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Alterations in the Expression and Gating of Drosophila Sodium Channels by Mutations in the *para* Gene

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Summary

Mutations in the *para* gene specifically affect the expression of sodium currents in Drosophila. While 65% of wild-type embryonic neurons in culture express sodium currents, three distinct mutations in the *para* locus resulted in a decrease in the fraction of cells from which sodium currents could be recorded. This reduction was allele-dependent: macroscopic sodium currents were exhibited in 49% of the neurons in *para*^{ts1} cultures, 35% in *para*^{ts2}, and only 2% in *para*^{ST76}. Voltage-clamp experiments demonstrated that the *para*^{ts2} mutation also affected the gating properties of sodium channels. These results provide convincing evidence that *para*, a gene recently shown to exhibit sequence similarity to vertebrate sodium channel α subunits, encodes functional sodium channels in Drosophila. The finding that one *para* allele (*para*^{ST76}) can virtually eliminate the expression of sodium currents strongly argues that the *para* gene codes for the majority of sodium channels in cultured embryonic neurons.

Introduction

The influx of sodium ions through voltage-gated channels is important in generating action potentials in many excitable cells. Drosophila is a powerful system in which physiological and molecular genetic techniques can be applied to examine structural features of sodium channel proteins involved in gating and to identify factors governing expression of these molecules. Prerequisite to these studies is identification of genes coding for sodium channels in Drosophila. One strategy that has been employed to locate putative ion channel genes has involved physiological analysis of mutants. Because electrical properties of a cell depend on the number, type, and distribution of ion channels in its membrane, these studies have focused on behavioral mutants that alter electrical excitability in nerve and muscle cells (for reviews see Papazian et al., 1988; Ganetzky and Wu, 1986; Salkoff and Tanouye, 1986; Tanouye et al., 1986). The cloning of a gene coding for a voltage-dependent potassium channel from the *Shaker* locus (Baumann et al., 1987; Kamb et al., 1987; Papazian et al., 1987; Timpe et al., 1988; Iverson et al., 1988), originally identified by its hyperexcitable mutant phenotype, demonstrated that mutations affecting excitability can be useful in localizing genes coding for ion channels.

One putative sodium channel gene, *para*, was defined by behavioral mutants exhibiting a reversible temperature-sensitive paralytic phenotype (Suzuki et al., 1971). Although the number of tetrodotoxin binding sites in membrane preparations from adult *para*^{ts} and wild-type flies was similar (Kauvar, 1982), physiological studies of *para*^{ts} flies revealed an increased excitation threshold of sodium-dependent action potentials in larval and adult nerve fibers at elevated temperatures (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980). In addition, cultured larval neurons from *para*^{ts} mutants exhibited a reduced sensitivity to veratridine (Suzuki and Wu, 1984), an alkaloid toxin that depolarizes excitable cells by opening sodium channels (for review see Catterall, 1980). These latter two results suggest that the density or function of sodium channels may be altered by this mutation. The recent cloning of *para* by P-element transposon tagging has also revealed that the *para* gene product shares extensive amino acid sequence similarity with the α subunit of vertebrate sodium channels (Ganetzky and Loughney, 1988, Soc. Neurosci., abstract). The analysis of the role of this gene in expression of functional sodium channels has been prevented by the difficulty of recording isolated sodium currents in Drosophila. However, we have recently described conditions under which it is possible to record voltage-clamped sodium currents from wild-type and mutant Drosophila neurons (O'Dowd and Aldrich, 1988). The present study was aimed at determining whether mutations in the *para* locus specifically affect functional sodium channels.

Our experiments revealed that sodium channel expression in cultured embryonic neurons was altered in three alleles of the *para* locus. All three genotypes were found to reduce the percentage of cells with macroscopic sodium currents, and one, *para*^{ts2}, also affected the voltage dependence of sodium currents expressed. In conjunction with the sequence similarity to vertebrate sodium channels (Ganetzky and Loughney, 1988, Soc. Neurosci., abstract), these results establish that *para* codes for sodium channel proteins expressed in embryonic neurons and demonstrate that these cells provide a favorable system for mutational analysis of sodium channel gating and regulation.

Results

Primary cultures were prepared from Drosophila embryos at midgastrulation (Seecof, 1979). To compare the percentage of cells with sodium current between different mutants and wild type, it was important to know whether the general properties of the cultures were similar. Cultures made from *para*^{ts1}, *para*^{ts2}, or *para*^{ST76} embryos contained a number of different cell types, including neurons. The distribution of cell types and the extent of their morphological differentiation were similar to

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Table 1. Cell Counts in Single Embryo Cultures

Genotype (n)	Number of Neurons	Number of Neurons in Clumps ≤10	Number of Myotube-like Cells	Total Number of Cells
Canton-S (6)	2243 (588)	715 (282)	493 (145)	2831 (678)
$para^{ST76}$ (4)	2505 (1277)	999 (231)	653 (194)	3253 (1121)

These counts represent the mean (± the SD) of half the number of cells differentiated from single mutant and wild-type embryos. There are no significant differences between the mutant and wild-type values in any of these categories.

wild type. The number of neurons, myotube-like cells, and total number of cells were not significantly different when 6 wild-type and 4 $para^{ST76}$ cultures were compared (Table 1). The cells chosen for physiological study were a subset of the total neuronal population and generally occurred in small clusters. Therefore the number of neurons in clusters of 10 cells or less was also counted and found to be similar in wild-type and mutant cultures (Table 1).

Mutations at the *para* Locus Reduce the Percentage of Cells Expressing Sodium Currents

Sodium currents are present in approximately 65% of the neurons in primary cultures of wild-type Drosophila embryos at 24 hr (O'Dowd and Aldrich, 1988). Sodium currents were recorded from neurons in cultures prepared from each of three mutant *para* genotypes. Figure 1 illustrates macroscopic sodium currents recorded from mutant neurons in response to a series of depolarizing voltage steps and the corresponding current-voltage (I-V) relationships. Sodium currents in $para^{ts2}$ mutant neurons were similar to sodium currents recorded from a number of different vertebrate and invertebrate preparations (Hille, 1984; O'Dowd and Aldrich, 1988). The variation apparent in the sodium current amplitudes illustrated in Figure 1 reflects the variations in the size of the cells from which the records were taken. The smaller amplitude sodium currents shown for the $para^{ts1}$ neuron were obtained from a relatively small cell, 1.7 pF, as compared with the wild-type and $para^{ts2}$ neurons, which had whole-cell capacitances of 2.6 and 2.9 pF, respectively. The current records shown for $para^{ST76}$ represent

