Pharmacological characterization of sodium channels in the primary culture of individual Drosophila embryos: neurons of a mutant deficient in a putative sodium channel gene

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Sodium channels in Drosophila embryonic neurons were characterized pharmacologically in the primary culture of individual gastrulae. In normal cultures, presence of sodium channels was demonstrated by neuronal degeneration in the presence of veratridine and ouabain, which was inhibited by tetrodotoxin. Embryonic neurons of Df(2R)M-c33a homozygotes that lack a putative sodium channel gene at 60E region showed normal neurotoxin sensitivity. Therefore, sodium channel genes other than 60E must be functional at this developmental stage. We also examined para++ and nap++ mutants and found that they were also sensitive to the neurotoxins. To determine the genotypes of single embryo cultures by histochemical staining, we utilized a special chromosome bearing a hsp70-lacZ fusion gene. The lacZ expression in the culture was studied in detail.

Drosophila melanogaster; Primary culture; Sodium channel

Introduction

The sodium channel is a voltage-sensitive transmembrane protein which controls the membrane permeability to sodium ions. This function is essential for generation of nerve action potentials (Hodgkin and Huxley, 1952). Recently, Salkoff et al. (1987) and our group (Okamoto et al., 1987) isolated putative sodium channel genes in Drosophila melanogaster, which were homologous to the eel sodium channel gene (Noda et al., 1984). Moreover, a potassium channel gene was cloned from the Shaker locus of Drosophila, which has been shown to encode a voltage-dependent potassium channel from different lines of electrophysiological evidence (Papazian et al., 1987; Tempel et al., 1987; Kamb et al., 1987; Baumann et al., 1987; Schwarz et al., 1988). These molecular and genetic approaches are expected to throw light upon the structure and function of ionic channels (Ganetzky and Wu, 1986; Tanouye et al., 1986).

We mapped the putative sodium channel gene (DIC60) to the right arm of the second chromosome (2R) in the region 60E by in situ hybridization to polytene chromosomes. Using the same method, we also found that the deficiency strain Df(2R)M-c33a, which lacks a chromosomal region
from 60E2-3 to 60E11-12, was indeed deficient in the DIC60 locus (Okamoto et al., 1987).

Questions remain as to whether DIC60 really encodes a functional sodium channel and whether there are other sodium channel genes in the Drosophila genome. In order to solve these problems, we examined pharmacologically the neuronal properties of Df(2R)M-c^33a homozygotes. Wu et al. (1983) reported that in the larval CNS culture, neuronal death was induced by 500 μM veratridine, a sodium channel-specific activator, and that the veratridine effect was inhibited by 1 μM tetrodotoxin (TTX), a specific sodium channel blocker (Narahashi et al., 1964). This cytotoxic effect is presumably due to forced opening of sodium channels by veratridine (West and Catterall, 1979). We applied the same method to cultured embryonic neurons, in order to examine whether Df(2R)M-c^33a homozygotes showed abnormal neuronal properties.

We also examined the cytotoxic effect of veratridine on the Drosophila paralytic mutants para^1st and nap^1st. These are known to have defective axonal conduction in larval and adult neurons at high temperatures (Suzuki et al., 1971; Wu et al., 1978; Burg and Wu, 1986).

Homozygous Df(2R)M-c^33a individuals which completely lack the chromosomal region covering DIC60 are lethal during development. To overcome this difficulty, we made primary cultures from early gastrulæ. Since such lethal mutants are maintained as heterozygotes with chromosomes which suppress crossing-over, we need to discriminate homozygous from heterozygous embryos present among the progeny. In order to determine the genotypes of individual embryos, we made primary cultures from single embryos with a cell marker on the chromosome. We used the special second chromosome CyO [ry^-hsp70-lacZ], into which a hsp70-lacZ fusion gene was introduced by the P element-mediated transformation method (Lis et al., 1983; Simon et al., 1985). Presence of this chromosome is identifiable by β-galactosidase staining in the embryonic culture. The staining pattern was carefully monitored to work out the optimum conditions for the histochemical reaction. The culture of a homozygous mutant embryo can be easily identified, since it is not stained for β-galactosidase activity whereas the heterozygous culture is clearly stained. Characterization of the hsp70-lacZ expression in primary culture cells of early gastrulæ could be useful for analyzing a wide variety of mutations, especially recessive lethals.

