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6. Complementary DNA's were prepared from poly(A)$^+$ RNA's of Tem-R larvae with an Amersham cDNA synthesis kit [V. Gubler and B. J. Hoffman, *Gene* **25**, 263 (1983)]. After addition of Eco RI linkers, cDNA's were introduced in phage λgt11 [R. A. Young and R. W. Davis, *Science* **222**, 778 (1983); *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983)] and plated on *E. coli* λ1090 (Promega Biotec). Among the 5×10^4 recombinants initially screened, 14 displayed a strong positive signal after four plaque purifications with esterase B1 antiserum but not with a preimmune serum or a serum raised against *Culex* esterase A1 (*5*). One of the recombinant phages, λgt-est, was used to lysogenize *E. coli* Y1089. The proteins from the lysogens, after their separation by sodium dodecyl sulfate (SDS)–polyacrylamide electrophoresis and immunoblotting [H. Towbin *et al.*, in (*5*); R. Hawkes, E. Niday, J. Gordon, *Anal. Biochem.* **119**, 142 (1982)] were shown to include a β-galactosidase fusion protein of 135 kD that was immunoreactive with esterase B1 antiserum. The DNA of λgt-est was digested with Eco RI and used to hybrid-select the mRNA of esterase B1 from poly(A)$^+$ RNA's extracted from Tem-R larvae. This selected mRNA (Fig. 1C) produced a 67-kD polypeptide comigrating with purified esterase B1 and immunoprecipitated by its specific antibody. λgt-est cDNA is therefore specific for esterase B1.
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11. Strains of *C. pipiens* [N. Pasteur, A. Iseki, G. P. Georghiou, *Biochem. Genet.* **19**, 909 (1981)] and *Musca domestica* [A. G. Clark and W. C. Dauterman, *Pestic. Biochem. Physiol.* **17**, 307 (1982); A. G. Clark, N. A. Shamaan, W. C. Dauterman, T. Hayaoka, *ibid.* **22**, 51 (1984)] resistant to OP insecticides through production of highly active enzymes, such as esterase A1 and glutathione transferase, respectively, had been shown to possess amplified sequences of genomic DNA that are absent from strains lacking such resistance mechanisms (*9*). No cross-hybridization has been obtained between these amplified sequences and the λgt-est probe. This is in agreement with the lack of serological relations between these enzymes (*5*).
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Occult *Drosophila* Calcium Channels and Twinning of Calcium and Voltage-Activated Potassium Channels

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In the membrane of the flight muscle cells of developing *Drosophila* a large calcium-sensitive potassium current, I_{Kc}, was found. It was present before the development of voltage-activated potassium channels and seems to be the first potassium current to develop in the membrane. Also present in these early cells were large numbers of occult (hidden) calcium channels, which remained inactive until the end of pupal development. These inactive calcium channels could be made to function by injecting adenosine triphosphate or ethyleneglycol tetraacetic acid into the early cells. I_{Kc} has kinetic properties resembling the later developing voltage-sensitive current I_{Kv}, and is distinct from the fast, transient calcium-dependent outward current I_{Ac}, which appears much later in development. I_{Ac} closely resembles the voltage-sensitive current I_{Av}, also present in these cells. Thus, both of the voltage-sensitive potassium channel types, I_{Av} and I_{Kv}, have similar calcium-sensitive counterparts, I_{Ac} and I_{Kc}, that are present in the same cells.

IN *Drosophila* DEVELOPMENT, THE LAST of three larval stages terminates with the formation of a puparium and the histolysis of larval muscles. In the following 4 days of pupal development, the adult flight muscles are made de novo. During this period these muscles are particularly accessible for the investigation of developing membrane electrical properties by voltage-clamp techniques (*1–3*). At the midpoint of pupal development a fast, transient voltage-dependent current appeared in the membrane and was mature at about 72 hours of pupal development (Fig. 1A). This current was termed the A current when first identified in molluscan neurons and is designated I_{Av} here. The maturation of I_{Av} is followed by the development of a slower, voltage-dependent K^+ current, I_{Kv}, which is mature at 96 hours of development (Fig. 1A). At the end of pupal development, when the adult is ready to eclose from the pupal case, a large calcium current, I_{Ca}, abruptly appears. There was evidence for the presence of a slow, Ca^{2+}-dependent K^+ current similar to I_{Kc} in other systems (*4*); we observed large, persistent currents in young adult cells after the addition of saline containing Ca^{2+} (6 m*M*) (Fig. 1B). These persistent currents (tails) had a reversal potential at the estimated K^+ equilibrium potential. The large, slow current tail present with Ca^{2+} (Fig. 1B, arrow 4) was absent when Ca^{2+} was removed (Fig. 1B, $-Ca^{2+}$); only a fast component due to I_{Kv} was evident. The current tail (arrow 4) is in the inward (downward) current direction because the K^+ reversal potential for this cell (approximately −60 mV) is more positive than the holding potential of −80 mV.

