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Selection of variant neuroblastoma clones with missing or altered sodium channels

(batrachotoxin/tetrodotoxin/scorpion toxin/sodium transport/electrical excitability)

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ABSTRACT Neurotoxins that cause persistent activation of voltage-sensitive sodium channels are highly cytotoxic to electrically excitable neuroblastoma cells. These toxins were used as selective agents to isolate variant neuroblastoma clones with missing or altered sodium channels. Of ten resistant clones analyzed, seven lacked functional sodium channels and one had a specific 40-fold increase in K_d for scorpion toxin and altered voltage dependence of scorpion toxin binding. The phenotypes of these cell clones were stable for more than 100 generations, indicating that they were the result of stable genetic change.

Neuroblastoma cells cultured *in vitro* are electrically excitable (1, 2) and generate sodium-dependent action potentials that are inhibited by tetrodotoxin, a specific inhibitor of sodium currents in nerves (2, 3). Three different neurotoxin receptor sites are associated with voltage-sensitive sodium channels in neuroblastoma cells. The lipid-soluble toxins veratridine, batrachotoxin, aconitine, and grayanotoxin bind to a common receptor site and cause persistent activation of sodium channels at the resting membrane potential (4–6). Polypeptide toxins from scorpion venom and from sea anemone nematocysts bind to a second receptor site and act cooperatively with the lipid-soluble toxins to activate sodium channels by an allosteric mechanism (5, 7–10). Tetrodotoxin and saxitoxin act at a third receptor site and inhibit ion transport by the sodium channel (4, 11). These three classes of toxins provide specific probes for functionally distinct components of the sodium channel. Our studies of the mechanism of action of these neurotoxins suggest the possibility that the sodium channel is a complex structure consisting of multiple interacting components.

Genetic methods involving selection and analysis of specific mutations have proven valuable in analyzing complex processes in prokaryotes and simple eukaryotes and in studying some metabolic pathways in mammalian cells in culture. It has proven more difficult to use this approach to analyze differentiated properties characteristic of specific cell types because these processes are often not required for cell growth. Genetic methods have been used successfully, however, to analyze the regulation of adenylate cyclase activity by hormones and other agents and to identify specific protein components of this system (12–15). These studies demonstrate the value of a genetic approach in analyzing complex physiological processes in cultured mammalian cells. In this report we describe the use of specific neurotoxins as selective agents to isolate variant neuroblastoma cell clones with missing or altered voltage-sensitive sodium channels and we present an initial characterization of the phenotypes of the neurotoxin-resistant clones.

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EXPERIMENTAL PROCEDURES

Materials. Chemicals and cell culture reagents were obtained from the following sources: the Dulbecco–Vogt modification of Eagle's medium and fetal calf serum from GIBCO; trypsin from Worthington; veratridine from Aldrich; and scorpion venom (*Leiurus quinquestriatus*) from Sigma. Batrachotoxin was supplied by John Daly and Bernhard Witkop (National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD). Scorpion toxin and [^{125}I]monoiodo scorpion toxin were prepared and purified as described (7, 16).

Cell Cultures. Clone N18 of mouse neuroblastoma C1300 was used for all studies. Cells were used between 22 and 40 subcultures after the original cloning. Stock and experimental cultures of N18 and the selected variant subclones were prepared as described (5).

Selection and Isolation of Variant Cell Clones. Cell suspensions were prepared after treatment with trypsin (5), and 10^5–10^6 cells were seeded in 100-mm culture dishes under selective conditions (50 nM scorpion toxin plus either 40 μM veratridine or 1 μM batrachotoxin) in 10% fetal calf serum/90% modified Eagle's medium. After 14–17 days, resistant colonies were encircled with porcelain cylinders sealed to the petri dish with sterile silicone grease and the cells were suspended after treatment with trypsin. The suspended cells were seeded at 30, 100, and 300 cells per 100-mm petri dish and allowed to attach for 1–3 hr. Individual cells were then located by microscope and encircled with a porcelain cylinder as before. The area within each cylinder was carefully scanned to ascertain that there was only a single cell in each. The cylinders were then filled with selective medium, the remainder of the plate was drained, and the cultures were returned to the incubator. Approximately 10% of cells encircled in this way grew to colonies. After the colonies had attained a size of 1000 cells or more, they were suspended by incubation with trypsin, seeded into larger culture dishes in nonselective medium, and grown as described for N18.

