Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8415–8419, November 1986
Neurobiology

A *Drosophila* mutation that eliminates a calcium-dependent potassium current

(ion channels/action potential/neurogenetics)

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Communicated by Julius Adler, July 17, 1986

ABSTRACT **A mutation of *Drosophila*, slowpoke (*slo*), specifically abolishes a Ca^{2+}-dependent K^+ current, I_C, from dorsal longitudinal flight muscles of adult flies. Other K^+ currents remain normal, providing evidence that I_C is mediated by a molecularly distinguishable set of channels. The pharmacological properties of I_C are similar to those of Ca^{2+}-dependent currents in some vertebrate cells. The muscle action potential was significantly lengthened in *slo* flies, indicating that I_C plays the major role in its repolarization.**

In the excitable membranes of neurons and muscles, K^+ channels comprise a variety of types as defined pharmacologically and electrophysiologically. These channels play an equal variety of roles in determining the natural signalling properties of excitable cells (1–5). Functions of K^+ currents include repolarizing action potentials, setting the frequency of endogenous pacemaking activity, and delaying the onset of spiking responses evoked by a depolarizing input (6). However, no molecular characterization of these channels has been achieved, primarily because high-affinity ligands for their purification are not yet available.

In *Drosophila*, previous voltage-clamp studies of the dorsal longitudinal flight muscles (DLMs) (7, 8) and the larval body wall muscles (9) have revealed three distinct outward K^+ currents: the voltage-dependent delayed rectifier I_K, and two early inactivating currents I_A and I_C. Although the two early currents share a similar time course, I_A is voltage dependent and I_C is Ca^{2+} dependent.

Here we describe a mutation, slowpoke (*slo*), that specifically eliminates I_C. The slo mutant provides a unique tool to examine the biological roles of this current. For example, the mutant was used to demonstrate directly that I_C is the current primarily responsible for repolarization of the muscle action potential. The *slo* mutation may also enable a molecular genetic analysis of the proteins mediating I_C.

MATERIALS AND METHODS

Isolation and Mapping of *slo*. The *slo* mutation was recovered among a collection of several thousand strains, which had been mutagenized with ethyl methanesulfonate, and made homozygous for the third chromosome (10). The flies were raised at 18°C and screened for those that became paralyzed when exposed to 38°C as described (11). The *slo* mutation is recessive and maps by recombination to the right arm of the third chromosome at position 86, just to the left of *Pr* (map position 3–90.0).

Assay of Flight Ability. Flies (n = 250–350) were dropped in at the top of a vertical 500-ml graduated cylinder whose inside wall (5-cm diameter) was coated with paraffin oil. When released into the cylinder, normal flies immediately initiate flight, striking the wall and becoming stuck in the oil near the top. Weak fliers drop farther before becoming stuck. Thus the distribution of flies over the vertical length of the cylinder is a function of their flying ability (12).

Physiological Techniques. Adult female flies 1–2 days after eclosion were chosen for all physiology experiments. For nerve stimulation, flies were immobilized to a pin with cyanoacrylate adhesive. The cervical connective containing the giant nerve fiber was stimulated with a pair of tungsten electrodes that were electrically isolated from ground (13). Resulting activity in DLM 45a or -b was recorded with KCl-filled glass electrodes inserted through the dorsal thoracic cuticle. An electrode in the abdomen served as reference.

For current and voltage clamping, DLMs 45a–45d (14) were removed, fully exposing DLMs 45e and -f to saline for experimentation. A wax barrier separated the saline from the lower half of the fly so that the spiracles could be aerated (15). Saline was composed of 1.8 mM $CaCl_2$/8 mM $MgCl_2$/128 mM NaCl/2 mM KCl/35.5 mM sucrose, and was buffered with 5 mM Hepes (Sigma) at pH 7.1. The drugs 4-aminopyridine (4-AP), apamine, quinidine, and tetraethylammonium were supplied by Sigma. Charybdotoxin was supplied by Christopher Miller. The preparation was cooled with a Peltier junction to 4°C for voltage clamping and to 18°C for current clamping. Conventional circuitry was used for two-microelectrode current and voltage clamping (16). A Dagan 5700 current/voltage clamp was used. Two intracellular KCl-filled glass electrodes were bevelled to a resistance of 10 MΩ. The current passing electrode was shielded to within a few millimeters above the surface of the saline. The second electrode recorded voltage. Total membrane current was collected by a Ag/AgCl bath electrode and was monitored by the current-to-voltage converter. Signals were displayed on a Tektronix storage oscilloscope and photographed with Polaroid film. Some measurements were made from a Nicolet 4094 digital oscilloscope.

