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

(ion channels/action potential/neurogenetics)

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**ABSTRACT** A mutation of *Drosophila*, slowpoke (*slo*), specifically abolishes a Ca\(^{2+}\)-dependent K\(^+\) current, *Ic*, from dorsal longitudinal flight muscles of adult flies. Other K\(^+\) currents remain normal, providing evidence that *Ic* is mediated by a molecularly distinguishable set of channels. The pharmacological properties of *Ic* 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 *Ic* 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 signaling 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 *Ig*, and two early inactivating currents *Ia* and *Ic*. Although the two early currents share a similar time course, *Ia* is voltage dependent and *Ic* is Ca\(^{2+}\)-dependent.

Here we describe a mutation, slowpoke (*slo*), that specifically eliminates *Ic*. 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 *Ic* 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 *Ic*.

**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 aereated (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 buffered with 5 mM Hepes (Sigma) at pH 7.1. The drugs 4-aminopyridine (4-AP), amphetamine, 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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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 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 19 ± 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. 3a), 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\(_{\text{c}}\)) 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\(_{\text{As}}\)) and by Ca\(^{2+}\) in the other (8). [We denote this current I\(_{\text{c}}\) rather than I\(_{\text{As}}\) as in ref. 8 for consistency with its designation in other systems (1, 4, 18).]

In agreement with earlier results, the I\(_{\text{As}}\) component was selectively eliminated by the drug 4-AP (9) or by the Sh\(^{K133}\) mutation (9, 19) (Fig. 3b and c). In normal flies treated with 4-AP or in Sh\(^{K133}\) 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\(_{\text{K}}\), the late noninactivating current, for the pulse to 0 mV (compare Fig. 3a 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\(_{\text{c}}\). This component becomes less prominent at a depolarizing step to 0 mV because the Ca\(^{2+}\) current that activates I\(_{\text{c}}\) becomes smaller as its reversal potential is approached and because the faster rise of the larger I\(_{\text{K}}\) obscures I\(_{\text{c}}\).

These results can be compared with the voltage-clamp records from slo under the same conditions (Fig. 3d–f). Note, in contrast to Sh\(^{K133}\) 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. 3d). The inward Ca\(^{2+}\) current in the same trace, however, remains normal. At more depolarized levels (Fig. 3d), a peak of early current appears but its amplitude relative to I\(_{\text{K}}\) is smaller than normal (cf. Fig. 3a). These results indicate that slo reduces or eliminates a component of early outward current insensitive to 4-AP and the Sh\(^{K133}\) mutation—namely, the Ca\(^{2+}\)-dependent K\(^+\) current, I\(_{\text{c}}\).

If our interpretation is correct, slo flies treated with 4-AP should retain little or no outward current. This, in fact, was observed (compare Fig. 3d with e). Only the slowly inactivating inward Ca\(^{2+}\) current and the delayed rectifier I\(_{\text{K}}\) were present (Fig. 3e), arguing that slo abolishes I\(_{\text{c}}\). As another test of this hypothesis, we constructed Sh\(^{K133}\) 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. 3f. 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\(^{K133}\). 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\(_{\text{As}}\) and slight blockage of I\(_{\text{K}}\) by 4-AP (ref. 7 and unpublished observations).

We examined other membrane properties of slo DLMs to determine whether the mutation specifically altered I\(_{\text{c}}\). 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 ± 0.4 nF (mean ± SEM; n = 6) in normal and 27 ± 4.8 nF (n = 8) in slo DLMs. I\(_{\text{A}}\) and I\(_{\text{K}}\) were compared in normal and slo flies using Ca\(^{2+}\)-free saline to eliminate the Ca\(^{2+}\) current and I\(_{\text{c}}\). The ratio of the amplitudes of current at the maxima of I\(_{\text{A}}\) and I\(_{\text{K}}\) during a 140-msec pulse from −80 to 0 mV was 1.0 ± 0.05 (n = 5) in slo flies and 1.04 ± 0.09 (n = 6) in normal flies. Activation of I\(_{\text{A}}\) and I\(_{\text{K}}\) was detectable near −40 mV and above for both genotypes.

The steady-state and kinetic properties of I\(_{\text{As}}\) and I\(_{\text{K}}\) have also appeared similar in slo and normal flies. For both genotypes, steady-state inactivation of I\(_{\text{As}}\) 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\(_{\text{K}}\) tail current following a repolarizing step from 0 to −80 mV was ≈100 msec.

**Pharmacology of I\(_{\text{c}}\).** 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\(_{\text{C}}\), I\(_{\text{A}}\), and I\(_{\text{K}}\) were all nearly eliminated. Two other drugs that are known to block Ca\(^{2+}\)-dependent K\(^+\) channels failed to block the DLM I\(_{\text{C}}\). I\(_{\text{C}}\) was unaffected by 1.5 μM apamin (3, 20), and by 1 μM quinidine (21). Charybdotoxin (22) did block I\(_{\text{C}}\) channels in the DLMs. In the example shown in Fig. 4a, we used a Sh\(^{K133}\) slo fly to isolate the I\(_{\text{C}}\) component of the outward current. The apparent K\(_{\text{m}}\) of charybdotoxin for blocking I\(_{\text{C}}\) was estimated from four experiments to be about 75 nM. If the conclusion that slo specifically eliminates I\(_{\text{c}}\) is correct, then it is expected that charybdotoxin would have no effect on outward currents in slo. As shown in Fig. 4b, this

![Graph](image-url)
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 $I_C$ pharmaceutically.

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

**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), ShKs133 (c), slo (d), slo with 5 mM 4-AP (e), and ShKs133 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 (c) is followed by an inactivating outward current (d). At $-20$ and 0 mV, a late outward current is evident that does not inactivate by the end of the voltage pulse (e). The early inactivating outward current is separable into $I_A$, which is removed by 4-AP or ShKs133 (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.
Neurobiology: the study of the nervous system. Minutes after addition of charybdotoxin (200 nM), the ShKSl33 DLM currents under voltage clamp. The pulse to −70 mV is below the Ca2+ current threshold. The pulse to −40 mV evokes the inward (downward) Ca2+ current, which is followed by the outward Ic. Ia is absent in this mutant, and Ic 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), Ic 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.

We do not yet know exactly how slo leads to the elimination of Ic in Paramecium, the only other organism in which comparable mutants are known, the genetic regulation of Ca2+-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, Ca2+-dependent K+ channels are known to be modulated by cAMP-dependent protein kinases (35, 36). Thus, slo could either affect the structural gene for Ic channels or alter some other factor responsible for their function or regulation. At whatever level slo exerts its effect, it apparently is specific for Ic 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 Ic channels and help elucidate their structure and regulation.

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FIG. 4. DLM currents before (Left) and 30 min after (Right) the addition of charybdotoxin (200 nM). (a) ShKSl33 DLM currents under voltage clamp. The pulse to −70 mV is below the Ca2+ current threshold. The pulse to −40 mV evokes the inward (downward) Ca2+ current, which is followed by the outward Ic. Ia is absent in this mutant, and Ic 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), Ic 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.

Other functions may also be ascribed to Ic. For example, using slo, the delayed excitation observed during current clamp of DLMs is also mediated by Ic (T.E. and B.G., unpublished observations). In addition, the existence of a Ca2+-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 Ca2+-dependent K+ current is reported to undergo a specific reduction during in vitro conditioning of type B photoreceptors in Hermisenda (31).