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The Major Delayed Rectifier in Both *Drosophila* Neurons and Muscle Is Encoded by *Shab*

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The delayed rectifier K^+ current in *Drosophila* is similar to the classical delayed rectifier, originally described by Hodgkin and Huxley. *Drosophila* provides unique tools of mutant analysis to unambiguously determine the genetic identity of this native K^+ current. We identified the *Shab* gene as the exclusive gene underlying delayed rectifier currents in both muscle and neurons. In muscles, a genetic mutation of *Shab* removes virtually all the whole cell delayed rectifier current (I_K), while leaving unaltered the transient A-current encoded by the *Shaker* gene. In neurons, the *Shab* mutation also removes the bulk of I_K, but leaves unaltered the transient A-current encoded by the *Shal* gene. Although most of the delayed rectifier current is the product of the *Shab* gene, the *Shaw* gene contributes a small "leak" current to most neurons and muscle cells. Thus, in contrast to the A-currents which are encoded by different genes in muscle and neuronal cell bodies (*Shaker* and *Shal,* respectively), the predominant I_K in both muscle and neurons is encoded by the same gene (*Shab*). With the genetic identity of I_K confirmed, all of the major K^+ currents in embryonic *Drosophila* neurons and muscle are now known.

[Key words:* Drosophila, *potassium channels, mutant analysis,* Shaker, Shal, Shab, Shaw, *embryonic neurons, excitable membranes,* I_K, *delayed rectifier, mutant]

The total voltage-dependent outward K^+ current in most neurons and muscles can loosely be divided into two components, a rapidly inactivating A-type current, I_A, and a much more slowly inactivating "delayed rectifier" (DR) component, I_K. These two components were originally identified as separable components based on pharmacology (Thompson, 1982). 4-Aminopyridine (4-AP) was found to rather selectively block I_A, while tetraethylammonium (TEA) preferentially blocks I_K. In *Drosophila* flight muscle, the genetic, developmental, and functional independence of these two components became obvious when they were examined at different stages of development in wild type and *Shaker* mutant flies. *Shaker* mutations remove I_A in *Drosophila* flight muscles, but not I_K (Salkoff and Wyman, 1981; Salkoff, 1983, 1985; Wu and Haugland, 1985; Solc et al., 1987; Zagotta et al., 1988; Broadie and Bate, 1993). Thus, I_A and I_K were shown to be genetically independent in muscles. I_A and I_K were also shown to be genetically independent in *Drosophila* neurons by a mutation of the *Shal* gene which eliminates I_A in neurons (Tsunoda and Salkoff, 1995), but leaves I_K unaltered.

I_A and I_K in muscle were also shown to have distinct developmental profiles: I_A appears first, reaching maturation 72 hr after pupariation, while I_K appears second, reaching maturation 96 hr after pupariation (Salkoff, 1985). I_A and I_K also appear on a similarly staggered developmental schedule in some vertebrate neurons such as quail chick neurons (Bader et al., 1985). Functionally, I_A and I_K also play different roles in *Drosophila* flight muscles, with I_A responsible for very rapid repolarization of the action potential, and I_K responsible for a slower phase of repolarization (Salkoff, 1985).

While the I_A currents in muscle and neurons have been genetically identified (*Shaker* and *Shal*), I_K currents in both cell types have not previously been genetically identified. Of the four cloned voltage-dependent K^+ channel genes, *Shaker, Shal, Shab,* and *Shaw,* I_K was most likely encoded by *Shab* and/or *Shaw,* because both express DR type currents in the *Xenopus* oocyte expression system (Wei et al., 1990). Because Shab currents heterologously expressed in *Xenopus* oocytes were the most similar to I_K in neurons and muscle cells, we suspected *Shab* to be a major contributor to I_K. To test this hypothesis, we examined neurons and muscle cells which are mutant for the *Shab* gene. We found that indeed virtually all of I_K was eliminated by a mutation of the *Shab* gene, thus showing that *Shab* does encode the predominant DR current component in both neurons and muscle cells.

In contrast to the other voltage-dependent K^+ channel genes, *Shab* appears to be a major contributor to the whole cell current in both neurons and muscle. *Shab* K^+ channel genes in mammals (Frech et al., 1989; Pak et al., 1991) are also expressed in a wide variety of tissues, including brain, heart, skeletal muscle, and many of the sensory systems (Trimmer, 1991; Hwang et al., 1992; Hwang et al., 1993a,b; Trimmer, 1993). Our study differs from the studies in mammalian systems in that it proved feasible to genetically delete the *Shab* gene in *Drosophila.* This enabled us to observe the remaining DR currents present after *Shab* removal. Our striking result is that there appears to be no other major DR current present in *Drosophila.*

Materials and Methods

Cell culture

Late-gastrula cell cultures were prepared in a manner similar to previous reports (Seecof et al., 1971; Tsunoda and Salkoff, 1995). Eggs were

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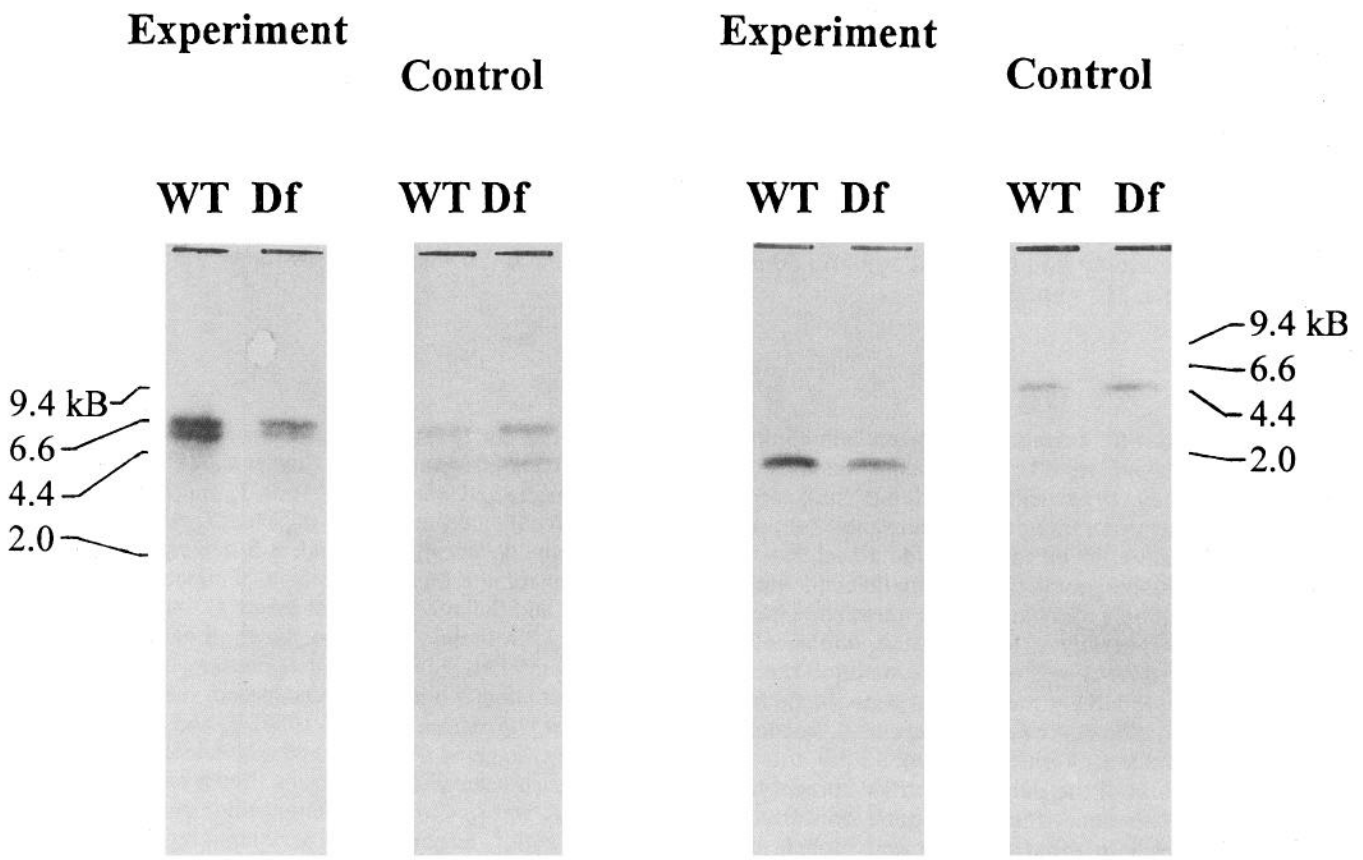

Figure 1. Southern analysis confirms that the *Shab* gene falls within the deficiency. Genomic DNAs from wild type (*WT*) and heterozygous deficiency, Df(3L)HR370 + Dp(3;3)pdp7/TM3 ftz-lacZ, (*Df*) flies were cut with EcoRI (*left pair*), and SalI (*right pair*). *Experiment,* Southern blots probed with the cDNA of the *Shab* gene; *Control,* same blots later probed with a control gene (see Materials and Methods). Labeled bands were quantified (see Materials and Methods). Bands labeled with the *Shab* cDNA were normalized to the intensity of bands labeled with a control gene. Heterozygous deficiency bands were 50% (EcoRI) and 67% (SalI) of wild type control. We expect DNA of the heterozygous deletion flies to label with an average intensity of 50% of the wild type control DNA.

collected over a 1–2 hr period and allowed to develop in a humidified chamber at room temperature for about 5.5–6 hr for neuronal recordings, and 7–8 hr for myotube recordings. Eggs were rinsed with 70% ethanol, dechorionated by rolling on double-stick Scotch tape, and covered with halocarbon oil. A sharp microelectrode was used to break through the vitellin membrane of single embryos and all contents were removed by suction. Single embryos were dissociated into a 20 μl drop of culture media similar to media previously described (Seecof and Donady, 1972): 18% fetal calf serum, 6 mU/ml insulin, 1% penicillin–streptomycin (from a stock of 10 kU penicillin and 0.5 mg/ml streptomycin in 0.9% NaCl), in Schneider's Medium. Cultures were prepared on glass coverslips. Cultures were grown at room temperature in a humidified chamber for 20–72 hr before being used for electrophysiology.