RESULTS

Behavior. At 38°C, *slo* flies are not completely paralyzed but are uncoordinated and unable to climb a vial. Upon returning *slo* flies to 22°C following a 4-min exposure to 38°C, they stand motionless for several minutes. Even when *slo* flies are not exposed to increased temperatures, they display abnormal locomotor behavior. Under ether anesthesia, they display leg-shaking behavior similar to Shaker (Sh) mutants (17), but less extreme. In addition, *slo* flies have greatly diminished flight ability and when placed in a large open

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Abbreviations: DLM, dorsal longitudinal flight muscle; 4-AP, 4-aminopyridine.

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container will usually walk rather than fly or will fly only in short hops. This decrease in flying ability was quantified as shown in Fig. 1.

Physiology. Intracellular records of DLM spikes in response to stimulation of the giant nerve fiber pathway revealed that DLM spikes were abnormally lengthened in *slo* flies. Measured at half-maximal amplitude, the duration of DLM spikes in *slo* flies was 19 ± 1 msec (mean ± SEM; n = 6) compared to 1.9 ± 0.2 msec (n = 12) in normal flies. The *slo* phenotype can be attributed to a defect in the muscle membrane itself because direct depolarization of the muscle in current-clamp experiments also elicited the broadened spikes (Fig. 2).

To examine further the ionic basis of the *slo* defect, standard two-microelectrode voltage-clamp experiments were carried out on the DLMs. The cell membrane was held at −80 mV, near the resting potential. Current measurements were made using a series of 140-msec step pulses in 20-mV increments from the holding potential (Fig. 3). As shown for a normal fly (Fig. 3*a*), the inward Ca^{2+} current was activated at −40 mV and was followed by a transient outward current. At depolarizing steps above −40 mV, the early outward current increased in amplitude and was followed by a second outward current (I_K) that did not inactivate. It has been suggested that the early transient current is composed of two separate currents with similar time course and amplitude but gated by voltage in one case (I_A) and by Ca^{2+} in the other (8). [We denote this current I_C rather than I_{Acd} as in ref. 8 for consistency with its designation in other systems (1, 4, 18).]

In agreement with earlier results, the I_A component was selectively eliminated by the drug 4-AP (9) or by the Sh^{KS133} mutation (9, 19) (Fig. 3 *b* and *c*). In normal flies treated with 4-AP or in Sh^{KS133} flies, the peak of the early outward current under voltage clamp was noticeably smaller than in normal controls. This reduction is most obvious when comparing the size of the early outward current to I_K, the late noninactivating current, for the pulse to 0 mV (compare Fig. 3 *a* with *b* and *c*). At steps to −40 mV and −20 mV, the remaining early outward current is still prominent and is believed to consist entirely of I_C. This component becomes less prominent at a depolarizing step to 0 mV because the Ca^{2+} current that activates I_C becomes smaller as its reversal potential is approached and because the faster rise of the larger I_K obscures I_C.

