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Characterization of Variant Neuroblastoma Clones with Missing or Altered Sodium Channels

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SUMMARY

Variant neuroblastoma cell clones were selected for resistance to the cytotoxic effects of neurotoxins that cause persistent activation of sodium channels. Three classes of variant clones were obtained: sodium channel-deficient clones, which have markedly reduced numbers of functional sodium channels; scorpion toxin-resistant clones, which have sodium channels with an altered interaction with scorpion toxin; and parental-type clones, which have functional sodium channels similar to the ones from N18 cells but have other heritable alterations that confer toxin resistance. The frequency of conversion to all three variant phenotypes was enhanced by treatment with the missense mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), suggesting that all three variant phenotypes are the result of mutational events. Incorporation of [^{35}S]methionine into the α-subunit of the sodium channel (M_r = 270,000; pI = 5.8 ± 0.2) was studied in normal and variant clones by affinity chromatography on wheat germ agglutinin/Sepharose followed by analysis of labeled polypeptides by 1- and 2-dimensional gel electrophoresis. Sodium channel-deficient clones do not synthesize the α-subunit of the sodium channel, suggesting that mutations in these clones block expression of the gene for this protein subunit. The scorpion toxin-resistant clone LV10 synthesizes an α-subunit which has a molecular weight and pI similar to those of the parental clones within the resolution of the methods used.

INTRODUCTION

Three groups of neurotoxins are known to bind with high affinity and specificity to distinct sites on the sodium channels responsible for action potentials in neuronal cells and, consequently, alter their properties (reviewed in ref. 1). Tetrodotoxin and saxitoxin block sodium channel ion transport by binding to a common receptor site on the sodium channel (2). Lipid-soluble toxins such as batrachotoxin and veratridine bind to a second receptor site and cause persistent activation of the channel (1). Polypeptide toxins like the toxin from the venom of the scorpion *Leiurus quinquestriatus* act cooperatively with the lipid-soluble toxins to cause persistent activation of sodium channels, inhibit the inactivation of the channel, and bind to a third receptor site on the channel in a membrane potential-dependent manner (1). The action of all of these toxins on the sodium channels can be studied by measurement of sodium flux across the membrane of cloned neuroblastoma cells (3).

Photoaffinity labeling of the scorpion toxin receptor and purification of the saxitoxin receptor from eel electric organ or rat brain each result in identification of a polypeptide of M_r of approximately 270,000, designated α, as an essential component of the sodium channel (4–6). In addition, a second polypeptide of M_r of approximately 38,000, designated β, is a component of the sodium channel from rat brain (5, 6). Genetic analysis of sodium channels can be complementary to biochemical experiments and can potentially elucidate how sodium channel subunits interact within the channel structure and what role each one plays in sodium channel function. Variant neuroblastoma clones with altered channels may provide useful tools in biochemical and electrophysiological studies. This genetic approach has been particularly successful in the study of another membrane-bound protein complex, hormone-stimulated adenylate cyclase (7–9).

Previously, West and Catterall (10) have found that neurotoxins that cause persistent activation of the sodium channels are cytotoxic to neuroblastoma cells. This enabled them to isolate variant clones with no functional sodium channels and one clone that has sodium channels with altered affinity for scorpion toxin. In the present work, we have studied the effect of mutagen treatment on the frequency and phenotype of toxin-resistant clones and have examined both parental and variant clones for the synthesis of the α-subunit of the sodium channel.

EXPERIMENTAL PROCEDURES

Materials. Chemicals and tissue culture reagents were obtained from the following sources: DMEM,[1] fetal calf

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[1] The abbreviations used are: DMEM, Dulbecco-Vogt modification

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serum, and newborn calf serum from Grand Island Biological Company (Grand Island, N. Y.); methionine-deficient medium from Irvine Scientific (Santa Ana, Calif.); trypsin from Worthington Biochemical Corporation (Freehold, N. J.); tetrodotoxin from Calbiochem (San Diego, Calif.); MNNG and veratridine from Aldrich Chemical Company (Milwaukee, Wisc.); scorpion venom (*L. quinquestriatus*) from Sigma Chemical Company (St. Louis, Mo.). Batrachotoxin was supplied by Dr. John Daly, Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health (Bethesda, Md.). Scorpion toxin was purified and iodinated as previously described (11). Radioactive isotopes were obtained from New England Nuclear Corporation (Boston, Mass.) and Amersham Corporation (Arlington Heights, Ill.). Ampholines were from LKB Instruments (Rockville, Md.). Radioactively labeled protein markers were from New England Nuclear Corporation.

Cell culture. Clone N18 of mouse neuroblastoma C1300 was used as the parental cell line for all studies. Cells were used between the 26th and 33rd subculture after the original cloning. Stock and experimental cultures of all clones were prepared as described previously (3). Newborn calf serum was comparable to fetal calf serum in all tests of plating efficiency and cell growth rate. Cell suspensions were prepared by incubation of monolayer cultures with 0.02% trypsin in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline followed by trituration in DMEM containing 5% newborn calf serum.