Materials and Methods

Flies, embryos, and culture medium

The normal strain Canton-S of Drosophila melanogaster was used for control. The deficiency strain Df(2R)M-c^33a / In(2LR)bw^32g was obtained from Umeå Drosophila Stock Center. CyO [ry^-hsp70-lacZ] / Bl Sp lΔWMG was made by Drs. J.T. Lis and C.A. Sutton (Simon et al., 1985), and provided by Dr. R.W. Aldrich. Df(2R)M-c^33a / CyO [ry^-hsp70-lacZ] from the cross of these two strains was used in this study. The second chromosomes In(2LR)bw^32g, CyO, and Bl Sp lΔWMG, which suppress crossing-over and are marked by mutations, have been previously described (Lindsley and Grell, 1968). Other mutant strains used are para^1st and nap^1st (Ganetzky and Wu, 1986). Both mutants have a genetic background of Canton-S.

Eggs were dechorionated and sterilized with 2.5% sodium hypochlorite for 1.5 min. Subsequently, they were washed with autoclaved distilled water and kept in culture medium (Koana and Miyake, 1982). We used the culture medium M3(BF), which was prepared according to Cross and Sang (1978). It contained penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml) (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD). Insulin (2.6 mU/ml) (Collaborative Research Inc., Bedford, MA) was added to the medium just before use.

Preparation of primary culture from individual embryos

The developmental stage of individual embryo was first determined under a stereoscopic microscope. An embryo at the early gastrula stage was selected as follows (Scoof et al., 1971; Koana and Miyake, 1982). The beginning of this stage is
characterized by the appearance of the ventral and cephalic furrows, and, at the end of the stage, the posterior invagination and dorsal folds appear, then the ventral furrow disappears. This period corresponds to stage 7—from 3 h 35 min to 3 h 45 min after fertilization at 22°C (Wieschaus and Nüsslein-Volhard, 1986).

The contents of an embryo were withdrawn with a glass capillary and plated directly onto a 35-mm culture dish (Falcon, Oxnard, CA) or a 15-mm round cover slip (Matsunami Glass Ind., Osaka, Japan) without poly-L-lysine coating. The cells were further dissociated with a capillary under a dissecting microscope. Each dish contained 1.5 ml culture medium. Yolk was removed from the medium as completely as possible (Koana and Miyake, 1982). All cultures, unless specified, were incubated at 25°C in a humid atmosphere of 5% CO₂ and 95% air with an incubator (Forma Scientific, Marietta, OH).

In the experiments with Df(2R)M-c²³a/Cyo, a single embryo was divided into two cultures on separate cover slips. The half-embryo cultures differentiated well and were stably maintained.

**Pharmacological methods**

The three neurotoxins used in this study are veratridine, TTX, and ouabain (Sigma, St. Louis, MO). Stock solutions of veratridine (3 mM in the culture medium), ouabain (5 mM in the medium), and TTX (100 μM in distilled water) were added to cultures to achieve the final concentrations indicated in the text. Veratridine and ouabain solutions were made just before use and sterilized by filtration through 0.22 μm or 0.45 μm filters (Nihon Millipore Ltd., Tokyo, Japan), and then heat-inactivated fetal bovine serum was added (10% by volume). Ouabain and TTX were applied 30 min prior to addition of veratridine. To compensate for the volume change caused by the additional medium, the same amount of medium was added to control cultures.

Morphology of the cultured cells was examined with phase contrast optics (DIAPHOT-TMD: Nikon, Tokyo, Japan). All cultures were rather uniform in that neurons mostly appeared in clusters of various sizes 4 days after plating. In order to record and quantify the effect of veratridine, each culture was photographed just before adding the neurotoxins. The cultures were kept for 2 days, and the same fields of each culture were photographed again. Most proximal neurites from the same neuronal clusters were counted and compared before and after the drug treatment. Distal branching of neurites was excluded from the count. Thick bundles of fasciculated neurites were resolved at high magnification. Since the culture itself is rather small (about 1–2 mm in diameter), the positive identification of the same neurites was always possible. Degenerate neurons appeared granular, and only clearly identifiable neurites were regarded as survived.