Measurement of Cytotoxicity of Neurotoxins. The cytotoxic effect of the neurotoxins was measured in two ways. In the first, the plating efficiency of cells was measured in normal growth medium and toxin-supplemented growth medium. Cells from stock cultures were suspended after treatment with trypsin and were seeded at various densities in 100-mm culture dishes. The cultures were incubated for 10 days without being disturbed. After this time, the dishes were drained, washed twice with isotonic saline, fixed by immersion in methanol, and stained with Giemsa. The colonies were then counted visually. Relative plating efficiency was calculated as the ratio of colonies observed with toxin to that observed without toxin multiplied by 100.

Cytotoxicity was also measured by estimating the fraction of cells in logarithmic growth phase killed by the toxins. In these experiments, cells were seeded at 3 × 10^4 per well in multiwell plates (1.6 cm diameter, Costar) and allowed to grow for 2 days. The growth medium was changed to one containing toxins and the cells were allowed to grow for 3 more days. The cultures were then washed with protein-free medium and suspended in 0.4 M NaOH, and cell protein was determined by the method of Lowry *et al.* (17). Although less rigorous than plating efficiency methods, this method required much less toxin and was more reproducible. It was therefore used when toxin concentration-effect relationships were studied.

Measurement of Sodium Permeability. The effect of batrachotoxin or veratridine plus scorpion toxin on sodium permeability of neuroblastoma cells was measured as described in refs. 6 and 7, respectively. The membrane potential dependence of scorpion toxin action was determined as in ref. 18. Protein was determined by the method of Lowry *et al.* (17).

Measurement of Scorpion Toxin Binding. Binding of [^{125}I]monoiodo scorpion toxin was measured as described (16).

RESULTS

Selection of variants

Inhibition of ion pumping in cells by ouabain is cytotoxic and has been used successfully to isolate variant cell clones resistant to ouabain (19). These studies suggest that alteration of normal ion gradients in cells is cytotoxic. Because neurotoxins that cause persistent activation of sodium channels in cells greatly increase sodium permeability, it seemed likely that these toxins might also be useful selective agents for variant cell clones.

The effect of different combinations of neurotoxins on the plating efficiency of N18 cells is presented in Table 1. Veratridine is a partial agonist, activating approximately 8% of sodium channels at saturation (6). Growth of cells in 40 μM veratridine has only a small effect on plating efficiency. Scorpion toxin does not activate sodium channels alone (7) and also has a small effect on plating efficiency. Incubation of cells with both 40 μM veratridine and 50 nM scorpion toxin activates 50% of sodium channels (6) and is highly cytotoxic, reducing plating efficiency to 0.03–0.07% of the control. The cytotoxic effect of veratridine plus scorpion toxin is completely inhibited by 1 μM tetrodotoxin, demonstrating that this is a specific effect due to activation of sodium channels.

Table 1. Effect of neurotoxins on plating efficiency of N18

Additions	% of control
Exp. I	
None	100
40 μM Veratridine	62
40 μM Veratridine + 1 μM tetrodotoxin	76
50 nM Scorpion toxin	67
40 μM Veratridine + 50 nM scorpion toxin	0.07
50 nM Scorpion toxin + 40 μM veratridine + 1 μM tetrodotoxin	86
Exp. II	
None	100
40 μM Veratridine + 50 nM scorpion toxin	0.03
1 μM Batrachotoxin + 50 nM scorpion toxin	0.004

Batrachotoxin is a full agonist, activating nearly 100% of sodium channels at saturation (6). Batrachotoxin plus scorpion toxin is even more cytotoxic than veratridine plus scorpion toxin, reducing plating efficiency to 0.004% of the control. Tetrodotoxin also blocks the cytotoxic effect of batrachotoxin plus scorpion toxin (not shown). These experiments demonstrate that persistent activation of sodium channels by neurotoxins is highly cytotoxic and suggest that these toxins might be useful tools to select variant neuroblastoma cells that lack sodium channels or have defective sodium channels.