Mutagenic treatment and isolation of variant clones. N18 cells were seeded at a cell density of $1–2 \times 10^6$ cells/100-mm diameter Petri dish containing DMEM and 10% calf serum. On the following day the cells were incubated with MNNG for 2 hr, rinsed with Dulbecco's phosphate-buffered saline, resuspended in fresh medium, and seeded into new culture dishes at cell densities of 10–50 cells/cm^2 to measure cell survival and 20,000–60,000 cells/cm^2 to prepare stock cultures for selection. After 7 days of growth to allow expression of altered phenotypes, cells in these stock cultures were suspended by treatment with trypsin and seeded in selective medium. The cytotoxic effect of toxins was highly dependent on the toxin concentration and on the cell density. Therefore, the following conditions were used for selection: 10^6 cells in 10 ml of DMEM, 10% calf serum, and 6-thioguanine (5 μg/ml); 5×10^4 cells in 10 ml of DMEM, 10% calf serum, 50 nM scorpion toxin, and 40 μM veratridine; 10^5 cells in 10 ml of DMEM, 10% calf serum, and 0.5–1 μM batrachotoxin. Cultures were incubated undisturbed for 10–15 days. The visible colonies were then counted. Each variant clone was isolated by encircling a single toxin-resistant colony with a porcelain cylinder sealed to the Petri dish with sterile silicone grease. Cells were resuspended in fresh medium, transferred to a 16-mm diameter well of a Costar plate, and allowed to grow in DMEM containing 10% calf serum. This cloning procedure was then repeated once more.

of Eagle's minimal essential medium; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate.

Measurement of Na^+ permeability. The procedures for measurement of the effects of neurotoxins on stimulation and inhibition of Na^+ flux have been previously described (3, 11). Extensive control experiments have shown that under the experimental conditions used the increase in Na^+ uptake is linearly proportional to the P_{Na}.

Protein concentrations were determined by the method of Lowry *et al.* (12).

Measurement of membrane potential. Membrane potentials were calculated from the equilibrium distribution of the lipid-soluble anion $^{35}SCN^-$ between the intracellular and extracellular compartments according to the Nernst equation (E_m = 61 mV log ($[^{35}SCN^-]_{in}/[^{35}SCN^-]_{out}$)), as described by Catterall *et al.* (13). The measurements of membrane potentials of N18 cells by this technique agree closely with measurements made by microelectrode impalement (13).

Measurement of scorpion toxin binding. Binding of mono-[^{125}I]iodoscorpion toxin was performed, as described previously (11), in the presence of 0.1–0.2 nM labeled toxin.

Polyacrylamide gel electrophoresis. Cell cultures growing in 100-mm Petri dishes were labeled in 2 ml of methionine-deficient DMEM supplemented with 5 μM L-methionine, 5% dialyzed fetal calf serum, and 100 μCi of [^{35}S]methionine (>600 Ci/mmole) for 3 hr. After three washes in phosphate-buffered saline, the cells from each plate were lysed in 5 ml of 10 mM Tris-HCl/1 mM EDTA (pH 7.5) and homogenized in a glass homogenizer with a Teflon pestle (30 strokes). The cell homogenate was centrifuged at 2,000 rpm for 2 min. The supernatant was collected and centrifuged at 18,000 rpm for 30 min. The pellet was solubilized in 1 ml of Solution A (400 mM choline chloride, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (adjusted to pH 7.4 with Tris base), 10 mM $CaCl_2$, 0.1% Triton X-100, and 0.02% phosphatidylcholine, supplemented with 1% Triton X-100. This suspension was applied to a column with 2 ml of WGA-Sepharose 4B, the column was washed with 5 volumes of Solution A, and the sample was eluted with Solution A containing 40 mM *N*-acetylglucosamine (6). Fractions containing measurable radioactivity were pooled, and 50 μg of bovine serum albumin were added as carrier protein. The protein was precipitated in 10% trichloroacetic acid for 30 min on ice and collected by centrifugation at 10,000 × *g* for 10 min. The precipitate was washed three times in acetone and dried. The pellet was resuspended in 15 μl of water and boiled in the presence of 2.5% β-mercaptoethanol and 0.8% SDS for 2 min. For 2-dimensional gel electrophoresis, 50 μl of isoelectric-focusing buffer were added and the sample was stored in liquid nitrogen until ready to use. Isoelectric focusing was carried out as described by O'Farrell (14), except that the concentration of Triton X-100 and ampholines in the isoelectric focusing buffer and gel were twice that suggested. The second dimension was run in 7% acrylamide SDS-gels.

One-dimensional polyacrylamide gel electrophoresis of the trichloroacetic acid-precipitated sample was carried out as described by Maizel (15). The gels were then treated for fluorography (16), dried, and exposed to Kodak X-Omatic film at −70°.

Synaptosomal proteins covalently labeled with azidonitrobenzoyl mono-[^{125}]iodoscorpion toxin were supplied by Dr. Daniel Beneski (5).

RESULTS

Effect of mutagen on frequency of resistant colonies. Neurotoxins that specifically activate sodium channels are highly cytotoxic to N18 cells when present in the growth medium (10). Tetrodotoxin blocks the cytotoxic effect of these toxins, indicating that they exert their toxicity by specifically altering the Na^+ permeability of the cells through activation of sodium channels. Using 40 μM veratridine plus 50 nM scorpion toxin as a selective medium, West and Catterall (10) isolated 10 toxin-resistant clones exhibiting 3 different phenotypes. Seven clones had no detectable Na^+ channels. One clone, named LV10, had functional Na^+ channels with a lower affinity for scorpion toxin and altered voltage dependence of toxin action. The two remaining clones had normal Na^+ channels but were resistant to the toxins.