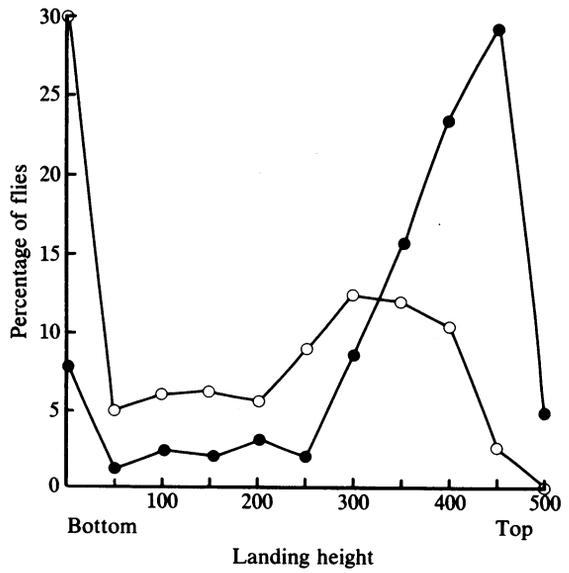

FIG. 1. Assay of flight ability in normal (●) and *slo* (○) flies. The points plotted represent the percentage of flies that became stuck in each 50-ml interval of a 500-ml graduated cylinder whose inside wall was coated with paraffin oil. Strong fliers get stuck near the top, whereas weak fliers drop farther before becoming stuck.

These results can be compared with the voltage-clamp records from *slo* under the same conditions (Fig. 3 *d–f*). Note, in contrast to Sh^{KS133} or 4-AP-treated wild-type flies, that the distinctive peak of early outward current following a step pulse to −40 mV is absent in *slo* (Fig. 3*d*). The inward Ca^{2+} current in the same trace, however, remains normal. At more depolarized levels (Fig. 3*d*), a peak of early current appears but its amplitude relative to I_K is smaller than normal (cf. Fig. 3*a*). These results indicate that *slo* reduces or eliminates a component of early outward current insensitive to 4-AP and the Sh^{KS133} mutation—namely, the Ca^{2+}-dependent K^+ current, I_C.

If our interpretation is correct, *slo* flies treated with 4-AP should retain little or no early outward current. This, in fact, was observed (compare Fig. 3 *d* with *e*). Only the slowly inactivating inward Ca^{2+} current and the delayed rectifier I_K were present (Fig. 3*e*), arguing that *slo* abolishes I_C. As another test of this hypothesis, we constructed Sh^{KS133} slo double mutants for voltage-clamp analysis. The few live double-mutant flies were weak compared with either single mutant and their locomotor activity was severely impaired. The current responses of DLMs in the double mutant are shown in Fig. 3*f*. As in the *slo* flies treated with 4-AP, there is no remaining early outward current, demonstrating clearly that *slo* eliminates the component of early current unaffected by Sh^{KS133}. Compared with the 4-AP-treated *slo* flies, the inward current in the double mutant decayed faster and the following outward current reached a plateau earlier during a depolarizing pulse to −40 mV. These differences may be attributed to incomplete removal of I_A and slight blockage of I_K by 4-AP (ref. 7 and unpublished observations).

We examined other membrane properties of *slo* DLMs to determine whether the mutation specifically altered I_C. The passive membrane constants were similar in normal and *slo* DLMs. Input resistances were 1–2 MΩ for both genotypes while membrane capacitance was 28 ± 6.4 nF (mean ± SEM; n = 6) in normal and 27 ± 4.8 nF (n = 8) in *slo* DLMs. I_A and I_K were compared in normal and *slo* flies using Ca^{2+}-free saline to eliminate the Ca^{2+} current and I_C. The ratio of the amplitudes of current at the maxima of I_A and I_K during a 140 msec pulse from −80 to 0 mV was 1.02 ± 0.05 (n = 5) in *slo* flies and 1.04 ± 0.09 (n = 6) in normal flies. Activation of I_A and I_K was detectable near −40 mV and above for both genotypes.

The steady-state and kinetic properties of I_A and I_K also appeared similar in *slo* and normal flies. For both genotypes, steady-state inactivation of I_A was half complete at about −50 mV. Recovery from inactivation following a conditioning pulse to −50 mV deviated from a first-order process and was half complete after 0.9 sec. The time constant of the I_K tail current following a repolarizing step from 0 to −80 mV was ≈100 msec.

