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ION CURRENTS IN *DROSOPHILA* FLIGHT MUSCLES

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SUMMARY

1. The dorsal longitudinal flight muscles of *Drosophila melanogaster* contain three voltage-activated ion currents, two distinct potassium currents and a calcium current. The currents can be isolated from each other by exploiting the developmental properties of the system and genetic tools, as well as conventional pharmacology.

2. The fast transient potassium current (I_A) is the first channel to appear in the developing muscle membrane. It can be studied in isolation between 60 and 70 hr of pupal development. The channels can be observed to carry both outward and inward currents depending on the external potassium concentration. I_A is blocked by both tetraethylammonium ion (TEA) and 3- or 4-aminopyridine. The inactivation and recovery properties of I_A are responsible for a facilitating effect on membrane excitability.

3. The delayed outward current (I_K) develops after maturation of the I_A system. I_K can be isolated from I_A by use of a mutation that removes I_A from the membrane current response and can be studied before the development of Ca^{2+} channels. I_K shows no inactivation. The channels are more sensitive to blockage by TEA than I_A channels, but are not substantially blocked by 3- or 4-aminopyridine.

4. The calcium current (I_{Ca}) is the last of the major currents to develop and must be isolated pharmacologically with potassium-blocking agents. I_{Ca} shows inactivation when Ca^{2+} is present but not when Ba^{2+} is the sole current carrier. When Ca^{2+} is the current carrier, the addition of Na^+ or Li^+ retards the inactivation of the net inward current. When the membrane voltage is not clamped, Ba^{2+} alone, or Ca^{2+} with Na^+ (or Li^+), produces a plateau response of extended duration.

5. The synaptic current (I_J) evoked by motoneurone stimulation is the fastest and largest of the current systems. It has a reversal potential of approximately -5 mV, indicating roughly equal permeabilities of Na^+ and K^+. During a nerve-driven muscle spike, I_J is the major inward current, causing a very rapid depolarization away from resting potential. An exceptionally large synaptic current is necessary to rapidly discharge the high membrane capacitance (0·03 μF/cell) in these large (0·05 × 0·1 × 0·8 mm) isopotential cells.

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INTRODUCTION

A combination of advantages makes it possible to analyse the neuromuscular electrical properties of the *Drosophila* flight muscles in a more comprehensive way than in any other insect neuromuscular system. These advantages include large isopotential muscle cells which may be voltage-clamped using a two-micro-electrode technique, and the ability to isolate the different current-carrying systems selectively by exploiting genetic tools and the developmental properties of the system. Because each current can be studied in isolation from the others, each should be amenable to genetic analysis.

The use of genetics as a tool in neurophysiology requires an organism which is suitable for both genetic and neurophysiological techniques. It has been suggested that *Drosophila* genetics could be a powerful tool for studying the structure and biophysical properties of voltage-sensitive ion channels (e.g. Siddiqi & Benzer, 1976; Jan, Jan & Dennis, 1977; Wu, Ganetsky, Jan, Jan & Benzer, 1978; Tanouye, Ferrus & Fujita, 1981). However, voltage-clamping is required for the identification of channels affected by mutants and for an accurate study of their biophysical properties. A recent study showed that the dorsal longitudinal flight muscles (d.l.m.) of *Drosophila* were suitable for voltage-clamping (Salkoff & Wyman, 1980). Use of this system for studying the development of membrane ion currents revealed that a single ion channel type developed before all other types (Salkoff & Wyman, 1981*a*). By virtue of its precocity, this current-carrying system (the fast transient potassium current called the A-current) could then be studied accurately in isolation from the other currents in the membrane. This allowed a further study which made a detailed comparison between A-currents in normal (wild-type) animals, and those in behavioural mutants carrying putative potassium channel defects. This study (Salkoff & Wyman, 1981*b*) revealed that the *Shaker* mutants, previously hypothesized to affect some type of potassium channel (Jan *et al.* 1977; Tanouye *et al.* 1981), did indeed affect a potassium channel, the A-current channel. Different mutations at the X-linked *Shaker* locus were found to affect either the kinetic properties of the A-current or the amount of current present. One class of these mutants, exemplified by Sh^{KS133}, completely removed the A-current system from the d.l.m. membrane. By using flies carrying this mutation at an early stage of development, delayed outward current can be studied by itself. Hence, because of its developmental properties and unique genetic tools, *Drosophila* is turning out to yield important advantages for studying excitable membranes. These advantages allow the selective isolation for study of each of the different current-carrying systems in the muscle membrane as well as the ability to alter the biophysical properties of the channels by induced mutations. We show here how each system is isolated, the characteristics of each system, and how each contributes to the electrical responsiveness of this neuromuscular system. Similar currents are present in other insect systems but are not subject to as many experimental manipulations (Rakowski, 1972; Ashcroft & Stanfield, 1981, 1982).

METHODS

The voltage-clamp equipment used was the same as that described by Dionne & Stevens (1975). Two intracellular electrodes were used for both voltage-clamp and constant-current experiments. Both voltage-recording and current-passing electrodes were filled with 3 M-KCl and had resistances of 3–10 MΩ. Voltage-clamp experiments were performed at 4 °C unless otherwise noted and constant-current experiments at 20 °C. The preparation was cooled with a Cambion Peltier plate. Preparations were wax-mounted on epoxy-coated slotted metal disks. Adult animals were sealed in the slot by sticky wax and their ventral portions were exposed to an air stream to facilitate respiration (see the following section). To expose the muscles to saline a small portion of the dorsal thorax was opened with microdissection needles.

Series resistance compensation was not considered necessary. A significant series resistance problem would distort the kinetic properties of the currents, with the amount of distortion depending on the magnitude of the currents. However, experiments showed that the decay of the A-current at a given test voltage occurred at the same exponential rate both before and after the magnitude of the current was experimentally lowered. Techniques for evoking and recording muscle responses via nerve stimulation, and regulating the amount of neurotransmitter release by use of the temperature-sensitive shi^{ts1} mutant, have been published previously (Ikeda, Ozawa & Hagiwara, 1976; Salkoff & Kelly, 1978). In the shi^{ts1} mutant the amount of neurotransmitter release depends on the precise temperature and the rate of stimulation. The Sh^{KS133} mutant (Jan *et al.* 1978; Salkoff & Wyman, 1981*b*) was used to study the properties of delayed rectification without contamination by the A-current. The Canton-S strain was used for observations in wild-type animals. The standard saline used contained NaCl (128 mM), KCl (4·8 mM), $CaCl_2$ (1·8 mM), and KH_2PO_4/K_2HPO_4 (0·2 mM), pH 6·8. Potassium currents were voltage-clamped in the following solution: choline chloride (128 mM), KCl (4·8 mM), EGTA (1·8 mM), $MgCl_2$ (10 mM), Tris HCl (2 mM), pH 7·0. The calcium current was voltage-clamped in two 2-hr-old adult animals in the following solution: Tetraethylammonium chloride (TEA; Kodak; 128 mM), 4-aminopyridine (4-AP; Sigma; 5 mM), KCl (4·8 mM), $CaCl_2$ or $BaCl_2$ (specified concentrations in text), Tris HCl (2 mM), pH 7·0.

The dorsal longitudinal flight muscles

The flight power muscles of the *Diptera* are a specially evolved type of muscle called fibrillar or asynchronous muscle. In order to achieve high frequencies of contraction and relaxation (over 200/sec), these muscles use a mechanism whereby a nerve-driven muscle spike allows calcium influx but does not, by itself, trigger a contraction. Instead, a quick stretch by an antagonist muscle triggers the contraction. These muscles are also special in having fibres of very large size. In *Drosophila* each of the bilateral d.l.m.s consists of only six fibres. Their shape is approximately a parallelepiped with sides 0·05 mm × 0·1 mm × 0·8 mm. Each fibre is innervated by a single motoneurone (Harcombe & Wyman, 1977; Ikeda, Koenig & Tsuruhara, 1980). The resting potentials of these fibres vary with age. In pupae, the resting potentials vary between −45 mV and −60 mV depending on developmental age (Salkoff & Wyman, 1981*a*). The polarization of adult fibres may exceed −90 mV (Ikeda *et al.* 1976). This is more negative than the estimated K^+ reversal potential of −50 to −60 mV. Resting potentials are commonly more negative than potassium reversal potentials in insect muscles fibres. This is probably because membrane potentials are maintained at a more negative level by active metabolic processes (Huddart & Wood, 1966; Rheuben, 1972). The resting potentials of adult fibres degenerate to the approximate K^+ reversal potential when respiration is blocked (Henon & Ikeda, 1981).