If all of these variant phenotypes result from mutational alteration of the cell genome, their frequency should be increased by treatment with a mutagen. For this test, we chose the mutagen MNNG, which is known to cause point mutations in prokaryotes (17). The effect of MNNG on plating efficiency of N18 cells is shown in Fig. 1. N18 cells are extremely sensitive to MNNG, with a 50% reduction in plating efficiency at 0.25 μg/ml under these conditions (Fig. 1). For best results, we found that the exposure to this mutagen should be for 2–4 hr. After MNNG treatment, the cells were incubated for at least 7 days to allow expression of altered phenotypes, plated in the presence of scorpion toxin and veratridine, and the plating efficiency under these selective conditions was measured. MNNG treatment increased the frequency of resistant colonies up to 16-fold in the concentration range from 0.25 to 2 μg/ml (Fig. 2). Since the measurement of mutation rate for different markers is known to vary widely, we also measured the frequency of 6-thioguanine resistance in the same cell population as a reference (Fig. 2). Resistance to 6-thioguanine is caused by mutations resulting in a loss or inactivation of the enzyme hypoxanthineguanine phosphoribosyl transferase (EC 2.4.2.8), which is coded by a gene located in the X chromosome (18, 19). The 6-thioguanine-resistant phenotype has been shown to result from mutational events (20, 21), and the mutation frequency at this locus has been well-documented for several cell lines. At the highest concentration of MNNG used, the frequency of 6-thioguanine resistance increased by at least 100-fold above the control (Fig. 2). These values compare well with published data from other cell lines (22).

The concentration dependence of the MNNG-induced increase in frequency of the 6-thioguanine-resistant and neurotoxin-resistant phenotypes is identical within experimental error (Fig. 2). These results provide strong evidence that the neurotoxin-resistant phenotype can arise from mutational events. In contrast, the absolute value of the frequency for conversion to 6-thioguanine resistance is much smaller than that for conversion to neurotoxin resistance. MNNG increases the frequency of 6-thioguanine-resistant colonies from less than 1.0×10^{-6} to 90×10^{-6} but increases the frequency of neurotoxin-resistant colonies from 4×10^{-5} to 66×10^{-5} (Fig. 2). Thus, the background rate of appearance of neurotoxin-resistant colonies is much greater than that for 6-thio-

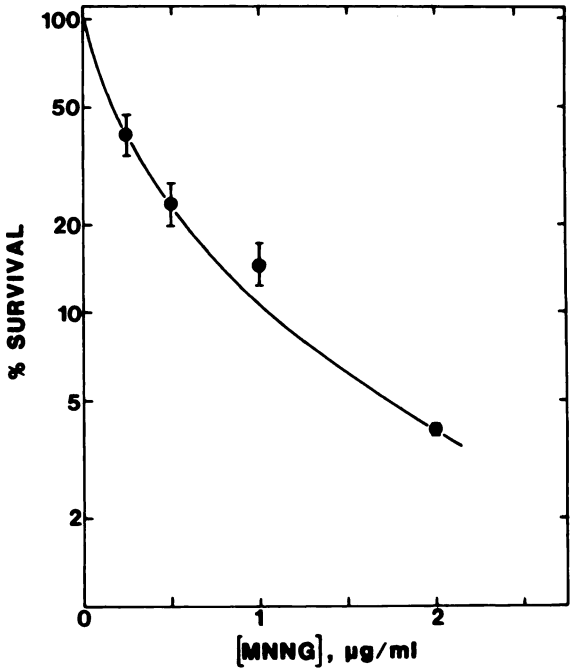

FIG. 1. *Effect of MNNG on survival*
N18 cells were mutagenized and plated as described under Experimental Procedures. Each point represents the mean of seven independent experiments. *Vertical bars* represent standard error of the mean.

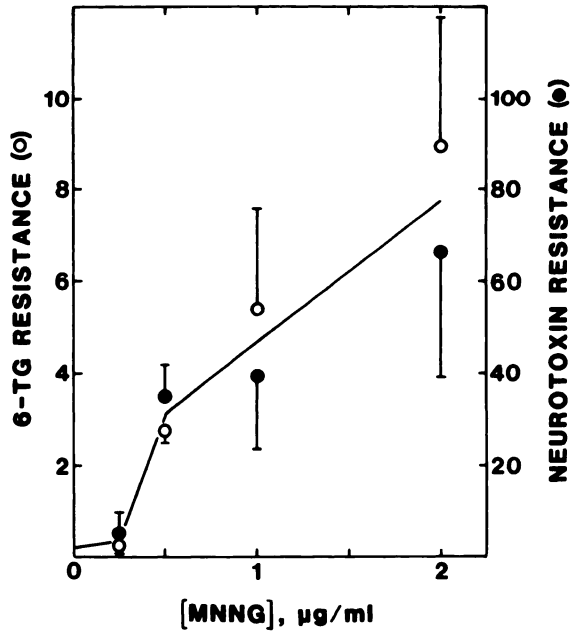

FIG. 2. *Effect of MNNG on frequency of resistant colonies*
Control and mutagenized cultures were incubated for 7 days to allow expression of the altered genome and subsequently plated in the appropriate selective medium, containing either 6-thioguanine (5 μg/ml) (○) or 50 nM scorpion toxin plus 40 μM veratridine (●). Each point represents the mean of four experiments. *Vertical bars* represent standard error of the mean. Toxin resistance is expressed as the number of resistant colonies per 10^5 cells plated.

guanine-resistant colonies whereas the effect of MNNG is comparable for both phenotypes.

In order to test whether the high rate of spontaneous conversion to the neurotoxin-resistant phenotype was due to heterogeneity in the cell culture population, several single-cell subclones were isolated from the parental cell line N18. In each case, these subclones exhibited a frequency for resistance to selective medium containing 40 μM veratridine and 20–100 nM scorpion toxin equal to N18. Thus, the high frequency of spontaneous conversion to the neurotoxin-resistant phenotype is characteristic of the majority of cells in the N18 cell population.

