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Voltage-Gated Currents and Firing Properties of Embryonic *Drosophila* Neurons Grown in a Chemically Defined Medium

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SUMMARY

This study reports the composition of a chemically defined medium (DDM1) that supports the survival and differentiation of neurons in dissociated cell cultures prepared from midgastrula stage *Drosophila* embryos. Cells with neuronal morphology that stain with a neural-specific marker are clearly differentiated by 1 day *in vitro* and can be maintained in culture for up to 2 weeks. Although the whole cell capacitance measurements from neurons grown in DDM1 were 5- to 10-fold larger than those of neurons grown in a conventional serum-supplemented medium, the potassium current densities were similar in the two growth conditions. A small but significant increase in the sodium current density was observed in the neurons grown in DDM1 compared with those in serum-supplemented medium. The majority of neurons

grown in DDM1 fired either single or trains of action potentials in response to injection of depolarizing current. Contributing to the observed heterogeneity in the firing properties between individual neurons grown in DDM1 was heterogeneity in the levels of expression and gating properties of voltage-dependent sodium, calcium, and potassium currents. The ability of embryonic *Drosophila* neurons to differentiate in a chemically defined medium and the fact that they are amenable to both voltage-clamp and current-clamp analysis makes this system well suited to studies aimed at understanding the mechanisms regulating expression of ion channels involved in electrical excitability. © 1995 John Wiley & Sons, Inc.

Keywords: ion channels, *Drosophila*, action potentials, defined medium, sodium currents.

INTRODUCTION

As in the mammalian nervous system, the electrical properties of *Drosophila* nerve and muscle cells are determined by the number, type, and distribution of voltage-gated ion channels within their membranes. A large number of *Drosophila* genes involved in controlling membrane excitability have been identified and studied through the use of mutants that alter the excitability of muscle cell membranes (Wu and Ganetzky, 1992). However, the role of genes uniquely expressed in neurons, such as sodium channel genes, have been more

difficult to assess because of the small size and inaccessibility of *Drosophila* neurons *in vivo*. Initial studies relied on identification of mutations that resulted in alterations in action potential conduction in the giant fiber system (Siddiqi and Benzer, 1976; Wu et al., 1978; Wu and Ganetzky, 1980) and changes in the number of tetrodotoxin (TTX) and saxitoxin binding sites in neuronal membrane preparations (Hall et al., 1982; Kauvar, 1982; Jackson et al., 1984, 1985a,b). More recently, use of the whole-cell recording technique in primary culture has allowed direct assessment of the effects of genetic mutations on the electrical properties of *Drosophila* neuronal membranes (Wu et al., 1983; Solc et al., 1987; O'Dowd and Aldrich, 1988; Baker and Salkoff, 1990). In particular, neurons differentiating in cultures prepared from midgastrula stage embryos have been useful in analyzing the func-

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tional role of both *para* and DSC, *Drosophila* genes, which are homologous to vertebrate sodium channel genes (O'Dowd et al., 1989; Germeraad et al., 1992). However, the embryonic culture system used in these studies has a number of limitations: the small size of neurons results in rapid internal perfusion with the solution in the patch pipet; neurons are typically clustered in groups of four or more cells, making individual neurons difficult to identify; and whole cell currents are small in amplitude, limiting the resolution of electrophysiological recordings.

These problems have been partially overcome in a culture system of giant *Drosophila* neurons differentiated from cell division-arrested embryonic neuroblasts (Wu et al., 1990). Some of the cells in this culture system were shown to have very large soma diameters, express sodium and calcium currents in addition to various potassium currents, and fire overshooting action potentials (APs) (Saito and Wu, 1991). To develop a system that combines the advantages of primary embryonic cultures and cytochalasin B-treated cultures, we experimented with media formulations that would support differentiation of single, large neurons in primary cultures from midgastrula stage *Drosophila*. These efforts focused on chemically defined media formulations that would be useful in future studies aimed at distinguishing between epigenetic (Hewes and Truman, 1994) and genetic factors that might influence the development of electrical membrane properties. The morphological and electrophysiological properties of large embryonic *Drosophila* neurons grown in a chemically defined medium are characterized in the present study.

METHODS

Cell Culture

Midgastrula stage embryos collected from Canton-S wild-type flies were dechorionated by immersion for 5 to 7 min in a 50% solution of bleach and rinsed in sterile water. Cells were removed from dechorionated embryos immersed in sterile medium with a sharp micropipet, and dispersed into drops of medium on uncoated glass coverslips. Cultures were grown in one of two media conditions: (1) Serum-supplemented medium that consisted of modified Schneiders medium (GIBCO) supplemented with 20% fetal calf serum; 50 $\mu\text{g/ml}$ insulin, or (2) *Drosophila* defined medium 1 (DDM1) that contained Ham's F-12 Delbecco's minimum essential medium DMEM (high glucose) supplemented with 1 mg/ml sodium bicarbonate; 20 mM *N*-2-hydroxyethyl-pepera-

zine-*N*-2-ethanesulfonic acid, 100 μM putrescine, 50 nM hydrocortisone, 30 nM sodium selenite, 20 ng/ml progesterone, 50 $\mu\text{g/ml}$ insulin, and 100 $\mu\text{g/ml}$ transferrin. Cultures were maintained in a 5% carbon dioxide environment at 22° to 26°C for up to 2 weeks.

Cell Counts

Cell counts were obtained from cultures prepared from single embryos grown in either serum-supplemented medium or DDM1. At 4 days, the cultures were fixed in 4% paraformaldehyde for 30 min at room temperature followed by a 1 h incubation with fluorescein-conjugated anti-horseradish peroxidase (HRP) antibodies (1:100, Organon Teknika). Coverslips were mounted on glass slides and neurons counted on a Nikon Optiphot fluorescence microscope.