Pharmacology of I_C. The pharmacological properties of the current blocked by *slo* were similar to those of Ca^{2+}-dependent K^+ channels in vertebrate systems. With a high concentration of tetraethylammonium (100 mM), I_C, I_A, and I_K were all nearly eliminated. Two other drugs that are known to block Ca^{2+}-dependent K^+ channels failed to block the DLM I_C. I_C was unaffected by 1.5 μM apamin (3, 20), and by 1 μM quinidine (21). Charybdotoxin (22) did block I_C channels in the DLMs. In the example shown in Fig. 4*a*, we used a Sh^{KS133} fly to isolate the I_C component of the early outward current. The apparent K_i of charybdotoxin for blocking I_C was estimated from four experiments to be about 75 nM. If the conclusion that *slo* specifically eliminates I_C is correct, then it is expected that charybdotoxin would have no effect on outward currents in *slo*. As shown in Fig. 4*b*, this

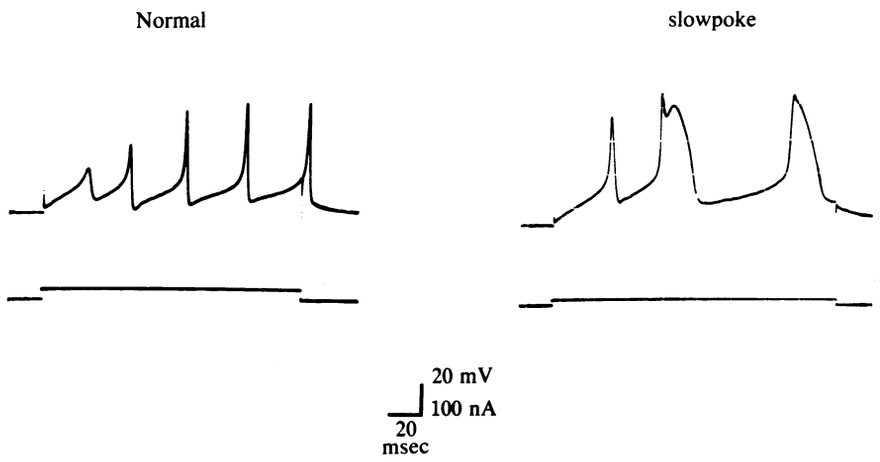

FIG. 2. Voltage responses to constant current injection into DLMs. (*Left*) normal fly; (*Right*) *slo* fly.

prediction is met, further indicating the specific loss of I_C in *slo*. I_C can thus be selectively eliminated in *Drosophila* DLMs both genetically and pharmacologically.

DISCUSSION

As in other insect muscle (23, 24), three distinct outward K^+ currents have been identified in *Drosophila* larval and adult muscle by voltage-clamp analysis (7, 8, 25). The functional roles of each current in different excitable cells and in different specialized membrane regions are not yet established. Mutations that disrupt I_A and I_K have already been found and analyses of their effects on membrane excitability have helped to resolve the functional roles of these currents. For example, Sh mutants are known to lack I_A and are defective in repolarization of action potentials in the giant axon of adults (26) and in the presynaptic terminal of the larval neuromuscular junction (27).

With the discovery of the *slo* mutation, this type of analysis can now be applied to study the biological roles of I_C. The *slo* mutation could prove uniquely helpful for these investigations because equally specific ways of blocking I_C pharma-