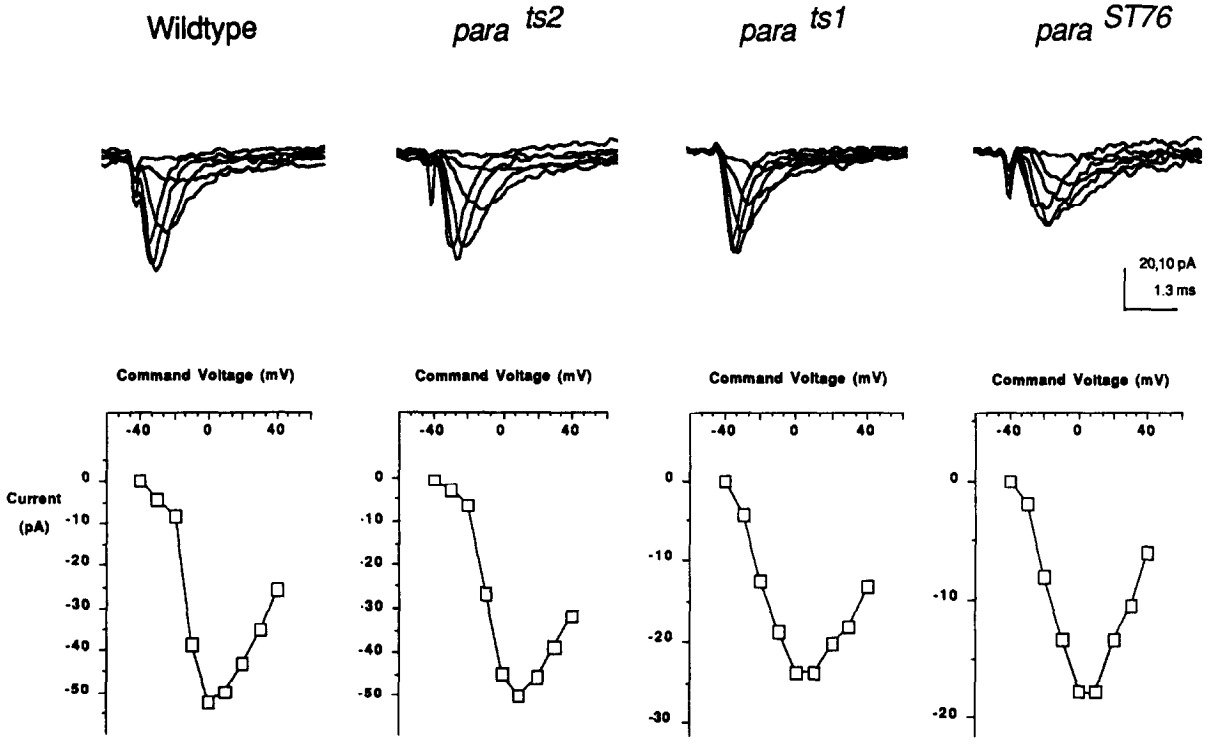

Figure 1. Macroscopic Sodium Currents Recorded from Wild-Type and *para* Mutant Neurons

Transient inward sodium currents were recorded from individual cells of each genotype at approximately 24 hr in culture at room temperature. The currents were elicited in response to a series of voltage steps between −30 and +20 mV from a holding potential of −70 mV. Scale bar is 20 pA for wild type and $para^{ts2}$ and 10 pA for $para^{ts1}$ and $para^{ST76}$. The corresponding I-V curves are shown for each cell.

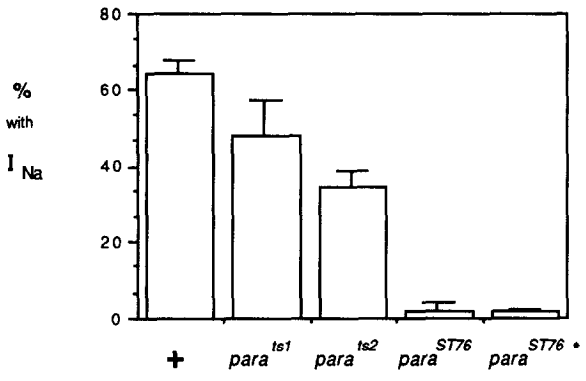

Figure 2. Allele-Dependent Reduction in the Percentage of Cells Expressing Sodium Currents

Each cell from which a stable whole-cell recording was obtained was examined for the presence of sodium currents. A cell expressing macroscopic sodium currents greater than or equal to 5 pA was scored as positive. The percentage of cells expressing sodium currents was determined on a per plating basis, with the minimum of 5 platings used to obtain the $para^{ST76*}$ data and the maximum of 58 platings used to obtain the wild-type data. The number of cells examined in each genotype are as follows: wild type, 225; $para^{ts2}$, 166; $para^{ts1}$, 42; $para^{ST76}$, 46; and $para^{ST76*}$, 39. Each of the *para* alleles exhibited a reduction in the proportion of cells with sodium currents.

Bars indicate SEM.

the sodium currents obtained from the only cell in this genotype that expressed sodium currents, and the density as well as the amplitude of the currents in this cell was small. The number of cells examined in the $para^{ST76}$ and $para^{ts1}$ genotypes was not sufficient to resolve differences in peak current density with respect to wild type.

Though sodium currents could be recorded from *para* neurons, the three *para* alleles examined exhibited a significant reduction in the fraction of neurons with sodium currents when compared with wild type (Figure 2). Given that the percentage of cells expressing sodium current was low in each of the *para* mutants examined, it seemed likely that mutations in the *para* gene were responsible for this change. However, the extreme phenotype of $para^{ST76}$ (1 out of 46 cells with sodium currents) led us to question whether the genetic background of these flies was partially responsible for the dramatic reduction in the proportion of cells with sodium currents. Previous work has demonstrated that mutations on other chromosomes can affect the phenotype of some *para* alleles (Ganetzky, 1984, 1986). Therefore male flies from the original $para^{ST76}$ stock were outcrossed for two generations to attached-X females and then made homozygous through a balancer stock to dilute out the contribution of possible second or third chromosome modifiers. Examination of the new stock of flies, designated $para^{ST76*}$, revealed a paralytic behavioral phenotype indistinguishable from $para^{ST76}$ (see section on elevated temperatures) and a similar low percentage of cells with sodium currents, 1 out of 39 (Figure 2). The lack of an increase in the percentage of cells with sodium currents in the outcrossed stock argues that the extreme phenotype of $para^{ST76}$ neurons is not due to modifiers on the autosomes, but it does not rule out the possibility that there may be additional mutations on the X chromosome which interact synergistically with $para^{ST76}$.