**β-galactosidase staining in primary cultures**

The expression of the hsp70-lacZ fusion gene in cultures was induced by activation of hsp70 promoter with a heat shock at 37°C for 1 h. The cultures were subsequently incubated at 25°C for 1.5 h. After removal of the culture medium, the cultures were fixed for 20 min at room temperature in PBS (0.13 M sodium chloride/8.0 mM disodium phosphate/1.8 mM sodium biphosphate, pH 7.4) containing 4% formaldehyde (Lazarides and Weber, 1974). They were rinsed thoroughly in PBS three times, and subsequently stained for β-galactosidase activity at 37°C with the following substrate solution (Hiromi et al., 1985): 0.2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Wako Pure Chemical Ind., Osaka, Japan), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2% gelatin in citric-phosphate buffer (2.8 mM citric acid/0.19 M disodium phosphate, pH 8.0).

**Results**

**Primary culture of individual embryos**

Each primary culture was individually prepared from an early gastrula of D. melanogaster. It has been reported that several cell types differentiate within 1 day in a culture (Shields et al., 1975; Cross and Sang, 1978; Koana and Miyake, 1982). For example, neurons, muscle cells, fat-body cells, hemocytes, and imaginal disc cells could be clearly identified. The presence of these cell types was
confirmed in each culture of normal and mutant embryos we used. We focused our attention on the morphology of neurons as well as of other cell types. In mature cultures, most neurons occurred in ganglion-like clusters, and some of them formed neural networks with their neurite bundles connecting cell clusters. In the pharmacological or histochemical experiments described below, 3–10-day cultures were used, since morphology of neurons appeared mature and stable at this stage.

*Sensitivity to sodium channel-specific neurotoxins*

In order to investigate the existence of sodium channels, we used the neurotoxins that are highly specific to voltage-dependent sodium channels. Their cytotoxic effects in the primary culture of the normal strain (*Canton-S*) embryo were examined prior to the analysis of the mutants.

Veratridine causes persistent activation of sodium channels at the resting membrane potential, by blocking sodium channel inactivation and by shifting activation to more negative potentials (Catterall, 1980). It causes depolarization of the membrane and entry of sodium ions. Other ions such as calcium may enter the cell or be released intracellularly as a secondary effect. Embryonic neurons in our cultures, however, did not show any noticeable morphological sign of cytotoxicity.
in the presence of 500 μM or 1.0 mM veratridine for several days.

In order to enhance the effect of veratridine, we applied ouabain 30 min prior to veratridine. We compared the identified neurons in the culture just before and 2 days after adding 1.0 mM ouabain and 500 μM veratridine (Fig. 1A and B). Remarkable neuronal degeneration was clearly observed. Degenerate neurons were readily recognized by their retraction of neurites. The survival ratio of neurites was 32.4 ± 17.5% (mean ± standard deviation; n = 4 cultures, x = 54 neurites counted before the treatment). During the course of degeneration, the neuronal process appeared like a string of beads. The effective concentrations of veratridine and ouabain were comparable with those reported by Catterall and Nirenberg (1973). In their experiments 100 μM veratridine and 5.0 mM ouabain were used. They showed that veratridine-dependent sodium ion influx in the presence of ouabain provides a specific and convenient means for determining the existence of sodium-dependent action potentials in cultured neuroblastoma and muscle cells. Ouabain prevents sodium ion efflux by specifically inhibiting ATP-dependent sodium-potassium pump.

Veratridine-induced neuronal degeneration was caused by sodium channels present in the embryonic neurons, since this effect was almost completely inhibited by 1 μM TTX (Fig. 1C and D). In this experiment, the survival ratio recovered to 73.4 ± 9.4% (n = 2, x = 26). This is significantly higher than that of veratridine and ouabain without TTX. Therefore, we conclude that TTX-sensitive sodium channels are expressed in embryonic neurons at this early developmental stage.