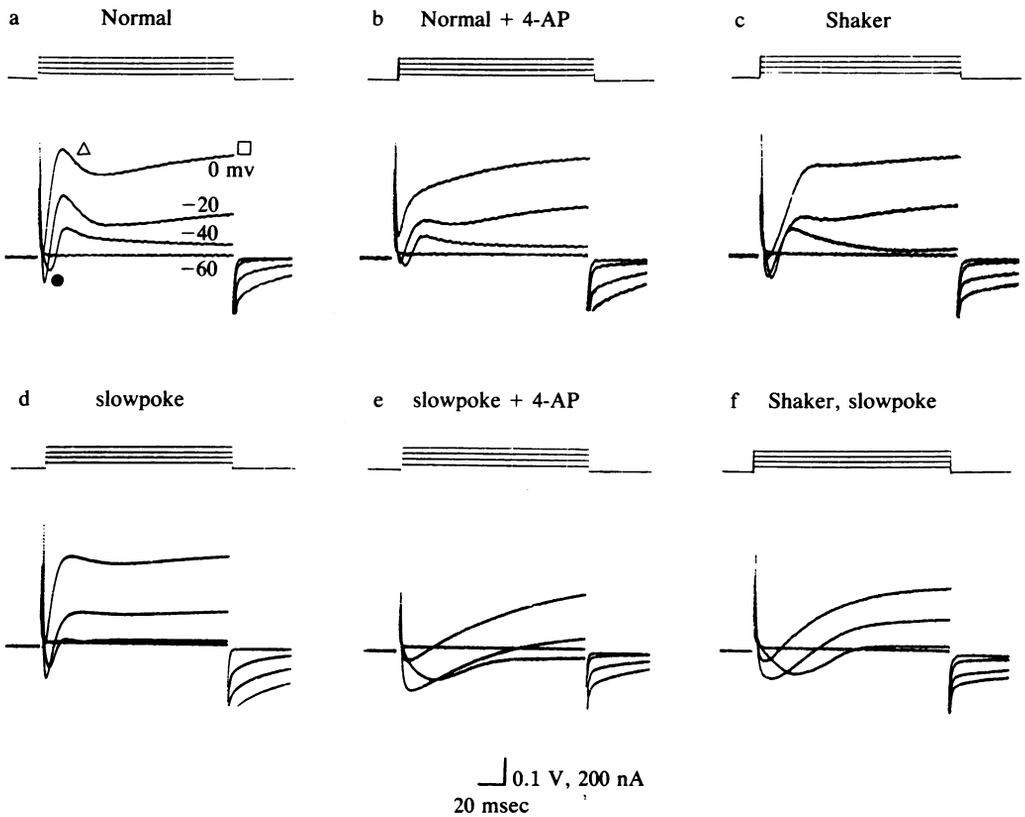

FIG. 3. Membrane currents in DLMs. Current responses (lower traces) to voltage-clamp steps (upper traces) from a normal muscle fiber (*a*), normal with 5 mM 4-AP (*b*), Sh^{KS133} (*c*), *slo* (*d*), *slo* with 5 mM 4-AP (*e*), and Sh^{KS133} *slo* double mutant (*f*) are shown. The membranes were held at −80 mV and stepped to −60, −40, −20, and 0 mV for 140 msec, as indicated. The pulse to −60 mV (*a*) activates only passive leakage current. At −40 mV, an inward (downward) Ca^{2+} current (●) is followed by an inactivating outward current (△). At −20 and 0 mV, a late outward current is evident that does not inactivate by the end of the voltage pulse (□). The early inactivating outward current is separable into I_A, which is removed by 4-AP or Sh^{KS133} (*b* and *c*) and I_C, which is removed by *slo* (*d*). See text for details. Temperature, 4°C; calibration, 0.1 V, 200 nA, 20 msec.