The fibres are isopotential. Fig. 1 shows the results of a three-electrode experiment where a current-passing and a voltage-recording electrode were inserted at one end of the muscle and another recording electrode was inserted at the other end of the muscle, about 0·5 mm away. It can be seen that the passive depolarization and hyperpolarization, as well as the active oscillatory response, is recorded with identical amplitude at both ends of the fibre. Pupae and young (0–6 hr post-eclosion) adults have vastly higher d.l.m. membrane resistance and are thus much more suitable for voltage-clamping than older adults. The input resistance declines from more than 2 MΩ prior to eclosion to less than 400 kΩ in old (i.e. 3-week) adults. (Note that even though Fig. 1 is from an older adult the muscle is still essentially isopotential.) For equal amounts of current injection, old adult muscle fibres have small oscillating responses rather than the large spike-like

responses seen in young adults. The electrode placement shown in Fig. 1 (bottom) can also be used to check isopotentiality in a voltage-clamp experiment. When this is done in pupae, the cells remain isopotential during step pulses exceeding 100 mV requiring 300 nA of current, indicating that an adequate space clamp is achieved by the two-micro-electrode voltage-clamp technique.

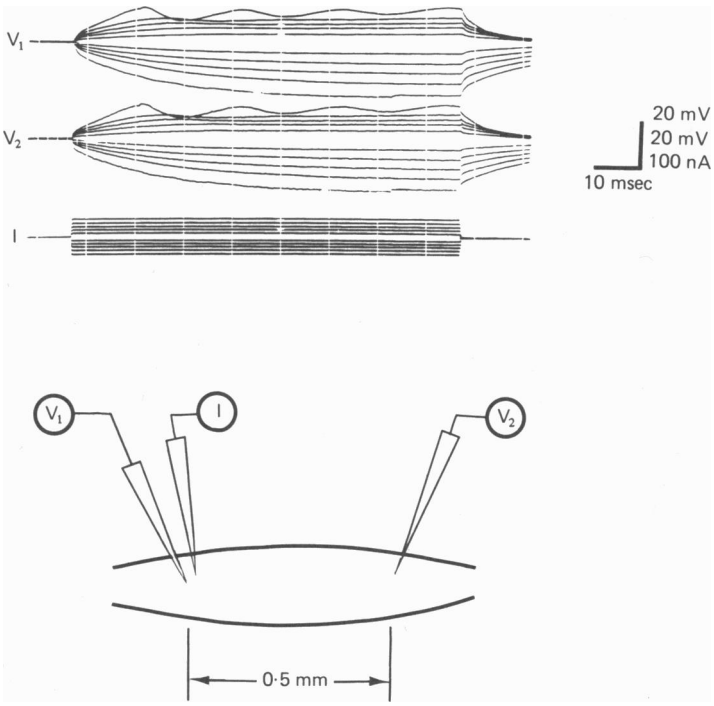

Fig. 1. Isopotentiality of d.l.m. fibre demonstrated by an experiment with three intracellular electrodes. The two recording electrodes, V_1 and V_2, are approximately 0·5 mm apart. The total fibre length is approximately 0·8 mm. Although the current-injection electrode, I, is within 50 μm of V_1, the voltage displacement recorded by V_1 and V_2 is virtually identical. The fly in this experiment was an older animal with an input resistance of approximately 500 kΩ.

RESULTS

There are three types of voltage-activated channels that are important in the muscle membrane's early response to depolarization. These are two outward current channels and one inward current channel. The currents are introduced here in the order of their developmental appearance in the membrane.

The A-current, I_A

The d.l.m. are formed during the pupal phase of development which lasts for approximately 96 hr at 25 °C (Bodenstein, 1965; Shafiq, 1963). At 55 hr of pupal development electrically activated channels are not present in the membrane (Salkoff & Wyman, 1981*a*). Soon afterwards, however, the first voltage-activated channels

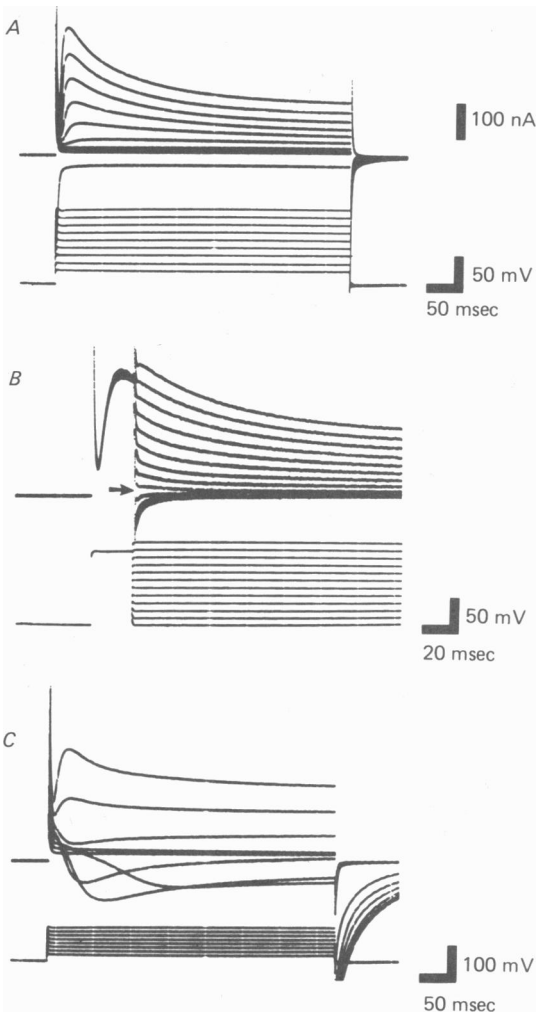

Fig. 2. Properties of the A-current in a 72 hr pupa. *A*, currents (upper traces) associated with depolarizing voltage steps (lower traces) from a holding potential of −80 mV. Superimposed sweeps are shown. The steps are from −60 mV to +20 mV at 10 mV intervals. The voltage recorded for the hyperpolarizing step (to −180 mV) is not shown. *B*, I_A current tail reversal. The membrane voltage is briefly stepped from −80 to +20 mV, and then stepped to the various other potentials. The resulting current tails reverse direction (arrow) at a potential between −50 and −60 mV. *C*, I_A observed in high external potassium (48 mM). The voltage steps are from −60 to +10 mV from a holding potential of −80 mV. I_A turns on initially as an inward current. At the higher voltage steps when the current reverses to become outward, inactivation appears to be partially suppressed.

appear. These first channels carry a fast transient outward current (Salkoff & Wyman, 1981*a*,*b*). They may be studied in isolation from other active channels by recording from the pupa at about 72 hr after pupariation, before the development of other channels (Fig. 2). Activation of the current begins at about −50 mV (Figs. 2 and 3). The channel is striking for the rapidity of its activation and for the fact that during

a maintained voltage step pulse it also inactivates rather rapidly. A similar current first fully characterized in *Mollusca* has been named the fast, transient potassium current or the A-current (Neher, 1971; Connor & Stevens, 1971*a*).