Fly stocks

(1) *Wild type flies: Oregon-R strain.*

(2) Shaker *mutants:* Sh^{102} *flies.* Molecular analysis of this mutation revealed a nonsense mutation closing the open reading frame between the S5 and S6 transmembrane spanning domains (Gisselmann et al., 1989).

(3) Shab *mutants: a genetic deficiency, Df(3L)HR370+Dp(3;3)pdp7.* This chromosome has a net deficiency of 63A to 63B8-9 (courtesy of Dr. Arthur D. Wohlwill; Wohlwill and Bonner, 1991). *Shab* was mapped to chromosomal band 63A on the left arm of the third chromosome (Butler et al., 1989). The deficiency, Df(3L)HR370, from 63A to 63D, combined with the duplication, Dp(3;3)pdp7, from 63B8-9 to 65 (Wohlwill and Bonner, 1991) results in a net deficiency of 63A to 63B8-9 which encompasses the *Shab* locus. We confirmed that *Shab* falls within this deficiency by genomic Southern analysis (Fig. 1). The *Shab* deficiency was maintained over a third chromosome balancer (either TM3 or TM6B) which carried the *lacZ* gene driven by the *ftz* promoter, which was essential for determining the genotype of a given culture.

(4) Shal *mutants: a genetic deficiency, Df(3L)JK18 or Df(3L)kto2 (Lindsley and Zimm, 1992).* This deficiency was shown to eliminate the transient Shal A-currents in neurons (Tsunoda and Salkoff, 1995).

Identifying neurons and myotubes

Although whole embryos containing many different cell types are dissociated, culture conditions have been shown to favor the survival of mainly muscle cells and neurons (Shields and Sang, 1970; Seecof et al., 1971). We identified cells which stained positive with FITC-conjugated anti-horseradish peroxidase (anti-HRP) (data not shown), an antibody which specifically recognizes CNS and PNS neurons in *Drosophila* (Jan and Jan, 1982). Positively staining neurons were cells with round cell bodies, 5–8 μm in diameter, and extended processes. These cells grew either singly or in clusters in culture and no difference in physiology was unique to single or clustered cells. Because of their distinct morphology, neurons could be reliably selected for electrophysiology in unstained cultures.

Myotubes were also identified by morphology for electrophysiology. These cells were observed to contract in culture. In addition, when cells from a strain containing the *lacZ* gene driven by the myosin heavy chain promoter, which has muscle specific expression, were stained with X-gal, myotubes like the ones chosen for electrophysiology stained blue.

Fixing and staining cultures

After recording, cells were immediately fixed for 2–4 min in 2% formaldehyde, 0.4% glutaraldehyde, in phosphate buffer solution (PBS).

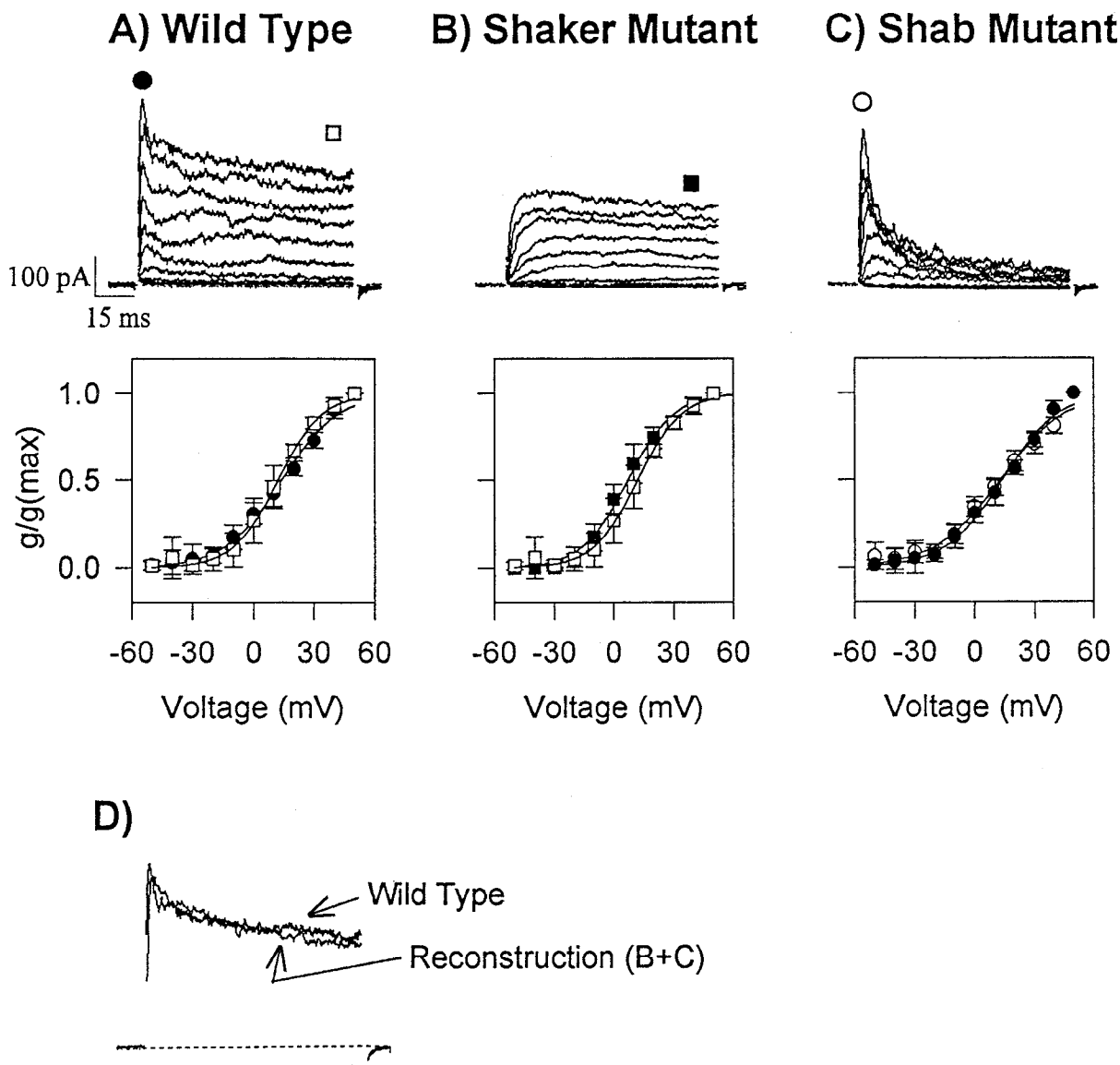

Figure 2. *Shab* Mutation Removes I_K in Myotubes. *A,* Representative whole cell currents from a wild type myotube. The steady-state component, measured 80 msec after jump onset, was 69.10 ± 10.17% of the total peak current (N = 13). Conductance–voltage relations (N = 5 cells) of peak current (●) and steady-state current (□) are shown below. SDs are indicated. A single Boltzmann equation, $g/g_{max} = 1/(1 + \exp((V - V_{½})/k$ was fit to each curve, where g/g_{max} is the normalized conductance, $V_{½}$ is the voltage at which g/g_{max} is half-maximal, and $k = RT/zF$. Best fit parameters are as follows: for peak current, $V_{½}$ = 13.75 ± 1.03 mV, z = 1.81 ± 0.11 mV/e-fold shift; for steady-state current, $V_{½}$ = 11.92 ± 0.56 mV, z = 2.31 ± 0.10 mV/e-fold shift. *B,* Representative whole cell current, I_K, in a Sh[102] myotube where the Shaker current (I_A) is removed. Below, conductance-voltage relation (■, N = 7) of I_K in Shaker[102] myotubes superimposed on the conductance–voltage relation of the steady-state current in wild type myotubes from *A* (□). g/g_{max} is plotted versus voltage and fit with a Boltzmann equation as described in *A*. Best fit parameters are $V_{½}$ = 6.9 ± 0.87 mV, z = 2.22 ± 0.14 mV/e-fold shift. *C,* Representative whole cell currents from *Shab*-deficient myotubes (N = 13). Most of the steady-state component was removed with only an estimated 23% remaining. This was only 15.59 ± 7.35% of the total peak current. Conductance–voltage relation of the transient Shaker current remaining in *Shab*-deficient myotubes is shown below (N = 6) (○) and is compared to the conductance–voltage relation of the peak current in wild type myotubes in *A* (●). Best fit parameters to Boltzmann equation are $V_{½}$ = 12.82 ± 1.28 mV, z = 1.57 ± 0.11 mV/e-fold shift. *D,* Whole cell currents at +50 mV reconstructed from a linear combination of *Shaker* mutant and *Shab*-deficient current traces are superimposed on a wild type current for comparison. Voltage jumps (80 msec) were from −50 to +50 mV in 10 mV increments from a holding potential of −90 mV. Whole cell conductance (g) was calculated from the equation $g = I/(V - E_r)$, where I is peak current elicited by voltage jumps to different test potentials, V is membrane potential, and E_r is the reversal potential for this current. E_r was calculated to be −84 mV from the Goldman-Hodgkin-Katz current equation, assuming that P_{Na}/P_K = 0.03 (Hille, 1992) and the solutions used. g/g_{max} values plotted are the averages, at each potential, of the normalized conductance for each cell.

