}; *Khc*^{6}/CyO (n = 1009), 4) *para*^{ts1}; *Khc*^{BD}/CyO × *para*^{ts1}; *Khc*^{6}/CyO; P{*Khc*$^{+}$ *w*$^{+}$}/+ (n = 405), and 5) *para*^{ts1}; *Khc*^{BD}/CyO × *para*^{ts1}; *Khc*^{6}/CyO; Dp(1,4)*para*$^{+}$ *f*$^{+}$/+ (n = 337). In none of the crosses was a maternal effect on viability or TS-paralysis observed. Genotypes for some classes of progeny from crosses 4 and 5 were determined by testing temperature-sensitive paralysis thresholds and by additional matings to expose the visible markers carried by the P-element and the duplication. The presence or absence of P{*Khc*$^{+}$ *w*$^{+}$} in the non-CyO progeny of cross 4 was revealed by mating to a *w*^{1} strain. The presence or absence of Dp(1,4)*para*$^{+}$ *f*$^{+}$ in the non-CyO progeny of cross 5 was revealed by mating to a *f*^{1} strain. Difficulties in strain construction prevented tests of the effect of the si-

multaneous presence of *Khc*$^{+}$ and *para*$^{+}$ in the *Khc-para*^{ts1} background.

Leg-shaking assay: To test for interactions between *Khc* mutations and potassium channel mutations the following matings were arranged: 6) *y w f Sh*^{KS133}; *Khc*^{BD}/CyO × *y w f Sh*^{KS133}; *Khc*^{6}/CyO; P{*Khc*$^{+}$ *w*$^{+}$}/+ and 7) *y w g eag*^{1}; *Khc*^{BD}/CyO × *y w g eag*^{1}; *Khc*^{6}/CyO; P{*Khc*$^{+}$ *w*$^{+}$}/+. In neither of the crosses was a maternal effect observed. Each mating produced two classes of double-mutant progeny (non-*Cy* wings): a control class that carried the wild-type *Khc* gene (pigmented eyes) and a test class that did not (nonpigmented eyes). Adult flies were collected after eclosion, then aged 1–3 days. They were anesthetized with ether until they ceased to climb the sides of the chamber and observed with a Nikon SMZ-2T stereo microscope and a 200 W fiber optic illuminator. Leg-shaking phenotypes were recorded with a Nikon 35-mm camera and UFX-IIA controller mounted on the microscope. Exposure times of ~1.5 sec were used with Ilford Pan-F Plus film (ASA 50). Images were digitized and transferred to a MacIntosh Quadra 800 (Apple Computer Co., Inc.), then manipulated to enhance contrast using Photoshop (Adobe Systems, Inc.). The degree of suppression of shaking seen in double-mutants depended on the age of the flies. Suppression was strongest in *Sh-Khc* flies aged 1–3 days and in *eag-Khc* flies aged 1–2 days.

Bang-sensitivity assay: Tests for shock-induced paralysis were conducted as described by SCHUBIGER *et al.* (1994). To test for interactions between *Khc* mutations and the sodium/potassium ATPase alpha-subunit mutation, the following mating was arranged: 8) *Khc*^{BD}/CyO; *Atpα*^{2206}/*Atpα*^{2206} × *Khc*^{6}/CyO; *Atpα*^{2206}/*Atpα*^{2206}. Viability of the double-mutant progeny was assessed by counting the number of progeny in each of the two genotypic classes produced (n = 296). Progeny that had straight wings were placed in clean glass vials and compared for bang-sensitivity with their curly winged siblings, or with *Atpα*^{2206}/*Atpα*^{2206} flies. The same flies were also tested for temperature-sensitive paralysis as described above.

RESULTS

Kinesin mutations cause temperature-sensitive paralysis: Mutations that reduce the activity of voltage-gated sodium channels in neuronal membranes cause reversible TS-paralysis (reviewed by WU and GANETZKY 1992). Flies that are homozygous for the *para*^{ts1} allele of the *paralytic* gene, which encodes the alpha-subunit of neuronal sodium channels (LOUGHNEY *et al.* 1989), become paralyzed when shifted from room temperature (~22°) to elevated temperatures (29°: SUZUKI *et al.* 1971; 32.5°: NELSON and WYMAN 1990; this report) and recover when shifted back down. It is not clear if the TS-paralysis caused by the *para*^{ts1} allele results from a missense alteration that makes the sodium channel protein thermolabile or a reduction in the expression of the *para* gene that leads to reduced sodium channel dosage in membranes (LOUGHNEY *et al.* 1989; B. GANETZKY, personal communication). However, strong evidence that reduced sodium channel dosage does cause TS-paralysis has come from studies of the *mle*$^{nap\text{-}ts1}$ allele of the *maleless* gene. Genetic and biochemical tests suggest that *mle* encodes an RNA-helicase and that the *mle*$^{nap\text{-}ts1}$ allele lowers sodium channel dosage in membranes by reducing the expression of the wild-type *para* gene (JACKSON *et al.* 1984; KERNAN *et al.* 1991). Flies that are homozygous for *mle*$^{nap\text{-}ts1}$ become reversibly paralyzed when shifted from room temperature to elevated temperatures (36–37.5°: WU *et al.* 1978; NELSON and WYMAN 1990; STERN *et al.* 1990; this report).