We investigated the developmental origin of this current with voltage clamping in pupal cells at the 72-hour stage (before I_{Kv} develops) (Fig. 1A). At this developmental stage, there is less net outward current. We determined the pattern of outward current in a 72-hour wild-type cell with no Ca^{2+} (Fig. 2, left) or 18 m*M* Ca^{2+} (Fig. 2, right) in the saline. The fast transient current, I_{Av}, was present in both conditions. However, in the right traces, I_{Kc} is also clearly present, as indicated by the rising outward current after the initial decline of I_{Av} and by the appearance of the large slow current tails after the termination of the voltage-clamp step pulse.

To eliminate I_{Av} from records at this stage

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of pupal development, we used muscle fibers from a *Drosophila* mutant *Shaker* allele that totally lacks I_{Av} (Fig. 1A) (*1–3*). At the 72-hour stage of development when I_{Av} is normally present in wild-type cells, Sh^{KS133} cells have only the large Ca^{2+}-dependent outward response and the large, slow current tails at the cessation of the step pulse characteristic of I_{Kc} (Fig. 2, bottom). The slow rise of the outward current in these traces is not a true reflection of the activation rate of the channels, but probably indicates that Ca^{2+} is slowly becoming available at the channel sites. The faster rate of rise of the small time-dependent current at the onset of the voltage-step pulse reflects the true activation time of the current (Fig. 2, *Sh*, $+Ca^{2+}$). The rapid activation of this small portion of I_{Kc} is probably due to a slight accumulation of intracellular Ca^{2+}. In other experiments with similar cells that had been injected with Ca^{2+} we found that I_{Kc} activates at a rate similar to I_{Kv}. At 0 mV, I_{Kv} takes approximately 190 msec to reach 90% of its peak value (*2*); the value for I_{Kc} was 200 msec in one calcium-injected cell. Similarly, the very slow decay phase of the current tails is probably not a true reflection of the channel closing time. The current tails were clearly biphasic with an initial fast phase (Fig. 2, arrow 1) that had kinetics similar to I_{Kv}, followed by a much slower phase (Fig. 2, arrow 2). This slow phase may be a reflection of the rate of calcium sequestration at the more polarized voltages. The I_{Kc} channels are apparently voltage-sensitive as well as Ca^{2+}-sensitive; in Ca^{2+}-free saline with Ca^{2+} injected directly into the cell, more channels open after depolarization than at rest. Thus, Ca^{2+} probably shifts the voltage sensitivity of activation to make openings more probable at a given voltage. I_{Kc} is eliminated by 25 m*M* tetraethylammonium chloride (TEA).