Cultures were rinsed with PBS after fixing and stained overnight at 30°C with an X-gal solution containing: 1 mg/ml X-gal (from a 8% stock made up in DMSO), 16 mM KFerrocyanide, 16 mM KFerricyanide, 2 mM $MgCl_2$ in PBS, pH 7.2–7.3. Cultures were rinsed with PBS and checked for blue staining.

Determining the genotype of a given culture

The following strategy was used to determine which single-embryo cultures were homozygous deficient for the *Shab* gene. The *Shab* deficiency is maintained over a balancer chromosome containing the *lacZ* gene.

Table 1. Comparison of I_K in muscle and neurons

	Muscle	Neuron
Activation		
$V_{1/2}$	7 ± 0.9	−1 ± 1.2
z	2 ± 0.1	2 ± 0.1
Steady-state inactivation		
$V_{1/2}$	−27 ± 0.4	−30 ± 0.3
z	7 ± 0.9	4 ± 0.2
Time to 90% maximum current		
at +50 mV	15 ± 1.2	14 ± 1.1
at +10 mV	31 ± 7.2	20 ± 5.3

Properties of the I_K current in muscle were examined in *Shaker* mutant myotubes, while those in neurons were examined in wild type neurons which were subjected to a (500 msec) prepulse to −50 mV in order to inactivate the I_A (Shal) current present. $N = 7$ for muscle activation curves; $N = 6$ for neuron activation curves; $N = 5$ for muscle steady-state inactivation curves; $N = 9$ for neuron steady-state inactivation curves; $N = 6$ for all time to 90% maximum current measurements. Averages are shown with standard deviations as noted.

The intercross of heterozygous *Shab*-deficient flies produce three genotypes in a 1:2:1 ratio: one-quarter homozygous for the balancer chromosome, one-half heterozygous, and one-quarter homozygous *Shab* deficiency. Cells homozygous and heterozygous for a balancer chromosome will synthesize β-galactosidase and stain blue when treated with X-gal. Only embryos homozygous for the deficiency will not stain.

Electrophysiology

The patch-clamp technique was used to record whole cell currents (Hamill et al., 1981). Electrodes were pulled using borosilicate glass and had resistances of about 20 MΩ in solutions used (below). Electrodes were Sylgarded for single channel recordings. Although series resistance errors affect the exactness of the voltages reported (such as in conductance–voltage relations), they do not affect the assays for currents used in these mutant analyses. Measured series resistance, 27 MΩ on average for neurons and 16 MΩ for myotubes, was 90% compensated (except for mutant data in Figs. 3 and 4, which were uncompensated). Uncompensated series resistance remaining, assuming a true series resistance of twice that of the electrode resistance (Marty and Neher, 1983) is about 16 MΩ for neurons and 26 MΩ for myotubes. Whole cell recordings which were digitized at 0.097 msec/pt for voltage jumps 80 msec in duration, and 0.488 msec/pt for voltage jumps 480 msec in duration. Whole cell recordings were lowpass filtered through an 80 dB Bessel filter with a cutoff frequency of 2 kHz.

Data was acquired using an Axopatch 200A amplifier (Axon Instruments). Data acquisition software used was CCURRENT (Dr. Keith Baker; Indec, Sunnyvale, CA). Analysis software included CQUANT (Dr. Keith Baker), NFIT (University of Texas, Galveston, TX), QPRO (Borland International, Inc., Scotts Valley, CA), and SIGMAPLOT (Jandel Scientific, Corte Madera, CA).

Solutions (in mM): internal, 140 K-gluconate, 2 $MgCl_2$, 11 EGTA, 10 HEPES; external, 140 Na-gluconate, 2 K-gluconate, 6 Mg-gluconate$_2$, 5 HEPES, 5 trehalose, 20–35 sucrose (to adjust osmolarity), and 100 nM tetrodotoxin. Some recordings were performed with aspartate as the major anion instead of gluconate; both anions gave similar results.

Southern analysis

Shab was confirmed to fall within the deletion, Df(3L)HR-370+Dp(3;3)pdp7 by Southern analysis (Fig. 1). Genomic DNA from wild type (OR-R) and heterozygous Df(3L)HR370 + Dp(3;3)pdp7/TM3 ftz-lacZ flies was isolated by homogenization of whole adult flies in 0.1% SDS in TE, followed by phenol/chloroform extractions and DNA precipitation; 3 μg of DNA was digested with restriction enzyme Eco RI, SacI, or SalI, and then run on a 1% agarose gel (EcoRI or SacI) or 0.7% agarose gel (SalI). DNA fragments were transferred to nitrocellulose and probed at high stringency with a fragment of the *Shab11* cDNA, encompassing S1 to S6 core region of the channel. Quantitation of DNA in each band was performed using a PhosphoImager (Molecular Dynamics, Inc.) and associated software called IMAGEQUANT. For the heterozygous deletion stock it was expected that restriction fragments labeled with a *Shab* specific probe would be half the intensity of control bands for genes present in two doses. Hence, to normalize quantitation for the amount of DNA in each lane, blots were later probed with the cDNA of the *Shal* gene (for cuts with EcoRI or SacI), or probed with the cDNA, 3-1AL, of the *Drosophila* Na^+ channel gene (for cuts with SalI). In the DNA containing the heterozygous deficiency of *Shab*, the labeled band indicating the single copy of *Shab* was quantified and determined to be half the intensity of a diploid control band (Fig. 1).

Results

Currents in embryonic muscle

Muscle from all stages of *Drosophila*, including pupal flight muscle, larval body wall muscle, and embryonic myotubes contain two separable voltage-dependent K^+ current components, I_A and I_K (Salkoff and Wyman, 1981; Salkoff, 1983; Wu and Haugland, 1985; Zagotta et al., 1988; Broadie and Bate, 1993). Muscle cells examined in this study are dissociated from 7 hr embryos, and allowed to develop in culture for up to 48 hr. Since the development from embryo to first instar larvae is normally complete in 24 hr, the cells used in this study probably correspond to the body wall muscles of the larvae.

I_A in pupal, larval, and embryonic muscle has been genetically identified as *Shaker* (Salkoff and Wyman, 1981; Salkoff, 1983; Wu and Haugland, 1985; Solc et al., 1987; Zagotta et al., 1988; Broadie and Bate, 1993), as well as I_A in *Drosophila* photoreceptors (Hardie et al., 1991). However, I_K was not identified in these previous studies. Under voltage clamp, both I_A and I_K current components are evident in wild type myotubes subjected to voltage jumps to depolarized potentials. Figure 2*A* shows wild type whole cell currents which quickly reach a peak current that partially, but rapidly, inactivates to a steady-state current. The partially inactivating component corresponds to I_A, while the steady-state current corresponds to I_K. Figure 2*B* shows removal of this component by a *Shaker* mutation. Figure 2*A* (bottom) shows conductance–voltage relations of the two components, peak current, corresponding primarily to I_A, and the steady-state current, corresponding to I_K. Conductance–voltage relations are similar with respect to both range and slope. Both I_K and I_A activate around −30 to −20 mV and reach half-activation at 14 mV (I_A) and 12 mV (I_K) ($N = 5$).