Effect of mutagen on the phenotype of neurotoxin-resistant clones. In the previous work using this selection method without mutagen treatment (10), 70% of the neurotoxin-resistant clones studied had no detectable sodium channels and only 10% of the clones (1 of 10) had altered sodium channels. Therefore, it was of interest to determine whether mutagen treatment increased the fraction of clones having altered sodium channels.

In several experiments using scorpion toxin and veratridine as selective agents as in Fig. 2, neurotoxin-resistant colonies were isolated, allowed to grow, and tested for three biochemical properties of sodium channels: (a) stimulation of $^{22}Na^+$ influx by the full-agonist batrachotoxin; (b) cooperative effect of 1, 50, and 200 nM scorpion toxin on $^{22}Na^+$ influx stimulated by veratridine, a partial agonist; and (c) binding of ^{125}I-labeled scorpion toxin. The results from these experiments on 36 clones are summarized in Table 1. In summarizing these phenotypes, which were described in detail in previous work (10), we have considered clones having less than 20% of parental batrachotoxin-stimulated $^{22}Na^+$ uptake and ^{125}I-labeled scorpion toxin binding to be sodium channel-deficient. Presumably, these clones survive the selection because they have a reduced number of sodium channels. Resistant clones having greater than 20% of the parental batrachotoxin-stimulated $^{22}Na^+$ uptake but with an altered value of $K_{0.5}$ for scorpion toxin action or a reduced maximal response to scorpion toxin are considered scorpion toxin-resistant. Finally, a small number of resistant clones have greater than 20% of the parental level of apparently normal sodium channels and are considered parental with respect to sodium channel phenotype. The data of Table 1 show that mutagen treatment does not markedly alter the relative frequency of each phenotype of variant clones under conditions where the occurrence of resistant colonies is increased 8- to 16-fold. In both the presence and absence of mutagen, most of the resistant clones are sodium channel-deficient, having lost from 80% to essentially 100% of their functional sodium channels. Similarly, in both untreated and treated populations, 10–20% of the resistant clones have sodium channels that are scorpion toxin-resistant, and approximately 10% of the resistant clones have a parental phenotype with respect to sodium channels. Presumably, these resistant clones with parental sodium channels have heritable changes in other cellular functions that allow them to grow in spite of the large increase in sodium permeability caused by toxins. Thus, the frequency of all three phenotypes of resistant clones is increased by mutagen treatment, suggesting that all three are the result of mutational events.

TABLE 1

Phenotype distribution among clones isolated in selective media

Mutagen treatment	No. of clones		
	Sodium channel-deficient	Scorpion toxin-resistant	Parental type
−	8	2	1
+	19	3	3

It is noteworthy that, in earlier work (10) and in this study of a larger number of clones, no variants were obtained that are specifically resistant to the alkaloid toxins, veratridine and batrachotoxin, but retain functional sodium channels as assessed by ^{125}I-labeled scorpion toxin binding. Since the selection utilizes both veratridine and scorpion toxin, we would have expected some clones to develop specific resistance to veratridine while retaining normal binding of ^{125}I-labeled scorpion toxin. The lack of such clones suggests that alterations at the alkaloid toxin receptor site are either very infrequent or have such serious functional consequences that a nonfunctional sodium channel results. In order to search directly for such clones, we used a selection medium containing 1 μM batrachotoxin, an alkaloid toxin that is a full agonist in activating sodium channels (3). Plating efficiency for N18 was reduced to 2×10^{-4} in this medium, indicating effective killing of parental cells. Fifteen resistant clones were isolated and the properties of their sodium channels analyzed as described for the clones of Table 1. None of these clones have sodium channels which are resistant to alkaloid toxin action but retain scorpion toxin binding, confirming that such alterations at the alkaloid toxin receptor site are either very infrequent or functionally damaging.

Characterization of new scorpion toxin-resistant clones. Three clones described in Table 1 with altered scorpion toxin affinity were subcloned and the properties of their sodium channels were studied in more detail. The affinity of the various neurotoxins for the sodium channel was measured by ^{22}Na flux experiments. Batrachotoxin stimulates flux in all three variant clones with a similar concentration dependence (Fig. 3A). In several experiments, analysis of ion flux data on double-reciprocal plots showed that the $K_{0.5}$ for sodium channel activation by batrachotoxin ranges from 0.6 μM to 1.5 μM for these four cell lines. In all three scorpion toxin-resistant clones, the number of functional channels is reduced as judged by the V_{max} for ^{22}Na uptake derived from double-reciprocal plot analysis of ion flux data: LV30 has approximately 68%, LV31 32%, and LV32 55%, respectively, of the $^{22}Na^+$ influx of N18. The effect of tetrodotoxin on normal and variant clones is illustrated in Fig. 3B. Cells were incubated with high concentrations of scorpion toxin (50 nM), and veratridine-stimulated uptake was measured in the presence of increasing concentrations of tetrodotoxin (Fig. 3B). The concentration of tetrodotoxin needed to block 50% of the stimulated uptake was approximately 12 nM in the variant clones as well as in N18 cells. Thus, these clones have normal toxin binding at two of the three neurotoxin receptor sites.