Electrophysiological Recordings

For physiological experiments, coverslip cultures were prepared at a density of two embryos per coverslip. Coverslips were transferred to a recording chamber mounted on a Nikon TMS inverted microscope and cells were visualized with Hoffman optics at a magnification of $\times 600$. Electrophysiological recordings were obtained using the whole cell configuration of the patch-clamp technique (Hamill et al., 1981). Recording pipettes had open pipette resistances of 2 to 5 M Ω . Composition of both internal and external recording solutions is listed in Table 1. Following formation of a high-resistance seal, the capacitance associated with the electrode and patch was subtracted electronically prior to rupture of the patch. On breaking into the cell, the whole cell capacitance was determined by integrating the area under the capacitative transient in a current record obtained by averaging the current responses elicited by 10 depolarizing voltage steps from -70 to -60 mV. Data were collected and analyzed using a List EPC-7 patch-clamp amplifier, a Dell 386 computer, and P-clamp software (Axon Instruments, version 5.5.1). All recordings were performed at room temperature.

RESULTS

A media formulation similar to one originally described by Bottenstein and Sato (1979) for growth of mammalian cells was found to support differentiation of cells in cultures prepared from midgastrula stage *Drosophila* embryos. The cultures could be routinely maintained for up to 2 weeks in this chemically defined medium (DDM1) in a 5% carbon dioxide environment at 22° to 26°C. These cultures contained a large number of differentiated cells, the majority of which appear to be neuronal

Table 1 Ionic Composition of Both Internal and External Solutions Used for Electrophysiological Recordings

	External Solution Composition (mM)								
	NaCl	MgCl ₂	HEPES	CaCl ₂	KCl	CoCl ₂	CdCl ₂	TEACl	TTX
AP	140	4	5	1	3	—	—	—	—
I _K ⁺	140	4	5	1	3	1	—	—	—
I _{Na} ⁺	140	4	5	1	3	1	—	4	—
	140	4	5	1	3	—	2	4	—
I _{Ca} ⁺⁺	140	4	5	1–5	3	—	—	—	10 ⁻⁷ M

	Internal Solution Composition (mM)									
	KGluc	KCl	KF	CsGluc	CsCl	CsF	HEPES	EGTA	MgCl ₂	CaCl ₂
AP, I _K ⁺	120	20	—	—	—	—	10	1.1	2	0.1
	—	70	70	—	—	—	10	1.1	2	0.1
I _{Na} ⁺ , I _{Ca} ⁺⁺	—	—	—	120	20	—	10	1.1	2	0.1
	—	—	—	—	70	70	10	1.1	2	0.1

All solutions were adjusted to pH 7.2 by addition of sodium hydroxide, potassium hydroxide, or cesium hydroxide. *Abbreviations:* CaCl₂ = calcium chloride; CdCl₂ = cadmium chloride; CoCl₂ = cobalt chloride; CsCl = cesium chloride; CsF = cesium fluoride; CsGluc = cesium gluconate; EGTA = ethylene glycoltetraacetic acid; KCl = potassium chloride; KF = potassium fluoride, KGluc = potassium gluconate; MgCl₂ = magnesium chloride; NaCl = sodium chloride; TEACL = tetraethylammonium chloride.

as judged from their morphology [Fig. 1(A)]. In addition, more than 90% of the differentiated cells in these cultures stained with fluorescently conjugated antibodies to HRP, which bind specifically to neurons (Jan and Jan, 1982). These findings suggest that DDM1 supports the growth and differentiation of neuronal cells, but it was not permissive for the growth of non-neuronal cell types. Although other media formulations were not explored extensively, it was noted that neurons could differentiate in DDM1 minus insulin. However, these cultures did not look as healthy as those grown with insulin and, therefore, all of the data reported in this study were obtained from cells grown in DDM1 that included 50 µg/ml insulin. In contrast to cultures grown in DDM1, serum-supplemented growth conditions supported differentiation of a variety of cell types, including neurons and myotubes [Fig. 1(B)]. One striking difference in the morphology of neurons grown in the two different media conditions was the level of fasciculation of the neuronal processes [Fig. 1(C,D)]. The neurons in serum-supplemented medium showed a high degree of fasciculation and individual clusters of neurons were connected by bundles composed of many closely apposed neurites [Fig. 1(D)]. In contrast, neurites from cells grown in DDM1 tended to have highly branched processes that extend radially from clusters of neurons with a much lower incidence of contact between processes in the same area [Fig. 1(C)].

The complement of neurons that differentiate in

the two culture conditions was also examined. Cultures prepared for quantitative analyses were plated at a density of one embryo per coverslip and allowed to differentiate for 4 days. The distribution of neurons was assayed by counting separately the number of neurons that were well isolated, appeared in groups of 2 to 4 cells, in clusters of 5 to 10, 11 to 20, 20 to 40, and more than 40; individual counts were summed to obtain a total number of neurons per embryo (Table 2). The total number of neurons per embryo in DDM1 and serum-supplemented medium was similar. However, there was a highly significant increase in the number of isolated neurons or neurons appearing in clusters of 2 to 4 or 5 to 10 cells in DDM1 compared with the serum-supplemented cultures. Examples of large isolated neurons in DDM1 typical of neurons examined electrophysiologically are illustrated in Figure 2.

Electrophysiological Properties of Neurons Grown in DDM1

Previous studies have characterized the properties of voltage-dependent sodium and potassium currents in wild-type embryonic *Drosophila* neurons grown in serum-supplemented medium in terms of their density, voltage dependence, and gating properties. In the present study several parameters obtained from DDM1 neurons were compared with the baseline data available from previous studies in the laboratory for neurons grown in serum-supple-

