Expression of Ion Channels and Mutational Effects in Giant *Drosophila* Neurons Differentiated from Cell Division–arrested Embryonic Neuroblasts

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A culture system of “giant” *Drosophila* neurons derived from cytokinesis-arrested embryonic neuroblasts was developed to overcome the technical difficulties usually encountered in studying small *Drosophila* neurons. Cytochalasin B–treated neuroblasts differentiated into giant multinucleated cells that displayed neuronal morphology and neuron-specific markers (Wu et al., 1990). Here, we report that these giant neurons express different excitability patterns and membrane channels similar to those reported in excitable tissues of *Drosophila*. Individual neurons exhibited distinct all-or-none or graded voltage responses upon current injection. Both current- and voltage-clamp recordings could be performed on the same neuron because of the large cell size, thus making it possible to elucidate the functional role of the individual types of channels. By using pharmacological agents and ion substitution, the following currents were identified in these giant neurons: inward Na^+ and Ca^{2+} currents and outward voltage-activated (the A-type and delayed rectifier) and Ca^{2+} -activated K^+ currents. In addition, we found a tetrodotoxin (TTX)-sensitive, Na^+ -dependent outward K^+ current and a persistent component of an inward Na^+ current, which have not been reported in *Drosophila* previously. This culture system can be used to analyze the mutational perturbations in ion channels and the resultant alterations in membrane excitability. Neurons from the mutant *slowpoke* (*slo*), which is known to lack a component of the Ca^{2+} -activated K^+ currents in muscles, exhibited prolonged action potentials associated with defects in the Ca^{2+} -activated K^+ current. This abnormality appeared to be more severe in the neurites than in the soma.

A growing number of genes important to the nervous system have been studied in *Drosophila melanogaster* (Hall, 1982). Genetic manipulation provides useful tools to analyze physiological functions of ion channels and to identify the genes important in controlling membrane electrical activities. However, our knowledge of mutational effects on membrane excitability in *Drosophila* is mainly based on studies of muscle membrane

(Ganetzky and Wu, 1986; Salkoff and Tanouye, 1986; Papazian et al., 1988; Wu and Ganetzky, 1988). To understand the genetic control of ion channel function in neurons, it is necessary to compare membrane properties in normal and mutant neurons. Such information may provide insights into problems unique to the nervous system, for example, neuronal integration and plasticity.

Because of technical difficulties, relatively few intracellular recordings have been obtained from *Drosophila* neurons *in situ*. Intracellular action potentials were first recorded by Ikeda and Kaplan (1970) from the thoracic ganglion to show a defect in *Hyperkinetic* mutant flies. The effects of *Shaker* mutations on action potentials in the cervical giant fibers have also been characterized (Tanouye et al., 1981; Tanouye and Ferrus, 1985). Recently, dissociated neuronal culture systems have been used to allow patch-clamp analysis of embryonic (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988; O'Dowd et al., 1989), larval (Wu et al., 1983; Sun and Wu, 1985; Solc et al., 1987; Solc and Aldrich, 1988), and pupal (Baker and Salkoff, 1990) neurons. However, there has been no study correlating current- and voltage-clamp results to determine the functional role of the different currents, presumably because action potentials in these small cells are extremely susceptible to internal dialysis by the electrode solution.

In order to facilitate the study of *Drosophila* neuronal function, we have developed a “giant neuron” culture system (Wu et al., 1990). Embryonic neuroblasts treated with cytochalasin B differentiated without cytokinesis into multinucleated giant cells with well-developed neurites. The giant neurons developed membrane excitability and expressed insect neuron-specific molecules (Wu et al., 1990). The use of these neurons allowed us to study active membrane responses such as action potentials and to correlate them with underlying current components.

In this study, we characterized the giant neurons using both current- and voltage-clamp methods to investigate two questions: first, whether the various ion channels underlying neuronal excitability are differentiated in the absence of cell divisions, and, second, whether the expression of mutations perturbing ionic channels can be analyzed in this culture system.

Some of the results described here have appeared in abstract form (Saito and Wu, 1989, 1990).

Materials and Methods

Cell culture. The culture method was similar to that described previously (Seecof et al., 1971; Salvaterra et al., 1987; Wu et al., 1990). Briefly, dissociated cells were prepared from early gastrulas of *Drosophila melanogaster*. Embryos were collected on agar plates (1–2 hr) and incubated for 3.5 hr at 25°C. To remove the chorionic membrane, embryos were

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treated with clorox/90% ethanol (1:1 by vol) solution for 1 min and then washed with 90% ethanol. After wash, embryos were homogenized in modified Schneider medium supplemented with 20% fetal bovine serum (FBS), 200 ng/ml insulin, penicillin (50 U/ml), and streptomycin (50 μ g/ml). To remove debris, dissociated cells were washed in the same medium and then collected by centrifugation (2500 rpm for 2 min). After being washed twice, cells were resuspended in medium containing 2 μ g/ml cytochalasin B and plated on glass coverslips. Cultures were kept in humidified chambers at room temperature (20–23°C). In most cases, cultures were washed to remove cytochalasin B on the second day. However, membrane excitability in giant neurons did not show detectable changes with or without the removal of cytochalasin B. A great majority of cells (more than 80%) that grew in cytochalasin B-treated cultures showed neuronal morphology. Cytochalasin B and insulin were from Sigma. Modified Schneider medium, FBS, penicillin, and streptomycin were from Gibco (Grand Island, NY).

Solutions and chemicals. For recording, cells on the coverslip were transferred to the bath solution containing (in mM) 128 NaCl, 2 KCl, 4 MgCl₂, 1.8 CaCl₂, and 35.5 sucrose, buffered with 5 HEPES at pH 7.1–7.2. To study the effects of Co²⁺ and Ba²⁺ on Ca²⁺ channels, these ions were substituted for Ca²⁺ in the bath solution, unless otherwise noted. Na⁺-free or K⁺-free solutions contained equimolar Tris-Cl in place of NaCl or KCl. For current-clamp recording or for voltage-clamp recording of outward currents, patch pipettes were filled with solution containing (in mM) 144 KCl, 1 MgCl₂, 0.5 CaCl₂, and 5 EGTA, buffered with 10 HEPES at pH 7.1–7.2. In voltage-clamp studies of inward Na⁺ and Ca²⁺ currents, CsCl was used as a substitute for KCl to remove K⁺ currents. Tetraethylammonium chloride (TEA) was added to the bathing solution without adjusting an osmotic change. Tetrodotoxin (TTX) and 4-aminopyridine (4-AP) were obtained from Sigma (St. Louis, MO). TEA was from Kodak (Rochester, NY).