I_K *in* Shaker *myotubes*

I_K is best separated from I_A using *Shaker* mutants which remove all the transient current from muscle. In Figure 2*B* where I_A has been removed by a mutation of *Shaker*, I_K can be observed without any contamination from I_A. I_K in *Shaker* mutant myotubes activates slowly around −30 to −20 mV, with half-activation at about +7 mV ($N = 7$, Fig. 2*B*, bottom). Figure 2*B*, (bottom) shows conductance–voltage relations of I_K in *Shaker* mutant myotubes compared with that of I_K measured as the steady-state current in wild type myotubes. The similarity of the data shows that I_K is unaltered by the *Shaker* mutation. The steady-state inactivation properties of I_K in *Shaker* mutant cells suggested that I_K consists primarily of one DR component, because the data is fit well by a single Boltzmann (data not shown). However, frequently accompanying the DR, there is a much smaller noninactivating current component present in some cells. In the steady-state voltage paradigm used for these experiments, cells were prepulsed to different membrane potentials, and then stepped to the same test potential (+50 mV). Steady-state inactivation curves are plotted as (normalized) peak current versus prepulse potential. In such experiments, data for the DR com-

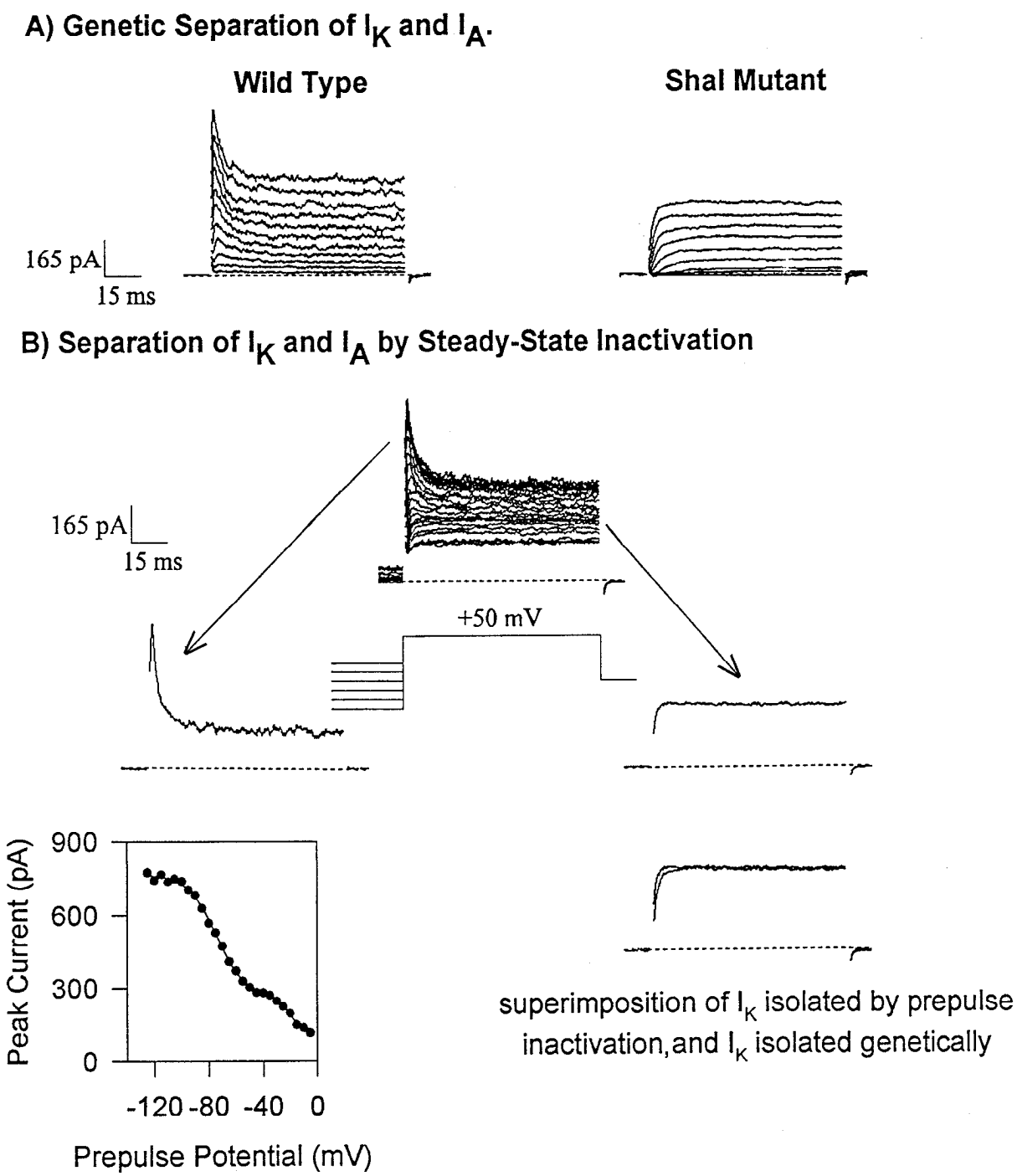

Figure 3. I_K separation from I_A in neurons: *A*, by genetics; *B*, by a physiological technique. *A*, Voltage dependent K^+ currents from a representative wild type (*left*) and *Shal*-deficient (*right*) neuron. Wild type currents consist of a transient Shal current as well as DR currents. In contrast, the *Shal* deficiency eliminates the transient Shal current and thus, reveals the DR currents present (*right*). *B*, Current traces in response to a steady-state inactivation voltage paradigm are shown from a representative wild type neuron (*middle*). Membrane was held (500 msec) at prepulse potentials from −125 to −5 mV, in 5 mV increments, before stepping to a test potential of +50 mV. Bottom (*left*), peak current is plotted versus prepulse potential. Data points were fit with a double Boltzmann function ($I = I_{max}/(1 + \exp((V - V_{½})/k))$, indicating two separable components with distinct voltage-operating ranges. I_K is represented by the component with the more depolarized steady-state inactivation curve (I_{max} = 147 pA, $V_{½}$ = −19.5 mV, z = 5.07 mV/*e*-fold shift); the transient Shal current is represented by the component with the more hyperpolarized steady-state inactivation curve (I_{max} = 510.8 pA, $V_{½}$ = −74.2 mV, z = 10.6 mV/*e*-fold shift). Thus, the Shal current can be inactivated with a prepulse to −45 mV, leaving primarily the DR current, I_K (*top, right*). *Bottom* (*right*), I_K isolated genetically in *A* is superimposed on I_K isolated in *B* with a prepulse to −45 mV. I_K isolated by these independent methods are indistinguishable.

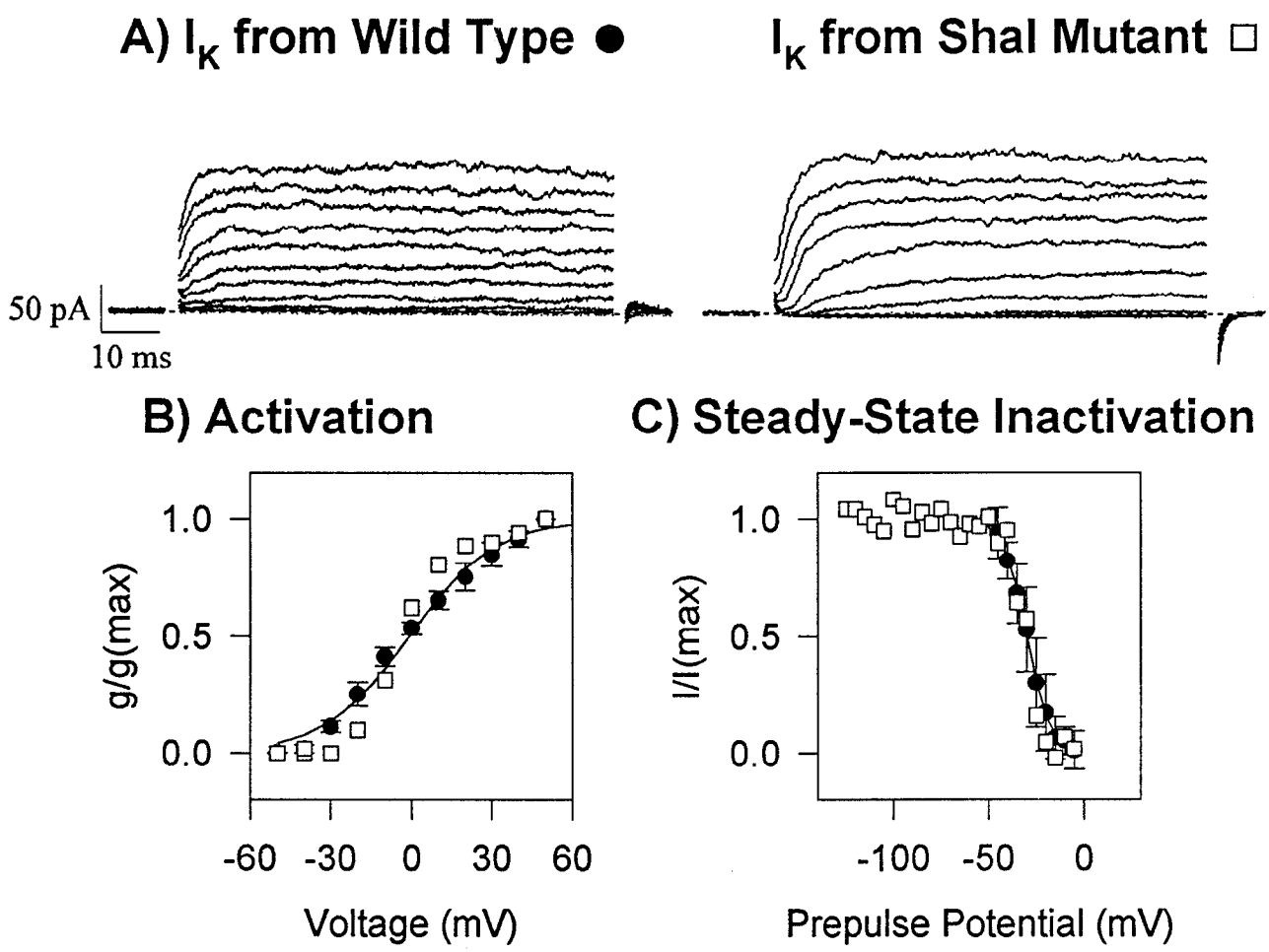

Figure 4. I_K in *Shal* mutant and wild type neurons. *A*, I_K remaining in a representative *Shal*-deficient neuron (*left*) and I_K isolated in a wild type neuron with a (500 msec) prepulse to −50 mV (*right*). Whole cell current traces are in response to 80 msec voltage jumps to potentials between −50 mV and +50 mV, in 10 mV increments. *B*, Conductance–voltage relations, and *C*, steady-state inactivation curves of I_K compared from wild type (●) and *Shal*-deficient neurons (□) (N = 6 for activation; N = 9 for steady-state inactivation). I_K is isolated from wild type by prepulse inactivation, and genetically in the *Shal*-deficient neuron. Conductance–voltage relations and steady-state inactivation curves are calculated, plotted, and fit with Boltzmann equation as described in Figure 2. Standard deviations are as indicated. Best fit parameters for conductance–voltage curve are $V_{½}$ = −1.05 ± 1/20 mV, z = 1.59 ± 0.10 mV/e-fold shift. Best fit parameters for steady-state inactivation curve are $V_{½}$ = −29.81 ± 0.28 mV, z = 4.19 ± 0.16 mV/e-fold shift.

ponent are fit with a single Boltzmann function (Fig. 2*B*), suggesting a single current component half-inactivated with a prepulse to −27 mV (N = 5) (Table 1).