Isolation and analysis of toxin-resistant variants

By use of the cloning procedure described under *Experimental Procedures*, 10 independently derived, toxin-resistant, single-cell clones were isolated. These resulted from two separate cloning experiments that gave similar results. The properties of these 10 resistant clones were tested after 10–20 generations of growth in nonselective medium. These data are presented in Table 2. Nine of the 10 resistant clones gave relative plating efficiencies greater than 10% (Table 2). These relative plating efficiencies represent a 500- to 3500-fold increase compared to N18. Veratridine- and batrachotoxin-stimulated $^{22}Na^+$ uptake and ^{125}I-labeled scorpion toxin binding were both reduced more than 95% in seven of the resistant clones (Table 2). These levels of toxin binding and sodium uptake are very low and, in some of these clones, may not be significantly greater than background. Thus, these seven resistant clones seem to have greatly reduced activity of voltage-sensitive sodium channels as measured in either ion-flux or ligand-binding experiments. In order to test the stability of this phenotype, we cultured clones LV9 and LB4 continuously for 4 months in nonselective medium and periodically tested them as in Table 2. The phenotype of these two clones remained stable with respect to both toxin resistance and sodium channel activities during this period of more than 100 cell generations. These seven resistant subclones seem to have undergone a stable, heritable loss of functional voltage-sensitive sodium channels. This loss might reflect a mutational event or a stable change in gene expression.

Two of the isolated clones, LV3 and LB1, had less than a 3-fold reduction in batrachotoxin-stimulated sodium uptake and retained measurable specific binding sites for ^{125}I-labeled

Table 2. Properties of toxin-resistant neuroblastoma clones

Cell line*	Relative plating efficiency	$^{22}Na^+$ uptake, nmol min^{-1} BTX (1 μM)	Veratridine (40 μM) + ScTX 0†	Veratridine (40 μM) + ScTX 2 nM†	Veratridine (40 μM) + ScTX 200 nM†	^{125}I-ScTX binding, fmol mg^{-1}
N18	0.02	83	31	85	116	21
LV9	71	4	<2	<2	3	<1
LV22	41	6	<2	3	7	<1
LV26	11	4	<2	<2	7	<1
LV2	46	5	<2	4	11	1.6
LV7	51	4	<2	3	12	<1
LB3	27	4	<2	<2	4	<1
LB4	16	4	<2	<2	5	<1
LV3	0.6	41	12	31	38	6.2
LB1	36	30	7	18	35	3.4
LV10	32	8	<2	6	32	<1

* Cell lines selected with scorpion toxin (ScTX) plus veratridine are labeled LV; those selected with scorpion toxin plus batrachotoxin (BTX) are labeled LB. The cell lines are grouped according to phenotype.
† Concentration of scorpion toxin.

scorpion toxin (Table 2). It is unlikely that the relatively small reduction in sodium channel activities observed for these two clones can account for their resistance to toxins. These clones may have other heritable changes that allow them to grow in the presence of toxins. These might include a more efficient sodium pump that can overcome the high level of sodium influx caused by toxins or a change in ionic requirements for a critical cellular process that allows cell function in the presence of high intracellular sodium. These two clones were not studied further in this work.

The last resistant clone, LV10, exhibited properties that differed from the others. Both batrachotoxin-stimulated sodium uptake and scorpion toxin binding were much reduced (Table 2). However, a significant increase in sodium uptake was observed in the presence of 200 nM scorpion toxin plus veratridine. The 5-fold increase in uptake caused by increasing the scorpion toxin concentration from 2 nM to 200 nM suggested that this cell clone might contain sodium channels with reduced affinity for scorpion toxin. This possibility was studied in two ways. The cytotoxic effect of various concentrations of scorpion toxin was measured for clones N18, LV9, LB4, and LV10 during the logarithmic phase of growth (see *Experimental Procedures*). Cells were cultured for 3 days in 40 μM veratridine plus increasing concentrations of scorpion toxin and the cell protein was measured. For N18 cells, the amount of cell protein was reduced 50% by 1 nM scorpion toxin (Fig. 1). For clones LV9 and LB3, which seem to lack voltage-sensitive sodium channels, no significant reduction in cell protein was observed at 100 nM scorpion toxin. Clone LV10 showed intermediate behavior, with 50% loss of cell protein at 50 nM. These results show that LV9 and LB3 have an absolute resistance to scorpion toxin whereas LV10 has a 50-fold shift in the concentration of scorpion toxin required for cytotoxicity. These results were obtained after more than 100 generations of growth under nonselective conditions.