Whole-cell recording and data analysis. Patch pipettes were made from glass capillaries (75- μ l micropipettes, VWR, Chicago, IL) using a micropipette puller (model PP-83, Narishige, Tokyo, Japan) and fire polished with a microforge (model MF-83, Narishige). They were coated with Sylgard (184 silicon elastomer kit, Dow Corning, Midland, MI) to reduce stray capacitance. The tip opening of the patch pipette was about 1.2 μ m, giving a resistance between 3 and 5 M Ω when filled with the solutions. Whole-cell recordings were made mainly from neurons 9–12 μ m in diameter using an Axon patch clamp (Axiopatch 2B, Axon Instruments, Foster City, CA). Junction potentials were nulled just prior to gigohm seal formation. Generation of current and voltage pulse paradigms and data collection were performed by using an IBM XT computer with the pCLAMP software program (version 5.03, Axon) and A/D and D/A converters (Labmaster, Axon). Unless otherwise noted, the membrane potential (V_m) was maintained at -55 ± 4 mV in the current-clamp studies and held at -80 mV in the voltage-clamp studies. Current- and voltage-clamp data were first filtered at 2 kHz (4-pole Bessel filter) and were digitized at 2–5 kHz. Except for experiments involving inactivating prepulses (shown in Figs. 4B, 5, 6), leakage and capacitive currents that could not be compensated electronically were digitally subtracted. This was achieved either by subtracting from the total current the linear current component scaled up from that induced by a 10- or 20-mV depolarization or, in some experiments, by using the P/4 protocol in the pCLAMP software.

In order to determine series resistance and whole-cell capacitance, a sequence of three voltage pulses stepping from -55 to -45 mV were applied immediately after establishing the whole-cell configuration. In some cells, the depolarizing pulses were applied from a more hyperpolarized potential to avoid eliciting active currents. The series resistance and membrane capacitance were calculated from the initial peak amplitude and the decay time constant of the averaged current trace. The average value of series resistance was 23.4 ± 9.1 M Ω (mean $\pm$ SD; $n = 39$). Series resistance compensation was about 40% in most experiments. Thus, in typical experiments, the error in membrane voltage measurements that resulted from the series resistance for a maximum current of 500 pA was estimated to be less than 10 mV. Seal resistance exceeded 5 (up to 50) G Ω before the rupture of the patch membrane. Neurons generally had an input resistance exceeding 800 M Ω (typically 1–4 G Ω) at rest, and this value decreased considerably in the presence of active currents. The average value of whole-cell capacitance determined in this manner was 38.5 ± 21.8 pF (mean $\pm$ SD; $n = 39$). This value is almost 10 times larger than that estimated for spherical cells of similar soma diameters (9–12 μ m), assuming a unit membrane capacitance of 1 μ F/cm². This indicates that a considerable portion of the membrane area is attributable to neurites, which makes it difficult to

achieve space-clamp conditions. In order to minimize the complications from the unclamped neurite membrane, we collected voltage-clamp data for quantitative analysis from cells of simple, similar morphology, that is, monopolar neurons with a shorter neurite. (More than 70% of current traces shown are from this type.) To determine the current–voltage (I – V) relation, the current density at different voltages was obtained by dividing current amplitude by membrane capacitance (pA/pF). Theoretical curve fitting for steady-state inactivation data (see Eq. 1, Fig. 5) was performed by using the SIGMAPLOT software (version 4.0, Jandel Scientific, Corte Madera, CA), which employs the Marquardt–Levenberg algorithm. All recordings were made at room temperature (20–23°C).

Results

As previously shown (Wu et al., 1990), *Drosophila* neuroblasts treated with cytochalasin B became multinucleated cells that displayed large soma (9–12 μ m in diameter, as compared to 3–8 μ m in untreated cultures) and well-developed neurites 1 d after plating (Fig. 1). Further growth of these neurons mainly involved lengthening and thickening of neurites with little change in the number of primary neurites. Figure 2 shows the distribution of neurons with regard to the number of their primary neurites compiled from cultures of different ages (>1 d). The majority of cells developed into monopolar neurons, which were most often used in this study (see Materials and Methods). Dissociated embryonic or larval neurons generally developed considerable swelling in the whole-cell configuration soon after the rupture of patch membrane, presumably due to internal dialysis by the patch pipette solution. In contrast, the soma of giant neurons usually showed little distortion in shape, even after completing the whole-cell clamp experiments (Fig. 1).

Outward and inward currents and their functional roles

Identification of the A-type current and its function

A large proportion of cells in this culture system developed all-or-none regenerative action potentials (see below). When the membrane potential was preconditioned at a level more negative than -55 mV, a significant delay was observed in action-potential initiation in response to positive current injection (Fig. 3). Depolarizing (or hyperpolarizing) current pulses before the stimulus pulse shortened (or lengthened) the delay of action-potential onset (Fig. 3A). This suggests the presence of an inactivating outward current that counterbalances the inward currents and thus delays action-potential onset. It is known that a transient outward K⁺ current, called A-current, may play a role in this process. Prepulses can influence the level of A-current inactivation to modify the delay in action-potential initiation (Gettings, 1983; Salkoff and Wyman, 1983). After current-clamp study, the neuron was examined for the presence of an inactivating outward current using the voltage-clamp method. We observed a prominent inactivating outward current that masked inward currents in this particular cell (Fig. 3C).

The following voltage-clamp experiments were performed to identify and characterize the A-type current in other cells: TTX (10^{-8} M) and Co²⁺ (replacing Ca²⁺) were used to eliminate Na⁺ and Ca²⁺-dependent currents (see below). Figure 4A shows the families of current traces before and after application of 2 mM 4-AP, which is known to block the A-current (Rudy, 1988). The difference between these two families of current traces gave the 4-AP-sensitive component, which showed the voltage dependence and inactivation rate typical of the A-type current (Fig. 4C). Before the application of 4-AP, a similar current component could also be demonstrated in the same cell by using inactivating prepulses. Experiments were carried out using a test

Figure 1. *Drosophila* giant neurons derived from cytochalasin B-treated embryonic neuroblasts. *A*, Neuronal clusters in normal dissociated embryonic cultures. Dissociated neuroblasts undergo asymmetric divisions and individually give rise to clusters of daughter cells (2 d in culture). *B*, Giant neurons differentiated from cell division-arrested neuroblasts. In cytochalasin B-treated cultures, individual neuroblasts grow into large single neurons with thicker neurites (3 d in culture). *C* and *D*, Comparison of cell morphology before (*C*) and after (*D*) recording. Whole-cell clamp recording caused no substantial change in morphology (4 d in culture).

pulse from different preconditioning voltages to -20 mV, at which activation of the delayed-rectifier K^+ current was minimized (see below). The test pulse evoked a maximum A-type current when the membrane was preconditioned at -100 mV. In contrast, the transient current was totally inactivated at -40 mV and could not be elicited by the test pulse (Fig. 4*B*). Figure 4*D* shows the steady-state inactivation of the transient current, that is, the ratio of the remaining current (I) at different preconditioning voltages over the maximum attainable current (I_0 , at -100 mV). The midpoint of steady-state inactivation in this particular neuron was about -70 mV. The I - V relation of the 4-AP-sensitive current was also plotted in Figure 4*D*, which indicates that this current could be inactivated at voltages where little or no activation occurs. The 4-AP sensitivity of this current has been confirmed in four other neurons, and the voltage-dependent inactivation has been observed in a number of neurons (see below).

There is evidence suggesting that at least two kinds of the A-type currents exist in *Drosophila*. In embryonic cultures, the voltage ranges of inactivation and activation of the A-type current in myotubes differ from those in neurons (Solc et al., 1987). In addition, inhomogeneity of A-current has been indicated in dissociated pupal neurons (Baker and Salkoff, 1990).