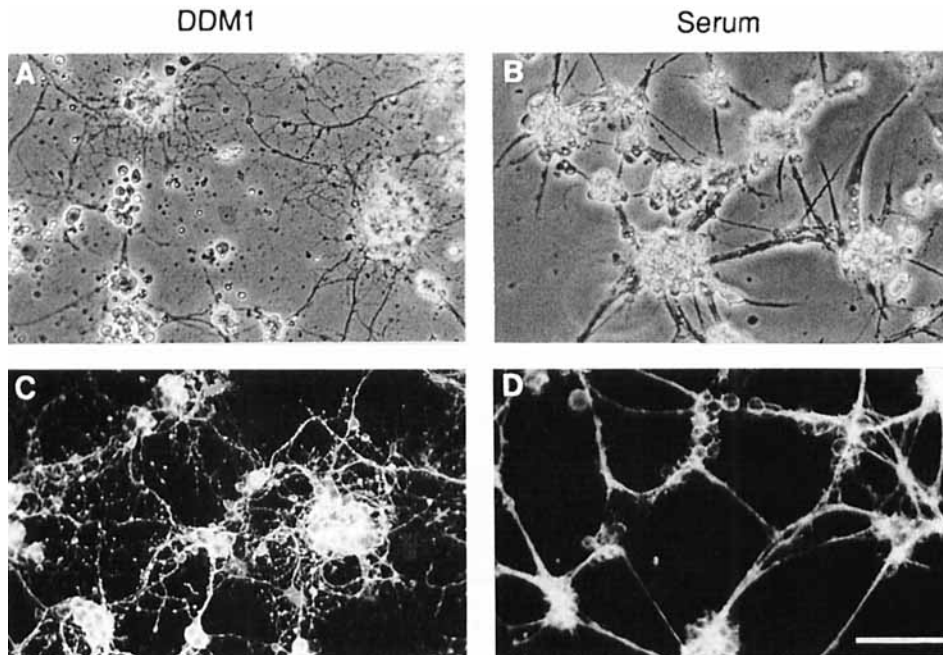


Figure 1 Embryonic *Drosophila* cells grown in DDM1 and serum-supplemented medium. (A) Low-power phase-contrast photomicrograph of embryonic *Drosophila* cells grown in a chemically defined medium (DDM1) for 4 days. Neurons with phase-bright cell bodies and phase-dark processes are generally found in clusters of 2 to 20 cells, but both isolated neurons and larger clusters are also seen. (B) Embryonic cultures grown for 4 days in serum-supplemented medium (+Serum) contain a variety of differentiated cell types, including neurons and elongated multinucleate, myotubes that appear phase-dark. Neurons are found most frequently in clusters ranging from 4 to greater than 40 cells. (C) Low-power fluorescence photomicrograph of a culture grown in DDM1 for 4 days, fixed, and stained with fluorescently conjugated anti-HRP antibodies. The vast majority of cells are fluorescently labeled, confirming their neuronal identity. (D) Cultures grown for 4 days in serum-supplemented medium also contain a large number of cells that are fluorescently labeled with anti-HRP antibodies. Note the high degree of fasciculation of the neuritic processes in the serum-supplemented medium. Scale bar = 50 μm .

mented medium (O'Dowd and Aldrich, 1988; O'Dowd et al., 1989) to determine whether the differentiation of electrical properties was altered by the growth conditions. A similar percentage of neurons displayed voltage-gated sodium and potassium currents when grown in either DDM1 or serum-supplemented medium [Fig. 3(A)]. In contrast, the average whole cell capacitance of neurons grown in DDM1 showed a much wider range and a fivefold larger mean value than neurons grown in serum-supplemented medium (11.4 ± 0.7 pF vs. 2.3 ± 0.05 pF (mean \pm SEM), respectively) [Fig. 3(B)].

Approximately 25% of the neurons expressed sodium currents that were under good voltage control defined by smoothly graded activation of current amplitude and kinetics over a 30 mV voltage range as illustrated in Fig. 3(C). Further biophysical analysis of sodium currents was restricted to this population of cells. The peak sodium current amplitudes recorded from neurons cultured in

DDM1 were much larger than those recorded from neurons grown in serum-supplemented medium [Fig. 3(C)]. To determine if there was a difference in the sodium current density, current amplitudes were normalized to whole cell capacitance and average current density-voltage curves for the population of neurons grown in DDM1 and serum-supplemented medium were compared [Fig. 3(D)]. Sodium current density in cells grown in DDM1 was approximately 60% larger than in neurons grown in serum-supplemented medium [Fig. 3(D)]. However, the shape of the two curves were similar, indicating that the voltage dependence of these currents was not altered by two different growth conditions.

The majority of neurons grown in both media conditions expressed a combination of transient and sustained potassium currents during prolonged depolarizing voltage steps. As seen with the sodium currents, the absolute size of the potassium

Table 2 All Cell Counts Were Performed on Cultures Prepared from Single Embryos Grown for 4 Days in Either Defined (DDM1) or Serum-Supplemented Medium

	Neurons/Embryo						Total
	Isolated	2-4	5-10	11-20	21-40	>40	
Serum (n = 27)	4 ± 1	76 ± 14	564 ± 76	860 ± 117	547 ± 87	123 ± 34	2150 ± 281
DDM1 (n = 47)	49 ± 4*	308 ± 17*	789 ± 53 [†]	620 ± 66	163 ± 29*	29 ± 13*	1958 ± 140

Cultures were fixed and stained with fluorescently conjugated anti-HRP antibodies and visualized on a Nikon Optiphot microscope. The number of neurons were determined for each coverslip. These numbers were summed to obtain the total number neurons per embryo. The mean values ± SEM reported in the table were obtained from the indicated number of cultures in the two conditions. Although there was no difference in the total number of neurons differentiating in the two growth conditions, there were significantly more cells appearing in isolation and clumps of two to four cells in DDM1 compared with serum. In contrast, there were significantly fewer cells appearing in large clumps of 21 to 40 or greater than 40 in DDM1 compared with serum.

* $p < 0.001$; [†] $p < 0.05$; Student's t-test.

currents recorded was larger in the population of DDM1 versus serum-supplemented neurons [Fig. 3(E)]. Comparison of average current density versus voltage relationships for the transient and sustained potassium currents indicated that there was no significant difference between cells in the two growth conditions [Fig. 3(F)]. Thus, with the ex-

ception of the slightly higher sodium current density, the electrical membrane properties of the large neurons grown in DDM1 were similar to those of smaller neurons that differentiated in the serum-supplemented cultures.