The values of $K_{0.5}$ for scorpion toxin enhancement of

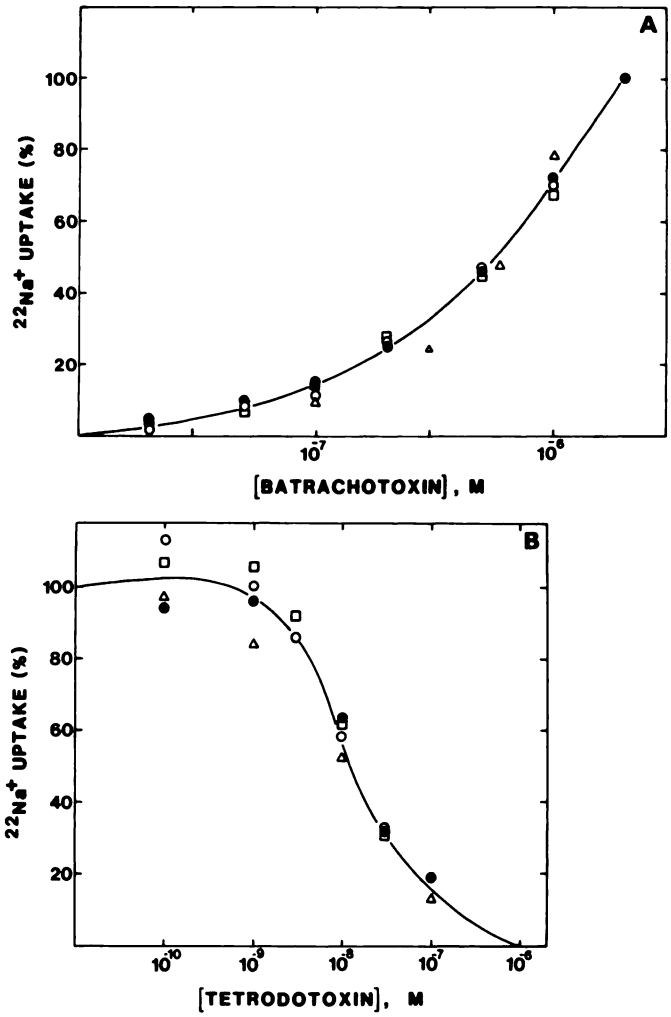

FIG. 3. *Effect of batrachotoxin (A) and tetrodotoxin (B) on ^{22}Na uptake by N18 and three variant clones*

A. $^{22}Na^+$ uptake assays were carried out as described in detail previously (3). Briefly stated, N18 cells were incubated for 30 min at 36° with the indicated concentrations of batrachotoxin in K^+-substituted medium to allow toxin binding and activation of sodium channels without alteration of ionic gradients. The initial rate of $^{22}Na^+$ influx was then measured for 30 sec at 36° in choline-substituted medium containing 10 mM $^{22}NaCl$ and 5 mM ouabain and the cells were washed at 0° in a choline-substituted wash medium. $^{22}Na^+$ uptake was determined by liquid scintillation counting, and the increase of $^{22}Na^+$ uptake by 2 μM batrachotoxin was taken as 100% stimulation. These values, in nanomoles per minute per milligram, were 125.1 for N18 (●), 85.4 for LV30 (□), 49.1 for LV31 (○), and 59.4 for LV32 (△).

B. $^{22}Na^+$ uptake assays were carried out as described in detail previously (11). Briefly stated, N18 cells were incubated for 30 min at 36° with 100 nM scorpion toxin in choline-substituted medium. The initial rate of $^{22}Na^+$ influx was then measured for 30 sec at 36° in choline-substituted medium containing 10 mM $^{22}NaCl$, 5 mM ouabain, 200 μM veratridine, and the indicated concentrations of tetrodotoxin. $^{22}Na^+$ uptake was determined by liquid scintialltion counting. Values of maximal ^{22}Na uptake in the presence of scorpion toxin and veratridine, in nanomoles per minute per milligram, were 85.8 for N18 (●), 35.2 for LV30 (□), 29.2 for LV31 (○), and 57.6 for LV32 (△).

veratridine-stimulated $^{22}Na^+$ flux are shown in Table 2. In clones LV30 and LV31, $K_{0.5}$ values are 27 and 10 times higher than in parental N18, respectively. In clone LV32 there is an increase of only 7-fold. Since the binding of scorpion toxin to sodium channels is directly dependent on membrane potential (11), this shift in scorpion toxin affinity could be caused by a more positive membrane potential in these clones. Previous studies on N18 cells have shown that a membrane depolarization from −30 mV to −20 mV would cause a change in K_D for scorpion toxin from 1 to 2.5 nM (11). As shown in Table 2, clones LV31 and LV32 have more positive membrane potentials than do N18. Thus, a fraction of the increase in $K_{0.5}$ for scorpion toxin action in these clones may be due to alterations in the resting membrane potential of the cells. In contrast, clone LV30 did not have an altered resting membrane potential, so the entire 27-fold increase in $K_{0.5}$ must be due to an alteration in sodium channel properties.

The affinity for scorpion toxin can also be measured in binding experiments with ^{125}I-labeled scorpion toxin (11), although the binding measurements are less sensitive than ion flux measurements for clones with high K_D values. For the parental clone N18, values for $K_{0.5}$ and K_D agree closely (ref. 11 and Table 2). In contrast, for the variant clones, values for $K_{0.5}$ tend to be greater than values for K_D (Table 2). Thus, the allosteric interaction of the scorpion toxin receptor site with the receptor site for veratridine and other alkaloid toxins may also be modified in the variant, scorpion toxin-resistant clones. For clone LV30, a 10-fold increase in K_D for toxin binding is observed together with a 27-fold increase in $K_{0.5}$ for toxin action (Table 2).