Because I_A in *Drosophila* is largely free of contamination from other currents at 72 hr, it is subject to experimental manipulations not normally available for studying this type of current. For example, the channels can be studied carrying either inward

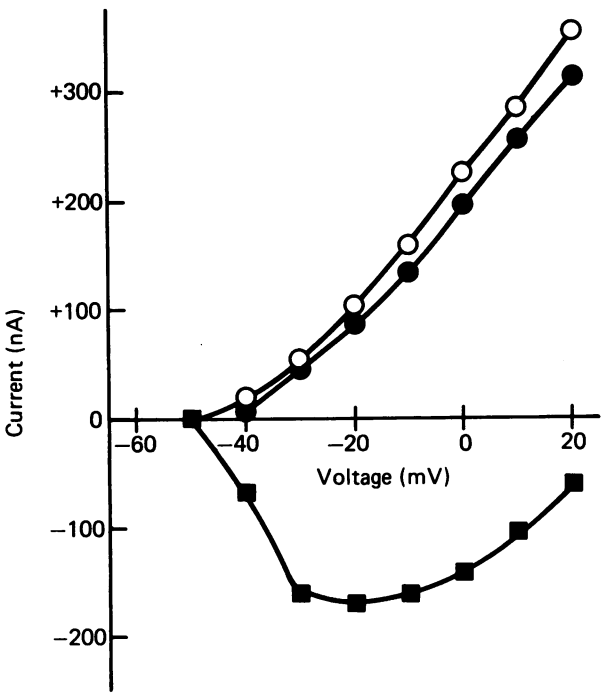

Fig. 3. Current–voltage relations from the three currents. To measure I_A, 72 hr pupae were used; for I_K, Sh^{KS133} 84–90 hr pupae were used. I_{Ca} was measured in 2 hr adults using 128 mM-TEA and 5 M-4-AP. Cells were stepped from the −80 mV holding voltage to the test voltage plotted on the abscissa. I_A (○) and inward current (■) were measured at their peak, while I_K (●) was measured after 400 msec at the test voltage. The leak, as measured from hyperpolarizing pulses, has been subtracted from all data.

or outward currents. By stepping the membrane voltage up to evoke a large outward current, and then immediately stepping down to various other voltage levels, tail currents result which are either in the inward or outward direction depending on the voltage level of the second step. Fig. 2*B* shows such an experiment in normal saline where the tail currents reverse from the outward to the inward direction at approximately −55 mV. However, analysis of I_A as an inward current is not limited to observations of tail currents. Because the K^+ reversal potential is more negative than the current threshold, I_A is normally observed turning on as an outward current (Fig. 2*A*). However, by increasing the external concentration of K^+ the current can be observed to turn on as an inward current (Fig. 2*C*). With elevated external K^+ the characteristics of I_A change significantly. The outward current seems to inactivate

less than in similar experiments with lower external K^+, but the inward current inactivates more completely than the outward current. Experiments in other systems have shown previously that the gating of potassium channels is dependent on the direction of current flow and the external concentration of K^+ (Stanfield, Ashcroft & Plant, 1981; Swenson & Armstrong, 1981). It is especially significant to mention that Swenson & Armstrong (1981) found that high external K^+ slowed the closing of K^+ channels carrying outward current.

The delayed outward current, I_K

Following the maturation of the A-current channels the delayed outward current begins to develop. By 90–96 hr of pupal development, just prior to eclosion of the adult from the puparium, delayed outward current is mature. At this stage there are two mature outward current-carrying systems in the membrane, and the membrane current response to voltage-clamp step pulses (Fig. 4*A*) is a composite response of these two systems. Note the early peaking component of I_A followed by the sustained outward current, I_K. Note, also the slow-current tails that are a distinguishing characteristic of I_K. The current tails are in the inward direction because the K^+ equilibrium potential is more positive than the holding potential of -80 mV. Both of the potassium currents in this composite response can be blocked with external TEA but they differ in their sensitivities to this agent. Fig. 4 *B* shows a composite I_A/I_K current response to a voltage-clamp step pulse immediately before (upper trace) and after (lower trace) the replacement of choline chloride with TEA. The maintained component of outward current, I_K, is reduced substantially more than the peaking component, I_A. The concentration of TEA used here (128 mM) eventually eliminated all of I_K while a vestige of I_A remained. The higher sensitivity of I_K to the blocking action of TEA was also shown in molluscan neurones (Thompson, 1977).

I_K can be studied in isolation from I_A by using either genetic mutations or a pharmacological agent, aminopyridine. The genetic techniques are the more satisfactory because aminopyridine drugs are not entirely specific. A number of mutations have been characterized, exemplified by Sh^{KS133}, where I_A is absent from the membrane current response of the pupal muscle (Salkoff & Wyman, 1981*b*). In animals carrying this mutation I_K may be studied in isolation from I_A by selecting animals for study at about 84–90 hr of pupal development (Fig. 4*C*). In these mutant animals the A-current is absent, the calcium current has not yet developed, and thus the delayed outward current channels are the only active channels present in the membrane. Note the absence in Fig. 4*C* of the early peaking component I_A present in Fig. 4*A*. By studying I_K without I_A it can be seen that I_K activates in a slightly higher voltage range than I_A (Fig. 3). The delayed current is a slower activating current that does not show any inactivation during depolarizing voltage-clamp step pulses maintained for as long as 10 sec.

Fig. 4*D* shows the isolation of I_K by pharmacological means. The Figure shows the result after applying 10 mM-4-AP to a normal wild-type preparation as in Fig. 4*A*. Originally both I_A and I_K were present. In Fig. 4*D* I_A is no longer apparent. I_K may also be reduced. 3-AP also has a similar effect but is not quite as effective. The relatively specific effects of 4-AP in blocking I_A were noted by Thompson (1977) in molluscan neurones.

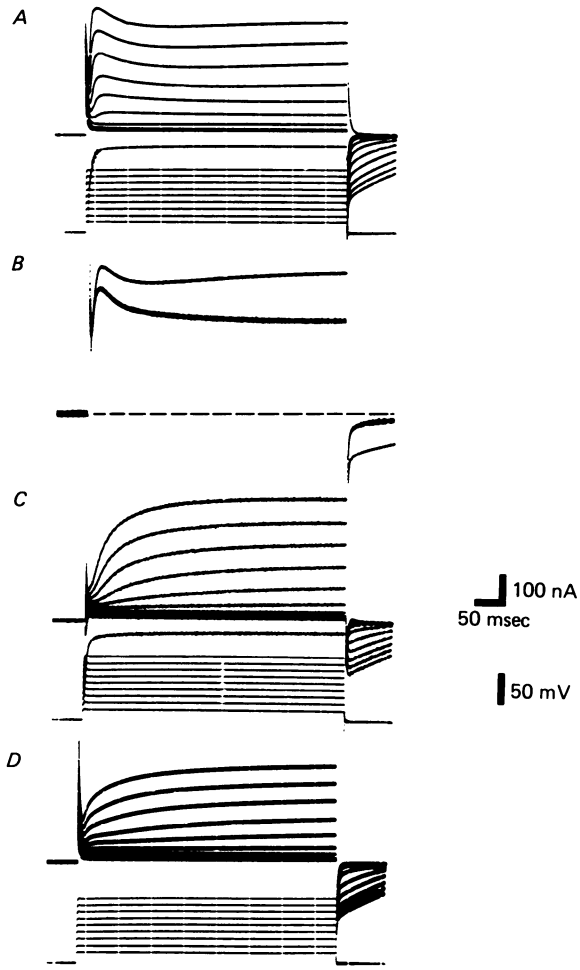

Fig. 4. Outward currents in 90 hr pupae. *A*, composite current response of I_A and I_K evoked by depolarizing voltage steps from a holding potential of −80 mV. The step pulses are from −60 to +20 mV at 10 mV intervals. The voltage record for the hyperpolarizing step to −180 mV is not shown. Note the large, slow inward current tails of I_K, resulting from the return of membrane voltage to holding potential. *B*, the effect of TEA on I_A and I_K. The membrane was transiently stepped to +20 mV from a holding potential of −80 mV, before (upper trace), and after (lower trace) the addition of TEA. The voltage record is not shown. See text for other details. *C*, I_K response in a Sh^{KS133} muscle cell which lacks I_A at this stage of development; pulse paradigm: as in *A* above. *D*, the effect of 4-AP on the composite (I_A and I_K) current response. The Figure shows the response after the addition of 4-AP. The current response before the addition of 4-AP was similar to *A*, above. Pulse paradigm: same as *A* and *C*. See text for other details.