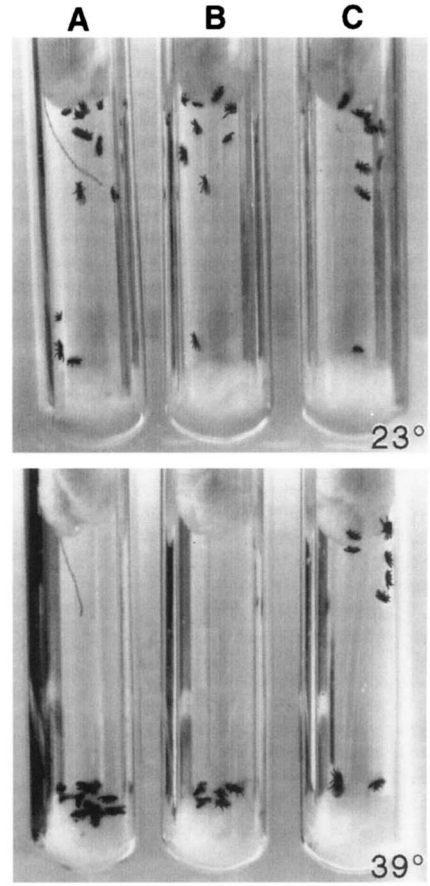

FIGURE 1.—Temperature-sensitive paralysis caused by kinesin mutations. Vials containing flies of three different genotypes are shown at 23° and after 2 min at 39°. The flies in vials A were homozygous *mle*$^{nap\text{-}ts1}$ mutants. The flies in B and C were *Khc*^{BD}/*Khc*^{6} mutants. The flies in C also carried P{*Khc*$^{+}$}, an ectopic copy of the wild-type *Khc* gene. Note that the *Khc* mutants were paralyzed at 39° (B), and that the paralysis was prevented by the presence of P{*Khc*$^{+}$} (C).

The best explanation of the TS-paralysis caused by reduced sodium channel activity is based on the observation that action potential propagation by axons is an inherently temperature-sensitive process (HODGKIN and KATZ 1949; reviewed by WU and GANETZKY 1992). As temperature increases, inward sodium currents decrease. When a "threshold temperature" is reached, action potentials fail (HODGKIN and KATZ 1949). Even wild-type flies can be reversibly paralyzed with a shift from 22° to 43° (NELSON and WYMAN 1990). A reduction in sodium channel activity caused by *para*^{ts1} or *mle*$^{nap\text{-}ts1}$ is thought to make action potentials even more sensitive to temperature elevation (WU and GANETZKY 1992). Support for this idea comes from physiological studies showing that *para*^{ts1} or *mle*$^{nap\text{-}ts1}$ cause a temperature-sensitive failure of action potentials in a subset of Drosophila neurons (ELKINS and GANETZKY 1990; NELSON and WYMAN 1990).

To determine if sodium channel activity is affected

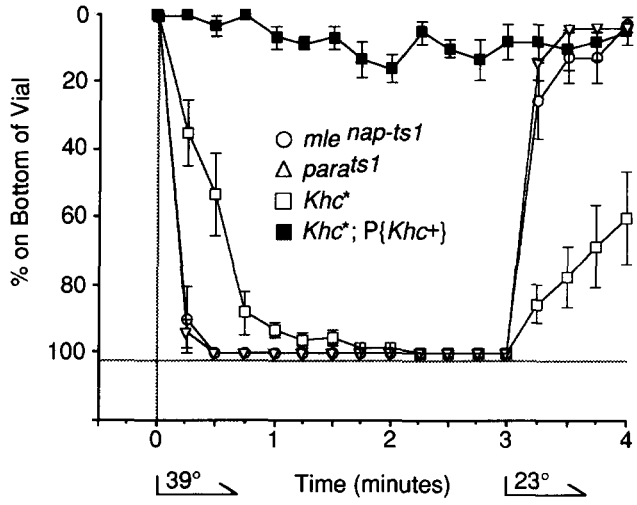

FIGURE 2.—Comparison of the kinetics of TS-paralysis and of recovery from paralysis of *Khc*, $para^{ts1}$, and $mle^{nap\text{-}ts1}$ mutants. Flies of the genotypes noted on the graph were agitated to induce them to climb the walls of their vials, then shifted from 23 to 39° at time 0. The onset of paralysis was marked by a loss of coordination that caused flies to fall to the bottom of the vial. The percentage of flies on the bottom of each vial is shown as a function of time at 15-sec intervals. After 3 min, the vials were shifted back to 23° and agitated to assess the rate of recovery from paralysis. Kinesin mutants righted themselves and began normal grooming behavior within 1 min of the shift down to 23° but often did not resume normal climbing behavior for some time. Each vial contained 10 flies. Data points shown the mean ± SE for five independent tests.