Synthesis of the α-subunit of the sodium channel in parental and variant clones. Protein components of the sodium channel have been identified by photoaffinity-labeling methods in neuroblastoma cells and rat brain membranes (5) and by purification from electric eel (4) rat skeletal muscle (23), and rat brain (6). In electric eel, rat brain, and neuroblastoma cells a polypeptide of $M_r \simeq 270{,}000$ has been shown to be an important component of the sodium channel (4–6). We have designated this subunit α to distinghish it from the smaller β-subunit ($M_r \simeq 38{,}000$), which is also a component of the sodium channel in rat brain (5, 6). Since α is an unusually large polypeptide, it seemed likely that it could be identified in 1- or 2-dimensional gel electrophoretic analyses of

TABLE 2

Membrane potential and affinity for scorpion toxin in N18 and variant clones

Clone	Membrane potential	$K_{0.5}$ for stimulation of $^{22}Na^+$ uptake[a]	K_D for binding	Expected $K_{0.5}$ due to membrane depolarization
	mV	*nM*	*nM*	*nM*
N18	−30.6 ± 2.8	0.7	0.6	
LV30	−34.1 ± 2.0	19.0	5	0.7
LV31	−20.1 ± 1.5	7.1	ND[b]	2.5
LV32	−21 ± 2.8	5.6	1.8	2.5

[a] $K_{0.5}$ for enhancement of veratridine activation of sodium channels was measured as described previously (11). Briefly stated, N18 cells were incubated for 30 min at 36° with increasing concentrations of scorpion toxin from 0.1 nM to 100 nM in choline-substituted medium. The initial rate of $^{22}Na^+$ uptake was then measured for 30 sec at 36° in choline-substituted medium containing 10 mM $^{22}NaCl$, 5 mM ouabain, 100 μM veratridine, and the same concentration of scorpion toxin.

[b] ND, No detectable binding.

neuroblastoma cell proteins. N18 cells have 156 fmoles of saxitoxin receptor sites per milligram of cell protein (24). Assuming that there is one mole of α per mole of saxitoxin receptor sites, then there should be 156 fmoles of α per milligram of cell protein (24), and α should comprise 0.004% of the cellular protein.

In order to detect protein(s) in such small amount, the cells were labeled with [^{35}S]methionine and the membrane glycoproteins were partially purified by affinity chromatography on a column of WGA-Sepharose 4B. This affinity chromatography procedure brings about 12-fold enrichment of the solubilized saxitoxin-binding activity from rat brain (6). The amount of radioactivity eluted from WGA-Sepharose-4B column by *N*-acetylglucosamine was approximately 1% of the total amount present in the initial cell homogenate and 10% of radioactivity in the soluble Tritron X-100 extract. This purified glycoprotein fraction was then subjected to 1- or 2-dimensional gel electrophoresis, and the [^{35}S]methionine-labeled proteins were detected by autoradiography as described under Experimental Procedures.

Figure 4 shows the autoradiogram of a single dimension SDS-polyacrylamide gel electrophoresis of labeled proteins from parental N18 and variant clone LV26, a sodium channel-deficient clone with no detectable neurotoxin-stimulated $^{22}Na^+$ uptake or neurotoxin binding (10). A marker sample of brain synaptosomes covalently labeled with a photoactivatable derivative of iodinated scorpion toxin as described by Beneski and Catterall (5) was run in parallel to identify the α- and β-subunits of the sodium channel. N18 cells (*Lane 3*) have a prominent band of [^{35}S]methionine-labeled protein which comigrates with the α-subunit of photoaffinity-labeled synaptosomes. In contrast, the sodium channel-deficient (10) clones LV26 (*Lane 2*) and LV9 (not shown) do not incorporate [^{35}S] methionine into the α-subunit. Thus, variant neuroblastoma cells which are sodium channel-deficient by functional criteria such as ion flux and neurotoxin binding lack the protein band corresponding to the α-subunit of the sodium channel.

In order to determine whether this band represents a single protein, [^{35}S]methionine-labeled glycoproteins of N18 cells and variant clones were analyzed by 2-dimensional gel electrophoresis. The saxitoxin receptor of the sodium channel from rat brain, consisting mainly of the α-subunit, is an acidic protein which adsorbs quantitatively to DEAE-Sephadex at pH 6.5 (6). Figure 5 shows that only a single protein spot, which is streaked markedly in the pH dimension, comigrates with α in the molecular weight dimension. This broad spot has a mean isoelectric point of 5.8 ± 0.2 in several gels run under these experimental conditions. We are unable to specify at present whether the apparent charge heterogeneity of α observed in these gels reflects a true heterogeneity in either the protein or carbohydrate composition of the subunit or incomplete solubilization of the hydrophobic α-subunit under the conditions of the pH gradient electrophoresis.

As in the case of 1-dimensional gel analysis (Fig. 4), the sodium channel-deficient clones are missing the spot corresponding to the α-subunit. Figure 5C illustrates this point for clone LV26. Two other sodium channel-deficient clones tested (LV2 and LV9) also failed to show