Heterogeneity in Electrical Membrane Properties of Embryonic Neurons in DDM1

Action Potentials APs were elicited in 69% (44 of 64) of the neurons grown in DDM1. Neurons capable of regenerative responses were divided approximately equally between the following two categories: single spiking (SS), which fired only a single action potential [Fig. 4(A)] and multiple spiking (MS), which produced more than one AP, in response to prolonged suprathreshold depolarizing current injection [Fig. 4(B,C)]. AP amplitude in these neurons was measured from threshold, defined by the inflection point in the rising phase of the impulse, to peak voltage. This method was chosen because injection of depolarizing current, in many cases, resulted in a large membrane charging curve on which small amplitude regenerative events were superimposed. Measuring AP amplitude from holding or resting membrane potential would greatly overestimate the size of these impulses. In SS neurons, the AP amplitude varied widely, ranging from 9 to 52 mV, with an average of 18 ± 2 mV (mean ± SEM, $n = 24$). Duration of the AP measured at half amplitude was also variable, ranging from 1.6 to 16 ms, averaging 6.1 ± 1 ms (mean ± SEM, $n = 24$). In some of the MS cells, the amplitude of the individual spikes in the train varied and there was often a pronounced delay between the

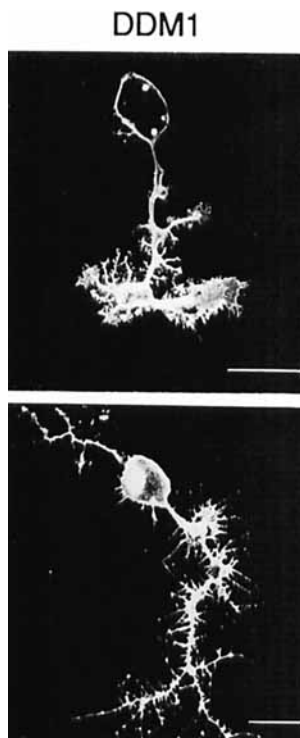


Figure 2 Morphology of large isolated embryonic neurons grown in DDM1. High-power photomicrographs of embryonic *Drosophila* neurons stained with fluorescently conjugated anti-HRP antibodies. Note large cell bodies, broad flat growth cones, and extensive filopodia. Scale bars = 25 μ m.

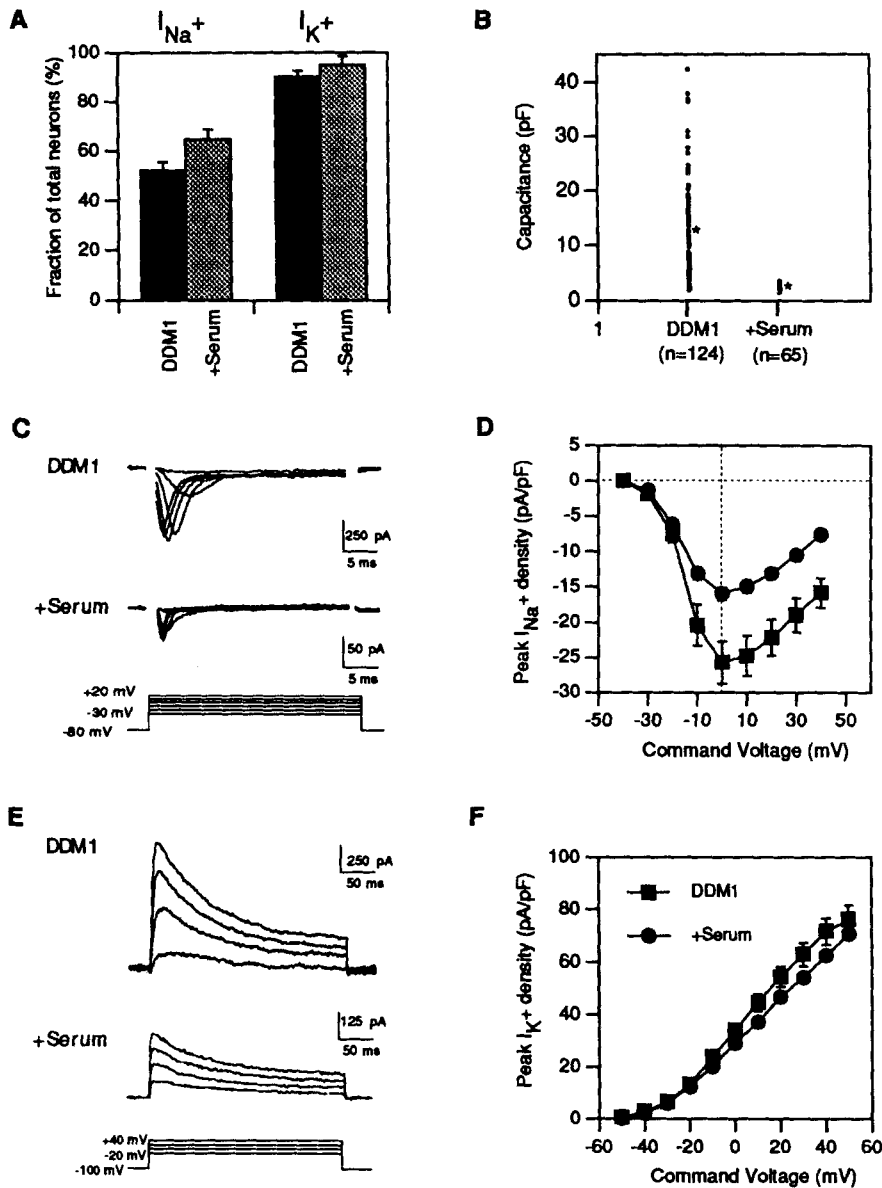


Figure 3 Comparison of neurons grown in DDM1 with those grown in serum-supplemented medium. (A) The fraction of the total number of neurons sampled expressing sodium or potassium currents was calculated on a per plating basis. The mean percent was calculated from data obtained from 84 platings in DDM1 and 51 platings in serum-supplemented medium, (+Serum). (B) Scattergram of individual whole cell capacitance values recorded from neurons in DDM1 or serum-supplemented medium. The mean whole cell capacitance of neurons grown in DDM1 was 5 times larger ($p < 0.0001$, Student's t test) than those grown in serum-supplemented medium (*mean). (C) Isolated sodium currents recorded from a neuron cultured in DDM1 and a neuron cultured in serum-supplemented medium. Note the much larger amplitude of sodium currents in DDM1. (D) Mean current density versus voltage curves were compiled from 36 neurons grown in DDM1 versus 46 in serum-supplemented medium. A 60% increase is observed in the peak sodium current density in the cells grown in DDM1 compared with those grown in serum-supplemented medium. The two curves are similar in shape, indicating that the voltage dependence of these currents has not been altered by the growth conditions. (E) Family of potassium currents elicited from a neuron grown in DDM1 and from a neuron grown in serum-supplemented medium. (F) Mean transient potassium current density versus voltage curves were compiled from 88 neurons in DDM1 and 19 neurons in serum-supplemented medium. Note that the cells in DDM1 are significantly larger than those grown in serum-supplemented medium, but there is no difference in the density versus voltage curve. Bars indicate SEM.