Alterations in Sodium Channel Gating in $para^{ts2}$ Mutants

In addition to changes in the number of cells expressing sodium currents, we were also interested in determining whether mutations at the *para* locus could cause alterations in the gating of sodium channels. Though the percentage of $para^{ts2}$ neurons that express sodium currents was low compared with wild type, the number of cells examined was large enough that channel gating could be studied. The number of cells from which voltage-dependent sodium currents have been recorded under conditions of adequate voltage clamp in $para^{ts1}$ (4) and $para^{ST76}$ (1) was too small to determine whether these mutations alter sodium channel gating. The drastic reduction in the number of cells with sodium currents makes analysis of sodium channel gating impractical in the case of $para^{ST76}$. We have therefore concentrated our analysis on sodium currents in $para^{ts2}$ neurons.

The voltage dependence, time to peak, and decay time constant of sodium currents generated by depolarizing voltage steps were analyzed along with the voltage dependence of prepulse inactivation. Because the amplitude of the sodium currents was quite small and current density and kinetics were variable in individual cells within each genotype, comparisons were made between mean values obtained from populations of wild-type and $para^{ts2}$ neurons. Data were obtained from cells of similar size in the two genotypes based on measurements of whole-cell capacitances, which were 2.5 ± 0.5 and 2.4 ± 0.4 pF for wild type and $para^{ts2}$, respectively.

Examination of the peak I-V relationship revealed a lower current density at each of the voltages between −30 and 0 mV in $para^{ts2}$ (n = 23) compared with wild type (n = 31) neurons (Figure 3A). At the higher voltages, the mutant and wild-type values begin to converge and the differences, evaluated in a point by point comparison, were not significant above +10 mV. There was also a shift of the mutant curve to more positive voltages, with the maximal current density occurring at +10 mV for $para^{ts2}$ as opposed to 0 mV for wild type. A number of possible changes in channel properties could underlie the alterations observed in the $para^{ts2}$ I-V relationship. The peak current elicited at each voltage is described by the equation $I = NP\gamma(V - E_{Na})$, in which N is the number of channels, P describes the probability of the channel being open, γ is the single-channel conductance, and $V - E_{Na}$ is the driving force on sodium ions. Alterations in the I-V relationship could in principle result from a change in any or all of the above parameters.

As the reversal potentials of the sodium currents in the $para^{ts2}$ neurons studied were similar to wild type, alterations in the I-V curve could not be attributed to changes in the driving force on sodium ions ($V - E_{Na}$). We examined the conductance-voltage (g-V) relationship to

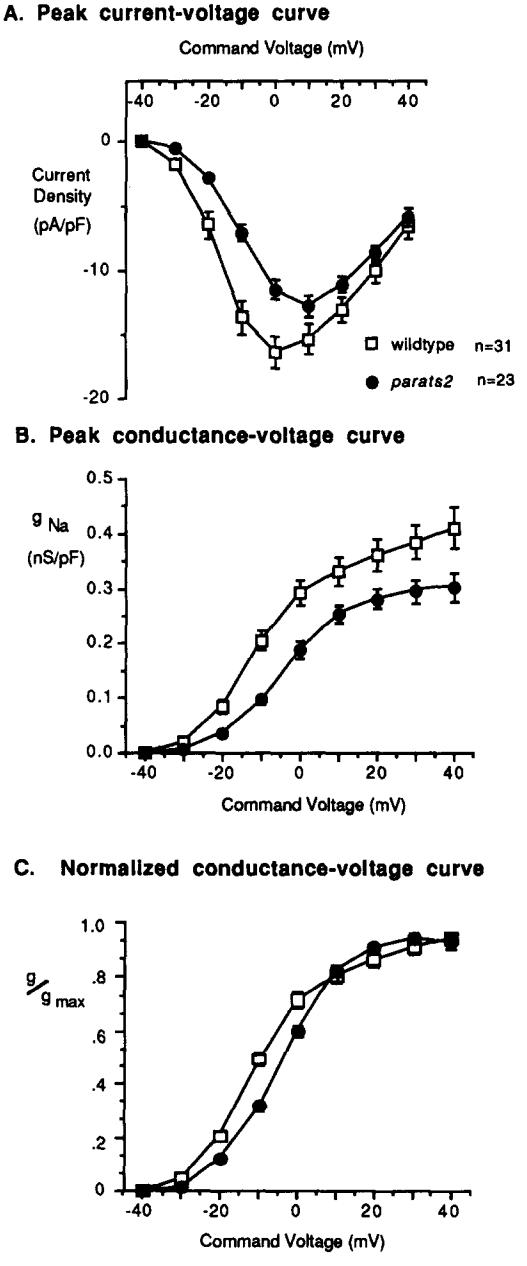

Figure 3. Alteration in the Gating Properties of Sodium Currents in *para*^{ts2} Neurons

(A) Sodium currents normalized to whole-cell capacitance were used to generate the peak sodium current I–V relationships. These curves represent the averaged data obtained from 23 *para*^{ts2} and 31 wild-type neurons. The values for mutant and wild type, between −30 and 0 mV, when evaluated in a point by point comparison, are significantly different at the $P < 0.05$ level. The mutant curve is shifted in the depolarizing direction, with the maximal current density value occurring 10 mV more positive than wild type.

(B) Peak g–V curves were constructed using the equation $g = I/(V - E_{rev})$, assuming a linear open channel I–V and using the E_{rev} extrapolated from the I–V curves for the individual cells. The *para*^{ts2} values at each voltage were significantly smaller than the wild-type values ($P < 0.04$).

(C) Normalized g–V curves were generated by expressing the conductance at each voltage as a function of the maximal conductance, on a cell by cell basis. These values were then averaged and plotted for the 23 *para*^{ts2} and 31 wild-type neurons examined. The half-maximal conductance in *para*^{ts2} neurons occurs 5–7 mV more positive than wild type. The normalized conductance values obtained from *para*^{ts2} neurons are significantly different from those obtained from wild-type neurons at −20, −10, and 0 mV ($P < 0.005$).