We also confirmed that general neuronal degeneration did not occur in the presence of either 1 μM TTX alone or 1.0 mM ouabain without veratridine. In order to confirm that this pharmacological effect is indeed mediated by sodium channels, we examined muscle cells that have no sodium channels (Tanouye et al., 1986). In the presence of 100 μM veratridine and 1.0 mM ouabain, the survival ratio of muscle cells was 79.9 ± 9.1% (n = 3, x = 124 muscle cells counted before the treatment). Some cells which disappeared or became spherical were regarded as not surviving. Spontaneous contractions of muscle cells were observed even after the addition of veratridine. Since this value is similar to that obtained in the TTX-inhibition experiment, it shows the nonspecific effect of drug treatments. Therefore, these data will serve as a criterion for sodium channel-deficient neurons.

We chose neurite survival rather than neuronal survival as an indicator of the pharmacological effect, since most neurons grow in clusters, and it is difficult to identify and discriminate individual neurons before and after the drug treatments. Fig. 2 shows the survival ratio of normal neurites, 2 days after the application of 1.0 mM ouabain with the indicated concentrations of veratridine. It was about 80% in the presence of 1.0 mM ouabain alone, which is comparable with the above two
values. Since half-maximal effect was obtained at 100 μM of veratrudine, and it could be the most sensitive concentration, the condition of 100 μM veratrudine and 1.0 mM ouabain was used in the subsequent experiments.

Identification of the genotypes of single embryo cultures

In the following experiments, we used the strain \(Df(2R)M-c^{33a}/CyO [ry^{+}.hsp70-lacZ]\). \(Df(2R)M-c^{32a}\) has been shown to lack the putative sodium channel gene \(DIC60\) by the in situ hybridization method (Okamoto et al., 1987). This strain carries the mutation of fairly strong \(Minute (M)\) and the rate of development is slow (Lindsley and Grell, 1968). Under our culture conditions, however, the growth and the differentiation of all cell types appeared normal, and all cultures were successfully maintained.

\(CyO [ry^{+}.hsp70-lacZ]\) is a second chromosomal balancer (crossing-over suppressor) with an insertion of \(hsp70-lacZ\) fusion gene introduced by the P element-mediated transformation method (Simon et al., 1985). This fusion gene has the upstream sequence of \(hsp70\), a \(Drosophila\) heat shock gene, fused in frame to the \(E. coli\) \(β\)-galactosidase gene. It is induced in the transformants by heat shock at 37°C (Li et al., 1983).

From the cross of \(Df(2R)M-c^{33a}/CyO\) and \(Df(2R)M-c^{33a}/CyO\) flies, three genotypes are expected among the progeny: \(Df(2R)M-c^{33a}/Df(2R)M-c^{33a}\), \(Df(2R)M-c^{33a}/CyO\), and \(CyO/CyO\). Since \(CyO\) homozygotes are larval lethal (Nüsslein-Volhard et al., 1984), they are expected to be present in embryonic cultures. However, the lethal phase of \(Df(2R)M-c^{33a}\) homozygotes is not yet defined. It is necessary to distinguish \(Df(2R)M-c^{33a}\) homozygotes among the progeny, in order to examine whether they occur in our cultures and whether the pharmacological properties are altered in the absence of \(DIC60\). We utilized \(E. coli\) \(β\)-galactosidase as a cell marker to unambiguously identify the genotypes of single embryo cultures. In the presence of the substrate, green staining spots appeared in 2 h in some of the cell clusters (Fig. 3A). The distribution pattern of spots did not change even after incubation at 37°C for 1 or 2 days, although the color of each spot became deeper. They were observed in a wide variety of cell types including small cell clusters. One-fourth of the developing embryos, derived from \(Df(2R)M-c^{33a}/CyO\) parents, were expected to be \(Df(2R)M-c^{33a}\) homozygotes which should not be stained for \(β\)-galactosidase activity; while the other 3/4 were to have the balancer chromosome and show positive staining. The results were consistent with this expectation. In two experiments, 5 out of 21 and 3 out of 9 cultures showed no activity (Fig. 3B). Consequently, this result shows that the lethal phase of \(Df(2R)M-c^{33a}\) homozygotes is after the early gastrula stage when
our primary cultures were made. The normal embryo (Canton-S, without hsp70-lacZ gene) never showed a false-positive staining, even after incubation at 37°C for 2 days.