Shab *encodes the major delayed rectifier current in embryonic myotubes*

We suspected that this major DR component is encoded by the *Shab* gene because it resembles Shab currents heterologously expressed in *Xenopus* oocytes. I_K currents, like Shab currents, are slowly inactivating and inactivate with prepulses to depolarized potentials (Wei et al., 1990). We investigated this hypothesis by performing mutant analysis, using a genetic mutation of the *Shab* gene (see Materials and Methods). To determine if *Shab* encodes I_K in embryonic myotubes, we characterized the component removed by the *Shab* mutation by comparing the DR currents in wild type myotubes to those in *Shab*-deficient myotubes. We first compared the amplitudes of I_K in wild type cells with I_K in the mutant. I_K was measured as the steady-state current remaining after I_A was inactivated (80 msec). We found that the amplitude of I_K in wild type cells was, on average, 69% (N = 13) of the peak I_A amplitude (Fig. 2*A*), but in *Shab*-deficient myotubes I_K was only 16% (N = 13) of the peak I_A amplitude (Fig. 2*C*). Thus, the *Shab* deficiency eliminates 77% of the DR current in wild type myotubes. Figure 2*C* shows the Shaker current remaining in *Shab*-deficient myotubes. Activation kinetics of the Shaker current are virtually identical to that of the peak I_A current in wild type myotubes (Fig. 2*C*, bottom), showing that I_A is unaltered by the *Shab* mutation. Figure 2*D* verifies that the *Shab* or *Shaker* mutations eliminate only a selective component without altering the remaining currents in the cell. Thus, the linear addition of currents remaining in a *Shaker* mutant plus the currents remaining in a *Shab* mutant, reconstitutes the wild type whole cell current.

Delayed rectifier currents in neurons

Drosophila neurons, like muscle, contain both a transient A-type K^+ current component, I_A, as well as a DR component, I_K. In order to isolate and analyze I_K in neurons, two independent techniques were used, a genetic technique which is unique to *Drosophila*, and a more conventional physiological approach. The

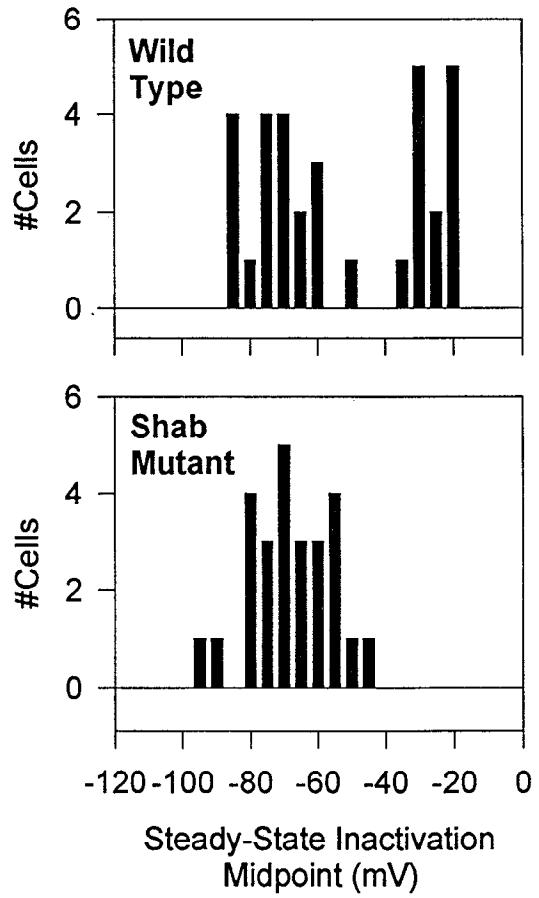

Figure 5. Distribution of steady-state inactivation midpoints in wild type and *Shab* mutant neurons. Wild Type, histogram of steady-state inactivation midpoints ($V_{½}$) of current components in wild type neurons. Two populations of current components are evident in the wild type neurons (N = 18): one population (*group of bars to the left*) has a more hyperpolarized steady-state inactivation midpoint, averaging −75 ± 8.7 mV, and always corresponds to the transient Shal current component; another population (*group of bars on the right*) has a more depolarized steady-state inactivation midpoint, averaging −29 ± 8.0 mV, and always corresponds to a slowly inactivating current component, I_K. 78% of wild type neurons contained these two separable components; 22% contained only the transient Shal current. *Shab-Mutant,* Histogram of steady-state inactivation midpoints ($V_{½}$) of current components in *Shab*-deficient neurons. In contrast to the wild type neuron histogram, only one component remains in *Shab*-deficient neurons (N = 26). The population remaining in *Shab*-deficient neurons corresponds to the transient Shal current components, having a more hyperpolarized steady-state inactivation midpoint of −70.52 ± 11.40 mV. The population of current components eliminated by the *Shab* deficiency corresponds to the slowly inactivating current I_K with a more depolarized steady-state inactivation midpoint. Steady-state inactivation midpoints ($V_{½}$) of current components are determined from fits of single and double Boltzmann equations ($I = I_{max}/(1 + \exp((V - V_{½})/k))$) to data points representing peak current versus prepulse potentials. Voltage paradigm used and representative fits to data points are shown and described in Figures 6 and 7.

genetic technique employs a genetic deletion of *Shal,* a gene which encodes most of the A-currents in neurons (Tsunoda and Salkoff, 1995). *Shal* mutant neurons typically contained only the slowly inactivating DR current, I_K. Figure 3*A* shows current traces of I_K from a representative *Shal* mutant neuron in response to voltage jumps to potentials between −50 mV and +50 mV, in 10 mV increments. I_K in these cells activates around −20 mV and reaches half-activation around −3 mV (Fig. 4*B,C*). Current rise times are voltage-dependent (Table 1).

Although the genetic technique described above is the most elegant technique for removing the transient current from a cell, an independent, more conventional physiological technique could also be employed for isolating I_K in wild type neurons. This method is based on the differences in steady-state inactivation properties between I_A and I_K in neurons, which is not present in muscles. I_A in neurons (*Shal*) has a much more hyperpolarized steady-state inactivation curve than I_K and thus is eliminated by more negative prepulses than I_K. On average, I_A was half-inactivated with a prepulse to −75 mV, while I_K was half-inactivated with a prepulse to −29 mV. Thus, when peak current in wild type cells is plotted versus prepulse potential, data points could usually be fit with a double Boltzmann equation, representing the two functionally independent components, I_A and I_K (Fig. 3*B*). We found that 100% of wild type neurons (N = 18) contained I_A, while 78% contained both I_A and I_K. When present in the same cell, the two components are sufficiently separated by their distinct voltage-operating ranges such that a prepulse to around −50 mV completely inactivates all transient Shal currents, leaving I_K fully active (Fig. 3*B*). Figure 3*B* (top, right) shows I_K isolated in a wild type cell in this manner.

When comparing I_K in neurons isolated by the two techniques, we find that I_K isolated in *Shal* mutant neurons appears virtually identical to I_K isolated in wild type neurons by prepulse inactivation. Thus, the genetic removal of Shal has no effect whatsoever on I_K. Figure 3*B* (bottom, right) shows I_K isolated by each technique, in two different cells, superimposed on one another. Figure 4 compares I_K isolated by both techniques. I_K in both wild type and *Shal* mutant neurons activates between −30 and −20 mV. The activation and steady-state inactivation of I_K isolated by both techniques shows a similar voltage dependence (Fig. 4*B,C*). In wild type neurons, I_K reaches half-activation at −1 mV (N = 6), and is half-inactivated by a prepulse to −30 mV (N = 9). Activation and steady-state inactivation data points from I_K in a representative Shal mutant neuron are shown superimposed on the same graphs as for I_K in wild type neurons to show the great similarity between I_K currents isolated by the two techniques.

Shab *encodes* I_K *in embryonic neurons*

Having analyzed the I_K component in neurons, we next compared its properties to I_K in muscle which we showed earlier in this study to be encoded by *Shab.* We found that, indeed, I_K in neurons is very similar to I_K in muscle, with respect to all properties (Table 1). Thus, we suspected that the neuronal I_K component was also encoded by *Shab.* This hypothesis is consistent with a previous study in *Drosophila* neurons which suggested that *Shab* encodes this DR current, a conclusion based on the similarity of single channel currents found in neurons to Shab single channel currents expressed in *Xenopus* oocytes (Tsunoda and Salkoff, 1995).