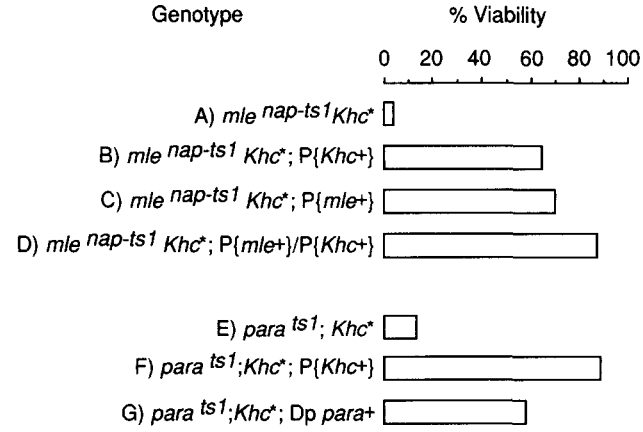

FIGURE 3.—Lethal interactions between kinesin and sodium channel mutations. Matings were arranged to produce *Khc-para* or *Khc-mle* double-mutant progeny either with or without relevant ectopic wild-type genes (Khc^+, $para^+$, or mle^+). The percent viability for each genotypic class of progeny was calculated by dividing the total number of adult progeny of that genotype by the total number expected for that genotype. The number of progeny expected for a given class was estimated from the number of progeny observed for the most viable sibling class. The designation *Khc** indicates the combination Khc^6/Khc^{BD}. The genetic details of the matings that produced these data are described in MATERIALS AND METHODS. The data in A and B are from cross 1, C and D are from cross 2, E are from cross 3, F are from cross 4, and G are from cross 5.

by impaired kinesin function, we compared the impact of elevated temperatures on flies that were mutant for either $mle^{nap\text{-}ts1}$, $para^{ts1}$, or *Khc*. Because severe *Khc* mutations lead to larval lethality, mild *Khc* mutations that have minimal effects on viability were used to allow the testing of adults. To our knowledge, none of the *Khc* alleles used encodes a kinesin heavy chain protein whose function is temperature labile. Nevertheless, Khc^4/Khc^5 or Khc^6/Khc^{BD} hetero-allelic mutant flies that had been cultured at 22° became paralyzed at 39° (Figure 1). This was above the thresholds for $mle^{nap\text{-}ts1}$ (36°) and $para^{ts1}$ (32°) but was well below the 43° threshold temperature expected for wild-type flies. The paralysis of *Khc* mutants at 39° was prevented by the presence of a wild-type copy of *Khc* carried on a transposon (Figure 1C). The temperature threshold for these "rescued" mutants was similar to that of wild-type flies (see Figure 5). It is clear that *Khc* mutations cause a distinct downward shift in the threshold temperature for paralysis. This indicated a shared phenotype between *Khc* and sodium channel mutations and suggests that sodium channel activity might be reduced by impaired kinesin function.

Although the TS-paralysis phenotype of *Khc* mutants was similar to that of $mle^{nap\text{-}ts1}$ and $para^{ts1}$ mutants, it was not identical. First, the temperature threshold for paralysis of *Khc* mutant flies was higher than that for $mle^{nap\text{-}ts1}$ and $para^{ts1}$ mutant flies. Also, the time course of paralysis and of recovery from paralysis was slower for *Khc* mutants than for the sodium channel mutants (Figure 2; see also GRIGLIATTI *et al.* 1973; WU *et al.* 1978). The $mle^{nap\text{-}ts1}$ and $para^{ts1}$ flies were paralyzed within 15 sec of the shift to 39°, rarely showed any movement after falling to the bottom of the test vial, and recovered full mobility within 15 sec of the shift back to room temperature. Similar fast kinetics for $mle^{nap\text{-}ts1}$ and $para^{ts1}$ flies were seen at 36 and 32° respectively. In contrast, *Khc* mutant flies required from 1 to 2 min at 39° for complete paralysis, and then after the shift back to room temperature required ≤1 min to right themselves. After righting themselves, the *Khc* mutant flies were sluggish for many minutes and would not climb up from the bottom of the test vial unless it was agitated. This sort of sluggish behavior is characteristic of *Khc* mutant adults (SAXTON *et al.* 1991), and it was observed before the shift to high temperature as well as after. In spite of this, it was clear that there were qualitative differences in the TS-paralysis of *Khc* and sodium channel mutant flies. This could be due simply to the required use of mild *Khc* alleles. On the other hand, the differences could indicate that the effects of impaired kinesin function in neurons are more complex than the discrete reductions in sodium channel activity caused by $mle^{nap\text{-}ts1}$ and $para^{ts1}$.