The calcium current, I_{Ca}

The d.l.m. membrane lacks voltage-sensitive sodium channels. Instead, calcium channels carry an inward current. I_{Ca} activates in a voltage range similar to that of the A-current (Figs. 3 and 5 *A*, left traces). The calcium currents, which are the fastest

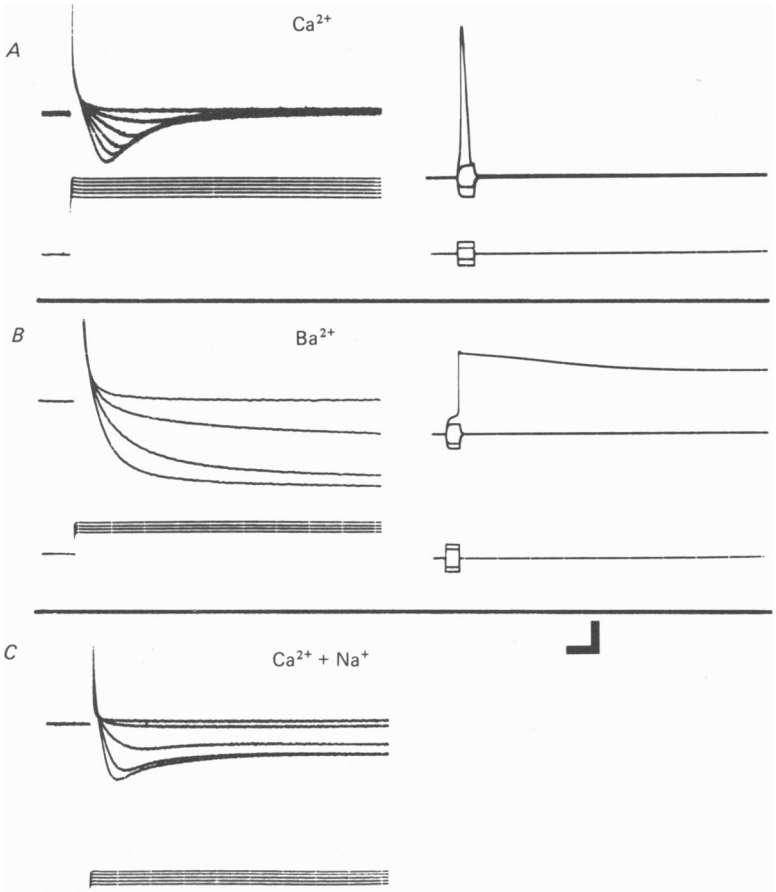

Fig. 5. Inward current responses in newly eclosed adults. *A*, evoked responses in 1·8 mM-Ca^{2+} (no sodium). Left traces, current responses to voltage-clamp step pulses from a holding potential of −80 mV. The threshold of the response is approximately −50 mV. Right traces, voltage responses (upper traces) to constant current injection (lower traces). *B*, evoked responses in 50 mM-Ba^{2+} (no Ca^{2+} or Na^{+}). Left traces, current responses to voltage-clamp step pulses from a holding potential of −80 mV. Right traces, voltage responses to constant-current injection. *C*, evoked current responses to voltage-clamp step pulses in saline containing Ca^{2+} (1·8 mM) and Na^{+} (400 mM). The holding potential is −60 mV. In *A*, *B* and *C*, outward currents have been blocked with TEA and 4-AP (see Methods). The time, current and voltage marks are as follows. *A*, left: 20 msec, 50 nA, 20 mV, right: 200 msec, 100 nA, 20 mV. *B*, left: 20 msec, 50 nA, 50 mV; right: 500 msec, 100 nA; 50 mV. *C*, 20 msec, 50 nA, 50 mV.

of the voltage-activated channels in the membrane, are the last to arise during development; they are mature only after adult eclosion (Salkoff & Wyman, 1981*a*). The calcium current can be isolated by using the K^{+} channel blockers TEA and AP in adult animals.

Like other systems where calcium channels are present the decay of the net inward current varies markedly under different experimental conditions (Connor, 1979; Brehm & Eckert, 1978; Eckert, Tillotson & Brehm, 1981; Hagiwara & Byerly, 1981).

Three examples showing different decay characteristics are shown in Fig. 5. In salines containing low concentrations of calcium (e.g. 1·8 mM) and no sodium (or lithium), the current declines rapidly after turning on during a maintained voltage-clamp step pulse (Fig. 5*A*, left traces). The decline of the current may be due to inactivation

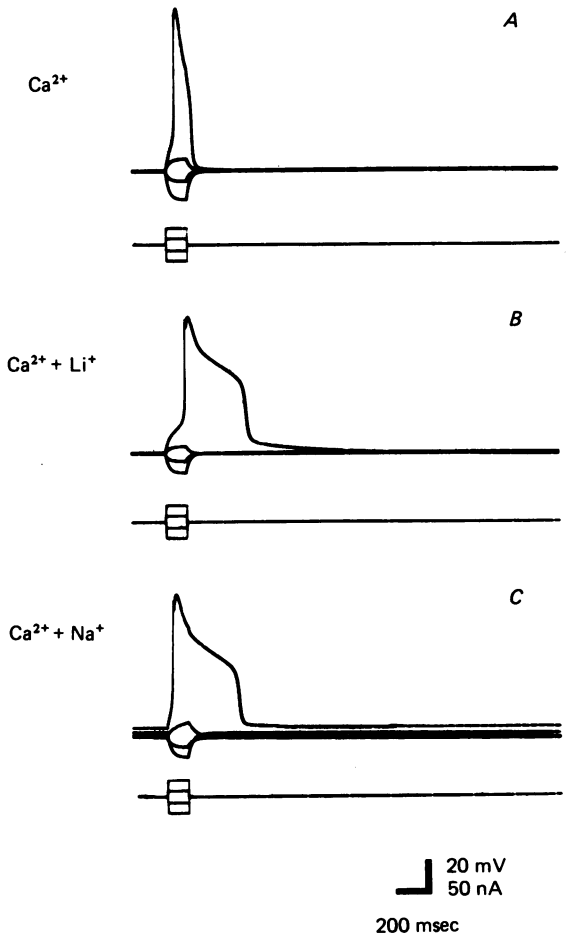

Fig. 6. Plateau responses induced by monovalent cation modulation of transient inward current. Voltage responses (upper traces) to constant-current injections (lower traces). *A*, Ca^{2+} (1·8 mM) only. *B*, Ca^{2+} (1·8 mM) + Li^{+} (128 mM). *C*, Ca^{2+} + Na^{+} (128 mM) after washing out Li^{+}. The membrane resting potential is approximately −70 mV but is undergoing a slight depolarization in *C*. Outward currents are blocked with TEA and 4-AP.

of the Ca^{2+} current or to the turning on of an outward current. A number of experiments in other systems suggests that Ca^{2+}-inactivation is caused by the internal accumulation of Ca^{2+} due to the influx of Ca^{2+} through the channels themselves (Connor, 1979; Brehm & Eckert, 1978; Eckert *et al.* 1981; Ashcroft & Stanfield, 1981). In *Drosophila*, as well as these other systems, the substitution of Ba^{2+} for Ca^{2+} as the current-carrying species reveals a non-inactivating inward current (Fig. 5*B*, left

traces). Although Ba^{2+} can substitute for Ca^{2+} as a charge carrier, Ba^{2+} may not function in the role required for closing the Ca^{2+} channels. (It is also possible that Ba^{2+} fails to activate a Ca^{2+}-dependent outward current). In Fig. 5*B* (left traces) the Ba^{2+} currents did not inactivate during step pulses maintained for as long as 10 sec.

When the membrane voltage is not clamped and the membrane stimulated by constant-current injection, the results when different ions are present might be predicted from the voltage-clamp data. With Ca^{2+} only present, as in Fig. 5*A* (right traces), only a transient spike-like response is seen because the net inward current declines rapidly. (Note that voltage-sensitive outward currents are blocked with TEA and 4-AP). However, when Ba^{2+} alone is present (Fig. 5*B*, right traces) a sustained plateau-like response is present that lasts for many seconds.