Bars indicate SEM; those in (C) are smaller or just slightly larger than the symbols.

determine whether the peak probability of a channel being open as a function of voltage was altered in the mutants. These curves were generated using the equation $g = I/(V - E_{Na})$, assuming a linear open channel I–V relationship. The g–V curves illustrated in Figure 3B reveal a reduction in peak conductance of mutant cells at each voltage compared with wild type. If a decrease in the number of channels (N) or in the single-channel conductance (γ) were responsible for the reduction in maximal conductance in the mutant cells, then the values should be decreased by a constant factor and the curves should superimpose when scaled to the same maximal conductance. Normalized g–V curves for cells in both genotypes were generated by expressing the conductance values at each voltage as a percentage of the cell's maximal conductance. As shown in Figure 3C, a shift of 5–7 mV in the positive direction at half-maximal conductance is seen in *para*^{ts2} mutants. From this result it is clear that alterations in the in g–V and I–V curves are not simply due to a decrease in channel number (N) or conductance (γ). These data are consistent with the *para*^{ts2} mutations causing a change in the voltage-dependent gating (P) of the channels. The results could also be explained by alterations in the open channel properties such as a voltage-dependent decrease in the ability of an open channel to conduct current at voltages more negative than +10 mV. Our data do not distinguish between these possibilities, nor do they rule out additional changes that also involve alterations in the channel number (N) or single-channel conductance (γ).

The time to peak current and the decay time constant of the sodium currents were shifted slightly in the positive direction in *para*^{ts2} neurons compared with wild type (Figure 4). However, the mean values obtained at most of the voltages were not significantly different when evaluated in a point by point comparison using the Student's t test. In Figure 4 the average voltage dependence of prepulse inactivation is illustrated for 15 wild-type and 13 *para*^{ts2} cells. Smooth curves were fitted by eye to inactivation data from individual cells using the following equations:

$$I/I_o = 1/(1 + \exp((V - V_{½})/Y))$$

$$Y = kT/ze$$

These equations, in which $kT/e = 25.4$ at room temperature, describe the probability of a channel being available for opening (noninactivated) at the end of a 500 ms prepulse (Hodgkin and Huxley, 1952). In wild type, the voltage at which half of the channels were inactivated, $V_{½}$, is -40 ± 1 mV and the equivalent gating charge, z,

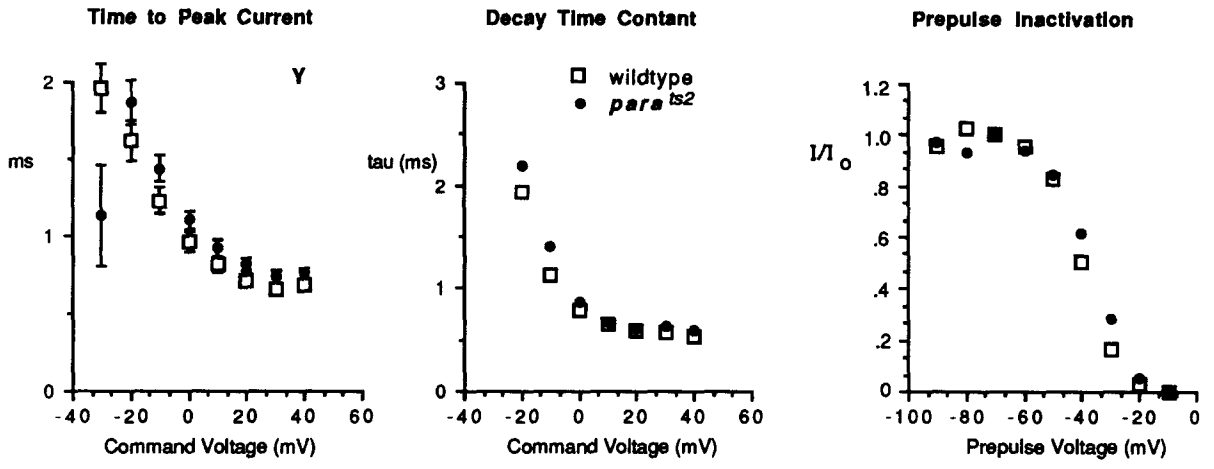

Figure 4. Comparison of Macroscopic Kinetic Properties in $para^{ts2}$ and Wild-Type Neurons

(A) Time to peak current was defined as the time from the onset of the voltage step to the peak of the current. The time to peak current decreases with increasing depolarization for both $para^{ts2}$ and wild type. None of the points on these curves from 25 wild-type and 23 $para^{ts2}$ neurons were significantly different when evaluated in a point by point comparison.

(B) The time constants of single exponential fits to the decaying phase of the transient current were used to describe the macroscopic time course of inactivation. The mutant and wild-type data are very similar.

(C) Prepulse inactivation was assayed by determining the amplitude of the current elicited at 0 mV when preceded by a 500 ms prepulse to voltages between −90 and −10 mV. The mutant and wild-type values were similar over the voltage range examined.

Bars indicate SEM.

is 4.2 ± 0.2. These values were not significantly different from those obtained from $para^{ts2}$, in which $V_{½}$ = −38 ± 1 mV and z = 3.8 ± 0.2.

Elevated Temperatures

The results presented so far have demonstrated that mutations at the *para* locus affect sodium channel expression in cultured embryonic neurons examined at room temperature. Previous studies have documented a temperature-sensitive paralytic phenotype in adult *para* flies (Suzuki et al., 1971), and both adult and third instar larvae exhibit altered neuronal excitability at elevated temperatures (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980). Therefore, we examined the ability of elevated temperatures to induce paralysis in intact animals at early developmental stages (similar to the age of the cultured neurons). Sodium channel gating was also monitored during acute exposure of the cultured neurons to high temperatures.

To determine the temperature dependence of adult paralysis in $para^{ts2}$, $para^{ST76}$, and $para^{ST76*}$ flies, the animals were placed in test tubes, confined to an approximately 2 ml air space with a cotton plug, and immersed in a water bath at a series of elevated temperatures. All of the animals from each of the *para* genotypes tested were immobilized at the bottom of the test tube following a 30 s exposure to temperatures of 35°C or above. The behavior of first instar larvae in two of the genotypes was observed following immersion in Ringer's solution at 35°C and 37°C or placement on agar dishes in an incubator at 35°C. Both wild-type and $para^{ts2}$ larvae remained mobile under these conditions for at least 5 min.

Though elevated temperatures did not appear to affect the behavior of the first instar $para^{ts2}$ and wild-type larvae, cultured neurons from these genotypes were exposed to high temperatures to determine whether alterations in sodium currents, in addition to those noted at room temperature, would be uncovered. Following the formation of a stable whole-cell clamp on an individual neuron in recording solution at 22°C, the temperature was raised, in 5 min or less, to between 27°C and 35°C. Identical voltage protocols were used to elicit sodium currents at both the initial and the elevated temperatures. Sodium currents were recorded from both wild-type and $para^{ts2}$ neurons at up to 35°C, a temperature that induces paralysis in adult flies. In response to voltage steps from −70 to 0 mV, the time to peak current was faster at the higher temperatures in all of the wild-type (n = 5) and $para^{ts2}$ (n = 8) neurons (Figure 5). In some cases the current amplitude also increased (4/5 wild type; 2/8 $para^{ts2}$). In the rest of the cases, the current amplitude either remained unchanged or decreased. Qualitatively, the $para^{ts2}$ sodium currents responded to elevated temperatures in a manner similar to wild type, but due to rapid deterioration of the seal resistance in the majority of the neurons subjected to this treatment, we were not able to analyze the currents quantitatively.