***Khc* mutations enhance sodium channel mutations:** If kinesin mutations do reduce neuronal sodium channel activity, then they should show genetic interactions with voltage-gated sodium channel mutations. To test this prediction, we constructed double-mutants using either

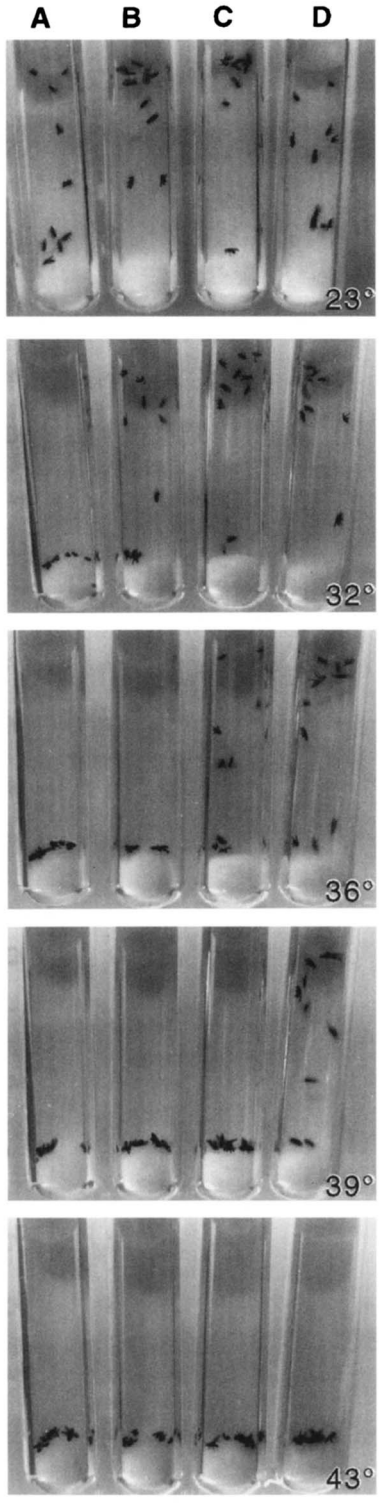

FIGURE 4.—TS-paralysis of *Khc-mle*$^{nap\text{-}ts1}$ double mutants. Matings were arranged to produce *Khc-mle* double-mutant progeny with or without *Khc*$^{+}$ and *mle*$^{+}$ transgenes. The four different classes of progeny were separated into vials and tested for paralysis at various temperatures. The flies in vial A were the simple *mle*$^{nap\text{-}ts1}$ *Khc*^{BD}/ *mle*$^{nap\text{-}ts1}$ *Khc*^{6} double mutants. The flies in the other vials contained that same *Khc-mle* double-mutant genotype with the addition of transposons carrying the following ectopic genes: (B) *Khc*$^{+}$, (C) *mle*$^{+}$, and (D) both *Khc*$^{+}$ and *mle*$^{+}$. Vials are shown 2 min after immersion in water baths maintained at the indicated temperatures. The genetic details of the matings that produced these flies are described in MATERIALS AND METHODS. The flies in A and B are from cross 1 and those in C and D are from cross 2.

para^{ts1} or *mle*$^{nap\text{-}ts1}$ and the hetero-allelic combination *Khc*^{BD}/ *Khc*^{6}. Parental strains and matings were arranged such that some progeny carried ectopic wild-type genes for *Khc, para,* or *mle.* When none of the wild-type genes were present, double-mutant flies showed very poor viability (Figure 3). In the *Khc-mle*$^{nap\text{-}ts1}$ tests, the presence of either of the *Khc*$^{+}$ or *mle*$^{+}$ transgenes raised the viability for double-mutant flies by a factor of 10. The simultaneous presence of both wild-type transgenes provided an additional increase to nearly normal viability. In the *Khc-para*^{ts1} double-mutant tests we observed similar results. These data indicate a specific semilethal genetic interaction (synthetic lethality) between kinesin mutations and mutations that reduce the activity of sodium channels. While synthetic lethality often indicates that two gene products interact or participate in the same pathway (KAISER and SCHEKMAN 1990), it can also arise from the combination of mutations in genes whose products are not understood to be in the same pathway. A strong argument that two proteins do participate in the same pathway can be made if double-mutant combinations produce synthetic lethality and if the respective single mutations share a phenotype (HUFFAKER *et al.* 1987). Although the kinetics shown in Figure 2 raised some doubt, the temperature-sensitive paralysis seen with mild *Khc* mutations (Figure 1) suggests a shared phenotype with sodium channel mutations.