In addition to the probable modulation of Ca^{2+} inactivation by Ca^{2+} itself and the possible involvement of Ca^{2+}-activated outward current, the system is further complicated by the apparent modulation of net inward current by monovalent ions such as Na^+ or Li^+. Fig. 5*C* shows the effect under voltage clamp of adding Na^+ when other experimental conditions are similar to those of Fig. 5*A*, i.e. 1·8 mM-Ca^{2+} is present. With added Na^+ the inward current does not fully decay as in Fig. 5*A*; a portion of the current persists for several seconds. Li^+ also has a similar effect. Again, predictably, when the membrane voltage is not clamped the persistent current that results from the addition of Na^+ or Li^+ elicits a plateau response (Fig. 6; cf. Patlak, 1976).

This persistent current resulting from the addition of Na^+ or Li^+ might be due to a direct modification of calcium-channel inactivation by these ions, or might be due to an effect these ions have on an additional channel system. For example Na^+ or Li^+ may block a persistent outward current. A third possibility is that an additional channel system permeable to Na^+ and Li^+ is present that carries a net inward current; such a Ca^{2+}-activated non-specific cation channel has been recently reported (Colquhoun, Neher, Reuter & Stevens, 1981; Yellen, 1982). These various possibilities are currently under study.

Steady-state inactivation of currents

Fig. 7 shows steady-state inactivation curves for the three currents. Both the net inward current and the A-current inactivate when the membrane voltage is moved to and held at a constant depolarized voltage. Of course, this is true for the inward current only when Ca^{2+} (and not Ba^{2+}) is the current-carrying ion. Delayed outward current shows no signs of inactivation for pulses lasting 10 sec or more. When the cell membrane is moved to a depolarized voltage the A-current and the inward current will reach a steady-state level of inactivation. This level is measured by the relative amplitudes of currents evoked by a voltage test step applied before and after the shift of steady-state voltage. Fig. 7 shows the amount of I_A (○) or I_{Ca} (■) which can still be activated at any given steady-state voltage level. I_K (●) is unaffected by the level of membrane voltage over the range shown.

Because of the steepness of the steady-state inactivation curves for I_A and I_{Ca} the precise resting potential of a cell will determine the level of inactivation for these currents. This will largely determine the response of the cells to excitation. Fig. 8 shows a series of constant current pulses applied to a muscle cell adjusted to three

different holding potentials by injecting small amounts of steady current. At a potential of −80 mV neither I_{Ca} nor I_A is inactivated. An excitatory current pulse, once threshold is reached, elicits an accelerating depolarization (due to I_{Ca}) and a rapid repolarization due primarily to I_A. As the membrane is held at more depolarized levels both the rising and falling phases of the responses slow due to the partial inactivation of I_{Ca} and I_A. Below a potential of about −40 mV, no spike-like response at all is evident.

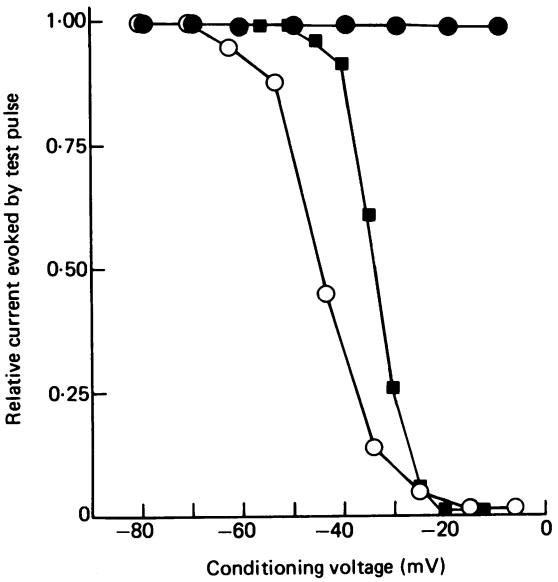

Fig. 7. Steady-state inactivation curves for I_A (○), I_{Ca} (■) and I_K (●). Currents were isolated as described in Fig. 3. The cells were stepped from a holding potential of −80 mV to the conditioning voltage (abscissa) for 1·8 sec. The currents elicited by the conditioning voltage closely approached a steady state of inactivation within this time. The amount of current still evokable was then measured by a further depolarization (test pulse) to +20 mV. Measured currents were normalized to the current elicited by a pulse from −80 mV to +20 mV. For I_A and I_{Ca} the peak current is plotted, while the amplitude of I_K was measured at the end of 400 msec of the test pulse. Current is measured on the ordinate and conditioning voltage on the abscissa.

The composite current response

The composite current responses of all three currents to voltage-clamp step pulses is shown in Fig. 9. Since the two transient currents I_{Ca} and I_A are activated at more hyperpolarized voltages than the delayed outward current, a small voltage-clamp step pulse evokes the two transient currents without the maintained outward current (Fig. 9, top, second trace). A larger voltage-clamp step pulse, however, evokes all three currents (Fig. 9, top, third trace).

Synaptic current

The six muscle fibres of the d.l.m. are each innervated by a single motor axon (Harcombe & Wyman, 1977; Ikeda *et al.* 1980). The axon branches on the surface of the fibre and makes its synaptic connexions in invaginations into the fibre (Shafiq,

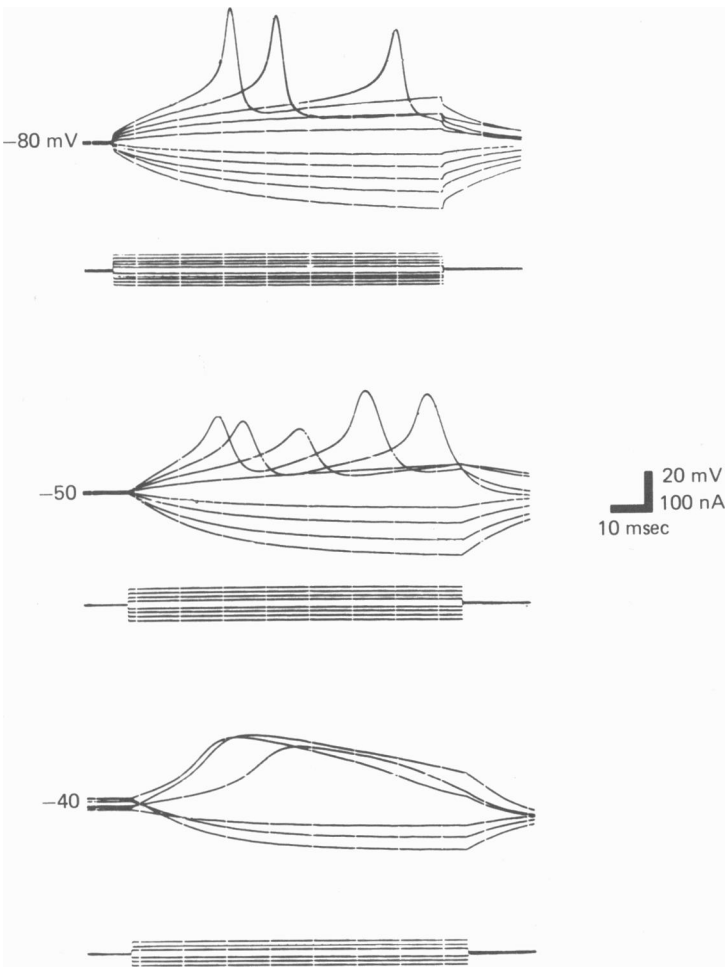

Fig. 8. Adult d.l.m. voltage responses to constant-current injection. Resting potentials were adjusted to the indicated voltages by injecting small amounts of d.c. current; this bias current is not shown. After adjustment to the indicated resting potentials, additional current injections (lower traces) evoked the voltage responses seen in the upper traces. The standard saline (see Methods) was used in these experiments.