Potassium Currents

Although the physiological evidence demonstrating that mutations in the *para* locus alter sodium current expression is consistent with it being a sodium channel structural gene, it was important to determine whether mutations in the *para* locus could also affect other membrane currents or whether they specifically alter sodium current expression. Therefore, potassium currents in *para*

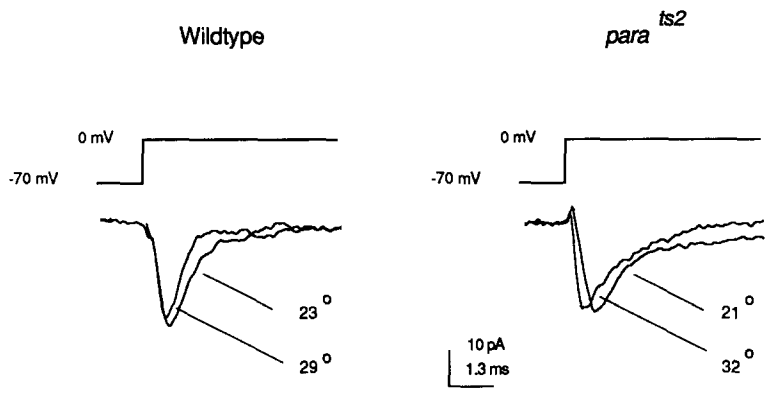

Figure 5. Sodium Currents in Mutant Neurons Respond to Elevated Temperatures in a Manner Similar to Those in Wild-Type Neurons

Following the formation of a whole-cell clamp, sodium currents were recorded from the individual *para*^{ts2} or wild-type cells at low (room) temperature. The currents displayed were elicited in response to voltage steps from −70 to 0 mV. The temperature of the recording solution was then raised, while maintaining the whole-cell clamp, and the current response to an identical voltage step was recorded. Sodium currents were recorded from wild-type and *para*^{ts2} neurons at both low and high temperatures. The time to the peak of the sodium current was faster in both mutant and wild-type neurons at the elevated temperatures, while the amplitude of the currents in these particular cells was not markedly altered.

neurons were compared with those in wild type. As seen in Figure 6, outward currents were recorded from embryonic neurons using a potassium solution in the pipette. Tail currents from both wild-type and *para* neurons at different potentials following a 30 ms pulse to +20 mV reversed between −80 and −90 mV (Figure 6). The equilibrium potential for potassium under these conditions is −97 mV, while the sodium and cloride equilibrium potentials are approximately +50 and 0 mV, respectively, indicating that the channels underlying the whole-cell outward currents are predominantly selective for potassium.

More than 90% of the wild-type cells had potassium currents (Figure 6). In contrast to the reduction in the percentage of cells expressing sodium currents, 90%–100% of the neurons in cultures bearing each of the *para* alleles also exhibited potassium currents (Figure 5B). Most cells expressed both inactivating and noninactivating currents during 300 ms depolarizing voltage steps between −40 and +40 mV. The relative contribution of each of these components to the whole-cell current varied from cell to cell; the range is illustrated in Figure 6 for wild-type neurons. These currents were similar to the potassium currents that have been described in both cultured larval and embryonic neurons (Solc and Aldrich, 1988; Byerly and Leung, 1988). The potassium currents in the different *para* mutants were similar to wild type (Figure 7). Comparison of the peak I–V relationship in a number of cells of each *para* genotype with wild type did not reveal any obvious differences (Figure 7). In addition, the voltage dependence of the noninactivating currents was also examined in the mutants by determining the mean current density at 290 ms after the initiation of the voltage step (Figure 7). There was no significant change in the *para* neurons.

These results demonstrate that *para* neurons with altered sodium channels have apparently normal potassium currents. This indicates that mutations in *para* specifically affect sodium channels without causing a more generalized membrane defect and the absence of functional sodium channels does not secondarily alter potassium channel expression.

Discussion

The recent cloning of the *para* gene, by means of P-element transposon tagging, has shown that it exhibits a high degree of sequence similarity to a vertebrate sodium channel α subunit (Ganetzky and Loughney, 1988, Soc. Neurosci., abstract). To determine whether the *para* gene codes for functional sodium channels requires demonstration of its role in their expression. This can be accomplished by showing that mutations in *para* modify functional sodium channels. Mutations in a channel gene may be expected to produce a range of effects, including alterations in the level of channel expression and changes in the gating properties of the channels present. Genetic alterations of the *Shaker* locus, which encodes a voltage-dependent potassium channel, have been observed to produce mutants that fall into both of these categories (Salkoff and Wyman, 1983; Papazian et al., 1987; Timpe et al., 1988; Kamb et al., 1987; Baumann et al., 1987; Iverson et al., 1988). Our results revealed that mutations in *para* specifically affected expression of functional sodium channels.

A consistent feature of all the *para* alleles tested was a decrease in the fraction of cultured embryonic neurons expressing sodium currents. The percent reduction was allele-dependent: 49% of the neurons in *para*^{ts1} cultures, 35% in *para*^{ts2} cultures, and 2% in *para*^{ST76} cultures exhibited macroscopic sodium currents, in contrast to 65% of the neurons in wild-type cultures. In *para*^{ST76} cultures sodium currents were recorded in only 1 of 46 cells. This severe reduction is not likely to be due to modifiers present on the second or third chromosomes, since the *para*^{ST76} chromosome produced simi-