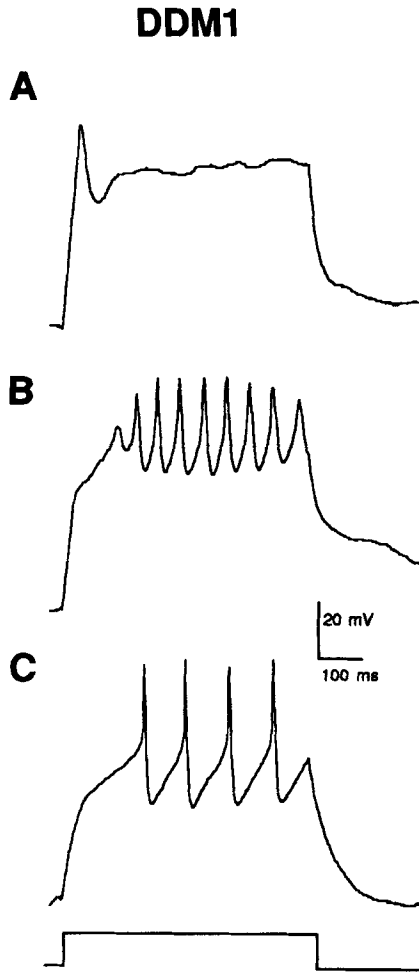


Figure 4 APs elicited from neurons grown in DDM1. (A) Example of a single spiking cell (SS) in which injection of a sustained suprathreshold depolarizing current pulse gives rise to only a single AP. (B) Example of a multiple spiking cell (MS) in which injection of a sustained depolarizing current pulse gives rise to a spike train in which the amplitude of the individual APs vary. Note delay between the onset of depolarizing current and firing of the first spike. (C) A second type of MS cell in which each AP in the spike train has a similar amplitude and duration. All three cells were grown in DDM1 and illustrated AP trains were elicited from a holding potential of -70 mV.

onset of the stimulus and appearance of the first spike [Fig. 4(B)]. In other MS cells all of the APs were of similar amplitude and duration [Fig. 4(C)].

Voltage-Gated Sodium Currents Not only was there heterogeneity in AP phenotypes among individual neurons grown in DDM1, but there was also a large variability in both current density and gating properties in the underlying sodium currents.

All quantitative measurements were obtained from cells in which the sodium currents were under good voltage control. The most prominent sodium current expressed was transiently activated during a 15 ms depolarizing voltage step with the peak of the current-voltage relationship near 0 mV [Fig. 5(A)]. Approximately 25% of the cells with transient sodium currents also expressed a sustained inward sodium current [Fig. 5(B)]. The peak density of this sustained current was small, on average $10.6 \pm 1\%$ ($n = 10$) of the transient current density in the same cell. The peak of the I-V relationship for the sustained currents was 5 to 10 mV more hyperpolarized than the transient currents. The apparent difference between the extrapolated reversal potential of the transient and sustained currents is most likely an artifact due to the technical problems of accurately measuring fast (transient) or small (sustained) currents at the more depolarized voltages. Prepulse inactivation protocols demonstrated that, although the transient currents were completely inactivated by an 800 ms step to -10 mV, there was little or no inactivation of the sustained currents [Fig. 5(B)]. It seems unlikely that this sustained inward current might be carried by calcium since the Ringer's solution contained Co^{2+} or Cd^{2+} to block voltage-gated calcium currents. Confirmation that the sustained current is carried by sodium was obtained by addition of 10^{-7} M TTX to the bathing solution which blocks both the transient and sustained sodium currents (Fig. 6). Although TTX blockade of the sodium currents in these neurons is partially reversible (data not shown), the off rate is very slow, as previously noted for sodium currents in *Drosophila* neurons grown in serum-supplemented medium (O'Dowd and Aldrich, 1988).

The variability in the density and gating properties of the transient sodium currents among individual cells is illustrated in Figure 7, where the mean and the standard deviation for each measured parameter are plotted for the population examined. The largest variability was observed in the sodium current density. First, approximately half of the cells examined did not express sodium currents [Fig. 3(A)] and among the cells that did express sodium channels, the peak sodium current density ranged from a high of 80 pA/pF to a low of 4 pA/pF [Fig. 7(A)]. Similar comparisons of the mean values and standard deviations of the measurements for steady-state inactivation [Fig. 7(B)] and time to peak current [Fig. 7(C)] curves illustrated a smaller cell-to-cell variability in these properties.

Voltage-Gated Calcium Currents Inward currents carried through voltage-gated calcium channels were observed in approximately 50% of neurons in