1963). During flight, motoneurone spikes cause a large, overshooting, stereotyped potential in the muscle cells. These potentials have the appearance of an all-or-none spike (Harcombe & Wyman, 1977). Electrical stimulation of the nerve reproduces this potential. Fig. 10 (top) shows the nerve-evoked muscle spike.

The neuromuscular transmitter in *Drosophila* as well as other insects is now

generally accepted to be glutamate (Jan & Jan, 1976*b*; Piek & Njio, 1979). Electrotonic depolarization of the presynaptic motoneurone terminal evokes a synaptic current in saline containing tetrodotoxin. In such an experiment removing sodium from the saline essentially eliminates the synaptic current, indicating that the post-synaptic inward current is primarily sodium. Jan & Jan (1976*a*,*b*) have shown, in a larval *Drosophila* muscle, that calcium and chloride do not make a

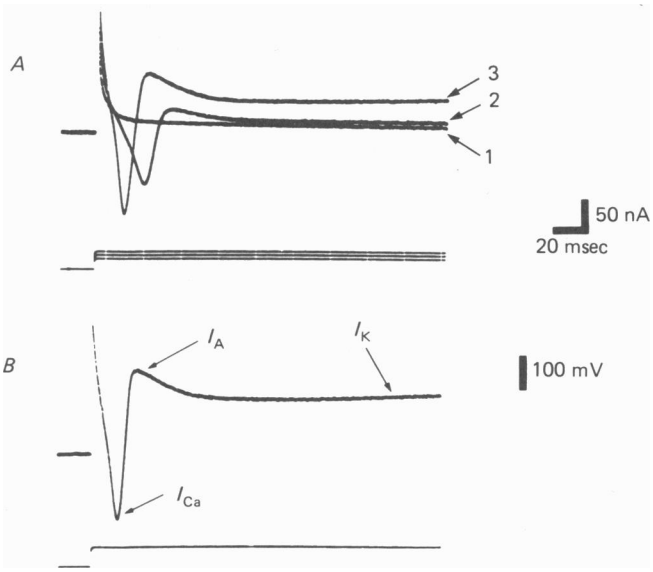

Fig. 9. Composite current response (I_A, I_K and I_{Ca}) in a newly eclosed adult muscle cell. *A*, current responses to voltage-clamp step pulses applied from a holding potential of −80 mV. The current response (arrow 1) to the lowest voltage step is purely passive. A voltage step to a slightly more positive level evokes the responses of the two transient currents (arrow 2), I_{Ca} and I_A, which have lower thresholds than I_K. A voltage step to a still higher level evokes all the three currents (arrow 3). *B*, prominent features of each current in the composite current response are labelled.

significant contribution to the synaptic potential. (However, the glutamate-activated channels are quite permeable to Mg^{2+}.) The reversal potential for the d.l.m. synaptic current in normal saline is approximately −5 mV (Fig. 11), Indicating that the channel must be about equally permeable to potassium and sodium, a conclusion also arrived at by Jan & Jan (1976*b*).

Under voltage clamp the synaptic current is seen to be extremely large and rapid. At room temperature (22 °C) the peak synaptic current can exceed 3 μA. At lower temperatures the synaptic current is reduced in size. Note, however, in Fig. 10 (I_J) that the rise time remains very rapid even at 4 °C. The effect of such a large and fast current when the cell is not under voltage clamp is to discharge the large cell capacitance (approximately 0·03 μF/d.l.m. fibre) rapidly, and thus depolarize the cell to about −5 mV in less than half a millisecond. This very rapid depolarization from resting potential contrasts with the slower, gradually accelerating responses evoked by direct current injection into the cell (Fig. 8). The synaptic potential normally

elicits an overshooting spike response. At about −5 mV a slowing of the rising phase of the potential occurs. The depolarization beyond this point is due to the contribution of I_{Ca}. Note in Fig. 10 the more rapid rise time of I_J relative to I_{Ca}. The times-to-peak of the various currents are plotted in Fig. 12. It can be seen that at the voltages

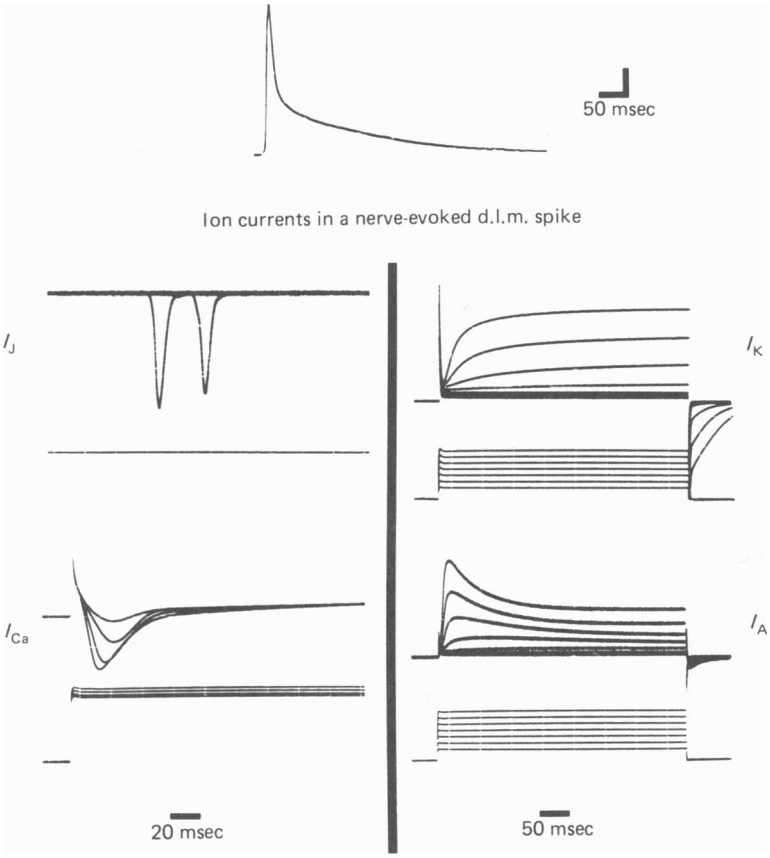

Fig. 10. Ion currents in a nerve-evoked d.l.m. spike. A normal nerve-evoked muscle spike is shown (top) along with the four ion currents under voltage clamp which contribute to the spike (bottom four illustrations). I_J and I_{Ca} are at 4 °C. I_A, I_K and the nerve-evoked spike are at 8 °C. Note that for comparison purposes I_A, I_K and the spike are on the same time base. Note also that the spike rises to approximately 0 mV and that the top traces of I_A and I_K also show the onset of the currents at this voltage. See text for a discussion of the relative roles of each potassium current in spike repolarization. Vertical calibration: spike voltage = 18 mV; current for I_J, I_{Ca}, I_K and I_A = 100 nA; voltage for I_{Ca} = 20 mV, for I_K and I_A = 50 mV. I_{Ca}, I_K and I_A were isolated in the same manner as for Fig. 3. I_A in this Figure is contaminated with a small amount of I_K. To record I_J a young adult animal was used in normal saline with the voltage-clamped at −80 mV. The nerve was then stimulated and the synaptic current measured.

involved in the rising phase of the spike (−80 to 0 mV) the synaptic current is always more rapid than the Ca^{2+} current. In addition the amplitude of I_J is larger (compare I_J and I_{Ca} in Fig. 10). Thus I_{Ca} will be dominated by I_J during the rising phase of the spike. However as the spike approaches the reversal potential of I_J (−5 mV) I_{Ca} takes over.