We sampled a number of cells to examine whether different A-type currents exist in subpopulations of giant neurons. We noticed significant variation in the voltage range in which the A-type current became inactivated (Fig. 5). Experiments were

carried out using a test pulse to -20 mV, as described above. The steady-state inactivation for each neuron could be described by Equation 1, derived from the Boltzmann distribution (Hille, 1984):

$$I/I_0 = 1/(1 + \exp((V - V_h)/S)), \quad (1)$$

Figure 2. Morphology of *Drosophila* giant neurons. Graph shows the distributions of giant neurons classified according to the number of primary neurites. (Data from three different cultures. Error bars indicate SD.)

Figure 3. Effect of the A-type transient current on the onset of action potentials observed in current- and voltage-clamp records from the same neuron. *A*, The delay of action-potential onset (injection of +35 pA) was shortened by a depolarizing prepulse (injection of +20 pA), which presumably inactivated the A-type current, but prolonged by a hyperpolarizing prepulse (injection of -20 pA), which removed inactivation of the A-type current. $V_h = -65$ mV. *B*, Injection of depolarizing currents (40 pA) caused gradual membrane depolarization and finally elicited action potentials. $V_h = -62$ mV. *C*, In voltage-clamp experiments, prominent transient outward currents similar to the A-type current were elicited by depolarization steps (-40 to 0 mV) from a holding potential (V_h) of -80 mV.

where V stands for the preconditioning voltage; V_h , the midpoint (voltages at which 50% of the current is inactivated); and S , the slope factor (which reflects the voltage sensitivity of channel inactivation). Among seven cells studied in the presence of TTX and Co^{2+} , V_h varied from -80 to -44.8 mV with a mean $\pm$ SD of -65.3 ± 11.0 mV (Fig. 5). S ranged from 4.1 to 8.0 mV (5.9 ± 1.1 mV). These values are within the ranges previously reported for the A-type currents in cultured larval and pupal neurons (Solc et al., 1987; Baker and Salkoff, 1990). Similar variation in V_h (ranging from -84.5 to -51.7 mV) has been observed in 13 additional neurons. Results from these neurons were excluded from the above analysis because the experiments were performed without TTX in the bath (see Figs. 11, 12).

Delayed-rectifier K^+ current

Another voltage-activated outward current usually encountered in giant neurons showed kinetics similar to that of the conventional delayed-rectifier K^+ current (Fig. 6*A*). TTX and Co^{2+} were present to block Ca^{2+} - and Na^+ -dependent currents. A sustained outward current could be isolated by applying 4-AP (Fig. 4*A*, broken line) or by manipulating the holding potential to inactivate the A-type current (Fig. 6*A*). The I - V relation of this sustained current is shown in Figure 6*B*. This current was activated at membrane potentials more positive than -30 mV and did not inactivate appreciably during the depolarizing pulse (up to 300 msec at 0 mV). From tail-current experiments, the reversal potential was estimated to be -74.6 ± 3.6 mV ($n = 5$) with normal K^+ concentration (2 mM) in bath. The sensitivity to TEA of this current varied from neuron to neuron. In some cases, 20 mM TEA blocked only half of the current, while in other cases, the same concentration of TEA completely eliminated the outward current (data not shown).

Figure 4. Inactivation and 4-AP sensitivity of the A-type current. *A*, The solid traces show the total outward current activated by pulses from $V_h = -80$ to +50 to +10 mV in 20-mV steps. Broken traces show after the addition of 2 mM 4-AP. *B*, Inactivation of the A-type currents elicited at -20 mV by 150-msec prepulses (-100 to -40 mV in 20-mV steps). *C*, 4-AP-sensitive current component. The differences in total outward currents between control (solid traces in *A*) and after treatment of 2 mM 4-AP (broken traces in *A*) are shown. *D*, I - V relation (open squares; data from *C*) and steady-state inactivation (solid squares; data from *B*). In this and the following figures, current density in the I - V relation was estimated by normalizing current amplitude to membrane capacitance (pA/pF). Steady-state inactivation was determined by the ratio of the remaining current (I) at different preconditioning voltages over the maximum attainable current (I_0 , at -100 mV). All data were taken from the same cell. The bath solution contained TTX (10^{-8} M) and Co^{2+} in place of Ca^{2+} .

Figure 5. Variation in steady-state inactivation of the A-type current. *A–C*, Examples of voltage-clamp traces from three different cells in which inactivation occurred in different voltage ranges. Steady-state inactivation was determined by applying preconditioning voltage pulses (500 msec; -100 to -40 mV in 10-mV steps) that preceded the test pulse to -20 mV. The A-type current in *A* inactivated at a more negative potentials with a midpoint $V_h = -80.0$ mV, compared with the currents shown in *B* ($V_h = -67.9$ mV) and *C* ($V_h = -44.8$ mV). *D*, Steady-state inactivation (I/I_0) of the A-type current at different preconditioning voltages. Each symbol represents a different cell. Data from the cells shown in *A–C* (symbols on the left side of traces *A–C*) were also plotted in this graph. The current amplitude (I and I_0) was measured as the difference between the peak and the remaining current at the end of the test pulse following the most positive pre-pulse.

Effects of K^+ channel blockers on action potentials and the outward current

Because giant neurons allowed performing current- and voltage-clamp recordings on the same cell, the effects of K^+ channel blockers on action-potential properties were first studied under current clamp, and the outward currents responsible for the change were further examined under voltage clamp. In the cell shown in Figure 7*A*, 20 mM TEA in normal saline lowered the current required to initiate action potentials. It also prolonged the duration of action potentials and induced repetitive firing during sustained current injection (Fig. 7*A*₂). Addition of 2 mM 4-AP further prolonged the action potential and induced a plateau at its falling phase (Fig. 7*A*₃). In the presence of TEA and 4-AP, the same neuron in voltage-clamp mode showed initial inward currents followed by outward currents (Fig. 7*B*₁). Apparently, TEA and 4-AP reduced K^+ outward currents, including the A-type current and delayed rectifier, and unmasked the inward currents responsible for the action potential. The recording solution was subsequently changed to normal saline (Fig. 7*B*₂). Subtracting B_1 from B_2 gives the 4-AP- and TEA-sensitive currents (Fig. 7*B*₂– B_1), which show both transient and sustained outward components. This experiment suggests that both current components contribute to action-potential repolarization.

Na^+ and Ca^{2+} inward currents

Because the blocking efficiency of TEA varied from neuron to neuron, outward K^+ currents were blocked by using patch pipettes filled with CsCl instead of KCl (Armstrong and Bezanilla, 1973). The remaining whole-cell currents consisted of mainly inward currents under this condition. The Na^+ current was observed with either Co^{2+} in place of Ca^{2+} in bath to block the Ca^{2+} current or subtraction of current recorded in Na^+ - and K^+ -free saline from that in K^+ -free saline. The Na^+ current could be activated by pulses to around -40 mV. A maximum peak current was elicited by a pulse to between -20 and 0 mV (Fig. 8*A*). Among 19 neurons studied, six of them showed a typical inward Na^+ current with nearly complete inactivation (Fig. 8*A*₁). The inward current in the other neurons, however, exhibited a

clear noninactivating component (Fig. 8*B*₁), which persisted during a 500-msec pulse. This persistent inward current was activated concurrently with the transient component and also peaked in a similar voltage range (Fig. 8*A*₂, *B*₂). It was most likely mediated by Na^+ channels because it was observed in K^+ -free bath solution and was blocked by 10^{-8} M TTX, as was the transient inward component (Fig. 8*A*₁, *B*₁). However, the per-

Figure 6. Delayed-rectifier-type outward current. *A*, Representative traces of delayed-rectifier outward K^+ current from a neuron in which the A-type current was totally inactivated by preconditioning at -40 mV (500 msec). *B*, I – V relation of delayed-rectifier currents. Data were pooled from four different neurons, in which the A-type current was removed by -40 -mV preconditioned pulses. The experiments were carried out in the presence of TTX (10^{-8} M) and Co^{2+} (1.8 mM in place of Ca^{2+}). Error bars indicate SEM.