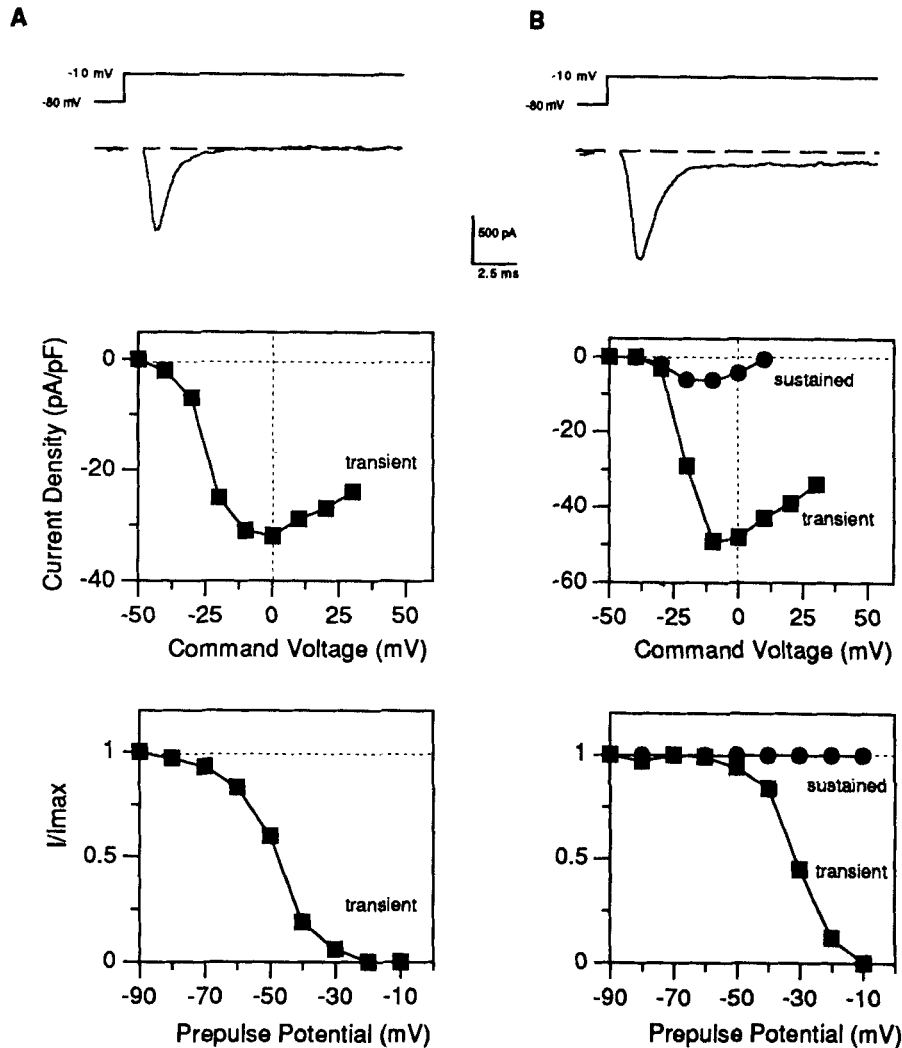


Figure 5 Transient and sustained sodium currents. Sodium currents recorded from two different neurons each grown for 2 days in DDM1. (A) Sodium current from a neuron that displayed only a transient component that decays baseline (dashed line) approximately 5 ms after current activation. Peak current density vs voltage curve and prepulse inactivation profile are shown below. Prepulse duration is 800 ms. (B) Sodium currents from a second neuron that exhibited both a transient and a sustained component that is maintained throughout the 15 ms voltage step. Peak current density versus voltage curve and prepulse inactivation profiles for the transient and sustained components are shown below. Note the low density and more hyperpolarized peak of the I-V curve for the sustained current. The prepulse inactivation profile demonstrates that prepulse voltages that inactivate the transient current almost completely (-20 and -10 mV) do not appear to inactivate the sustained component at all.

which the sodium and potassium currents were blocked ($n = 13$). The peak calcium current density was relatively low, 5 ± 1 pA/pF (mean \pm SEM; $n = 7$) compared to the peak sodium current density of 25.6 ± 3 pA/pF (mean \pm SEM; $n = 36$). Calcium currents shown in Figure 8 illustrate the differences in both the voltage dependence of gating and kinetic properties seen among individual cells. In the first cell [Fig. 8(A)] inward calcium currents acti-

vated around -60 mV and the peak inward current occurred at -40 mV. Calcium currents from a second cell illustrated in Figure 8(B) activated at a much more depolarized voltage and the peak inward current occurred at 0 mV as opposed to -40 mV. Both activation and inactivation kinetics of the calcium currents were markedly slower in cell A than in cell B, suggesting that there are at least two different types of calcium channels expressed

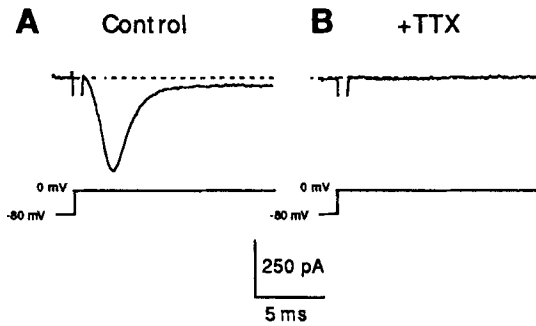


Figure 6 TTX blocks both transient and sustained sodium currents. (A) A dual component sodium current recorded from a neuron grown in DDM1. (B) Addition of 5×10^{-7} M TTX to the bathing solution blocked both the transient and sustained components.

in these embryonic neurons. Both transient and sustained currents recorded under these conditions were blocked by addition of cadmium or cobalt to the external bathing solution.

Voltage-Gated Potassium Current Similar to our findings regarding inward currents, there was a great deal of heterogeneity in the properties of the voltage-gated potassium currents between individual neurons grown in DDM1. For example, striking differences were apparent in the relative proportions of transient to sustained voltage-activated potassium currents present in different cells. Figure 9(A) illustrates current records obtained from a neuron in which the majority of the current was carried through transiently activated potassium channels. A prepulse inactivation profile shown to the right demonstrated that approximately 50% of the channels were inactivated by a prepulse to -65 mV [Fig. 9(A)]. A second cell in which the contribution of transient and sustained potassium components were approximately equal is shown in Figure 9(B). In a third cell the outward current was dominated by potassium flowing through noninactivating potassium channels [Fig. 9(C)]. These results demonstrate that two or more types of voltage-gated potassium channels underlie the macroscopic potassium currents expressed in the embryonic neurons and that the relative abundance of the different channel types varies among individual neurons.

DISCUSSION

This study characterizes the electrical membrane properties of embryonic *Drosophila* neurons

differentiating in a chemically defined medium. Single ectodermal precursor cells of *Drosophila* embryos at an early gastrula stage (stage 7) already have the capability of developing as neuroblasts or epidermoblasts as assayed by their division pattern, morphological phenotype, and expression of cell