Several experiments were performed to rule out the possibility that the observed spot pattern was an experimental artifact. Prolonged heat shock for 3 h did not affect the pattern. Although there were some variations in the ratio of stained cells and in the staining intensity from individual to individual, the presence or absence of stained cells was clear and distinct to determine the genotype of each embryonic culture. In order to obtain further evidence, we divided a single embryo into two cultures on separate cover slips and cultured them under the same condition. The twin cultures always exhibited similar staining patterns, which indicates that staining intensity depended on individual embryos. Hence, the staining is a reliable indication of the culture genotype, but not due to trivial experimental variations.

A culture in a pair fixed for 5 min showed a pattern similar to that of its counterpart fixed for 20 min. However, longer fixation tends to inhibit β-galactosidase activity. We could not detect the positive staining after 40-min fixation, whereas the other culture of the pair with 20-min fixation was stained. We also tried to freeze and thaw some of the twin cultures on dry ice (no fixation) to make cell membranes permeable. There was no change in the staining pattern as compared with the fixed counterpart. We did not adopt this freeze–thaw procedure for our routine experiments, because it caused marked morphological damage. Nevertheless, this experiment suggests that staining patterns do not depend on the fixation methods and that inaccessibility of the substrate should not be the cause of the spot pattern.

Pharmacological effects on homozygous Df(2R)M-c33a neurons

Based upon these results, we examined the pharmacological property of homozygous Df(2R)M-c33a neurons. Twenty-one primary cultures were made from individual F1 embryos by using Df(2R)M-c33a/CyO [ry+ hsp70-lacZ] as parents. Each culture was divided into two and plated on separate cover slips. One from each pair was tested for β-galactosidase staining to identify its genotype (Fig. 4A and 4D); the other was determined for its sensitivity to veratridine. Five out of 21 cultures were judged to be Df(2R)M-c33a homozygotes by the absence of β-galactosidase activity. The morphology of neurons in each culture was recorded on photographs. Neuronal degeneration was observed in all 21 cultures in the presence of 100 μM veratridine and 1.0 mM ouabain (Fig. 4B and C; 4E and F). The survival ratio of Df(2R)M-c33a homozygote neurites was 22.2 ± 4.7% (n = 5, x = 184), while that of Df(2R)M-c33a/CyO heterozygote or CyO homozygote was 24.5 ± 6.8% (n = 16, x = 362). Concerning neuronal sensitivity to veratridine, there is no significant difference among Df/Df and Df/CyO or CyO/CyO.

In another experiment, we further added 1.0 μM TTX to the cultures with veratridine and ouabain, in order to exclude the trivial possibility that Df(2R)M-c33a homozygotes were hypersensitive to either veratridine or ouabain, even though they were originally suspected to lack the sodium channel. TTX almost completely blocked the veratridine effect. The survival ratio of Df/Df was 76.2 ± 8.0% (n = 2, x = 66), while that of Df/CyO or CyO/CyO was 75.3 ± 17.3% (n = 2, x = 60). Therefore, we conclude that sodium channels are present even in the embryonic neuron of Df(2R)M-c33a homozygotes at this early developmental stage.

Other mutations affecting sodium channels

We further examined the other mutations para11 and nap15 that are proposed to affect sodium channels (Ganetzky and Wu, 1986). Under our culture conditions, the embryonic cultures of para11 and nap15 appeared normal and were successfully maintained. They also showed neuronal degeneration in the presence of 100 μM veratridine and 1.0 mM ouabain. The survival ratios of para11 and nap15 neurites were 39.8 ± 8.9% (n = 4, x = 130) and 40.3 ± 6.7% (n = 5, x = 118), respectively; while that of normal was 41.6 ± 8.6% (n = 3, x = 103).