Figure 7. Effects of K^+ channel blockers on the action potentials and membrane currents in the same neuron. *A*₁, Control action potential. *A*₂, TEA (20 mM) prolonged action-potential duration. *A*₃, Subsequent addition of 4-AP (2 mM) further prolonged the action potential. $V_r = -50.3$ mV in *A*₁ and -56.2 mV in *A*₂ and *A*₃. Depolarizing current (36 pA) was injected in all experiments. *B*₁, In the subsequent voltage-clamp measurement, this neuron showed an inward current followed by an outward current in the presence of 4-AP and TEA. *B*₂, Total membrane current after washout of TEA and 4-AP. The difference between *B*₂ and *B*₁ (*B*₂ - *B*₁) gives the 4-AP- and TEA-sensitive component. No TTX or Co^{2+} was present in this experiment. $V_H = -80$ mV.

sistent current component might represent the activation of a separate population of Na^+ channels, because it was less sensitive than the transient component to inactivating prepulses (Fig. 8*C*₁, *C*₂). A 500-msec preconditioning pulse to -30 mV inactivated more than 80% of the transient component but exerted little effect on the persistent component.

Ca^{2+} currents were studied with solution in which Na^+ was replaced with Tris-Cl (Byerly and Leung, 1988). The amplitude of Ca^{2+} current was much smaller than that of Na^+ current (cf. Fig. 8*A*₁, *B*₁; 9*A*, *B*). The Ca^{2+} current was consistently abolished by either replacing Ca^{2+} with Co^{2+} or adding 50–100 μM Cd^{2+} or 100–200 μM La^{3+} to the bath (not shown). The I - V relation for the peak Ca^{2+} current is shown in Figure 9*C*. Some variation in kinetics was observed in the neurons studied (more than 20). Figure 9*A* shows a Ca^{2+} current consisting of inactivating and noninactivating components. Another example is shown in Figure 9*B*₁, in which the inward current inactivated almost completely during the 100-msec pulse. When Ca^{2+} was replaced with Ba^{2+} , which is permeable through the Ca^{2+} channel, the resultant currents did not show any inactivation (Fig. 9*B*₂). This is consistent with the report that inactivation of Ca^{2+} current could be associated with accumulation of internal Ca^{2+} (Tillotson, 1979).

Ca^{2+} -activated K^+ currents

Ca^{2+} -activated K^+ channels have been shown to regulate action-potential repolarization and to modulate repetitive firing (Blatz and Magleby, 1987; Latorre et al., 1989). Replacing Ca^{2+} with Co^{2+} resulted in a delay in repolarization and a slight reduction in the hyperpolarizing afterpotential (Fig. 10*A*), both of which could be attributed to a block of Ca^{2+} -activated outward current.

Under voltage-clamp conditions, the presence of Ca^{2+} -acti-

vated outward currents was reflected by a reduction, instead of an increase, in the net outward current when the inward Ca^{2+} current was blocked. Families of current data were collected before (Fig. 10*B*) and after (*C*) replacing Ca^{2+} with Co^{2+} . TTX (10^{-8} M) was used to block Na^+ inward current. The differences between Figure 10, *B* and *C*, consist of inward Ca^{2+} current and Ca^{2+} -activated outward current. In the majority of neurons examined, except for a brief inward transient immediately following the depolarizing step, the inward current was overwhelmed by the ensuing outward currents (Fig. 10*B*, *C*). Because all outward currents were abolished by internal Cs^+ (cf. Figs. 8, 9), this outward component in the Ca^{2+} -dependent current (Fig. 10*B*, *C*) is most likely carried by K^+ . The time course of the total current (Fig. 10*B*, *C*) also suggested the presence of a transient and a sustained phase in the Ca^{2+} -activated outward current. The transient and sustained components were both activated at membrane potentials similar to the Ca^{2+} current (cf. Fig. 9). In 18 neurons examined, 15 showed net outward Co^{2+} -sensitive currents. Among them, nine displayed a transient peak followed by a late component (e.g., Fig. 10*D*, *E*), and six showed no transient peak. The net total current in the remaining three neurons was inward during a depolarizing pulse (Fig. 10*F*).

TTX-sensitive outward current

TTX is thought to block specifically Na^+ channels (Narahashi et al., 1964; Hille, 1984). To our surprise, TTX not only abolished the Na^+ inward current, but also reduced the outward current in many of our experiments. Figure 11 shows one example in which the effects of TTX were studied with both current- and voltage-clamp techniques in the same cell. Current injection evoked action potentials that were completely blocked by TTX at 10^{-8} M (Fig. 11*A*). In the subsequent voltage-clamp

Figure 8. Voltage-activated Na⁺ inward currents in Ca²⁺- and K⁺-free saline. Patch pipettes were filled with CsCl to block K⁺ current. Ca²⁺ current was blocked by the substitution of Ca²⁺ with Co²⁺. *A₁* and *B₁* were taken from two neurons with different proportions of transient and persistent components. Both components were blocked by 10⁻⁸ M TTX. *A₂*, *I-V* relation of peak transient Na⁺ currents. Data are from a different set of three neurons. *B₂*, *I-V* relation of persistent Na⁺ currents. Data are from three different neurons. *C₁*, Preconditioning at -30 mV (500 msec) inactivated only the transient component but not the persistent component. *Broken line* indicates the holding current level. *C₂*, Steady-state inactivation of the transient (solid circles) and persistent (open circles) Na⁺ inward currents. Data are from two different neurons. Error bars in the graphs indicate the range.

recording after TTX was washed out (17 min), there was recovery not only in the inward current, but also in the outward current. Further application of TTX again abolished the inward current and part of the outward current (cf. Fig. 11*B₁*, *B₂*). Both currents showed partial recovery after 2 min of wash (Fig. 11*B₃*). This TTX-sensitive outward current was observed in 11 out of 12 neurons that showed a clear initial inward component (e.g., Fig. 11*B₁*).

It is likely that the outward component blocked by TTX was activated by accumulation of internal Na⁺. The effect of reducing Na⁺ influx by removal of Na⁺ in the bath solution was therefore examined. Figure 12*A* shows an example of reduction of external Na⁺ that led to a decrease in the inward current and also in the outward current. Partial recovery of both inward and outward currents was observed after returning to normal saline. The majority of neurons examined (eight out of nine) showed a decrease in outward current amplitude after removal of Na⁺

from the recording solution. Consistent with the results of TTX treatment, removal of Na⁺ in saline caused a 30–50% reduction in outward current amplitude.