To determine whether *Shab* encodes I_K in neurons, we compared currents in wild type neurons to those in *Shab* mutant neurons. As in muscle, the *Shab* deficiency removed I_K. We found that *none* of the *Shab*-deficient neurons (N = 26) contained the current component corresponding to I_K, as described in the previous section. *Shab*-deficient neurons had a steady-state inactivation curve that was always fit with a single Boltzmann component characteristic of that of the Shal current, while

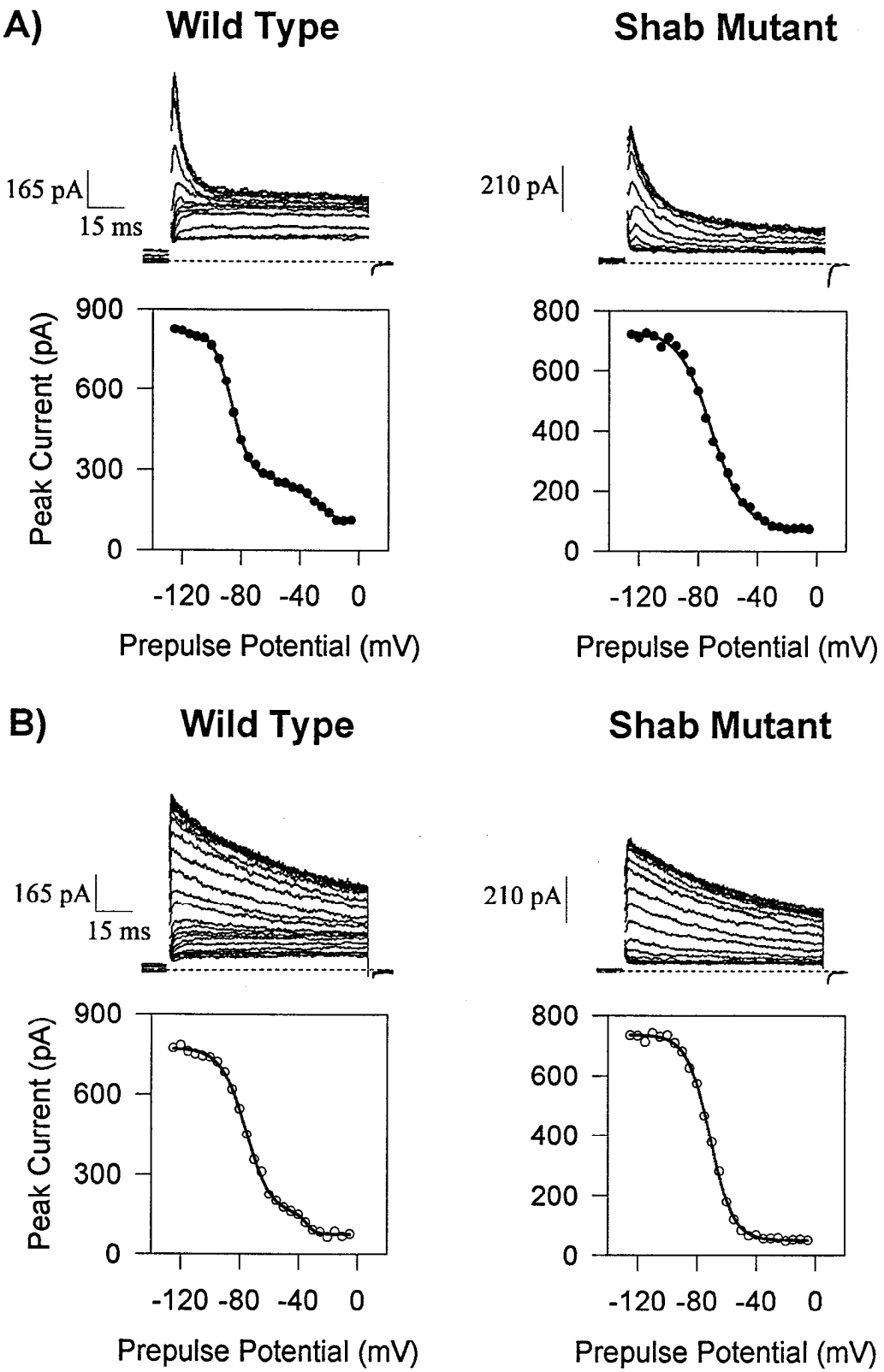

Figure 6. Selective removal of I_K in neurons by *Shab* mutation. *A: Left* (*Wild Type*), fast I_A, Current traces in response to a steady-state inactivation voltage paradigm from a representative wild type neuron containing a Shal current with a very fast inactivation rate. *Right* (*Shab-Mutant*), fast I_A, Current traces in response to the same steady-state inactivation voltage paradigm from a representative *Shab*-deficient neuron containing a similar fast transient Shal current. Current traces shown were in response to a test jump were to +50 mV. Each test jump was preceded by a 500 msec prepulse to potentials between −125 and −5 mV, in 5 mV increments. Traces are shown in response to prepulses every 10 mV. Below current traces, peak current is plotted versus prepulse potential at 5 mV intervals. Data points are best fit with either one or the sum of two Boltzmann equations: $I = I_{max}/(1 + \exp((V - V_{½})/k)) + \text{baseline}$, where $V_{½}$ is the potential at which current is half-inactivated, k is RT/zF, baseline is offset current due to "leak" current and noninactivating (Shaw) currents present. Wild type currents shown were best fit with the sum of two Boltzmann equations as shown. The transient Shal current component is fit with the parameters, I_{max} = 544.98 pA, $V_{½}$ = −86.2 mV, z = 6.19 mV/e-fold shift;

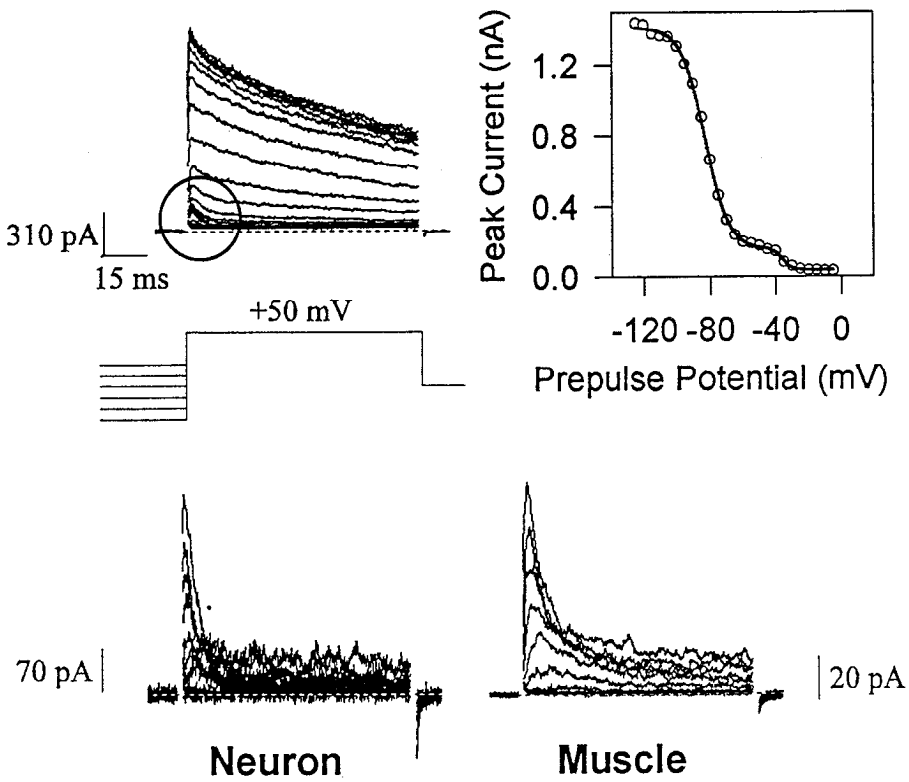

Figure 7. Shaker-like current revealed in *Shab* mutant neurons. *Top,* Current traces in response to a steady-state inactivation voltage paradigm from a *Shab*-deficient neuron (*left*). Membrane was prepulsed (1 sec) to potentials between −125 and −5 mV, in 5 mV increments, prior to stepping to a test potential of +50 mV for 80 msec. Peak current is plotted versus prepulse potential (*right*). Unlike most Shab-deficient neurons, data points are fit with the sum of two Boltzmann equations, $I = (I_{max}/(1 + \exp((V - V_{½})/k))) + (I_{max2}/1 + \exp((V - V_{½}/k_2)))$ as previously described, indicating two separable components. Unlike wild type neurons, both current components (*left*) are transient A-type currents. The larger transient component has a hyperpolarized steady-state inactivation curve characteristic of Shal currents in these neurons. Best fit parameters for this component are I_{max} = 1.3 nA, $V_{½}$ = −82.7 mV, z = 7.00 mV/*e*-fold shift. The smaller transient component has a more depolarized steady-state inactivation curve characteristic of Shaker currents seen in muscle. Best fit parameters for this component are I_{max} = 100 pA, $V_{½}$ = −35.5 mV, z = 2.98 mV/*e*-fold shift. *Bottom,* Whole cell Shaker-like current in neurons (*left*) and Shaker current in a representative *Shab*-deficient myotube (*right*). Neuronal Shaker-like current shown is from the same cell as above. Membrane was prepulsed to −50 mV to inactivate the larger transient Shal current and voltage jumps were taken to potentials between −50 and +40 mV, in 10 mV increments. Similar voltage jumps were taken from a holding potential of −90 mV for the *Shab*-deficient myotube.

most wild type neurons (78%) had a steady-state inactivation curve that was fit with the sum of two Boltzmann functions, one corresponding to Shal, and one corresponding to Shab, as seen in muscles. Figure 5 shows steady-state inactivation midpoints from wild type and *Shab*-deficient neurons plotted in separate histograms. Two populations of inactivation midpoints appears to be present in wild type neurons: one hyperpolarized, averaging −75 mV, that corresponds to I_A (Shal), and a second depolarized, averaging −29 mV, that corresponds to I_K. In contrast, only the more hyperpolarized population of inactivation midpoints, averaging −71 mV, appears to be present in *Shab*-deficient neurons. Thus, the *Shab* deficiency eliminates the currents with a more depolarized steady-state inactivation midpoint, I_K, while leaving the component corresponding to the transient Shal current unaltered.