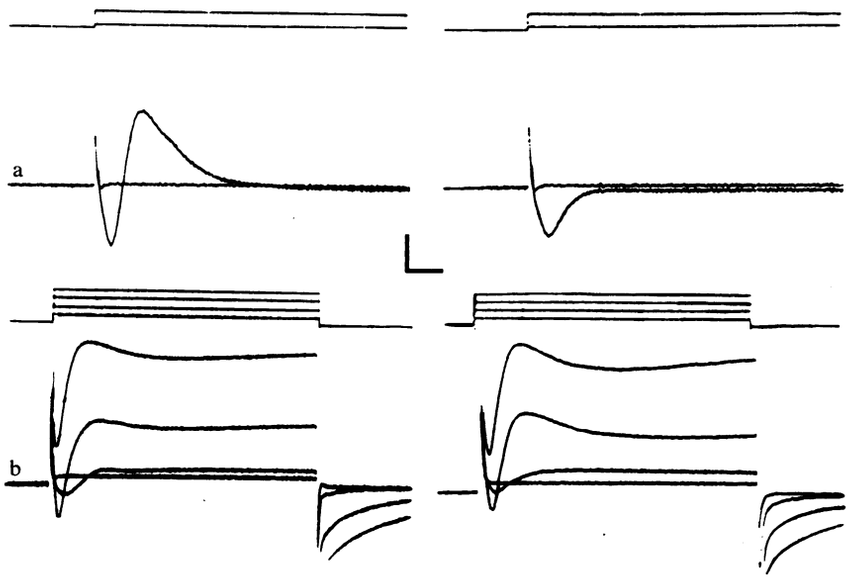

FIG. 4. DLM currents before (*Left*) and 30 min after (*Right*) the addition of charybdotoxin (200 nM). (*a*) Sh^{KS133} DLM currents under voltage clamp. The pulse to −70 mV is below the Ca^{2+} current threshold. The pulse to −40 mV evokes the inward (downward) Ca^{2+} current, which is followed by the outward I_C. I_A is absent in this mutant, and I_K is not evident at these potentials. Thirty minutes was allowed for diffusion of the drug through the muscle stack. After addition of the drug (*Right*), I_C was severely reduced. (*b*) *slo* DLM currents under voltage clamp. Thirty minutes after addition of charybdotoxin (*Right*), outward currents remain unaffected. Temperature, 4°C; calibration, 0.1 V, 100 nA (*a*), 200 nA (*b*), 20 msec.

cologically are not available. For example, even though charybdotoxin can partially block I_C, it was not possible to phenocopy the *slo* defect completely, because at high doses the Ca^{2+} current was reduced by charybdotoxin as well (not shown).

At least one role for I_C is clearly revealed in the present study—namely, the early repolarization of muscle action potentials. It was reported for certain vertebrate sympathetic neurons whose inward currents include both Ca^{2+} and Na^+ influx that a Ca^{2+}-dependent K^+ current plays a role in spike repolarization (1, 2, 4). In those neurons, treatments that block Ca^{2+} entry caused slight broadening of action potentials by reducing the rate of repolarization. A similar effect was seen in the *Drosophila* giant axon (25). In the DLMs, where the inward current is carried entirely by Ca^{2+}, the effect of *slo* on action potential repolarization was dramatic, prolonging the depolarized phase by at least an order of magnitude. Thus, in these cells I_C is the major repolarizing current of the action potential. This idea is supported by the lack of a comparable effect produced by blocking I_A. Note, however, that the first response in *slo* DLMs either to current injection or repetitive nerve stimulation is a spike of shorter duration than those that follow (Fig. 1). If *slo* DLMs were exposed to 5 mM 4-AP, the initial spike became as prolonged as those that followed. Similar results were observed in Sh^{KS133} slo double mutants. Thus, it appears that I_A is able to repolarize the first spike in the absence of I_C but then becomes inactivated.

Other functions may also be ascribed to I_C. For example, using *slo*, the delayed excitation observed during current clamp of DLMs is also mediated by I_C (T.E. and B.G., unpublished observations). In addition, the existence of a Ca^{2+}-dependent K^+ current that plays a role in repolarizing the presynaptic terminal of the larval neuromuscular junction has been inferred (28, 29). Whether or not this current is also affected by *slo* remains to be determined. It would also be of interest to examine whether conditioned responses (30) are altered in *slo* flies since a Ca^{2+}-dependent K^+ current is reported to undergo a specific reduction during *in vitro* conditioning of type B photoreceptors in *Hermissenda* (31).

We do not yet know exactly how *slo* leads to the elimination of I_C. In *Paramecium*, the only other organism in which comparable mutants are known, the genetic regulation of Ca^{2+}-dependent K^+ currents is complex. For example, mutations of at least two different genes eliminate this current (32, 33) and mutations of a third gene enhance the current (34). In other organisms, Ca^{2+}-dependent K^+ channels are known to be modulated by cAMP-dependent protein kinases (35, 36). Thus, *slo* could either affect the structural gene for I_C channels or alter some other factor responsible for their function or regulation. At whatever level *slo* exerts its effect, it apparently is specific for I_C channels, since other K^+ currents are not detectably altered. Molecular cloning and characterization of the *slo* gene product should help to resolve the question of how *slo* affects I_C channels and help elucidate their structure and regulation.

We gratefully acknowledge Frank Haugland for his advice and assistance, Chris Miller for his gift of charybdotoxin, and various colleagues for comments on the manuscript. This work was supported by Grants NS15390 and Research Career Development Award NS00719 to B.G., NS00675 and NS18500 to C.-F.W., and NSGM07133 to the Laboratory of Genetics, all from the U.S. Public Health Service. T.E. also received support from L. M. Passano and the Department of Zoology. This is paper no. 2857 from the Laboratory of Genetics, University of Wisconsin, Madison.

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