Repolarization of the nerve-evoked spike

I_A has a larger role in spike repolarization than I_K. This can be seen in Fig. 10 which shows the relative time courses of spike repolarization, and the activation time course of I_K and I_A. These are shown at the same temperature and on the same time scale in this Figure. The spike rises to approximately 0 mV. The top trace of I_K and I_A

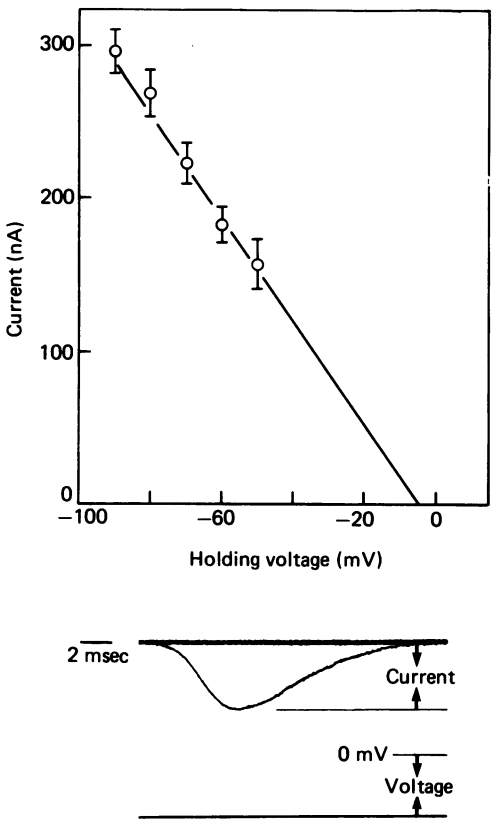

Fig. 11. Reversal potential of synaptic current. The muscle cell was voltage-clamped at the holding voltage indicated on the abscissa. The motorneurone was stimulated once to elicit a synaptic current. The peak amplitude of the synaptic current is plotted on the ordinate. Standard saline (see Methods) was used. The preparation was cooled to 4 °C to reduce the speed and size of currents. A straight line fitted to the measured currents was extrapolated to cross zero at a holding potential of approximately −5 mV, the estimated reversal potential. Holding potentials more positive than approximately −40 mV were avoided because they caused a change in the potassium reversal potential due to ion redistribution resulting from increased potassium conductance. Error bars shown are ± one standard deviation.

are also shown at 0 mV to compare activation times at this voltage. Note that much of the spike is repolarized well before I_K is fully activated. The slow afterdepolarization of the spike is probably due to the turn-off of I_K which is visible in voltage-clamp experiments as slow current tails following the return of the membrane to the holding potential. (Note that E_K is more positive than the resting potential.)

Previous discussion of the physiological function of I_A has focussed on the modulation of interspike interval length in repetitively firing cells (Connor & Stevens, 1971*b*). It is generally presumed that spike repolarization is due to delayed rectification. However, the d.l.m. muscle is not known to engage in repetitive firing (Harcombe & Wyman, 1978). Here I_A functions as the agent of the fast phase of spike repolarization. I_K actually retards repolarization by causing a depolarizing afterpotential.

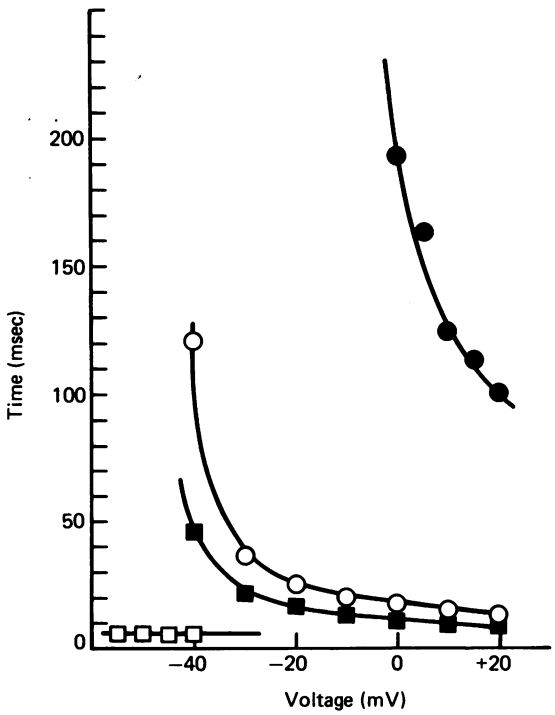

Fig. 12. Relative activation times of d.l.m. ion currents. Currents were isolated as for Fig. 3. Membrane was stepped from a holding potential of −80 mV to the test potential shown on the abscissa. For I_A (○), I_{Ca} (■) and I_J (□), the time to reach peak value is plotted on the ordinate. For I_K (●) the time to reach 90% of its maximum value is plotted. All experiments were at 4 °C.

Variability of active responses due to A-current plasticity

Because of its large amplitude and rapid turn-on time, I_A is a potent force for repolarizing the membrane during depolarizing activity. Two properties of the current, however, can diminish its effectiveness as a repolarizing force. The first of these is the attainment of steady-state inactivation if the membrane resting potential moves to values more positive than −60 mV. This has already been discussed with regard to the variable electrical responses evoked by direct current injected into the muscle at different resting potentials (Fig. 8). A second property of I_A which leads to variability in membrane electrical responsiveness is the rate of I_A recovery from inactivation. Following an initial activation of the A-current, a period of time must

elapse before the current can be fully activated again. The time period required for recovery varies with the voltage at which recovery is occurring. Fig. 13 shows the rates of inactivation and recovery from inactivation (τ_h) plotted with respect to voltage. The relation is a steep bell-shaped one with the recovery rate being longest at about −55 mV, the approximate reversal potential for the current. The A-current in other organisms exhibits similar properties. There is a similar bell-shaped relation

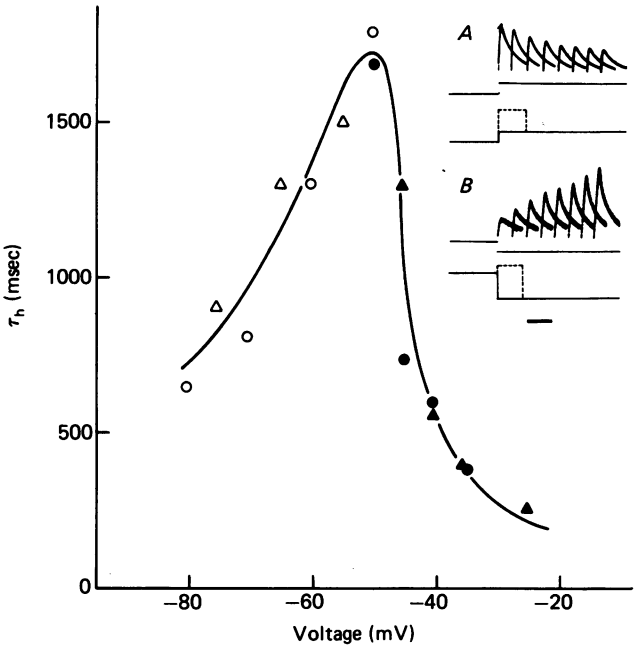

Fig. 13. The time course of development and removal of inactivation (τ_h) plotted on the ordinate *vs.* conditioning membrane voltage, plotted on the abscissa. Circles and triangles are two different animals. Filled symbols, time constants of exponential inactivation onset as measured by a two-pulse experiment as in *A*. Open symbols, time constants of recovery from inactivation as measured in *B*. *A* and *B* show superimposed current traces. For *A* and *B* voltage traces are drawn below the actual currents for clarity. Only one test pulse (dashed line) is shown for each. The test pulse was moved to the right in each subsequent trace. Test pulses were to +20 mV. All experiments were at 4 °C. Time mark = 200 msec.

for τ_h and V_m in *Helix* (Neher, 1971) and *Renilla* (Hagiwara, Yoshida & Yoshii, 1981). but not in *Anisodoris* (Connor & Stevens, 1971; C. F. Stevens, personal communication), where the relation is flat. Significantly, an exception has also been found in a mutant of *Drosophila* which makes τ_h practically insensitive to voltage (Salkoff, 1982). The significance of this mutation with regard to an alteration of channel structure is currently under investigation.

The relatively long time required for I_A to recover from inactivation is a significant cause of variability during repetitive muscle stimulation. A prior publication dealt with the effect of A-current inactivation during repetitive current injection into the muscle (Salkoff & Wyman, 1980). During such stimulation an initial depolarizing stimulus is largely shunted by the rapid turn-on of I_A, thus aborting a large active

response. However, a repeat of the same stimulus before the A-current fully recovers, elicits a much larger active muscle response.