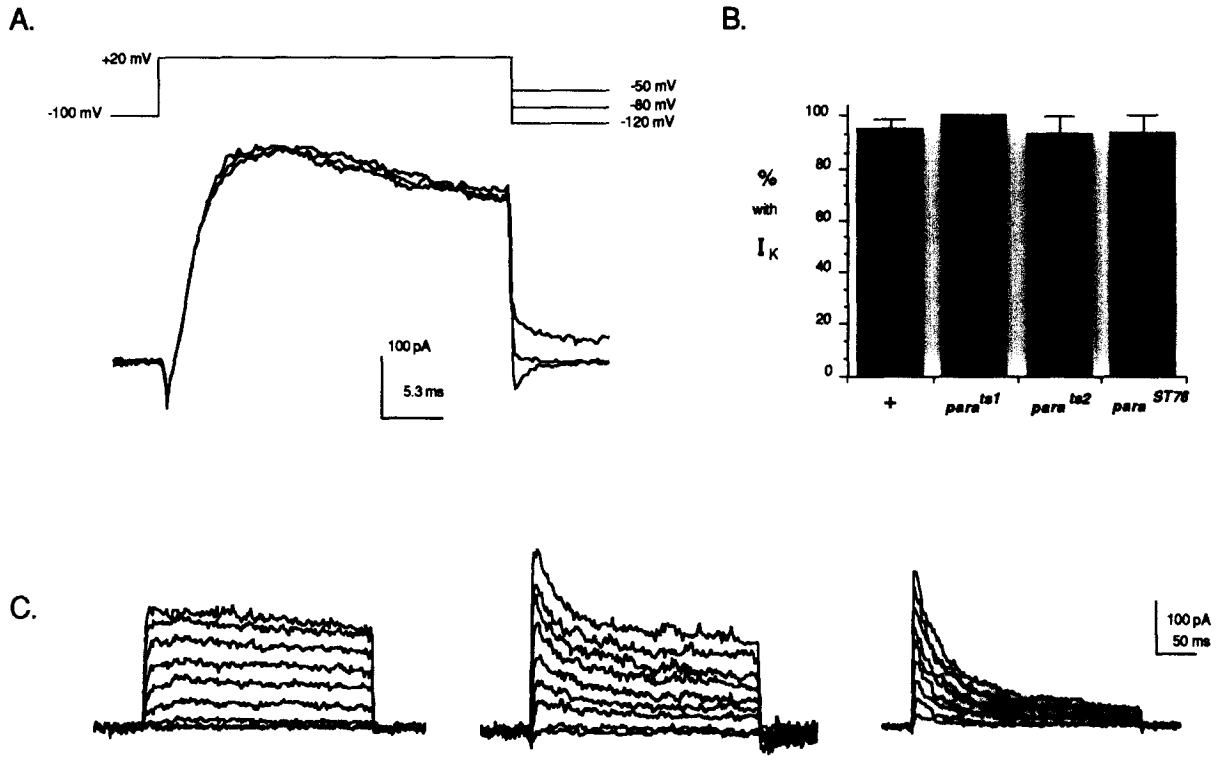

Figure 6. Potassium Currents in Embryonic Neurons

(A) Tail currents recorded from a wild-type neuron with 140 mM potassium in the pipette (intracellular) and 3 mM potassium in the extracellular solution. The voltage was stepped from −100 to +20 mV for 30 ms and then returned to postpulse voltages of −120, −80, and −50 mV. The currents reversed between −80 and −90 mV, which is near the calculated reversal potential for potassium (-97 mV). This indicates that the outward currents are carried primarily by potassium ions.

(B) The percentage of cells expressing macroscopic potassium currents was determined in a manner similar to that used to calculate the percentage expressing sodium currents. Each cell on which a whole-cell clamp was obtained was scored for the presence of potassium currents if a maximal current of 10 pA or greater was elicited by a step from −100 to 0 mV. The percentage of cells with potassium currents was calculated on a per plating basis. Almost all of the neurons in wild-type cultures expressed macroscopic potassium currents, and this property was not altered by mutations in the *para* locus. Bars indicate SEM.

(C) Whole-cell potassium currents in cultured embryonic neurons range from primarily noninactivating to primarily inactivating. Current families were elicited from three different wild-type neurons in response to voltage steps between −40 and +40 mV following a 500 ms prepulse to −100 mV.

lar reductions in two genetic backgrounds. These results suggest that mutations in the *para* gene are responsible for the reduction in the number of cells with sodium currents. In contrast, the differentiation and viability of mutant cells and their expression of neuronal potassium currents appeared unaffected, suggesting that changes due to mutations in the *para* gene are specific for the sodium channels. Voltage-clamp experiments on $para^{ts2}$ demonstrated positive shifts in both the I–V and the g–V relationships. These changes are consistent with this mutation modifying voltage-dependent gating or conductance properties of the channel. Because a relatively large percentage of $para^{ts1}$ cells express sodium currents, it should also be feasible, by examining more cells, to assess whether this mutation affects gating properties of sodium channels. Analysis of single sodium channels will be important in defining the specific defects in channel function that are responsible for the alterations in the macroscopic currents.

Are There Genes in Addition to *para* That Code for Drosophila Sodium Channels?

The changes induced by mutations in the *para* locus on the level of sodium channel expression and on the voltage-dependence of sodium currents are consistent with the *para* gene coding for a sodium channel structural component. Could there be other genes that also code for sodium channels in Drosophila? In rat brain there appear to be at least three distinct genes encoding polypeptides that are similar to the α subunit of the sodium channel protein originally cloned from eel electroplax (Noda et al., 1984, 1986; Kayano et al., 1988). Recent studies have indicated that the expression of these genes in a particular cell type can be selective, as undifferentiated PC12 cells appear to express type II but not type I sodium channels and NGF can specifically induce the type II gene (Mandel et al., 1988).

In Drosophila there exists at least one gene, in addition to *para*, with sequence similarity to vertebrate so-