If kinesin and sodium channels act through independent pathways to generate TS-paralysis, then their temperature thresholds should be independently derived and double-mutant flies should show the threshold of a sodium channel single mutant. The TS-paralysis of the *Khc-mle*$^{nap\text{-}ts1}$ double-mutant flies, with or without the relevant wild-type transgenes, is shown in Figures 4 and 5. The threshold temperature for paralysis of the double-mutants (32°) was markedly lower than that of the *Khc* or the *mle*$^{nap\text{-}ts1}$ single mutants (compare Figure 5, B, D and E). The presence of either *Khc*$^{+}$ or *mle*$^{+}$ eliminated this enhanced sensitivity to temperature. The simultaneous presence of both wild-type transgenes brought the threshold temperature back to the wild-type level. In the case of the *Khc-para*^{ts1} double-mutants, similar results were observed (Figure 5). The threshold temperature for the double-mutants was 28° as compared with 32° for *para*^{ts1} single mutants. This synergistic and specific effect of *Khc* and sodium channel mutations in lowering the temperature threshold for paralysis suggests that the TS-paralysis is indeed a shared phenotype and that kinesin is active in the "pathway" that leads to the sodium currents of neuronal action potentials.

***Khc* mutations suppress potassium channel mutations:** Potassium channels shape and terminate action potentials by controlling the repolarization of the axo-

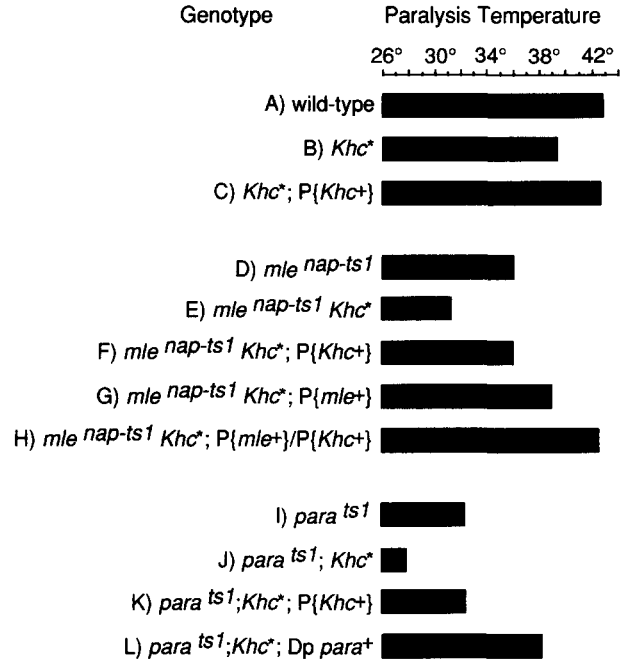

FIGURE 5.—Threshold temperatures for paralysis of *Khc*-sodium channel double mutants. Matings were arranged to produce *Khc-mle*$^{nap\text{-}ts1}$ or *Khc-para*^{ts1} double-mutant progeny with or without relevant ectopic wild-type genes. Different classes of progeny were tested for paralysis over a range of temperatures. The bars in the graph extend out to the threshold temperature for paralysis determined for each genotype. The threhold temperature was defined as the lowest temperature at which 100% of the flies of a given genotype were paralyzed within 2 min. Note that the sodium channel-kinesin double mutants became paralyzed at a temperature well below that of the relevant single mutants. The designation *Khc** indicates the combination *Khc*^{6}/*Khc*^{BD}. The genetic details of the matings that produced these data are described in MATERIALS AND METHODS. The data in E and F are from cross 1, G and H are from cross 2, J are from cross 3, K are from cross 4 and L are from cross 5.

nal membrane. Null mutations in the *Shaker* and *ether-a-go-go* genes, which encode potassium channel subunits, cause rapid spasmodic leg shaking when flies are anesthetized with ether (KAPLAN and TROUT 1969; TROUT and KAPLAN 1973; KAMB *et al.* 1987; PAPAZIAN *et al.* 1987; WARMKE *et al.* 1991). The leg shaking is thought to reflect an imbalance between normal inward sodium currents and reduced outward potassium currents across excitable membranes. This imbalance causes neurons to become hyperexcitable, and they spontaneously propagate repetitive action potentials that elicit leg shaking (JAN and JAN 1979; TANOUYE and FERRUS 1985).