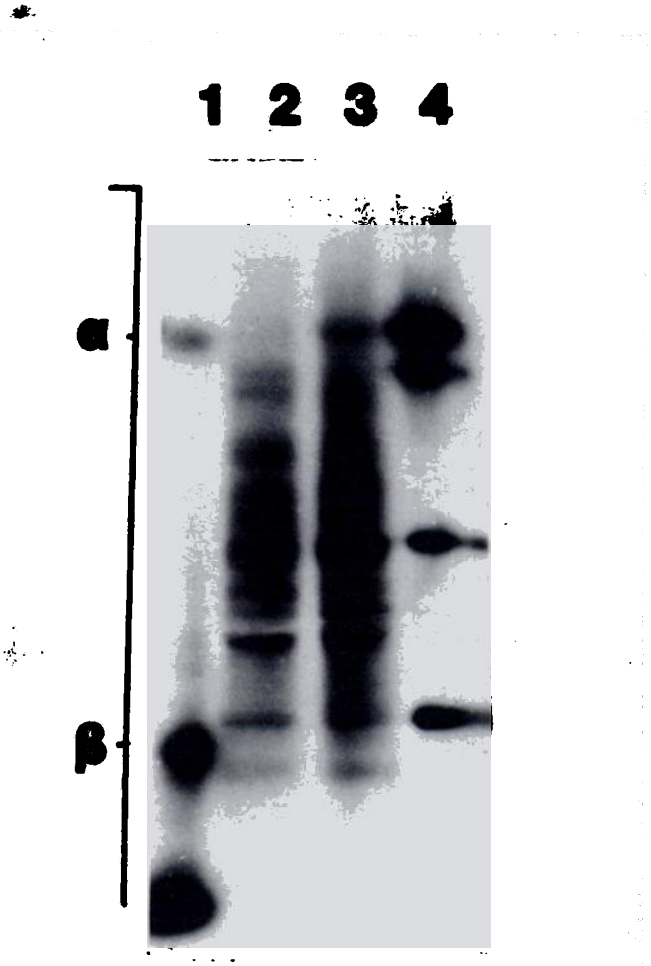

FIG. 4. *SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins*

Partially purified proteins of [^{35}S]methionine-labeled cultures from LV26 (*Lane 2*) and N18 (*Lane 3*) were prepared and subjected to electrophoresis as described under Experimental Procedures. Approximately 2.3×10^4 cpm were loaded in to each well. Two types of protein standards were run in parallel: rat synaptosomal proteins covalently labeled with azidonitrobenzoyl-[^{125}I]iodoscorpion toxin (*Lane 1*) and a mixture of [*methyl*-^{14}C]methylated proteins (*Lane 4*) that included, from *top* to *bottom*, filamin (M_r = 280,000), myosin (M_r = 200,000), phosphorylase (M_r = 93,000) and ovalbumin (M_r = 46,000). Gels were treated for fluorography and exposed to Kodak X-Omat film for 10 days.

this spot. Comparison of Fig. 5A and C shows that other protein spots are missing or changed in amount in LV26. These differences were not as apparent in the 1-dimensional gel analysis of Fig. 4. Thus, the mutational events that give rise to sodium channel-deficient variants apparently can affect multiple membrane glycoproteins. However, in analyzing several such clones, the spot at M_r of approximately 270,000 and pI of approximately 5.8 corresponding to the α-subunit of the sodium channel was the only one consistently missing. Therefore, we can conclude that the sodium channel-deficient phenotype most likely results from failure to synthesize the α-subunit of the sodium channel, perhaps along with the other subunit components.

It was also of interest to analyze the other two variant phenotypes isolated by our selection procedure. Figure

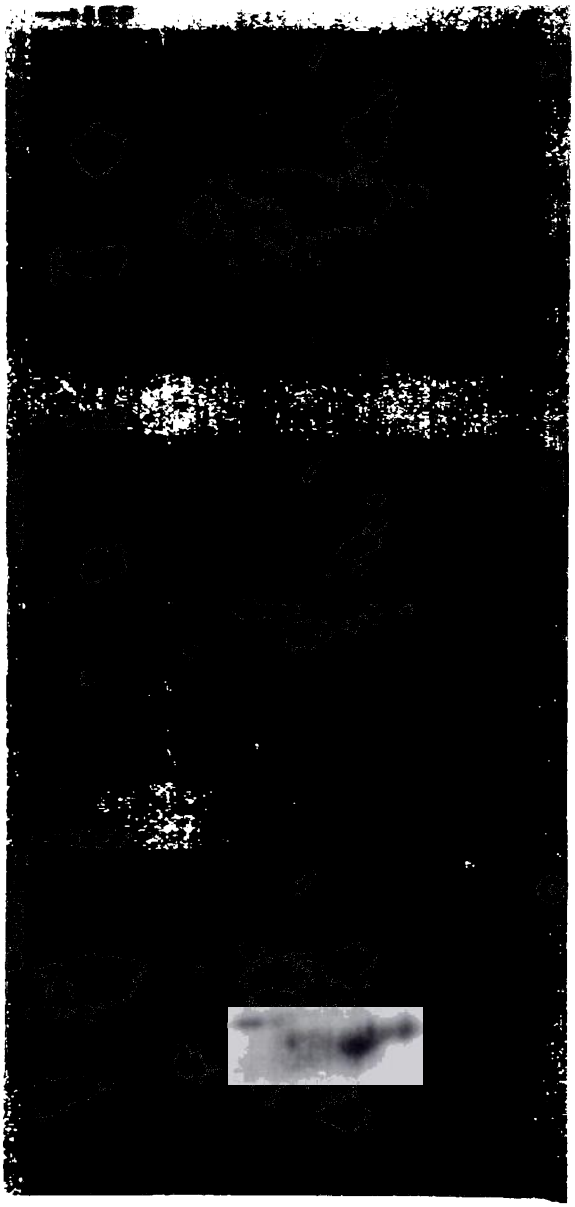

FIG. 5. *Isoelectric focusing of [^{35}S]methionine-labeled proteins*
N18 (A), LB1 (B), and LV26 (C) cultures were labeled with [^{35}S] methionine. Membrane proteins were partially purified through WGA-Sepharose and subjected to isoelectric focusing (*IEF*) followed by SDS-polyacrylamide gel electrophoresis. Each sample loaded into isoelectric-focusing gels contained approximately 7×10^4 cpm. Gels were treated for fluorography and exposed to Kodak X-Omat film for 42 days.