In order to examine whether the neuronal properties of para11 and nap15 are altered at high temperature, we incubated embryonic cultures at 30°C throughout the experiment. All cultures of
Fig. 4. Pharmacological effects on neurons in the deficiency cultures. (A, D) Six-day cultures of individual embryos, derived from Df(2R)M-c-c13α/CyO [ry + -hsp70-lacZ] parents. They were stained histochemically for β-galactosidase activity (4-h incubation). The culture in panel A is not stained, indicating a Df(2R)M-c-c13α homozygote; in contrast, that in panel D is stained, indicating either a Df(2R)M-c-c13α/CyO heterozygote or a CyO homozygote. A single embryo was divided into two cultures; A, B and D, E are pairs. (B, E) Seven-day cultures just before neurotoxin addition. (C, F) Nine-day cultures, 2 days after adding 100 μM veratridine and 1.0 mM ouabain to the cultures shown in panels B and E, respectively. The veratridine-induced neuronal degeneration was observed in both cultures. Arrows and arrow heads indicate the corresponding positions of neurites in the culture prior to addition of toxins. Bar represents 100 μm.
para<sup>++</sup> and nap<sup>++</sup> did not appear different from normal even at 30°C. We found marked neuronal degeneration in only 1 day after adding 100 μM veratridine and 1.0 mM ouabain. The survival ratio of normal neurites was 40.7 ± 9.4% (∑ = 4, x = 118) under the same condition; while those of para<sup>++</sup> and nap<sup>++</sup> were 36.0 ± 7.9% (∑ = 4, x = 87) and 33.4 ± 13.5% (∑ = 4, x = 49), respectively. We further confirmed that at 30°C the scores of these strains were 69.8 ± 12.5% (∑ = 2, x = 37) and 79.8 ± 6.8% (∑ = 2, x = 25), respectively, in the presence of 1.0 mM ouabain alone without veratridine.

Discussion

Identification of the genotypes of single-embryo cultures

Our single-embryo culture system renders easy access to in vitro examination of the earliest neuronal development. The presence of ionic channels and their functions can be examined both pharmacologically and electrophysiologically. This system also allows us to study lethal mutations that affect essential neuronal properties.

We reported here a genotype identification method, which is essential for precise analysis of mutational effects. Koana and Miyake (1982) previously reported a histochemical method using Zw<sup>+</sup>, an X-linked recessive mutation that causes deficiency in glucose-6-phosphate dehydrogenase (G6PD). The mutation to be analyzed must be located on the same chromosome as the marker Zw<sup>+</sup> in order to select for the homozygous mutant which showed no G6PD activity. Thus their method could be applied to X-chromosome mutations. Extending it to autosomes, we had used the second chromosomal balancer CyO bearing fitz-lacZ fusion gene (Hiromi et al., 1985). However, the fitz promoter was difficult to use for this purpose, because it is expressed only in a specific subset of neurons (Doe et al., 1988). In the study reported here, we used a dominant marker hsp70-lacZ deliberately introduced into the balancer chromosome CyO. The same chromosome was also used by S. Germeraad, D.K. O'Dowd, and R.W. Aldrich of Stanford University to identify the embryos of homozygous deficiencies in their 'zero-dose' screening approach (personal communication).

This genotype identification method is applicable to the analysis of mutations on all chromosomes. Insertion of the hsp70-lacZ fusion gene into a particular balancer chromosome can be made by using the P-element-mediated gene transfer method (Rubin and Spradling, 1982). Subsequently, the activity of the E. coli β-galactosidase in an embryonic cell culture can be readily visualized histochemically, which indicates the presence of at least one balancer chromosome bearing the marker insert.

The staining for β-galactosidase activity was detected as green spots distributed over some, but not all, cells. We confirmed that the uneven distribution was not caused merely by artifacts, such as uneven fixation or inaccessibility of the substrate to all cells. Twin half-embryo cultures always showed similar patterns of variation in the staining intensity among cell clusters. It remains unknown whether this variegation depends on the chromosomal location of the inserted gene. Nevertheless, this method appears very sensitive in detecting the enzyme activity, and some small cell clusters often exhibit intense staining. Although endogenous β-galactosidase is present in Drosophila, it had no detectable enzyme activity under our experimental condition, because the normal culture never showed any positive staining at all. This particular contrast in staining makes our method very efficient in determining the genotypes of embryonic primary cultures.

Pharmacological effects on embryonic neurons in primary cultures

In the present study, we observed very prominent morphological change of neurites induced by 100 μM veratridine and 1 mM ouabain. Since this effect was blocked by 1.0 μM TTX, existence of sodium channels was clearly demonstrated in embryonic cultures. We found the neurotoxin sensitivity in both normal and homozygous Df(2R)M- c<sup>33a</sup> cultures. These results indicate no difference in the density of sodium channels between normal and deficiency cultured neurons. If there is no sodium channel gene other than DIC60, homo-
zygous Df(2R)M-c$^{33a}$ neurons should lack sodium channels and exhibit a reduced sensitivity to veratridine. Since this was not the case, there must be other genes coding for TTX-sensitive sodium channels, functional at this early stage.