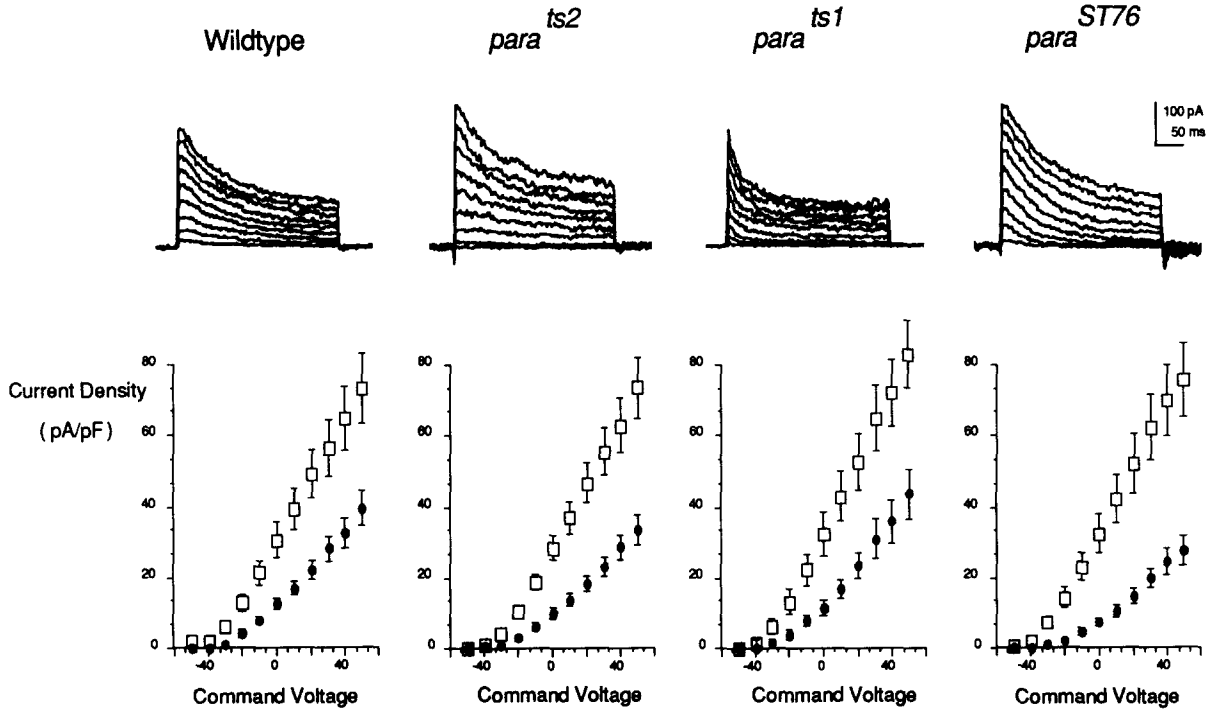

Figure 7. *para* Mutations Do Not Affect Potassium Currents

Potassium currents were recorded from neurons bearing each of the *para* alleles in response to a series of depolarizing voltage steps between −40 and +40 mV from a holding potential of −100 mV. The mean current density at the peak determined from 10 or more cells of each genotype was plotted as a function of voltage and is indicated by the open squares in each graph. The mean current density at 290 ms after the onset of the step, indicated by closed circles in each graph, was also determined in each of the genotypes. The values from each mutant genotype were compared with wild type, and no significant differences were found. Bars indicate SEM.

dium channel α subunits (Salkoff et al., 1987; Okamoto et al., 1987). This gene is located at position 60D-E on the right arm of the second chromosome and has been shown by Northern blot analysis to be expressed in both early and late embryos and pupae (Salkoff et al., 1987). Based on these findings, it is possible that this gene may code for some of the channels in each of the embryonic neurons or all of the channels in some of the embryonic neurons. However, we have two lines of evidence suggesting that *para*, and not the gene at 60D-E, is coding for the majority of the functional sodium channels expressed in cultured embryonic neurons. First, one *para* allele ($para^{ST76}$) virtually eliminated the expression of sodium currents in the cultured neurons. The finding that sodium currents were present in one of the $para^{ST76}$ neurons examined leaves open the possibility that the gene at 60D-E codes for sodium channels in a small fraction of the cells or a very small number of channels (<5 pA of macroscopic current) in each cell. Second, in the absence of recognized mutations in the gene at 60D-E, we have developed a technique to physiologically examine homozygous deficiencies to test the effect of eliminating both copies of this gene (Germeraad, O'Dowd, and Aldrich, unpublished data). We found that sodium currents recorded in neurons with zero copies of the gene at 60D-E appeared similar to wild type, suggesting that it was not necessary for expression of sodium channels in the majority of the cells. Neither of these results exclude the possibility of additional genes that interact with *para* to form embryonic sodium channels.

Cessation of neural activity in the giant fiber system at temperatures that induce behavioral paralysis has been documented in *para* mutants (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980; Benshalom and Dagan, 1981; Nelson and Baird, 1985, Soc. Neurosci., abstract). However, it has also been shown that evoked sodium-dependent action potentials could be elicited at 40°C (Nelson and Baird, 1985, Soc. Neurosci., abstract). One explanation for this heterogeneous temperature-dependent increase in excitation threshold is that some of the fly's sodium channels are not affected by the *para* mutations. The fact that $para^{ST76}$ is not a lethal genotype, despite our findings of drastically reduced numbers of functional sodium channels in cultured embryonic neurons, supports the suggestion that there may be genes, such as the one at 60D-E, that encode sodium channels expressed in cell populations different from those expressing the *para* gene product. Tissue-specific expression of the R_I and R_{II} sodium channel subtypes has been documented in rat (Gordon et al., 1987). It is also possible that different genes code for sodium channels at different developmental stages. In mammals, the family of globin genes provides an example of related but distinct genes that code for embryonic, fetal, and adult forms of hemoglobin (Maniatis et al., 1980).

Three additional Drosophila genes, *nap*, *sei*, and *tip-E*

(Wu et al., 1978; Kulkarni and Padhye, 1982; Jackson et al., 1985) have also been proposed to be important in sodium channel expression based on physiological and biochemical analysis of mutants (for reviews see Papazian et al., 1988; Ganetzky and Wu, 1986; Salkoff and Tanouye, 1986; Tanouye et al., 1986). Examination of voltage-clamped sodium currents in *sei*^{ts1} and *tip-E* confirmed that these mutations altered sodium channel expression in embryonic neurons (O'Dowd and Aldrich, 1988). In the absence of amino acid sequence data it was not possible to determine whether these genes code for structural components of the sodium channel or for products involved in regulating the number, localization, or function of these channels.

The *para* Phenotypes Observed Are Consistent with Mutations in a Structural Gene

There are at least two alterations in sodium channel expression that could reduce the fraction of cells expressing sodium currents. First, it is possible that each mutant completely eliminates sodium channels in a specific subpopulation of cells, while the sodium channels in the rest of the cells are unaltered. Alternatively, each mutant genotype may reduce the level of sodium channel expression in all of the cells, some of which then fall below the limits of detection (<5 pA of macroscopic current). Though our experiments do not allow us to distinguish between these two possibilities, there are a number of ways that mutations in a structural gene coding for sodium channels could result in the *para* phenotypes described.

The three *para* alleles may represent alterations in either the coding or controlling sequences of the gene. Changes in either of these regions could reduce the efficacy with which the gene is transcribed or the protein is processed and inserted into the membrane, thereby reducing the percentage of cells expressing sodium currents in each of the *para* mutants. The *para*^{ts2} mutation most likely occurs in the coding region of the gene, as we have noted changes in the gating properties of the sodium currents expressed. Changes in the coding region, such as those affecting the voltage dependence of slow inactivation, could also be manifest as a decrease in the percentage of cells expressing sodium currents by reducing the number of channels activated during the conventional stimulus protocol.