Not only current injection, but also repetitive nerve stimulation elicits variable responses. In this case the variability is manifested as a broadening of spikes in a burst (Fig. 14*A*). Such spike bursts frequently occur during the initiation of flight in *Drosophila*. Since the A-current partially inactivates but does not fully recover

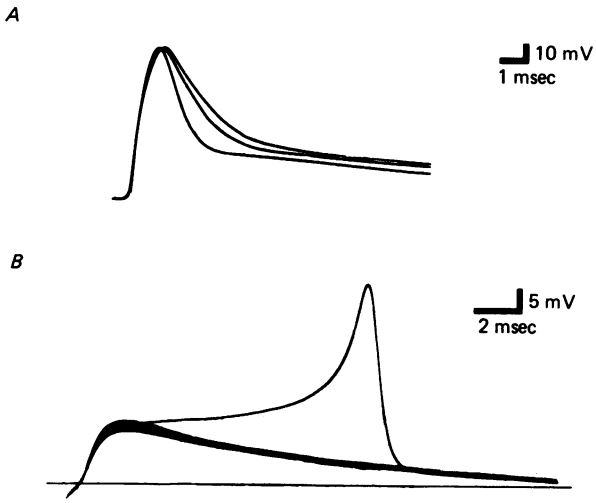

Fig. 14. Plasticity of nerve-evoked muscle responses. *A*, spontaneous burst of muscle spikes at approximately 20 Hz produces spike broadening in wild-type d.l.m. cell. Similar records are produced by electrical stimulation of the motoneurone at this frequency. *B*, increasing effectiveness of a synaptic potential after partial inactivation of I_A. See text for method of lowering e.p.s.p. amplitude. Eight consecutive (superimposed) e.p.s.p.s were evoked by stimulating the motoneurone to the muscle cell. The amplitudes of all eight are the same. The first seven sweeps show subthreshold e.p.s.p.s. 200 msec prior to the eighth and final sweep a conditioning pulse (not shown) was delivered to the muscle. (The conditioning pulse was 50 msec in duration and of sufficient current to elicit an active response). The eighth e.p.s.p. then evoked the spike-like response shown.

during the interspike interval, it accounts for at least part of this broadening. In molluscan neurones a similar spike broadening has been ascribed to the inactivation of a delayed outward current (Aldrich, Getting & Thompson, 1978). As previously described, however, the delayed outward current in the *Drosophila* d.l.m. does not inactivate, and thus cannot participate in spike broadening.

Synaptic plasticity

In Fig. 14*A* the rapid depolarizing phase does not vary significantly because the synaptic current depolarizes the muscle so rapidly that I_A is not activated sufficiently to shunt the spike. Thus, the rate of rise of the nerve-evoked spike does not depend on the state of the A-current. However, by use of a temperature-sensitive mutant that depresses neurotransmitter release (Ikeda *et al.* 1976; Salkoff & Kelly, 1978) the size of the synaptic current can be reduced to a level near the threshold of the inward current. In this situation it can be demonstrated that the state of I_A inactivation

can have a profound effect on the effectiveness of synaptic transmission. Fig. 14*B* demonstrates that, if a stimulus is near threshold for a response, the A-current can be the critical factor in determining the presence or absence of a spike-like response. In Fig. 14*B* the synaptic current has been reduced by use of the temperature sensitive mutation *shi*^{ts} so that it is below threshold for eliciting any spike-like response of the membrane. Note that eight superimposed identical excitatory junction potentials (e.p.s.p.s) are shown. All but the last one failed to elicit a spike-like response. Just before the last nerve stimulus a conditioning current pulse was delivered intracellularly to the muscle (not shown in Figure). The size of the pulse was large enough to inactivate a sufficient amount of the A-current to lower the threshold of the spike-like response effectively (Salkoff & Wyman, 1980). It can now be seen that the final e.p.s.p. activates sightly more inward current than outward current. The result is a slowly rising membrane potential that accelerates into a spike-like response. Hence, the A-current may act as a post-synaptic switch for turning synaptic transmission on or off (Daut, 1973).

It is worthwhile noting that in Fig. 14*B*, even though the spike-like response is synaptically evoked, the slow rate of the initial rise more closely resembles the response when the muscle is directly stimulated by current injection (Fig. 8) than the synaptically evoked spike shown in Figs. 10 (top) and 14*A*. This is due to the fact that the inward calcium current is turning on rather slowly at its threshold in Fig. 14*B* while in Fig. 14*A* most of the rising phase of each spike is due to the synaptic current.

DISCUSSION

In the *Drosophila* d.l.m. electrical responses are highly variable. The type of response seen in the d.l.m. depends on many diverse factors such as the resting potential of the cell, the age of the fly, and whether the excitatory input is from a motorneurone or from micro-electrode current injection. Some of the first observations of graded responsiveness in electrically active cells were made in insect muscle cells (Cerf, Grundfest, Hoyle & McCann, 1959; Piek & Njio, 1979). Until recently little was known about the mechanisms underlying this phenomenon. Piek & Njio, in their recent review (1979), can only state that graded activity 'is supposed to be the result of two currents, an inward current of calcium ions and an outward current of potassium ions.' In the *Drosophila* d.l.m. there are two voltage-sensitive potassium channels. The A-current channels, which carry the fast transient outward current, are particularly responsible for the variation in response (Salkoff & Wyman, 1980). As shown, this current activates rapidly and opposes the depolarizing force of the inward calcium current. Also, the amount of current carried by this system varies greatly, depending on the resting potential of the cell as well as the prior history of electrical activity. An additional feature which enhances graded responsiveness is the long time constant of the cell. Injecting current directly into a cell produces slowly rising depolarizing responses as the high membrane capacitance is slowly discharged. In contrast to these responses, stimulation of the motorneurone supplying this muscle produces an extremely rapid depolarization, because of the extremely large and fast synaptic current.

Many vertebrate muscle cells have regenerative sodium channels which rapidly drive the membrane potential to a depolarized level (Nastuk & Hodgkin, 1950). The reason for this spiking mechanism is, presumably, to propagate excitation throughout the cell for releasing internal Ca^{2+} sotres. In *Drosophila* flight muscle, regenerative sodium channels do not exist. Since the cells are isopotential, they do not need a propagating action potential. However, apparently a rapid depolarization is still needed. As a substitute for sodium channels a very large synaptic current rapidly depolarizes the membrane. Calcium channels themselves are not the primary mechanism for depolarization. One reason for this might be that the density of calcium channels in the membrane is determined by the need for particular levels of calcium flux. The density of Ca^{2+} channels in the membrane does not match the density of channels that would be needed for a very rapid depolarization of the membrane. Unfortunately, the details of the functioning of insect muscles are poorly understood (Piek & Njio, 1979), and so the reasons for requiring each of the ion-channel types present in the *Drosophila* d.l.m. can only be guessed at.

The value of studying this system extends far beyond one's interest in fibrillar flight muscles. The preparation offers an opportunity to study calcium and potassium channels which are apparently involved in modulating synaptic transmission (Daut, 1973; Shapiro, Castellucci & Kandel, 1980), In spontaneous and rhythmic neural activity (Smith, Barker & Gainer, 1975) and in behaviour and learning (Byrne, 1980). Significantly, the molecular structure of none of these channels is yet understood. Identification of the genes coding for these channels (Salkoff & Wyman, 1981*b*; Tanouye *et al.* 1981) opens up the possibility of using molecular biological techniques to obtain and study these molecules. Because it now offers so many advantages in fields as diverse as neurophysiology and molecular biology, *Drosophila* may well play a role in unravelling the functioning of these channels.

Note added in proof. Since completion of this paper I_A in adult animals has been found to have a Ca^{2+}-dependent component in addition to the voltage-dependent component. In the pupa the Ca^{2+}-dependent component is not active. In this paper the term I_A in adults refers to the sum of both components.

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