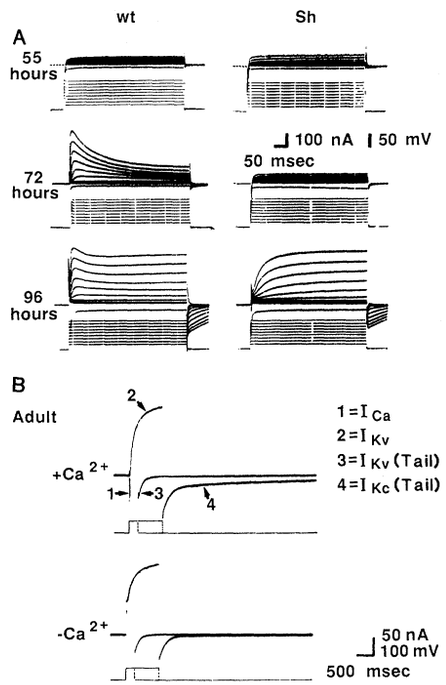

Fig. 1. (A) Voltage-clamp experiments showing the development of two voltage-sensitive, Ca^{2+}-sensitive currents. The hours given are measured from the beginning of pupal development; wt, normal wild type; Sh, *Shaker* mutant lacking I_{Av}. At 72 hours of development, I_{Av} was mature in wild type but absent in *Shaker*. At 96 hours, both I_{Av} and I_{Kv} were present in wild type. However, only I_{Kv} was present in *Shaker*. Voltage steps to +20 mV were made from a holding potential of −80 mV. Saline (see below) contains no Ca^{2+}. (B) Voltage-clamp experiments showing I_{Kc} tail current in young adult Sh^{KS133} cells. I_{Av} is not present in Sh^{KS133} cells. Composite current response to short (200 msec) and long (800 msec) voltage-step pulses under voltage clamp in the presence ($+Ca^{2+}$) (6 m*M*) and absence ($-Ca^{2+}$) of Ca^{2+} in the saline. For each figure, current is shown by the upper traces, voltage by the lower traces. Arrow 1, I_{Ca}; arrow 2, I_{Kv}; arrow 3, I_{Kv} tail current; arrow 4, I_{Kc} tail current. In the presence of Ca^{2+}, there was a delayed appearance (compare arrows 3 and 4) of an additional slower component of the tail current in response to the longer step pulse. The additional component of tail current reflects the activation of I_{Kc} in addition to I_{Kv}. After removal of Ca^{2+} ($-Ca^{2+}$) the slower component of the tail current was absent. A larger fast component is evoked by the longer (800 msec) voltage-step pulse because more I_{Kv} is evoked by the longer pulse. Voltage steps to −10 mV were made from a holding potential of −80 mV. The composition of the saline was 125 m*M* choline chloride, 5 m*M* KCl, 2 m*M* EGTA, 10 m*M* $MgCl_2$, and 5 m*M* Hepes, *p*H 7.0. Additions of $CaCl_2$ or $BaCl_2$, as noted in the text, were made in the absence of EGTA. All experiments were performed at 4°C.

The presence of an early developing, Ca^{2+}-sensitive current would logically require the presence of Ca^{2+} channels. Although I_{Ca} is not evident in the pupal stage until its abrupt appearance at the time of adult eclosion (*1*), a smaller less prominent Ca^{2+} current might be present in early cells. We again used muscle cells of the *Shaker* mutant Sh^{KS133}, which lacks I_{Av}. If not eliminated, I_{Av} could obscure the presence of an inward current having a similar time course. We compared the membrane voltage responses of these mutant cells from the two developmental stage (Fig. 3) before (72 hours) and after (young adult) the Ca^{2+} current normally becomes apparent. In normal saline (with Ca^{2+} present), the responses of the 72-hour pupal cell appeared almost completely passive, while the young adult cell showed large overshooting action potentials. The contrast between excitable properties was also evident in the membrane properties under voltage clamp in saline containing Ba^{2+} and TEA (Fig. 3). Although the 72-hour cell again appears completely passive, the young adult cell exhibits a large inward current. Nevertheless, Ca^{2+} channels might still be present at the 72-hour stage but be inactive. Since many Ca^{2+} channels are known to be inactivated by intracellular Ca^{2+} (*5*), perhaps the intracellular Ca^{2+} concentration in these developing cells was above a level that permits the Ca^{2+} channels to function. To investigate this we injected cells with ethyleneglycol tetraacetic acid (EGTA) to lower the intracellular Ca^{2+} concentration, or injected adenosine triphosphate (ATP) to increase active Ca^{2+} sequestration (*6*). Both of these techniques unmasked large inward currents in cells that had showed no trace of inward current