Previous reports (Dryer et al., 1989; Haimann et al., 1990) of Na⁺-activated K⁺ current in other species have demonstrated that Li⁺ can permeate Na⁺ channels but does not activate this K⁺ current. We studied the effect on giant neurons of replacing external Na⁺ with Li⁺. In two out of three neurons, Li⁺ decreased the outward current but left the inward current intact (Fig. 12*B*). The above results indicate that an Na⁺-activated K⁺ current, similar to that described in other species (Bader et al., 1985; Dryer et al., 1989), appears to be present also in *Drosophila* neurons.

Excitability in different cell categories

Previous voltage-clamp studies indicate considerable inhomogeneity in membrane currents in cultured embryonic and larval

Figure 9. Voltage-activated Ca^{2+} inward currents observed in Na^{+} - and K^{+} -free saline with CsCl-filled electrodes. *A* and *B*, were taken from two neurons with different proportions of transient and sustained components. *B*₁, Ba^{2+} current in the same neuron as in *B*, observed with Ba^{2+} in place of Ca^{2+} in saline. Note that Ba^{2+} removed channel inactivation. *C*, *I-V* relation of peak Ca^{2+} inward currents. Data are from four different neurons. Error bars indicate SEM except for data points marked by asterisks, which show the range of two samples.

CNS neurons of *Drosophila* (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988; Solc and Aldrich, 1988). Giant neurons derived from dissociated early gastrulas also exhibited variation in membrane currents under voltage clamp and various patterns of regenerative potentials under current clamp. We made an attempt to correlate the variety of membrane potentials with underlying currents.

Categories of regenerative responses

In current-clamp experiments, several distinct patterns of responses were induced by depolarizing current steps. From a total of 149 cells, we tentatively classified them into three major types according to properties of the regenerative membrane potentials: (1) *All-or-none* (62 out of 149). These neurons produced all-or-none action potentials with a clear threshold (Fig. 13*A*; see also Figs. 3, 7, 10, 11). (2) *Graded*. Regenerative potentials

in this group of neurons increased in amplitude with increasing strength of the injected current. They could be further subdivided into two types. Some neurons (27 out of 149) displayed oscillations with either increasing or decreasing amplitudes [Fig. 13*B*, graded MP (graded, multiple peak)]. Other neurons (34 out of 149) in this group showed a single graded response only at the beginning of the current pulse [Figs. 4; 13*C*, graded SP (graded, single peak)]. The graded response varied in peak amplitude, and some could reach the size of all-or-none action potentials. However, they differed from all-or-none action potentials in that the depolarizing phase often lacked a clear inflection point and the repolarizing phase was less rapid. (3) *Nonregenerative* (26 out of 149). These cells showed outward rectification but no clear sign of regenerative potentials (data not shown).

Both Ca^{2+} and Na^{+} inward currents can support membrane depolarization to generate spikes of different time courses. We

Figure 10. Current- and voltage-clamp studies of Ca^{2+} -activated outward current. *A*, Substitution of Co^{2+} for Ca^{2+} in saline induced delay of action-potential repolarization, suggesting the presence of a Ca^{2+} -activated K^{+} current. The action potential was induced by 13-pA constant depolarizing current. *B*, Voltage-clamp traces in the presence of TTX. *C*, Traces from the same neuron with Ca^{2+} substituted by Co^{2+} in saline containing TTX. *D*, Subtraction of *C* from *B* ($B - C$), representing Ca^{2+} -dependent inward (arrow) and outward currents. Note the transient and delayed outward components. *E* and *F*, Net Co^{2+} -sensitive current extracted from two other wild-type neurons, illustrating variation in Ca^{2+} -dependent current. Net inward currents were seen in a few neurons (*F*), suggesting a lack of Ca^{2+} -activated K^{+} currents. *G*, Co^{2+} -sensitive currents from a *slo* neuron displaying purely inward currents.

Figure 11. TTX-sensitive current. *A*, Action potentials evoked by 10-pA depolarizing current injection (*A*₁) were blocked by 10^{-8} M TTX (*A*₂). $V_r = -53.7$ mV in *A*₁, -52.7 mV in *A*₂. *B*, The same neuron was subjected to voltage-clamp study after TTX was removed by wash (*B*₁). Note inward currents (arrow) preceding outward currents. Application of 10^{-8} M TTX blocked part of the outward current as well as the inward current (*B*₂). After 2 min wash. Note partial recovery of outward currents (*B*₃).

were interested in the relative contributions of Ca^{2+} versus Na^{+} currents in generating the graded and all-or-none types of regenerative responses (Fig. 14*A*₁, *B*₁). At a concentration of 10^{-8} M, TTX blocked both all-or-none action potentials and graded regenerative potentials (Fig. 14*A*₂, *B*₂), implicating a major role of Na^{+} currents in generating electric activity in these neurons. However, the Ca^{2+} current may still play a role in initiating and shaping the electrical responses even though it cannot sustain regenerative potentials by itself. The effect of the Ca^{2+} current on membrane depolarization was revealed by reducing K^{+} currents. With the addition of 10–20 mM TEA to the bath solution containing TTX, graded type neurons were capable of producing slow but large voltage oscillations (Fig. 14*B*₃). These regenerative potentials were enhanced by replacing Ca^{2+} with Ba^{2+} and were blocked by 1 mM Co^{2+} in the bath solution (not shown). In contrast, all-or-none type neurons showed little or no hint of regenerative responses even with the presence of a higher concentration of TEA in the bath solution (Fig. 14*A*₃).

The above results suggest that the contribution of Ca^{2+} currents is greater in the graded type than in the all-or-none type of regenerative potentials. The fact that all-or-none spikes had

Figure 12. Na^{+} -dependent outward current. *A*, Both inward and outward currents were reduced at a low ($1/4$) external Na^{+} concentration. The remaining inward current (primarily Ca^{2+} current) had a slower time course. After returning to normal solution, both inward and outward currents showed partial recovery. $V_H = -80$ mV; test pulses to 0 mV. *B*, Replacing Na^{+} with Li^{+} reduced Na^{+} -dependent outward current without affecting inward current. $V_H = -80$ mV; test pulses to -10 mV.

a faster time course than graded potentials in these neurons is also consistent with the observations in other species that Ca^{2+} spikes are generally slower in time course than Na^{+} spikes (Hagiwara and Byerly, 1981).

Categories of net membrane currents

A series of experiments was performed without the use of any channel-blocking agent to correlate the total membrane currents with the resultant regenerative responses. As already shown, the total membrane current was a mixture of inward currents and voltage-activated and cation-activated (Ca^{2+} or Na^{+}) outward currents. In all cells studied, outward currents appeared to be the dominant component and often masked the inward current completely (Fig. 15). Voltage-clamp records of the net total current in 122 cells were classified into three categories according to their characteristic kinetic features: (1) *Fast transient* (36 out of 122). Following the initial inward current, a large transient outward current rose and decayed rapidly (within 10–30 msec) to a sustained plateau of outward current (Fig. 15*A*). The time course of this transient outward component appeared to be faster than that of the A-type current and coincided more closely with the transient phase of Ca^{2+} -activated K^{+} current in some neurons (cf. Figs. 4, 5, 10). Neurons in the remaining two categories showed little net inward current. (2) *Slow transient* (70 out of 122). A slowly decaying outward current was prominent that followed a time course similar to that of the A-type current (Fig. 15*B*). (3) *No transient* (16 out of 122). The net current was

Figure 13. Types of regenerative potentials in giant neurons under current clamp. *A*, All-or-none; $V_r = -55.2$ and 55.7 mV. *B*, Graded, multiple-peak (graded MP); $V_r = -57$ and 56 mV. *C*, Graded, single-peak (graded SP); $V_r = -55.7$ and 58.8 mV. Responses were induced by injecting depolarizing currents: 50 and 60 pA in *A*, 40 and 50 pA in *B*, and 40 and 60 pA in *C*.

dominated by a sustained outward current that showed little decay after the initial rise. It is likely that the major outward component consisted of either the delayed rectifier or a sustained cation-activated K^+ current (Fig. 15C).