5B illustrates a 2-dimensional gel analysis of glycoproteins in clone LB1, a neurotoxin-resistant clone that has apparently normal sodium channels by toxin-binding and ion flux criteria (10). This clone has a prominent spot corresponding to the α-subunit of the sodium channel, as expected from its phenotype. Thus, neurotoxin resistance resulting from mechanisms other than loss of sodium channels is not associated with loss of the protein spot corresponding to the α-subunit.

We have also studied the scorpion toxin-resistant clone LV10 by 1- and 2-dimensional gel electrophoresis. From our ion flux and neurotoxin-binding data, we have concluded that this clone synthesizes a reduced number of sodium channels with altered scorpion toxin-binding characteristics (10). Two-dimensional gel analysis (not shown) revealed a faint, broad spot corresponding to the α-subunit of the sodium channel. The breadth of the spot in the pH dimension did not allow us to determine whether a pH shift had occurred. Thus, we can conclude from these experiments that scorpion toxin-resistant clone LV10 synthesizes an α-subunit with molecular weight similar to that of parental N18. Further studies with increased resolution and more purified materials will be required to determine whether any of the variant cell lines with altered K_D values for scorpion toxin synthesize sodium channel subunits with altered pI values indicative of mutational change in charged amino acid residues.

DISCUSSION

The results presented continue the analysis of neuroblastoma clones selected for resistance to the cytotoxic effects of neurotoxins that activate sodium channels. Previous work (10) showed that the resistant clones obtained from nonmutagenized cell populations were of three phenotypes: sodium channel-deficient clones which lack functional sodium channels, scorpion toxin-resistant clones with modifications at the scorpion toxin receptor site, and resistant clones having sodium channels with parental properties. In this work, we have found that the point mutagen MNNG increases the frequency of all three of these toxin-resistant phenotypes up to 16-fold. The concentration dependence of MNNG action is the same for increase in frequency of neurotoxin-resistant variants and of 6-thioguanine-resistant variants which result from mutations in the structural gene for the enzyme hypoxanthine-guanine phosphoribosyltransferase (19–22). These results support the conclusion that all three resistant phenotypes result from mutational events.

Although our results suggest a mutational origin for the neurotoxin-resistant clones, we cannot conclude that these mutations occur in the structural genes for the sodium channel subunits. It seems likely that the sodium channel-deficient phenotype is due to heritable changes in expression of the sodium channel genes rather than to mutations within them. In the three sodium channel-deficient clones we have analyzed by 1- or 2-dimensional gel electrophoresis, we found no evidence for synthesis of the α-subunit of the sodium channel consistent with the conclusion that the gene coding for this protein is not expressed or poorly expressed in the sodium channel-deficient clones. Thus, our results suggest that mutational events can lead to lack of expression of the sodium channel genes in this cell line with a spontaneous frequency of 3×10^{-5}/cell plated.

The scorpion toxin-resistant phenotype is the best candidate for alteration of the structural genes of the sodium channel subunits. In previous work (10), one such clone was isolated from nonmutagenized cell populations and shown to have sodium channels with altered properties, including an increased K_D and altered voltage dependence of scorpion toxin binding. In this study, three more scorpion toxin-resistant clones were analyzed. How-

ever, two of these may have resulted from membrane potential alterations (Table 2). Therefore, of the 46 clones analyzed in the two studies, only two have sodium channels with clearly altered properties. Analysis of these by 2-dimensional gel electrophoresis demonstrated the presence of the α-subunit spot of the sodium channel. The breadth of the α-subunit in the pH dimension and the small amount of sodium channel protein present in these cell lines prevented an unequivocal assessment of a possible charge shift of the variant α-subunit as compared with N18 cells. Two-dimensional gel experiments with more highly purified sodium channel preparations and better resolution in pH gradient electrophoresis will be required to determine whether scorpion toxin-resistant clones have charge alterations in the α- or β-subunits of the sodium channel.

There is a marked quantitative difference between the frequency of conversion to neurotoxin resistance and the frequency of mutations in the structural gene for hypoxanthine-guanine phosphoribosyl transferase as assessed by 6-thioguanine resistance. The frequency of appearance of neurotoxin-resistant variants is at least 40 fold-higher in the absence of MNNG and 14-fold higher in the present of MNNG, 2 μg/ml. This difference in frequency may result from the presence of multiple mechanisms for generation of the neurotoxin-resistant phenotype in addition to mutations in the structural genes for the sodium channel. Only two scorpion toxin-resistant clones of the 46 variant clones characterized are likely to have mutations in the structural genes for the sodium channel. Thus, the frequency of appearance of variants with structural gene mutations in the sodium channel may be comparable to that for 6-thioguanine resistance. However, the majority of resistant clones are sodium channel-deficient and seem to result from other classes of mutations which reduce or block expression of the sodium channel genes. A similar high frequency of variant clones is also observed for the adenylate cyclase-deficient (*cyc*$^-$) phenotype of S49 lymphoma cells (7). This variant results from loss or inactivation of the guanine nucleotide-binding component required for hormone stimulation of adenylate cyclase (8). The high frequency of variant deficient in sodium channels or adenylate cyclase may reflect the fact that they are cell-surface proteins involved in differentiated functions and are not required for nutritional survival of the cells, in contrast to hypoxanthine-guanine phosphoribosyl transferase and most other markers studied.

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