It has been shown that a reduction in neuronal sodium conductance can specifically suppress the leg shaking of potassium channel mutants by restoring a more normal balance between inward and outward currents. Thus, *mle*$^{nap\text{-}ts1}$ or certain alleles of *para* suppress the leg shaking caused by *Shaker*^{KS133} or *ether-a-go-go*^{1} (GANETZKY and WU 1982a,b; STERN *et al.* 1990; WU and GANETZKY 1992). A screen of 2500 mutagenized second chromosomes for recessive suppressers of *Shaker*^{KS133} produced a single mutation that was designated *2-60* (M. STERN, unpublished results). The chromosome that carries *2-60* did not cause lethality when homozygous. However, complementation tests showed that it did cause lethality when placed over a chromosome that carried a severe *Khc* allele (*Khc*^{8}) or a deletion that removed the *Khc* locus [Df(2R)Jp7]. This lethality was completely rescued by the presence of a P{*Khc*$^{+}$} transposon and was mapped by genetic recombination to the interval between *curved* and *Black cell* that contains the *Khc* locus. In addition, *2-60*/*Khc*^{8} larvae showed the sluggishness and posterior paralysis characteristic of *Khc* mutations (SAXTON *et al.* 1991). These data indicate that *2-60* is a mild allele of *Khc*. We have designated this allele *Khc*^{BD}.

To determine if the suppression of *Shaker* by homozygous chromosomes carrying *2-60* was due to the *Khc*^{BD} mutation and if the interaction was either allele- or locus-specific, genetic interactions between various potassium channel mutations and *Khc* alleles were studied. Fly strains were constructed that carried either *Shaker*^{KS133} or *ether-a-go-go*^{1} on homozygous *X* chromosomes and various *Khc* mutant alleles on balanced second chromosomes. Identical strains were constructed with the addition of a third chromosome that carried a wild-type copy of *Khc* (P{*Khc*$^{+}$}). Matings between these strains produced flies that were doubly mutant for a potassium channel subunit and for Khc. Siblings produced by each mating either carried P{*Khc*$^{+}$} or did not. Figure 6 shows that the mild hetero-allelic combination of *Khc*^{BD}/*Khc*^{6} suppressed the leg shaking caused by either of the potassium channel mutations, and that this suppression was reversed by the presence of the wild-type *Khc* transgene. Additional tests showed that homozygous *Khc*^{1ts} suppressed leg shaking when cultured at restrictive temperature (29°) but not at permissive temperature (25°) and confirmed that homozygous *Khc*^{BD} suppressed leg shaking as well. The fact that different *Khc* mutations suppressed the leg shaking caused by mutations in two different potassium channel subunits showed that the interaction was neither allele- nor locus-specific. Therefore reduced kinesin activity, like reduced sodium channel activity, counteracts the hyperexcitability caused by impaired outward potassium currents. This provides another strong argument that impaired kinesin activity restricts sodium channel activity.

***Khc* mutations do not mimic or enhance a sodium/potassium ATPase mutation:** The neuronal sodium/potassium ATPase creates and maintains the transmembrane ion gradients that provide the driving force for sodium and potassium ions during an action potential. A mutation in the Drosophila sodium/potassium ATPase alpha-subunit gene (*Atpα*^{2206}) has been isolated recently by means of a transposon insertion (SCHUBIGER *et al.* 1994). The insertion, which appears to reduce the expression of an otherwise normal protein, causes

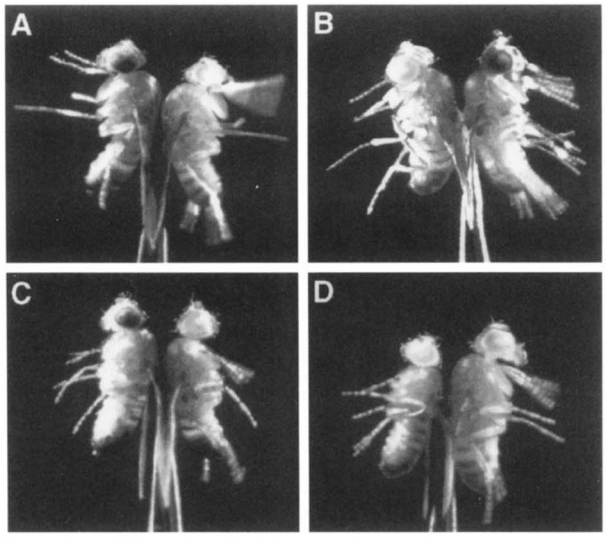

FIGURE 6.—Suppression of potassium channel mutations by kinesin mutations. The leg-shaking behavior of flies under ether anesthesia is shown with 1.5-sec film exposures. Flies were etherized and observed in pairwise combinations: (A) wild type (left) and homozygous mutant *Shaker* (right); (B) *Shaker-Khc* double mutant (left) and *Shaker-Khc* double mutant plus P{Khc^+} (right); (C) wild type (left) and homozygous mutant *ether-a-go-go* (right); (D) *ether-a-go-go-Khc* double mutant (left) and *ether-a-go-go-Khc* double mutant plus P{Khc^+} (right). Note that the kinesin mutations suppressed the leg shaking caused by the potassium channel mutations and that this suppression was relieved by the presence of P{Khc^+}. The genetic details of the matings that produced these flies are described in MATERIALS AND METHODS (crosses 6 and 7).

paralysis of adult flies when they are subjected to severe, repetitive physical stimulation. This "bang-sensitivity" is thought to result from a degradation of transmembrane ion gradients during sustained neuron firing (SCHUBIGER *et al.* 1994).