Different categories of current- and voltage-clamp responses observed in neurons with different numbers of primary neurites are summarized in Figure 16, *A* and *B*. Histograms correlating voltage- and current-clamp responses collected from the same cells were also constructed (Fig. 16C). We did not find a striking correlation except the tendency that cells (16 out of 17) exhibiting a total current of the fast transient type showed regenerative potentials under current clamp (Fig. 16C) and that a higher

proportion of multipolar cells showed all-or-none spikes (Fig. 16B).

We noticed that unclamped action currents were often associated with neurons displaying the slow transient-type total current and/or all-or-none action potentials. Conversely, unclamped action currents have not been seen in a total of 52 cells displaying graded or nonregenerative types of voltage responses. The above results suggest that in some all-or-none type cells the regenerative currents occurred in remote neuritic areas where membrane potentials were difficult to control by the patch electrode positioned at the soma. Under voltage clamp, fast transient-type neurons (30 out of 34) displayed clear net inward currents, which presumably induced the subsequent cation-activated K^+ outward currents (Fig. 15). Some of these cells were also studied under current clamp, and a majority of them (11 out of 17) showed graded-type responses. This suggests that channels mediating inward currents may be more abundant in the soma than the neurites in neurons of the fast transient category.

Expression of *slowpoke* mutant phenotypes

The mutation *slowpoke* (*slo*) has been shown to eliminate the transient Ca^{2+} -activated K^+ current in adult and larval muscles (Elkins et al., 1986; Singh and Wu, 1989; Komatsu et al., 1990). Consequently, *slo* muscle fibers are more excitable, displaying broadened action potentials (Elkins and Ganetzky, 1988; Singh and Wu, 1990). It is important to determine the role of the *slo* gene product in neurons.

Neurons in *slo* cultures showed all three types of voltage responses to current injection (Fig. 17A) as described above for wild-type neurons. In addition, several kinds of irregular regenerative responses not seen in wild-type neurons have been observed in a subset of *slo* neurons. Figure 18 shows examples of such responses, including oscillations of irregular amplitudes and tiny spikes riding on top of large, slow waves of depolarization (Fig. 18A₁, A₂), which were observed even in neurons with relatively simple (monopolar) morphology. It was also evident that some all-or-none type neurons in *slo* cultures exhib-

Figure 14. Effects of TTX and TEA on neurons displaying all-or-none or graded regenerative responses. *A*, All-or-none action potentials induced by 20 pA current injection (*A*₁; $V_r = -55.7$ mV) were blocked by 10^{-8} M TTX (*A*₂; 25 pA; $V_r = 53.2$ mV). No significant change in excitability was observed after further addition of 20 mM TEA (*A*₃; 34 pA; $V_r = 56.6$ mV). *B*, Graded regenerative potentials (*B*₁; $V_r = -57.6$ mV) were suppressed by 10^{-8} M TTX (*B*₂; $V_r = -62$ mV). Large membrane oscillations could be induced following further addition of 20 mM TEA (*B*₃; $V_r = -59.6$ mV). All cells received 40-pA injection.

Figure 15. Categories of total membrane currents according to kinetics of the net outward currents under voltage-clamp conditions. *A*, Fast transient. *B*, Slow transient. *C*, No transient. $V_H = -80$ mV; test pulses to -40 , -20 , and 0 mV.

ited regenerative potentials of an increased duration. Such effect was variable, ranging from no apparent change to about a four-fold lengthening in action-potential duration (Fig. 18*B*, *B*). Variation in action-potential broadening may reflect differences in the relative contributions between voltage-activated and Ca^{2+} -activated K^+ currents to membrane repolarization. This notion is supported by the following observations.

In *slo* neurons that displayed relatively normal spikes, partial inactivation of the A-type current by membrane depolarization (-45 mV) broadened action potentials and shortened the delay to their initiation (Fig. 19). In wild-type neurons, only decrease in onset time but not spike broadening was induced by sub-threshold membrane depolarization (Fig. 19; see also Fig. 3). Prolonged current injection further inactivated the A-type current and tended to increase the duration of action potentials in *slo* but not in wild-type neurons (Fig. 18*B*, *B*). The above results are consistent with a reduction of Ca^{2+} -activated K^+ current in *slo* neurons. Repolarization of neuronal action potentials thus appears to depend on both Ca^{2+} -activated and voltage-activated K^+ currents, and the effect of *slo* became more evident when the A-type current was inactivated.

Voltage-clamp experiments employing the paradigm shown in Figure 10 suggested that, compared to wild-type neurons, a lower percentage of *slo* neurons had Ca^{2+} -activated outward K^+ current. The differences between membrane currents before and after addition of Co^{2+} to saline (containing 10^{-8} M TTX) were determined. In 6 out of 16 *slo* neurons examined, the net Co^{2+} -sensitive current was purely inward, whereas only 3 out of 18 wild-type neurons displayed a purely inward net current (Figs. 10*F*, *G*; 17*B*). The remaining 10 *slo* and 15 wild-type neurons

Figure 16. Summary of current- and voltage-clamp data collected from neurons with different morphology. *A*, Correlation between number of primary neurites and types of voltage-clamp responses. *B*, Correlation between number of primary neurites and types of current-clamp responses. *C*, Correlation of current- and voltage-clamp data collected from the same neuron. See Figures 13 and 15 for categories of current- and voltage-clamp responses.

showed outward net current. However, the outward component in these *slo* neurons was significantly reduced (3.43 ± 3.39 pA/pF, mean $\pm$ SD, as compared with 8.12 ± 7.61 pA/pF in wild-type neurons at the transient peak evoked by a depolarizing step to 20 mV). Although not all wild-type neurons express Ca^{2+} -activated current uniformly (Fig. 10*D–F*), the above results do suggest that *slo* affects the Ca^{2+} -activated current at least in a subpopulation of neurons, in which both the transient and sustained components of the Ca^{2+} -activated current were either eliminated or significantly reduced.

It is interesting to note that the action-potential broadening by the *slo* mutation appeared to be more pronounced in the neurites. In two successful attempts to perform recordings from neurites, successive action potentials induced by sustained cur-

Figure 17. Summary of current- and voltage-clamp studies from *slo* neurons in comparison with wild-type data. **A**, Distributions of different types of responses observed under current clamp in wild-type and *slo* neurons. Note the absence of the irregular type of response in wild-type neurons. **B**, Percentage of neurons exhibiting purely inward Co^{2+} -sensitive currents under voltage clamp. Note a higher proportion of such neurons in *slo* culture. MP, multiple peak; SP, single peaks.

rent injection increased the duration to a much greater extent compared to the soma action potentials (see Fig. 18*B₂*, *B₃*; note difference in time scales). The duration of neurite spikes could reach more than 100 msec, whereas those in the soma never exceeded 30 msec in *slo* neurons examined.