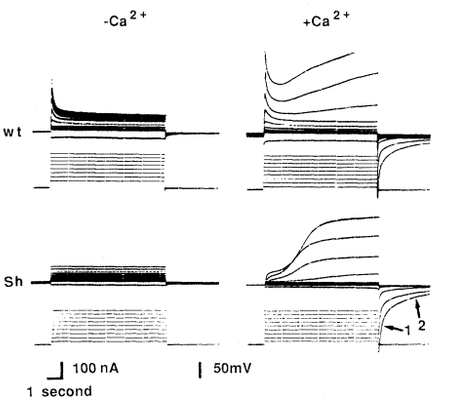

Fig. 2. I_{Kc} in 72-hour pupae. Composite current responses to 6-second voltage-step pulses under voltage clamp in cells from 72-hour pupae, in the presence ($+Ca^{2+}$) and absence ($-Ca^{2+}$) of high extracellular concentrations of Ca^{2+} (18 m*M*). At this stage in development, I_{Av} was fully developed in wild-type cells (transient component in top traces), but was absent in Sh^{KS133} cells (bottom traces). In the presence of high extracellular Ca^{2+}, I_{Kc} was slowly evoked by depolarizations (right traces). Upon repolarization, I_{Kc} has a biphasic current tail (see text for an explanation of arrows 1 and 2). As expected for a K^+ current, the reversal potential for the current tails shifts to more positive values when the extracellular K^+ concentration increased. In one experiment the reversal potential for the tails shifted from approximately −50 mV to −20 mV when the extracellular KCl concentration was increased from 5 to 25 m*M*. Experiments substituting SO_4^{2-} for Cl^- were not done. The presence (top traces) or absence (bottom traces) of I_{Av} had no effect on I_{Kc}. Cells were held at −80 mV and successively stepped to a maximum of +10 mV. The leak current traces in the inward direction were in response to a hyperpolarizing step pulse to −130 mV. In wild-type cells ($+Ca^{2+}$), I_{Av} appears larger than in wild type ($-Ca^{2+}$) because the leak (and therefore instantaneous current) has increased over the course of the experiment. This is reflected in the shift of the baseline of the holding current.

before injection (Fig. 3). In fact, the amplitude of currents evoked from these 72-hour cells was similar to that measured in young adult cells, often exceeding 150 nA of net inward current. ATP seemed to be more effective than EGTA in unmasking inward current; of ten cells that showed no inward current before ATP injection, eight produced large inward currents after injection. Only about half the cells injected with EGTA showed inward current. Either Ba^{2+} or Ca^{2+} could serve as the current-carrying ion; Ca^{2+} currents were smaller and more transient than Ba^{2+} currents. Cells often required periods of ATP injection as long as 10 minutes before maximal inward current appeared (Fig, 3).

To investigate whether I_{Kc} or I_{Ca} was present at even earlier developmental times, we used wild-type cells. For this purpose, the early developmental appearance of I_{Av} in wild-type cells was advantageous; the absence of I_{Av} in young (55-hour) wild-type cells was a reliable developmental marker with which to judge the maximum age of each preparation. Both I_{Kc} and I_{Ca} could be evoked in these young cells (Fig. 4); both were observed before there was any trace of I_{Av}. The maturation time of these currents, however, apparently overlaps the developmental onset of I_{Av}, because both I_{Kc} and I_{Ca} are larger at 72 hours than at 55 hours.