Sodium Currents at Elevated Temperatures

The *para*^{ts} mutants, like several other temperature-sensitive mutations, were originally proposed to affect sodium channels based on the observation of behavioral paralysis and altered membrane excitability at elevated temperatures (for review see Ganetzky and Wu, 1986). From these previous studies it seemed possible that the number and function of sodium channels in *para* flies at permissive temperatures were normal and that elevation of the temperature induced a conformational change in the channel protein that inhibited conduction. Our results have demonstrated that mutations at the *para* locus alter sodium channel expression at room temperature. A reduction in the number of cells with sodium channels or the density of sodium channels within a cell could easily account for alterations in impulse activation and conduction at elevated temperatures, especially in the polysynaptic pathways that have been used to demonstrate temperature-dependent spike failure (Siddiqi and Benzer, 1976) without invoking a thermolabile protein. At low temperatures, the kinetics of sodium and potassium channel activation may permit initiation of the impulse despite a mutationally induced reduction in sodium current expression. A rise in temperature, which normally speeds up channel gating kinetics, could result in rapidly activating potassium currents overcoming the reduced sodium currents in the mutants, thus blocking initiation of action potentials in all or a subset of cells in the nervous system. This mechanism of spike failure would be expected at higher temperatures even in cells with a normal complement of sodium channels, as has been documented in the squid giant axon (Hodgkin and Katz, 1949). The finding that wild-type flies also paralyze, at temperatures 5°C–10°C higher than the mutants (Jackson et al., 1985), is consistent with this mechanism.

Sodium currents can also be recorded from *para*^{ts2} mutant neurons at the adult paralytic temperatures, though it should be noted that the mobility of first instar larvae was not discernibly altered at these temperatures. These experiments suggest that whatever the basis for adult paralysis is, it does not affect larvae to the same extent. In addition, the fact that *para*^{ST76} animals are mobile at room temperature, even though virtually none of the cultured neurons examined express sodium currents, may reflect a difference between the subset of neurons studied in culture and the population of neurons important in locomotory behavior.

This system, in which modification of a structural component of the sodium channel can be monitored biophysically in its native environment, is well suited to studies aimed at identifying structural domains of the molecule important in function. Alleles, such as *para*^{ts2}, that alter the voltage-dependence or kinetics of the whole-cell currents can be studied at the single-channel level to determine the underlying changes in gating that correspond to defined alterations in the amino acid sequence. These cultures also provide a favorable system in which to examine the set of genes involved in the expression of functional sodium channels in embryonic Drosophila neurons.

Experimental Procedures

Fly Stocks

Fly stocks were maintained at 22°C on cornmeal–yeast–dextrose–sucrose medium. *para*^{ts1} and *para*^{ST76} stocks were obtained from B. Ganetzky. *para*^{ts2} was provided by T. Grigliatti. The *para*^{ST76*} stock was prepared by outcrossing the *para*^{ST76} stock to attached-X females for two generations followed by crossing to a balancer stock to make the mutant X homozygous. Canton-S was used as the wild-type strain. Since *para*^{ts1} and *para*^{ts2} were generated in an Oregon-R background and only *para*^{ST76} was made in Canton-S, there are some uncontrolled differences in the genetic background of the mutants. Each of the stocks was tested periodically for the paralytic phenotype.

Cell Culture

Cultures were prepared according to the procedures developed by Seecof (1979) and described by O'Dowd and Aldrich (1988). Coverslips used for voltage-clamp experiments were prepared with 2–4 embryos per coverslip. For experiments in which the numbers of neurons and myotubes were counted, *para*^{ST76} and wild-type embryos were collected simultaneously and cultures were prepared from single embryos of each genotype under identical conditions. At 24 hr, the cells were fixed with 2% paraformaldehyde, stained with hematoxylin–eosin, and coded so that the cultures were counted blind with respect to genotype. One-half of each culture was counted at 400× under bright-field illumination. Cells with round somata and definite processes, or those in clumps that extended processes, were classified as neurons. Flattened cells were classified as myotube-like cells, and when it was possible to see multiple nuclei in fused cells, each nucleus was counted as a separate cell.

Solutions

Solutions used for experiments in which both sodium and potassium currents were examined contained the following: (internal) 70 mM KF, 70 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES (pH 7.2); (external) 140 mM NaCl, 3 mM KCl, 0–2 mM TEA-Cl, 4 mM $MgCl_2$, 1 mM $CoCl_2$, 5 mM HEPES, 1 mM $CaCl_2$ (pH 7.2). Solutions used for experiments in which only sodium currents were examined contained the following (internal) 70 mM CsCl, 70 mM CsF, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES (pH 7.2 with 25 mM NaOH); (external) 140 mM NaCl, 2 mM TEA-Cl, 4 mM $MgCl_2$, 1 mM $CoCl_2$, 5 mM HEPES, 1 mM $CaCl_2$ (pH 7.2). The osmolarity of all solutions was routinely adjusted to between 320 and 330 mosm with 50 mM sucrose.

Electrophysiology

Currents were recorded using the tight-seal, whole-cell recording technique (Hamill et al., 1981). Patch electrodes were coated with Sylgard and fire-polished to a resistance of 6–14 MΩ when filled with the standard solutions. Pipette potentials were nulled just prior to seal formation. After establishing a high resistance seal, the pipette capacitance was electronically compensated to less than 0.2 pF. The whole-cell capacitance was determined immediately upon breaking into the cell by integrating the area under the capacitative transients in a current record obtained by averaging the current responses elicited by 64 depolarizing voltage steps from −70 to −50 mV. All data were collected from cells with whole-cell capacitances of 1–5 pF. Series resistance compensation was not routinely attempted, as the maximal series resistance errors from the largest sodium currents recorded were estimated to be less than 2 mV. Each cell with a sodium current greater than or equal to 5 pA was scored as positive in determining the number of cells with sodium currents. The percentage of cells with sodium currents was calculated on a per plating basis. All data used for I-V curves and kinetic measurements were obtained from cells with sodium currents under good voltage control and with extrapolated reversal potentials of less than +70 mV (O'Dowd and Aldrich, 1988).

Data Acquisition and Analysis

Data were collected and analyzed using a List EPC-7 patch clamp amplifier and a Digital PDP 11-73 computer. Current records were sampled at 50 or 100 μs intervals and filtered with an 8-pole Bessel filter at 2.5 kHz unless otherwise indicated. A linearly scaled leak current obtained by averaging 64 depolarizing steps from the holding potential of −70 to −50 mV was subtracted from each of the current records prior to analysis. Data were collected from both wild-type and mutant flies over the same time period with identical media and recording solutions. A two-tailed Student's t test for unpaired data was used for statistical comparison of wild-type and mutant values. The temperature of the recording media was controlled by a thermocouple device with a feedback circuit.

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