The apparent affinity of sodium channels in clone LV10 for neurotoxins was measured directly in ion-flux experiments. In Fig. 2, concentration-effect curves for activation of sodium channels by batrachotoxin are illustrated. The ordinate axes have been adjusted so that the two sets of data appear superimposable. The maximum sodium uptake observed with LV10 is only 20% of that observed with N18, but the apparent K_d is 0.6 μM in each case. Concentration-effect curves for scorpion toxin are illustrated in Fig. 3. As with batrachotoxin, the maximum uptake observed with clone LV10 is approximately 20% of that observed with N18. In contrast, however, the concentration-effect curve for scorpion toxin is shifted 40-fold to higher concentration in LV10. These results suggest that a major change in scorpion toxin affinity has occurred in LV10 cells. Concentration-effect curves for inhibition of sodium channels in N18 and LV10 by tetrodotoxin are presented in Fig. 4. In each case, 50% inhibition is observed at approximately 3 nM. Thus, we find that clone LV10 has a major change in affinity at the scorpion toxin receptor site but no detectable change in affinity for ligands at the tetrodotoxin or batrachotoxin receptor sites.

In N18 cells, the K_d for scorpion toxin is voltage dependent and increases 10-fold for each 31 mV depolarization (16, 18).

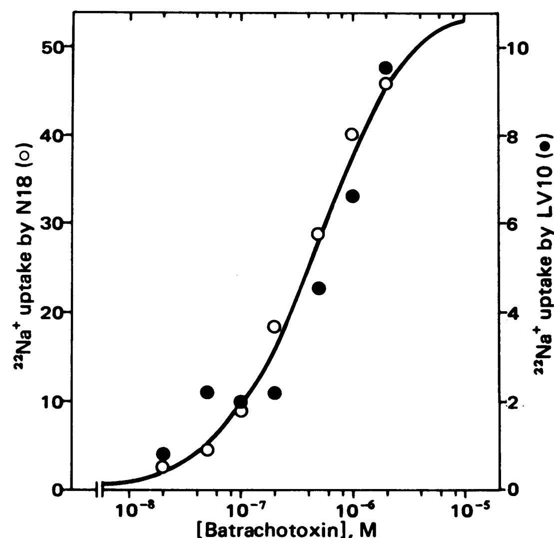

FIG. 2. Effect of batrachotoxin on $^{22}Na^+$ uptake by LV10 and N18 cells. N18 (○) or LV10 (●) cells were incubated for 30 min with the indicated concentrations of batrachotoxin; initial rates of $^{22}Na^+$ uptake were measured. The increment over control $^{22}Na^+$ uptake is plotted in nmol min^{-1} mg^{-1}. Note the different ordinate axes for N18 and LV10.

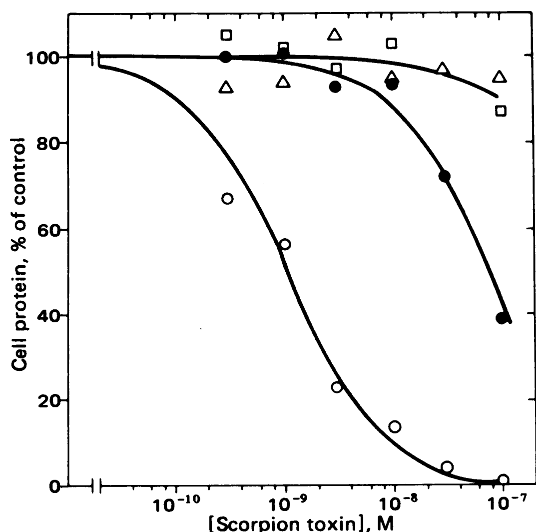

FIG. 1. Effect of scorpion toxin concentration on inhibition of cell growth. Cells of clones N18 (○), LV9 (△), LV10 (●), and LB4 (□) were seeded at 3 × 10^4 cells per well in multiwell plates. Growth inhibition by scorpion toxin in the presence of 40 μM veratridine was measured.