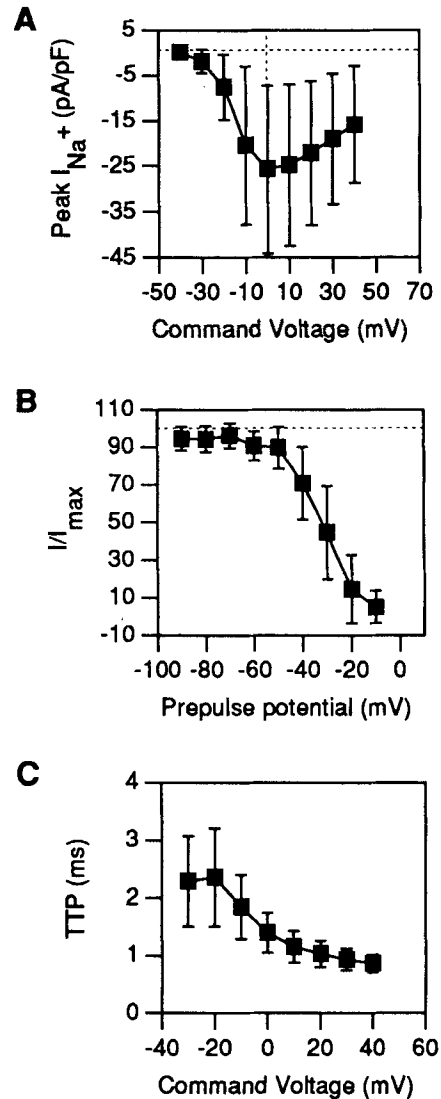


Figure 7 Variability in gating properties and kinetics of transient sodium currents. (A) Range in the peak sodium current density versus voltage curves where population mean values are indicated by the symbols and bars indicate standard deviation for each of the points. The large standard deviation indicates the wide range in the sodium current density observed among individual cells. (B) A smaller range in the prepulse inactivation (800 ms) and the average time to peak current curve for the transient sodium current (C) was observed. Values obtained from 36 individual cells in which sodium currents were under good voltage control. Bars indicate standard deviation.

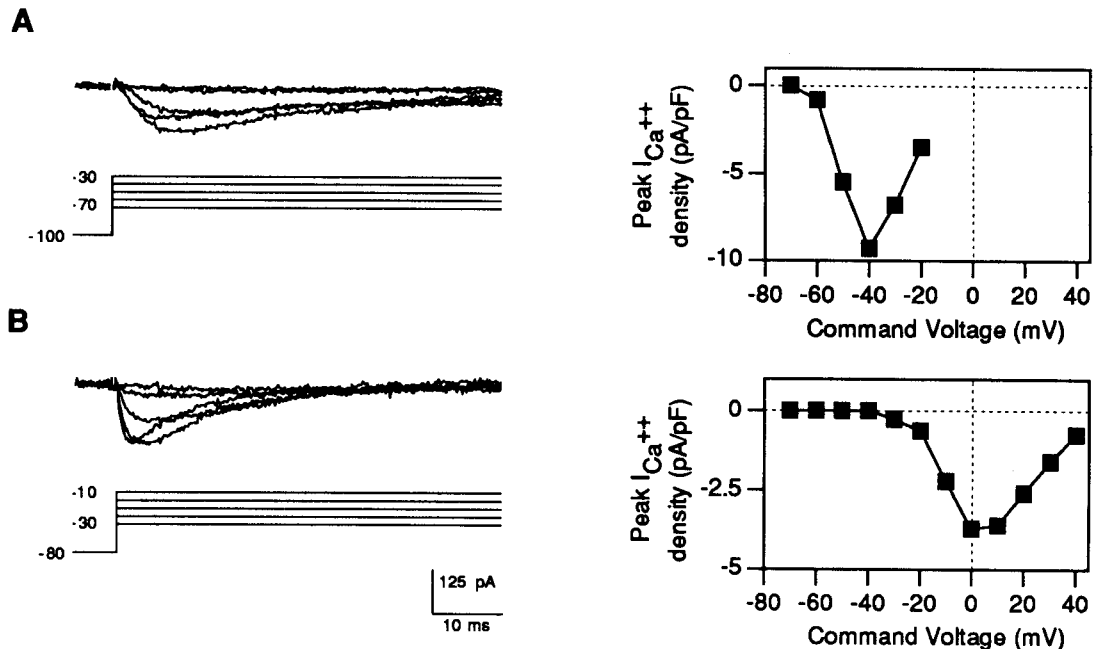


Figure 8 Calcium currents with distinct voltage-dependent and kinetic properties. (A) Slowly activating and inactivating macroscopic calcium currents recorded from a neuron in DDM1. Current density versus voltage curve to right demonstrates a low activation threshold for this current (-60 mV) with a peak around -40 mV. (B) Macroscopic calcium currents recorded from a second cell in DDM1 that activates and inactivates more rapidly than the first cell. As shown in the I-V curve to the right, the current in this cell also activates at a much more depolarized voltage and peaks around 0 to 10 mV.

specific antigens, when grown in primary culture in a serum-based, conditioned medium (Luer and Technau, 1992). Our results extend these findings in several ways. First, we have shown that differentiation of embryonic *Drosophila* neurons can proceed in the absence of growth factors or other agents present in serum supplemented or conditioned media. Although a previous study has reported that embryonic cells can survive in a chemically defined medium, they found that extracts from adult *Drosophila* were necessary for normal proliferation and differentiation (Wyss, 1982). Second, because very few non-neuronal cells are present in DDM1 cultures, it seems likely that factors secreted into, or removed from, the medium by other cell types are not required for differentiation. Analysis of the electrophysiological properties of individual neurons grown in separate wells, as has been done in the case of embryonic *Xenopus* neurons (Henderson and Spitzer, 1986), will be necessary to determine how factors released by other neurons might influence the development of individual neuronal properties. Finally, we have shown that the electrophysiological properties of these neurons can develop in the absence of physical

contact with other cells in culture, either neuronal or non-neuronal. Cell-cell interactions are important in differentiation of neuroblasts from the early neuroectoderm in insect embryos (Doe and Goodman, 1985; Technau and Campos-Ortega, 1986; Chu-LaGriff and Doe, 1993; Doe and Technau, 1993) and for specification of photoreceptors in the *Drosophila* visual system (Reinke and Zipursky, 1988; Tomlinson, 1988; Kramer and Cagan, 1994). If similar intercellular interactions are important in determining the electrophysiological properties of the neurons that we have described in this study, they must occur at, or prior to, midgastrulation (stage 7c-8a, 4-5 hours of development at 25°C) when the cells are removed from the embryo and dissociated.