To determine if the reduction in sodium currents caused by impaired kinesin function is due to reduced ion gradients across neuronal membranes, the phenotypes of *Khc* and sodium/potassium ATPase single-mutants were compared. Flies carrying the hetero-allelic combination Khc^{BD}/Khc^{6} and flies homozygous for $Atp\alpha^{2206}$ were tested for bang sensitivity and TS-paralysis in parallel. After being vortexed in a vial for 10 sec, the $Atp\alpha^{2206}$ flies were paralyzed for 5–10 sec and proceeded through staggering uncoordination to normal behavior over the course of 1–2 min (see also SCHUBIGER *et al.* 1994). In contrast, vortexing of the *Khc* mutant flies caused no paralysis or uncoordination. When tested for TS-paralysis, $Atp\alpha^{2206}$ mutant flies showed a threshold of ~41.5°. However, the testing procedure caused the flies to become highly agitated so it was unclear if the paralysis was due to the temperature shift or overstimulation. The *Khc* mutants showed the expected threshold of 39°.

The possibility that the kinesin and sodium/potassium ATPase mutations might generate a synthetic phenotype was pursued by constructing *Khc*-$Atp\alpha^{2206}$ double mutants. The number of double mutants that developed to adulthood was 46% of the number expected by comparison with the most viable class of single mutant siblings. This raised the possibility of a mild synthetic lethal interaction. However, the double mutants that were recovered showed the TS-paralysis and bang-sensitivity phenotypes of the respective single mutants. No evidence of synergistic effects of kinesin and sodium/potassium ATPase mutations on the behavioral phenotypes was seen. These results suggest that impaired kinesin function does not have a major impact on the electrochemical gradient that drives inward sodium currents during neuronal action potentials.

DISCUSSION

We have shown that mutations in the microtubule-based motor protein kinesin mimic the effects of mutations in two different genes, *para* and *mle,* that are known to reduce the function or the dosage of neuronal voltage-gated sodium channels in Drosophila. Various partial-loss-of-function alleles of *para* and $mle^{nap\text{-}ts1}$ cause TS-paralysis. When a mutant *para* allele and $mle^{nap\text{-}ts1}$ are combined, they cause synthetic lethality or a synergistic decrease in the threshold temperature for TS-paralysis depending on the severity of the *para* mutation (WU and GANETZKY 1992). Our results indicate that partial-loss-of-function *Khc* alleles cause TS-paralysis in single mutants. When combined in double mutants with either $para^{ts1}$ or $mle^{nap\text{-}ts1}$ *Khc* mutations cause synthetic lethality and a synergistic decrease in the thresholds for TS paralysis . It has been demonstrated that if mutations in two genes share a phenotype and if they generate synthetic lethality when combined then their gene products are likely to participate in the same pathway or process (HUFFAKER *et al.* 1987; KAISER and SCHEKMAN 1990).

The shared TS-paralysis phenotype and the genetic interactions between kinesin and sodium channel mutations might still be due to a nonspecific weakening of flies caused by impaired kinesin function. Two independent lines of evidence indicating that kinesin mutations affect sodium channel activity argue against this point of view. First, our data show that kinesin mutations can suppress the leg shaking caused by null mutations in the *Shaker* and *ether-a-go-go* genes that encode potassium channel subunits. In a screen of 2500 second chromosomes, *Khc* was the only locus that produced a suppresser of *Shaker* (M. STERN, unpublished). The other known mutations that can suppress *Shaker* are various alleles of *para,* the nap-ts1 allele of *mle, tipE* (see below) and mutations at two uncharacterized loci that were identified in a screen of 35,000 mutagenized *X* chromosomes (STERN *et al.* 1990). Thus, suppressers of potassium channel mutations are somewhat rare, and the *Shaker* suppressers other than *Khc* that have been characterized (*para, mle* and *tipE*) have direct effects on the dosage or function of the sodium channel α-subunit.

A study of compound action potential propagation in

the segmental nerves of *Khc* mutant Drosophila larvae provides a second line of evidence that the shared phenotypes and genetic interactions of kinesin and sodium channel mutations are significant. Like *para*^{ts1} and *mle*$^{nap\text{-}ts1}$ (reviewed by WU and GANETZKY 1992), kinesin mutations inhibit the propagation of compound action potentials (GHO *et al.* 1992). Therefore, kinesin and sodium channel mutations share three phenotypes: TS-paralysis, suppression of potassium channel mutations, and inhibition of compound action potential propagation. In combination with the synergistic effects seen in kinesin-sodium channel double mutants, these data show that kinesin and sodium channels both participate in the process that produces the inward sodium currents of neuronal action potentials.