Discussion

Our previous study (Wu et al., 1990) showed that cytokinesis-arrested *Drosophila* neuroblasts in dissociated culture can develop neuritic arbors and neuron-specific antigens recognized by anti-HRP (Jan and Jan, 1982; Salvaterra et al., 1987; Snow et al., 1987). Furthermore, cytochalasin B applied after different cycles of neuroblast divisions does not alter the cell lineage of the development of specific neurotransmitters in culture (Huff et al., 1989). These results suggest that gastrula neuroblasts are already determined to differentiate certain basic neuronal properties in the absence of further cell–cell interaction. In this article, we present further support for the above idea, demonstrating that cell division-arrested neuroblasts in dissociated cultures also express a variety of ion channels that have been reported in excitable cells of *Drosophila*. Inward Na^+ and Ca^{2+} currents and various K^+ outward currents observed in this preparation showed properties similar to those described in adult and larval muscles (Salkoff and Wyman, 1983; Wu and Haugland, 1985; Wei and Salkoff, 1986; Timpe and Jan, 1987; Gho and Mallart, 1986; Singh and Wu, 1989) and in cultured larval and embryonic neurons (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988; Solc and Aldrich, 1988). In addition, we reported a persistent Na^+ inward current and a TTX-sensitive, Na^+ -dependent K^+ current, which have been observed in other species but not previously reported in *Drosophila*.

This is the first report of a whole-cell current-clamp study of all-or-none and graded regenerative responses from cultured *Drosophila* neurons. The pattern of regenerative responses observed here reflects the variety of all-or-none action potentials and graded responses that have been recorded intracellularly from *in situ* preparations of different insects, including *Drosophila* (Ikeda and Kaplan, 1970), *Cicada* (Hagiwara and Watanabe, 1956), the cockroach (Christensen et al., 1988), and the locust (Burrows, 1979; Goodman and Heitler, 1979; Laurent, 1990).

In primary cultures derived from *Drosophila* whole embryos and larval CNS, a major difficulty in studying mutational effects on neurons is the small cell size compounded with inhomogeneity in membrane currents expressed in different cell types. Previous studies of mutant effects relied on statistical analysis of large samples (O'Dowd and Aldrich, 1988; Baker and Salkoff, 1990). We found it possible to correlate current- and voltage-clamp records from the same giant neuron in cytochalasin B-treated cultures, which has not yet been achieved in untreated neurons. This approach enables us to determine, within individual neurons, the role of various currents in the generation of action potentials and regulation of membrane excitability, circumventing some of the problems imposed by cellular inhomogeneity. As the experiments on *slo* giant neurons demonstrated, current-clamp analysis may provide a sensitive screening method to detect the range of mutational effects on membrane excitability in different neurons and to determine the alterations in the corresponding currents.

Inward Na^+ and Ca^{2+} currents

By using Na^+ -free solution or applying TTX, we isolated the Ca^{2+} inward current that was sensitive to inorganic blockers such as Co^{2+} , Cd^{2+} , and La^{3+} . Giant neurons producing graded regenerative potentials appear to show greater Ca^{2+} -dependent depolarizations than those displaying all-or-none action potentials (Fig. 14). In voltage-clamp experiments, some neurons showed exclusively inactivating Ca^{2+} currents, while the others displayed a substantial noninactivating component (Fig. 9), suggesting the possibility of Ca^{2+} channel subtypes. The existence of two different Ca^{2+} channels in *Drosophila* embryonic neurons has been recently demonstrated by using spider toxins (Leung et al., 1989). It will be important to determine the roles of Ca^{2+} currents with different inactivation kinetics in shaping the regenerative response.

Inward Na^+ current isolated by replacing Ca^{2+} with Co^{2+} showed voltage dependence similar to the Na^+ current in cultured embryonic neurons (O'Dowd and Aldrich, 1988). More than 50% of the neurons generated a transient inward Na^+ current with a delay of a few milliseconds following the voltage steps. The delay decreased as depolarization increased, indicating that the main source of the Na^+ current was localized in

Figure 18. Abnormal regenerative responses in *slo* neurons under current clamp. *A*, Examples of irregular regenerative potentials from two *slo* neurons not seen in wild-type neurons (*A*₁: $V_r = -61.5$ mV; 40 pA injection; *A*₂: $V_r = -49.8$ mV; 10 pA). *B*, All-or-none responses in different neurons showed variable degrees of abnormality. *B*₁, Action potentials with similar durations seen in wild-type neurons. $V_r = -56$ mV; 50 pA. *B*₂, Action potentials with durations significantly longer than that of wild-type neurons. $V_r = -53$ mV; 18 pA. *B*₃, Action potentials recorded from neurites that showed progressive broadening in their duration. $V_r = -64.0$ mV; 80 pA.

neurites where voltage was not satisfactorily controlled. Similar observations regarding Na^+ channel distribution have also been reported in cultured embryonic neurons in culture (O'Dowd and Aldrich, 1988). In contrast, we did not encounter such difficulty in voltage-clamp measurements of Ca^{2+} currents. This difference suggests that the soma expresses a higher density of Ca^{2+} channels, whereas Na^+ channels are more abundant in neurites.

About 70% of giant neurons expressing inward Na^+ current showed a persistent component (Fig. 8*B*). The transient and persistent components showed marked differences in steady-state inactivation but were both blocked by TTX and depended on external Na^+ (Fig. 8*B,C*). Persistent Na^+ currents (or conductances) have been reported in neurons of insects (Yawo et al., 1985; Christensen et al., 1988; Lapied et al., 1989), crustaceans (Davis and Stuart, 1988), and mammals (Stafstrom et al., 1985; Reklung and Laursen, 1989; French et al., 1990). Molecular studies have shown that small subunits (e.g., β -subunit)

Figure 19. Effects of the resting membrane potential on action-potential broadening in a wild-type and a *slo* neuron. The membrane potential was set at different values as indicated. Inactivation of the A-type current by membrane depolarization shortened the delay in action-potential onset in both wild-type and *slo* neurons. This also prolonged the action potentials in *slo* but not wild-type neurons. The amplitudes of the current pulses in these sequential recordings were 20 and 13 pA in the wild-type and 40 and 30 pA in the *slo* neuron.

may contribute to the regulation of Na^+ channel inactivation (Auld et al., 1988; Krafte et al., 1988). In addition, there may be multiple types of the α -subunit that coexist in neurons. In fact, more than one gene in *Drosophila* have been implicated to code for Na^+ channels (Okamoto et al., 1987; Salkoff et al., 1987; Loughney et al., 1989; Ramaswami and Tanouye, 1989) or to affect Na^+ channel function (Ganetzky, 1986; Jackson et al., 1986; O'Dowd and Aldrich, 1988). In particular, the *para* gene may produce multiple types of Na^+ -channel α -subunit by an alternative splicing mechanism (Loughney et al., 1989). It will be of interest to determine whether or not the two components reported here represent two Na^+ channel populations in *Drosophila*.