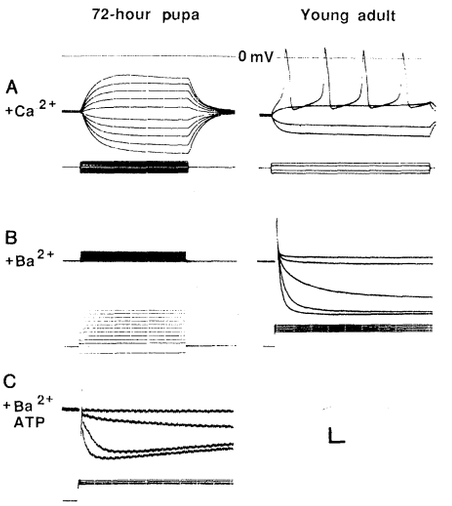

Fig. 3. Unmasking I_{Ca} in pupal muscle in Sh^{KS133} cells. (A) Voltage responses to intracellular current injections for 72-hour pupal (left) and young adult (right) cells, in the presence of saline containing 2 m*M* Ca^{2+}. The top line indicates 0 mV. In response to intracellular current injection (bottom trace) 72-hour pupal cells are inexcitable (left traces), showing only minor deviation from a linear current-voltage relation. In contrast, young adult cells (right traces) generate a train of broad, overshooting action potentials in response to a similar stimulus. (B) Same cells as above under voltage clamp in the presence of Ba^{2+} (50 m*M*). The large Ba^{2+} current present in the adult cell was absent in the 72-hour cell. (C) A large inward current carried by Ba^{2+} evoked after the injection of ATP into a 72-hour cell. Holding potential of −80 mV used for all voltage-clamp figures. For voltage clamp of inward current, TEA was substituted for choline chloride. Current-clamp experiments were at 20°C, voltage-clamp records at 4°C, ATP injections were made by intracellular iontophoresis from a current electrode filled with 1*M* ATP and 5 m*M* Hepes, *p*H 7.0. An average experiment required −25 nA of steady injected current for 10 minutes before a maximum current appeared. Voltage, current, time scale: Upper traces, 20 mV, 100 nA, 20 msec; middle and lower traces, 50 mV, 50 nA, 50 msec.

In the nerve and muscle cells of many animals, a Ca^{2+} current appears early in the development of membrane electrical properties (*7*). In *Drosophila* flight muscles, however, the Ca^{2+} current abruptly becomes prominent at the end of pupal development after the development of voltage-dependent outward conductances (*1*). Our observations now show that many Ca^{2+} channels are, indeed, present more than 2 days before. These observations make *Drosophila* development more consistent with the overall picture presented by Spitzer (*7*) of early appearing Ca^{2+} channels in membrane electrical development. The reasons for Ca^{2+} channel inactivity in young fibers and the action of ATP in inducing activity are unknown. Internal perfusion of cultured chick sensory neurons and molluscan neurons with ATP or EGTA stabilizes Ca^{2+} channel activity (*8*). Since EGTA or ATP unmask *Drosophila* Ca^{2+} channels, intracellular Ca^{2+} concentration may normally be high at early stages of muscle development. However, other more direct effects on the channels have not been ruled out.

The role for the early appearance of I_{Kc} is unclear, but it could function to repolarize cells to shut down Ca^{2+} channels and prevent unwanted Ca^{2+} influx. This may be particularly critical before the development of other repolarizing currents and adequate Ca^{2+} buffering capacity. I_{Kc} was undetected previously because of the high concentration of extracellular Ca^{2+} necessary to activate it. Although the intracellular Ca^{2+} concentration in early cells may be high enough to block I_{Ca}, I_{Kc} apparently requires an even higher level for activation at the voltages in these experiments. The finding of I_{Kc} increases the total number of identified, independent current-carrying systems in this membrane to seven (*1*). The picture that now emerges of membrane electrical properties is one of redundant channel properties and staggered channel development extending from early pupal into adult life. The two fast transient outward currents, I_{Av} and I_{Ac}, are genetically separable (*3*) and thus probably fully independent; it is likely that I_{Kc} and I_{Kv} are also fully independent. Some *Shaker* alleles have no I_{Av} and yet this does not produce a lethal phenotype; thus, there is at least a partial overlap of function by separate current systems. In addition, occult or "masked" Ca^{2+} channels may be present in other systems.

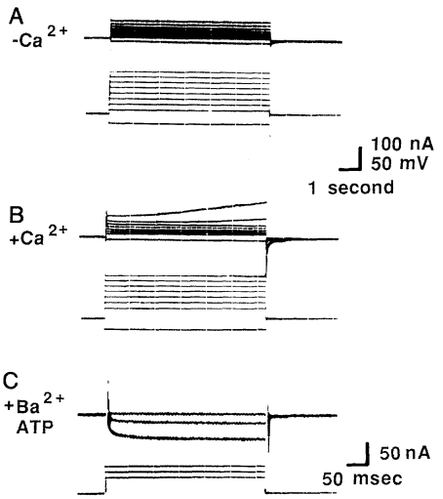

Fig. 4. Early development of I_{Kc} and occult Ca^{2+} channels. (A) Voltage-clamp records of wild-type pupal muscles at about 50 hours of development. There was no evidence of the later appearing I_{Av}. (B) After addition of Ca^{2+} at this same developmental stage, I_{Kc} was seen as a large delayed outward current. The small biphasic current tail produced upon repolarization was larger when increasing amounts of current were evoked. (C) Occult Ca^{2+} channels are unmasked at this same early stage by intracellular injection of ATP in the presence of 20 m*M* Ba^{2+}.

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