How might kinesin, a motor for anterograde axonal transport, influence inward sodium currents? Three factors control the sodium conductance of an excitable membrane: the driving force on sodium ions created by the transmembrane electrochemical gradient, single sodium channel conductance properties, and the number of sodium channels per unit area of the membrane (HILLE 1992). Any or all of these could be altered by kinesin mutations. The driving force might be reduced if kinesin is involved in the anterograde transport of the sodium/potassium ATPase or other proteins that are responsible for establishing and maintaining gradients of positive ions across the axonal membrane. Single channel conductance might be reduced if kinesin is involved in the anterograde transport of proteins that regulate channel function (CATTERALL 1992). Finally, the number of sodium channels in the axonal membrane might be reduced if kinesin is involved in the anterograde transport of the channel subunits themselves.

It is unlikely that the kinesin mutations that we have tested reduce the driving force on sodium ions. Although our results showed a mild synthetic lethal interaction between the chromosomes that carry the kinesin and sodium/potassium ATPase mutations, the single mutants did not share either the bang-sensitive or the TS-paralysis phenotypes. In addition, kinesin-sodium/potassium ATPase double mutants showed no synergistic effect on either bang sensitivity or TS-paralysis. A second argument against an effect on ion gradients and the driving force by kinesin mutations rests on the observation that sustained repetitive stimulation of segmental nerves in *Khc* mutant larvae does not lead to an increase in compound action potential failures (M. GHO, B. GANETZKY, and W. SAXTON, unpublished results). Such an increase in failures would be expected if *Khc* mutations significantly compromised ion gradients or the activity of the sodium/potassium ATPase.

It remains possible that kinesin mutations alter the dosage or activity of proteins that regulate sodium channels. Mutations in Drosophila genes that encode proven channel regulatory proteins have not, to our knowledge, been reported. However, a good candidate for this sort of function is encoded by the *tipE* locus (GANETZKY 1986; JACKSON *et al.* 1986; FENG *et al.* 1995). Mutations in *tipE* cause TS-paralysis (KULKARNI and PADHYE 1982). When combined with *mle*$^{nap\text{-}ts1}$ or certain *para* alleles, they cause synthetic lethality as well as a synergistic lowering of the temperature thresholds for TS-paralysis (GANETZKY 1986; JACKSON *et al.* 1986). In addition, *tipE* mutations weakly suppress the leg shaking phenotype caused by *Shaker* mutations (JACKSON *et al.* 1986). These effects parallel those of *para*, *mle*$^{nap\text{-}ts1}$, and *Khc* mutations. This suggests that impaired kinesin function could reduce sodium conductance through effects on the transport of the product of the *tipE* locus or other similar proteins.

Kinesin could influence sodium conductance by reducing the number of channels present in axonal membranes. We have shown that *Khc* mutations cause defects that are similar to those caused by *mle*$^{nap\text{-}ts1}$, a mutation that is thought to reduce the dosage of sodium channels by reducing the expression of the *para* gene (reviewed by WU and GANETZKY 1992). While it is unlikely that kinesin is involved in the regulation of the expression of the *para* gene, kinesin could be involved in the delivery of sodium channels to the plasma membrane. It has been shown that the application of colchicine to isolated neuronal cell bodies prevents the appearance of sodium currents across their plasma membranes (BRISMAR and GILLY 1987). This suggests that a microtubule-based process is required for the delivery of sodium channels to the membrane. In addition, voltage-sensitive sodium currents appear when vesicles from axoplasm are fused with artificial lipid bilayers (WONDERLIN and FRENCH 1991). This suggests that sodium channels are carried from the cell body to the axon in vesicles. In the context of these studies and given that kinesin is a motor for microtubule-based anterograde vesicle transport, the simplest interpretation of our results is that kinesin is required for the transport of vesicles that bear sodium channels from the cell body to the axonal membrane.

Kinesin activity is probably also required for the axonal transport of other proteins. The differences in the kinetics of TS-paralysis between *Khc* and sodium channel mutations indicate that a loss of kinesin activity has more complex effects than just a loss of sodium channel activity. This is supported further by our analysis of the effects of *Khc* mutations on nerve terminal structure and function (GHO *et al.* 1992; D. HURD and W. SAXTON, unpublished data). Those studies indicate that kinesin activity is required in neurons not only for excitibility, but also for neurotransmitter release and terminal development. Given the abundant and widespread expression of kinesin in nonneuronal tissues (HOLLENBECK 1989; SAXTON *et al.* 1991) as well as the results of other studies of kinesin inhibition (BLOOM and ENDOW 1994), it would be surprising if a loss of kinesin activity halted the movement of only a single subtype of transport organelle. Therefore, while the distinct effects of *Khc*

mutations on sodium channel activity encourage speculation about a unique relationship between kinesin and sodium channels, it is possible that neurons are simply more sensitive to decreased transport of sodium channels than they are to decreased transport of potassium channels and other axonal proteins.

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