The larger sodium current densities observed in neurons grown in DDM1 when compared with neurons differentiating in serum-supplemented medium could reflect differences in several parameters, including an increase in the number of channels, a higher probability that the channels will open in response to a given depolarization, or an increase in the number of the channels localized at or near the cell body. The observation that the

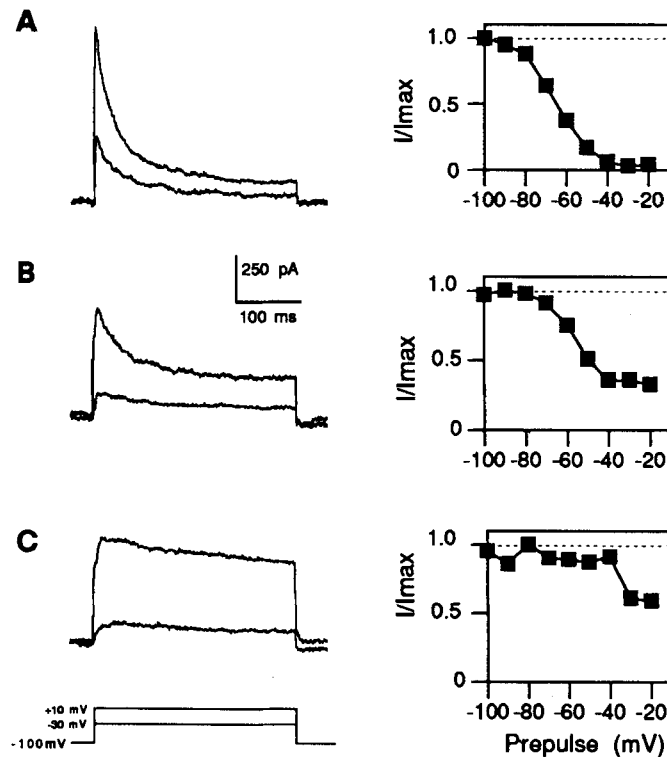


Figure 9 Variability in the ratio of transient and sustained potassium currents between individual cells. Macroscopic potassium currents recorded from three different cells grown in DDM1 in response to voltage steps to -30 and 10 mV from a holding potential of -100 mV. Prepulse (500 ms) inactivation profile from each neuron shown to the right. (A) Potassium currents dominated by a transient component with half the channels inactivated by prepulses to -65 mV. (B) Potassium currents with approximately equal contributions of transient and sustained components. (C) Potassium currents dominated by a sustained component. Prepulses up to -40 mV do not significantly inactivate this current.

function of rat brain sodium channels is modulated by both cyclic adenosine monophosphate-dependent (Gershon et al., 1992; Li et al., 1992; Smith and Goldin, 1992) and protein kinase C-dependent phosphorylation (Numann et al., 1991; West et al., 1991; Li et al., 1993), also raises the possibility that a growth condition-related change in the basal phosphorylation state of the sodium channels could underlie the differences observed in whole cell current density. Since there have been no recordings of sodium currents from embryonic *Drosophila* neurons *in vivo*, we do not know if the current density in the neurons grown in DDM1 or serum-supplemented medium is more representative of neurons developing *in situ*.

There was no significant difference in the density of either the transient or sustained voltage-gated potassium currents in neurons grown in the two media conditions. The similar current densities observed in the neurons grown in DDM1 that are five to six times larger than those grown in se-

rum-supplemented medium suggest a tight regulation between membrane surface area and the number of active channels under these two different growth conditions. This is in contrast to cytochalasin B-treated neurons, in which the average sodium and potassium current densities reported are significantly smaller than is observed in either serum-supplemented medium or DDM1 growth conditions (Saito and Wu, 1991) (Figs. 6 and 8). It is possible that the intracellular machinery that regulates channel density is disrupted by the exposure to cytochalasin B. Interestingly, the mean calcium current density appears to be similar in the cytochalasin B-treated neurons (Saito and Wu, 1991) and the large DDM1 neurons reported here.

The majority of neurons grown in DDM1 were capable of firing APs in response to depolarizing current injection. Although cells are classified into two groups based on the ability to fire either one or trains of APs, there is heterogeneity within these groups based on AP duration, amplitude, and fir-

ing frequency. The range of firing patterns in our study is similar to those previously reported for the cytochalasin B-treated giant neurons (Saito and Wu, 1991) as well as responses recorded *in situ* from *Drosophila* (Ikeda and Kaplan, 1970) and from other insect preparations (Ikeda and Kaplan, 1970; Goodman and Heitler, 1979; Burrows, 1987; Christensen et al., 1988; Laurent, 1990).

A high degree of heterogeneity in the density and gating properties of potassium, calcium, and sodium currents was seen among individual cells grown in DDM1. As previously reported for cells grown in serum supplemented medium (O'Dowd et al., 1989), there are both transient and sustained voltage-gated potassium currents expressed in neurons grown in DDM1. Although the majority of neurons express both current types, some have a clearly dominant transient component, whereas others express little transient current relative to the sustained component. Although we did not examine calcium currents extensively, we did note both transient and sustained components of the currents, which had different relative contributions to the whole cell currents in different cells, consistent with previous studies demonstrating multiple calcium channel subtypes in *Drosophila* (Greenberg et al., 1989; Leung et al., 1989; Pelzer et al., 1989). A wide range in the density and a somewhat smaller range in the gating properties of the transient sodium currents between individual neurons was also observed. Some of the neurons grown in DDM1 expressed, in addition to a transient sodium current, a TTX-sensitive sustained inward current, similar to a current characterized in division-arrested embryonic neurons. These findings demonstrate that two or more types of voltage-gated channels are likely to underlie the macroscopic sodium, calcium, and potassium currents expressed in the embryonic neurons and that the relative abundance of the different channel types varies among neurons that contributes to the heterogeneity in individual firing patterns.

What controls the number, type, distribution, and function of ion channels in these cells? To answer questions about the regulation of channel expression at the molecular level, it will be important to identify the genes encoding each of these channel types in the embryonic neurons. Using this culture system and single cell polymerase chain reaction, we have recently shown that alternative splicing of the *para* gene, which encodes functional embryonic sodium channels, is correlated with sodium current expression in these cells (O'Dowd et al., 1995). Further analysis at both the molecular and biophysical level of these primary neurons

should provide important information about the regulation of ion channel gene expression and ultimately how this regulation affects the electrical excitability of individual neurons.

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