Since Shal currents have been shown to have different rates of inactivation (Tsunoda and Salkoff, 1995), we wanted to confirm that the *Shab* mutation eliminates I_K regardless of the kinetics of the Shal current present. Figure 6 shows representative wild type and *Shab*-deficient neurons containing fast (very rapidly inactivating) and slow (less rapidly inactivating) Shal currents. Steady-state inactivation analysis reveals that I_K is eliminated with the *Shab* deficiency, regardless of the kinetics of the Shal current (I_A). Thus, we conclude that Shab encodes I_K in

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the slowly inactivating current component I_K is fit with the parameters, I_{max} = 180.48 pA, $V_{½}$ = −30.0 mV, z = 9.31 mV/*e*-fold shift, baseline = 91.69 pA. In contrast, the *Shab*-deficient neuron was fit with only a single Boltzmann equation with the following parameters for the transient Shal current: I_{max} = 655.55 pA, $V_{½}$ = −71.0 mV, z = 10.94 mV/*e*-fold shift, baseline = 74.75 pA. *B: Left* (*Wild Type*), slow I_A, Current traces in response the same a steady-state inactivation voltage paradigm from a representative wild type neuron containing a Shal current with a slower inactivation rate. *Right* (*Shab-Mutant*), slow I_A, Current traces in response the same steady-state inactivation voltage paradigm from a representative *Shab*-deficient neuron containing a Shal current similar to the one of the wild type neuron shown. Steady-state inactivation curve from the wild type neuron is best fit with a double Boltzmann equation while the steady-state inactivation curve from the *Shab*-deficient neuron is best fit with a single Boltzmann equation. For the wild type neuron, the transient Shal current component, best fit parameters are I_{max} = 624.59 pA, $V_{½}$ = −75.4 mV, z = 8.50 mV/*e*-fold shift; the slowly inactivating current component I_K has best fit parameters of I_{max} = 72.64 pA, $V_{½}$ = −34.1 mV, k = 3.08 mV/*e*-fold shift, baseline = 75.68 pA. For the *Shab*-deficient neuron, the transient Shal current component is best fit the parameters I_{max} = 682.47 pA, $V_{½}$ = −71.1 mV, z = 7.77 mV/*e*-fold shift, baseline = 51.71 pA.

neurons as well as in muscle, and that the *Shab* encoded component is completely independent of the transient component in the cell.

Shaker *revealed in* Shab *mutant neurons*

Although the predominant A-current in embryonic neurons is encoded by *Shal,* previous work has suggested that *Shaker* might also encode an A-current in a small percentage of pupal neurons (Baker and Salkoff, 1990). However, it was not apparent that any wild type neurons in our preparation derived from the late embryo contained two separable A-currents. Nevertheless, I_K was eliminated with a genetic deficiency of *Shab, two* transient currents became apparent in a small percentage of cells. In most *Shab*-deficient neurons, only the transient Shal current, with its characteristically hyperpolarized steady-state inactivation curve, was present, but in 11% of the *Shab*-deficient neurons ($N = 26$), two A-currents were observed. These two components had distinct voltage-operating ranges; steady-state inactivation curves were fit with a double Boltzmann function, corresponding to one transient current with a hyperpolarized steady-state inactivation curve, and a second transient current with a depolarized steady-state inactivation curve (Fig. 7). The larger of the two components always had a hyperpolarized steady-state inactivation curve, characteristic of Shal currents. The smaller component had a steady-state inactivation midpoint of about −35 mV, which was unlike Shal (Tsunoda and Salkoff, 1995), but quite similar to Shaker (Solc et al., 1987; Baker and Salkoff, 1990).

The reason that this second component was difficult to detect became apparent when it was realized that it had a steady-state inactivation curve very similar to that of the Shab DR current (Figs. 4, 5). Thus, the small A-current was masked by the larger DR Shab current in experiments designed to separate components based on differences in steady-state inactivation properties. Thus, its presence could only be detected when the Shab current was eliminated.

The steady-state inactivation midpoint of this small A-current (−35 mV) is similar to that of Shaker currents in embryonic myotubes, which have a $V_{½}$ of −30 mV (Solc et al., 1987). To isolate and examine this A-current in neurons, we inactivated the large Shal current with a prepulse to −50 mV and took voltage jumps to potentials between −50 and +40 mV, in 10 mV increments. This A-current was not activated until potentials of −20 mV and above, another property similar to Shaker currents in other cell types (Salkoff, 1983b; Wu and Haugland, 1985; Solc et al., 1987; Broadie and Bate, 1993). This is markedly unlike Shal currents which activate between −50 and −40 mV (Tsunoda and Salkoff, 1995). This small A-current also had activation kinetics and inactivation rates that were similarly voltage-dependent to Shaker currents in other cell types (data not shown). Figure 7 compares this Shaker-like current to Shaker currents observed in *Shab*-deficient myotubes. Because this Shaker-like current is present in such a small percentage of *Shab*-deficient neurons, the verification by mutant analysis that this current was encoded by the *Shaker* gene was not practical. However, because of its great similarity to Shaker currents observed in muscle cells, we suggest the likelihood that it is.

Shaw *encodes a small steady-state current in both neurons and muscle*

In neurons, we previously found that *Shaw* encodes a noninactivating K^+ channel with distinctive properties, which include an unusually low voltage sensitivity, a short mean open time, and a large conductance (Tsunoda and Salkoff, 1995). These single channel properties give rise to a whole cell current which behaves much like a small, leak-type current in neurons. Since *Shab* appears to encode most, but not quite all, of the DR current in myotubes, we suspected that *Shaw* might be responsible for the remaining steady-state current in myotubes, as it is in neurons.

We found that myotubes do indeed contain a channel that is very similar in conductance and behavior to the Shaw channels identified in neurons. Figure 8 shows representative records of Shaw channels in neurons compared to the Shaw-like channels observed in myotubes. Shaw-like channels in myotubes, like neuronal Shaw channels, also appear to have brief open times and a low voltage sensitivity. Current–voltage relations, revealed by single channel activity during a voltage ramp (Fig. 8), predict a similar large conductance of Shaw channels in myotubes as well as neurons: 62 pS in myotubes, and 48 pS in neurons. Thus, *Shaw* appears to be responsible for a small steady-state current that remains in *Shab*-deficient myotubes.

Discussion

The same gene, Shab, *encodes the major delayed rectifier current in* Drosophila *neurons and muscle*

Historically, voltage-dependent K^+ currents have been generally classified as either a transient A-type current or a delayed rectifier (DR) type current (review by Rudy, 1988: Hille, 1992). However, although DR currents in both neurons and muscle cells have been described, the genetic identity of these currents is rarely known unambiguously. Previous studies in *Drosophila* have identified I_K and I_A current components, but their genetic identity was not determined (Byerly and Leung, 1988; Saito and Wu, 1993). Here we show by mutant analysis that the major DR current in both *Drosophila* neurons and muscle is encoded by the same gene, *Shab,* and that *Shab* appears to be virtually the exclusive gene encoding DR currents. It is possible that mammalian *Shab* genes play a similarly important role. Although numerous mammalian *Shaker* homologs express DR-type currents in the *Xenopus* oocyte expression system, there is little evidence that they actually have similar kinetics in mammalian neurons.