Voltage-activated K^+ outward currents

Previous studies in *Drosophila* muscle fibers (Salkoff and Wyman, 1983; Wu and Haugland, 1985; Haugland and Wu, 1990) and dissociated neurons (Byerly and Leung, 1988; Solc and Aldrich, 1988) demonstrated two different types of voltage-activated K^+ currents, which can be separated on the basis of pharmacological or physiological differences. Our results from giant neurons indicate that the same two currents, the 4-AP-sensitive, inactivating, A-type current and the delayed rectifier, could be isolated in the presence of Na^+ and Ca^{2+} channel blockers (Figs. 4–6). Current-clamp experiments suggested that the A-type current appeared to be involved in regulating action-potential initiation, repolarization, and repetitive firing in the giant neuron. Partial inactivation of the channels by depolarizing holding currents led to more rapid action-potential initiation (Fig. 3) and higher firing frequencies (M. Saito and C.-F. Wu, unpublished observations). In the case of larval motor axon

terminals, it has been reported that removal of the current by 4-AP or *Shaker* mutations prolongs transmitter release because of prolonged action potentials and repetitive firing at the terminal. This phenotype becomes more extreme at reduced Ca^{2+} concentrations, probably due to a more important role of the A-type current in membrane repolarization when the Ca^{2+} -activated K^+ current is weakened at lower Ca^{2+} levels (Jan et al., 1977; Ganetzky and Wu, 1982, 1983; Mallart et al., 1991). In adult cervical giant fibers, blockade or modifications of the A-type channels by *Shaker* mutations also results in prolonged action potential and, in some case, repetitive firing (Tanouye et al., 1981; Tanouye and Ferrus, 1985).

Our data suggest that A-type channels have nonuniform voltage dependence and inactivation kinetics (Fig. 5). Some variation might be derived from factors affecting voltage-clamp measurements, for example, differences in the proportions among cell input resistance, seal resistance, and series resistance (see Materials and Methods). A more detailed study in the soma of dissociated pupal thoracic neurons has also revealed significant variation in inactivation properties of the A-type current and the coexistence of two populations of channels in some cells (Baker and Salkoff, 1990). It is possible that the A-type channels inactivating at more negative potentials may be involved in the delay of action-potential initiation and regulation of firing rate (Connor and Stevens, 1971; Getting, 1983; Hille, 1984), whereas those inactivating at more positive potentials may be more important to action-potential repolarization.

TTX-sensitive K^+ current

Na^+ -activated K^+ currents have been observed in whole-cell voltage-clamp studies as a transient outward current following the Na^+ inward current in chick neurons (Bader et al., 1985; Dryer et al., 1989) and crayfish motor neurons (Hartung, 1985). This type of channel appears to be activated by Na^+ influx through the voltage-gated Na^+ channel. In cell-free patches, the channel activity is proportional to the Na^+ concentration on the cytoplasmic side without signs of inactivation (Kameyama et al., 1984; Haimann et al., 1990). The following lines of evidence suggest the presence of a similar Na^+ -activated K^+ channel in *Drosophila* giant neurons: First, TTX not only blocked the inward current, but also significantly decreased the outward current. Second, in Na^+ -free saline, the outward current was also reversibly reduced (Fig. 12A). Third, substitution of Na^+ with Li^+ in bath left the inward current intact but reduced the outward current (Fig. 12B). Apparently, Li^+ could pass through Na^+ channels but failed to activate this TTX-sensitive K^+ current (see Bader et al., 1985; Dryer et al., 1989). Finally, the major charge carrier for this current is most likely to be K^+ for the following reasons: This outward current was blocked by internal Cs^+ (cf. Fig. 8), and its tails reversed at about -80 mV (data not shown), which was close to the Nernst potential for the K^+ gradient (2 mM/144 mM). In different cells, the amount of Na^+ -activated outward current varied in terms of the absolute and relative amplitude of the transient and sustained components, which may reflect activation by the transient and persistent components of Na^+ current. It will be important to investigate interactions between the Na^+ current and the Na^+ -activated K^+ current. Regulation of these two currents, analogous to the interplays between the Ca^{2+} and Ca^{2+} -activated K^+ currents, may provide neurons with additional handles to enrich their signaling capability.

Ca^{2+} -activated K^+ currents and effects of the *slo* mutation

The transient and the sustained components of Ca^{2+} -activated K^+ currents have been described in *Drosophila* adult and larval muscles (Salkoff, 1983; Wei and Salkoff, 1986; Gho and Mallart, 1986). These two components represent two distinct channel populations because *slo* eliminates the transient component (I_{CT}) but not the sustained component (I_{CS}) of Ca^{2+} -activated K^+ currents (Elkins et al., 1986; Singh and Wu, 1989, 1990). In addition, current components similar to these two have been observed in a variety of neurons in other species (Meech, 1974; Blatz and Magleby, 1987; Rudy, 1988; Latorre et al., 1989). A noninactivating Ca^{2+} -activated K^+ current has been reported in *Drosophila* embryonic neurons in culture (Byerly and Leung, 1988). Our study indicates that a transient Ca^{2+} -activated K^+ current is likely to be present in *Drosophila* neurons, as well (Fig. 10).

The Co^{2+} -sensitive outward component was clearly reduced in *slo* giant neurons. However, the expression of Ca^{2+} -activated transient K^+ current does not appear uniform (Fig. 10D–F), and the *slo* mutation did not eliminate all Co^{2+} -sensitive outward components in at least a subset of neurons (Fig. 17B). In addition, *slo* appears to affect the late component as well as the early peak of the Co^{2+} -sensitive outward current, contrasting to the selective elimination of the transient Ca^{2+} -activated K^+ current in muscle. Recent DNA sequence analysis indicates that the *slo* gene codes for a channel polypeptide (Robertson et al., 1991). However, a previous study demonstrates a lack of clear gene-dosage effect of the *slo* mutations (Komatsu et al., 1990), raising the possibility that the Ca^{2+} -activated K^+ channel may contain additional subunits from different genes. Therefore, discrepancies in *slo* effects on nerve and muscle could be explained by the *slo* gene product being utilized differently in neurons and muscle cells.

Current-injection experiments suggest that, in cells generating all-or-none action potentials, *slo* prolonged the action potential, especially when the A-type current was inactivated (Fig. 19). Similar conclusions have been drawn from previous intracellular recordings in adult (Elkins et al., 1986) and larval (Singh and Wu, 1990) muscles of *slo* mutants. Complex regenerative activity was observed in some *slo* giant neurons (Fig. 18A), suggesting that the lack of Ca^{2+} -activated K^+ current may result in distinct activity patterns under different circumstances. In principle, differential distribution of membrane channels mediating various inward and outward currents in different subcellular regions can give rise to complex electric interactions. It will be interesting to determine whether the channels affected by *slo* have a nonuniform distribution (e.g., neurites vs. somata, as suggested by Fig. 18B₃).

The present study indicates that this giant neuron system is suitable for physiological, pharmacological, and cell biological analyses. Further studies of *slo* and additional mutants using giant neurons may provide insights into mechanisms of channels and related proteins, their expression in subcellular regions, and their roles in determining membrane excitability.

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