The expression of DIC60 in the embryonic culture has not been determined previously. Although the present study suggests that it is not functional or does not code for a dominant species of sodium channels in the early embryo, it may still be actively expressed at other stages (Salkoff et al., 1987). Another possibility is that this gene does not code for a sodium channel, but for other channels with a different ionic selectivity. Recently, a putative calcium channel gene was cloned from rabbit skeletal muscle, which was shown to be similar in structure to the sodium channel (Tanabe et al., 1987). Therefore, at this time it is difficult to establish the functional role of DIC60, despite its homology to the vertebrate sodium channel gene. The structural domain of the gene that is essential for the ionic selectivity must be first determined in future studies in order to clarify the function of this gene.

Recently, D.K. O'Dowd, S. Germeraad, and R.W. Aldrich of Stanford University have examined electrophysiologically the homozygous deficiencies in the 60C–60F region (personal communication). They recorded sodium currents by whole cell voltage-clamp method in cultures of early gastrulae. Consistent with our results, normal sodium current was recorded even in the cultured neurons of Df(2R)M-c$^{33a}$ homozygotes. However, there is a difference in the developmental stages of the neuron in the two studies. O'Dowd et al. used cultures of a very early stage before neurite elongation. This is necessary for voltage-clamp experiments, because subsequent neurite elongation increases the space constant of the cultured neuron. In contrast, we examined the pharmacological properties of mature neurons with neurites. Since it is expected that sodium channels are more abundant in neurite membrane than in soma, our pharmacological approach provides further information complementary to the electrophysiological studies.

The fact that neuronal degeneration in embryonic cultures was not induced by veratridine alone is in contrast to the results from cultured larval neurons reported by Wu et al. (1983). It could be simply explained by assuming that the number of sodium channels in the cultured embryonic neurons is less than that in the cultured larval neurons. The fewer sodium channels there are, the less sodium ion influx would be caused by veratridine. Therefore, the presence of ouabain was needed to obtain veratridine-induced degeneration of embryonic neurons.

We also demonstrated that para$^{ts1}$ and nap$^{ts}$ embryonic neurons showed normal sensitivity to veratridine at both 25°C and 30°C. These mutations have been suggested to affect the voltage-sensitive sodium channel on the basis of physiological, pharmacological, and toxin binding studies (Ganetzky and Wu, 1986). Wu et al. (1983) as well as Suzuki and Wu (1984) reported an increased resistance to veratridine in para$^{ts1}$ and nap$^{ts}$ primary cultures of larval neurons. The discrepancy between their result and ours may be due to the sensitivity of the experiments. Since the temperature-sensitive mutations, such as para$^{ts1}$ and nap$^{ts}$, must be regarded as weaker alleles of these genes, their phenotypes cannot be expected as marked as deficiencies. In these mutants, the channel function may be partially retained and veratridine could affect them with ouabain in our experiments. Thus our result should not be taken as the evidence that para$^{ts1}$ and nap$^{ts}$ do not affect sodium channel functions in embryonic neurons.

A more interesting possibility is that the para$^{ts1}$ and nap$^{ts}$ mutations affect a type of sodium channels that become abundant only at a later stage of development. It is known that double mutants of para$^{ts1}$ and nap$^{ts}$ lead to lethality even at temperatures permissive for each single mutant (Wu and Ganetzky, 1980). It is probably due to the unconditional failure of this double mutant to propagate nerve impulses (Ganetzky and Wu, 1986). The lethality occurs during larval development because the double mutant can survive up to a period of the late first instar larva (Ganetzky, 1984). Therefore, the fact that reduced sensitivity to veratridine was found in the third instar larval culture of para$^{ts1}$ and nap$^{ts}$ but not in the embryonic culture could be explained by multiple types of sodium
channels that vary in abundance at different developmental stages.

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References


Seeof, R.L., N. Alléaume, R.L. Teplitz and J. Gerson: Differentiation of neurons and myocytes in cell cultures made