Genetic and functional independence of the major delayed rectifier current in Drosophila

We have found that although multiple K^+ channel genes are expressed at the same time, and in the same cells of *Drosophila* neurons and muscles, K^+ currents, such as I_K and I_A, appear to be genetically, as well as functionally, independent of one another. This concept of independent K^+ current systems was originally suggested in a previous report (Covarrubias et al., 1991). Subunits from all the subfamilies of cloned K^+ channel genes (*Shaker, Shal, Shab, Shaw*) form homomultimers, but Covarrubias et al. showed that there is a "barrier" to the formation of heteromultimers from subunits encoded by members of different subfamilies. For instance, coexpression of *Shab* and *Shaker* in *Xenopus* oocytes results in two separate currents expressed by *Shab* and *Shaker* homomultimers, rather than a current carried by a heteromultimeric channel containing mixtures of Shab and Shaker subunits. Consistent with these results, we find that the major DR current in both neurons and muscle is encoded by a single gene, *Shab,* and that it is unaffected by the expression of other K^+ channel genes. These results should not be confused

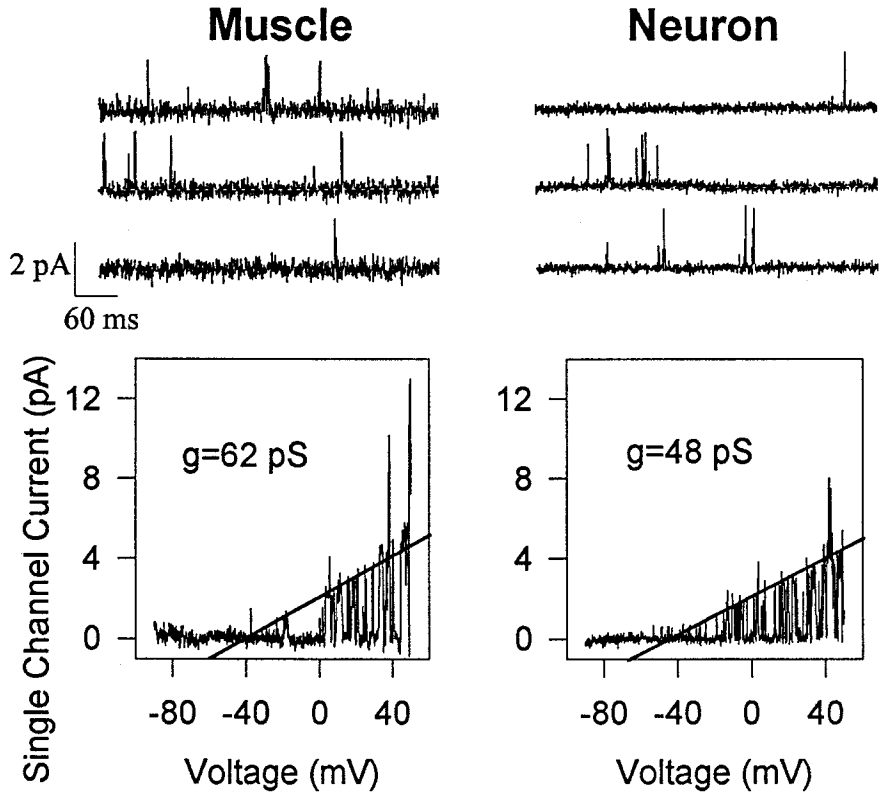

Figure 8. Shaw channels in neurons and muscle. *Top,* Representative traces of Shaw-like channels present in myotube (*left*) and identified Shaw channels in neurons (*right*) in outside-out patches. Traces are in response to voltage jumps to 0 mV from a holding potential of −60 mV. *Bottom,* Channel openings in response to a voltage ramp from −90 mV to +50 mV. A line drawn by eye through channel openings predicts a conductance of 48 pS for Shaw channels in neurons and 62 pS for the Shaw-like channels in myotubes. Data was digitized at 0.488 msec/pt and filtered at 2 kHz.

with the results of Sheng et al. (1993) and Wang et al. (1993), which showed that, in mammals, subunits encoded by members of the same subfamily do form heteromultimers.

Drosophila provides unique genetic tools with which to study the multiple K^+ currents present in *Drosophila* cells. The fact that a particular DR current is eliminated with a deficiency of *Shab* suggests that the *Shab* gene encodes the essential subunit of the channel that carries this current. Furthermore, the fact that deficiencies of other K^+ channel genes, such as *Shal* in neurons (Tsunoda and Salkoff, 1995) and *Shaker* in muscle (Salkoff and Wyman, 1981; Broadie and Bate, 1983; Salkoff, 1985; Wu and Haugland, 1985; Zagotta et al., 1988), do not affect this same DR current suggests that none of these other K^+ channel genes contribute subunits to the channel that carries this current. Thus, the diversity of voltage-dependent K^+ currents in *Drosophila* neurons and muscle appears to be largely explained by the combination of currents which are genetically and functionally independent.

Shaw *encodes a "leak"-like current in neurons and muscle*

Shaw encodes a current with similar distinctive properties in both neurons and muscle. In previous studies we showed that Shaw channels in neurons have a very low open probability and an unusually low voltage sensitivity (Baker, 1992; Tsunoda and Salkoff, 1995), and we suggested that they contribute a macroscopic current that probably functions much like a "leak" current in neurons. This small whole cell current component is sometimes detectable in *Shab*-deficient neurons when the predominant Shal current is inactivated. In the present study, we find that channels very similar to Shaw channels in neurons, are also present in muscle. Thus, while *Shab* encodes the major DR current in both neurons and muscle, *Shaw* appears to encode a minor leak-type current in both cell types. Since Northern analysis suggests that Shaw has only one splice form which is expressed throughout development and into adulthood (Tsunoda and Salkoff, 1995), Shaw may play the role of a leak-type channel in multiple cell types throughout development.

A genetically defined system

Many K^+ channel genes from *Drosophila* and mammals have been characterized in heterologous expression systems. However, little is known about the currents which are expressed by these genes in their normal cells. Northern analysis and even PCR amplification of channel genes from identified tissues, or even single cells, can only provide a correlation of gene expression with electrophysiology, rather than a certainty of which gene expresses which current. In this study and in recent work (Tsunoda and Salkoff, 1995), we use unique genetic tools only available in *Drosophila* to examine native K^+ currents in wild type cells and cells which contain genetic deficiencies for each of the known K^+ channel genes cloned in *Drosophila.* In this way, we can unambiguously identify the currents of the cloned K^+ channel genes in their native cells.

Shaker currents were the first ion currents to be genetically characterized. *Shaker* was shown to encode the A-type current in muscle at developmental stages from the embryo to the adult (Salkoff and Wyman, 1981; Broadie and Bate, 1983; Salkoff, 1983; Salkoff, 1985; Wu and Haugland, 1985; Solc et al., 1987; Zagotta et al., 1988). More recently, we showed that the A-type currents in the soma of neurons are encoded by the *Shal* gene (Tsunoda and Salkoff, 1995). Now, in our current study, we show that, in both neurons and muscle, the major delayed rectifier current is encoded by *Shab,* while a minor, noninactivating current is probably encoded by *Shaw.* Thus, all of the major whole cell current components can be genetically assigned to each of the cloned genes, *Shaker, Shal, Shab,* and *Shaw,* perhaps suggesting that the "set" of genes encoding voltage-dependent K^+ currents is virtually complete.

At the single channel level, virtually all currents, in both neurons and muscle, can now be assigned to each of the cloned genes from *Drosophila.* In neurons, we previously showed that only three types of single channel currents are apparent at the cell soma of embryonic neurons. Two of these single channel currents were shown by mutant analysis to be encoded by *Shal* and *Shaw* (Tsunoda and Salkoff, 1995). A third single channel current, similar to single *Drosophila* Shab channels heterologously expressed in *Xenopus* oocytes (Tsunoda and Salkoff, 1995), was assumed to underlie the slowly inactivating DR current, I_K. In this study, we show that I_K is indeed encoded by *Shab.* Thus, all the single channel currents in embryonic neurons can now be accounted for. In a previous study, similar single K^+ channel currents were observed in larval neurons (Solc and Aldrich, 1988). Although these currents were not identified genetically, their similarity to the identified channels in embryonic neurons make it likely that they are expressed by *Shal, Shaw,* and *Shab.*

In muscle, most single channel currents can also be accounted for by the cloned genes. Of the four types of single channel currents that have been described by Zagotta et al. (1988), three are voltage dependent. The first, A_1, is rapidly inactivating and was shown to be encoded by *Shaker* (Zagotta et al., 1988). The second, called the K_D channel, has properties of a delayed rectifier current, I_K, which we believe underlies the whole cell current shown in our study to be encoded by *Shab.* In addition, consistent with our study, K_D channels were observed in both neurons and muscle (Zagotta et al., 1988; Solc and Aldrich, 1990), just as we find that whole cell Shab currents are present in both neurons and muscle. The third voltage-dependent K^+ channel described by Zagotta et al. (1988) is the K_0 channel, which we suggest is encoded by *Shaw,* based on its similarity to Shaw channels previously identified in neurons (Tsunoda and Salkoff, 1995). From *Drosophila,* we now have a unique culture system in which to study native ion channels in a system that is almost completely genetically defined.

Voltage-dependent K^+ channels in *Drosophila* have now been studied at multiple levels, the results of which all contribute to a consistent picture. The delayed rectifier currents in neurons and muscle are encoded by the only two cloned K^+ channel genes in *Drosophila* which express currents with delayed rectifier type properties in the *Xenopus* oocyte expression system, *Shab* and *Shaw.* The transient A-type currents observed in *Drosophila* neurons and muscle are encoded by *Shal* and *Shaker,* respectively, the only two cloned *Drosophila* genes which express A-type currents in *Xenopus* oocytes. Each gene appears to express a current which is independent from all the others. And finally, all of the voltage-dependent K^+ currents present in *Drosophila* neurons and muscle can be accounted for by these four genes alone.

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