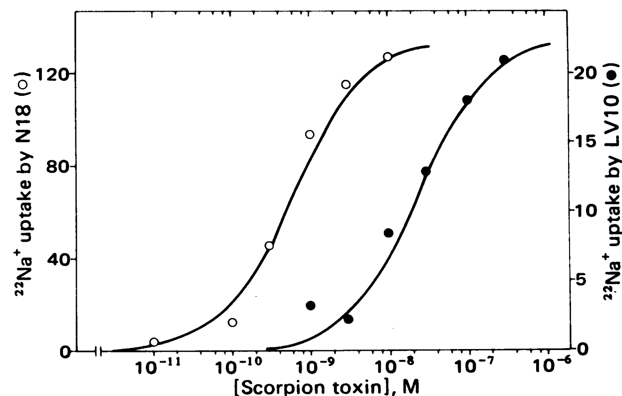

FIG. 3. Effect of scorpion toxin on veratridine-stimulated $^{22}Na^+$ uptake by LV10 and N18 cells. N18 (○) or LV10 (●) cells were incubated for 30 min with the indicated concentrations of scorpion toxin; initial rates of $^{22}Na^+$ uptake were measured in the presence of 200 μM veratridine. The increment over veratridine-stimulated uptake is plotted in nmol min^{-1} mg^{-1}. Note the different ordinate axes for N18 and LV10.

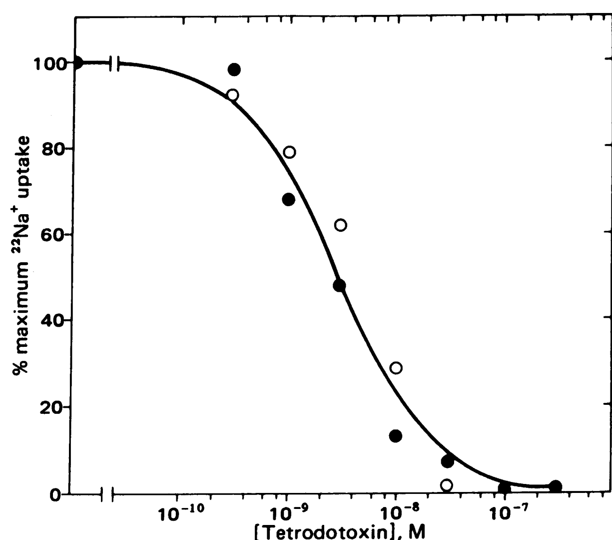

FIG. 4. Effect of tetrodotoxin on toxin-stimulated sodium uptake by LV10 and N18 cells. N18 (O) or LV10 (●) cells were incubated for 30 min with 100 nM scorpion toxin plus the indicated concentrations of tetrodotoxin; initial rates of $^{22}Na^+$ uptake were measured in the presence of 200 μM veratridine and the same concentrations of tetrodotoxin. Data are presented as the percent of the maximum toxin-stimulated $^{22}Na^+$ uptake in the absence of tetrodotoxin.

In view of these earlier results, it was important to determine whether clone LV10 cells have a large change in resting membrane potential that might account for the 40-fold change in apparent K_d. From our earlier work (16), a 50 mV depolarization of LV10 relative to N18 would be required to account for the observed difference in K_d. Membrane potential and apparent K_d for scorpion toxin were measured in companion experiments for LV10 and N18. Membrane potentials were calculated from the equilibrium distribution of the lipid-soluble anion $^{35}SCN^-$ between the intracellular and extracellular compartments as described (18). Membrane potentials determined by this technique compare favorably with measurements made by microelectrode impalement of N18 cells (18). In these comparison experiments, a 50-fold difference in apparent K_d was observed whereas the membrane potential of the N18 cells was -28 ± 2 mV compared to -23 ± 4 mV for LV10. We conclude that the small difference in resting membrane potential cannot account for the large difference in apparent K_d and, therefore, that clone LV10 cells contain sodium channels having a scorpion toxin receptor site with reduced affinity for scorpion toxin.

In order to test the voltage dependence of scorpion toxin action in LV10 cells, we incubated cells with scorpion toxin at different membrane potentials by varying the extracellular K^+ concentration (18). Then veratridine-stimulated sodium uptake was measured at the normal membrane potential in 5 mM K^+. A concentration of scorpion toxin near the apparent K_d (0.5 nM for N18 and 25 nM for LV10) was used so that changes in K_d due to depolarization would give a similar reduction in $^{22}Na^+$ uptake for the two cell lines. The results of these experiments (Fig. 5) indicate that the effect of scorpion toxin on sodium uptake in LV10 is voltage dependent, as in N18 (16, 18), but the dependence on membrane potential is less steep than for N18. Thus, the change in the scorpion toxin receptor site in clone LV10 results in both altered affinity for toxin and altered voltage dependence of binding. Because we are unable to measure ^{125}I-labeled scorpion toxin binding directly in LV10 cells, we cannot, at present, make an accurate quantitative measurement of the dependence of K_d on membrane potential.

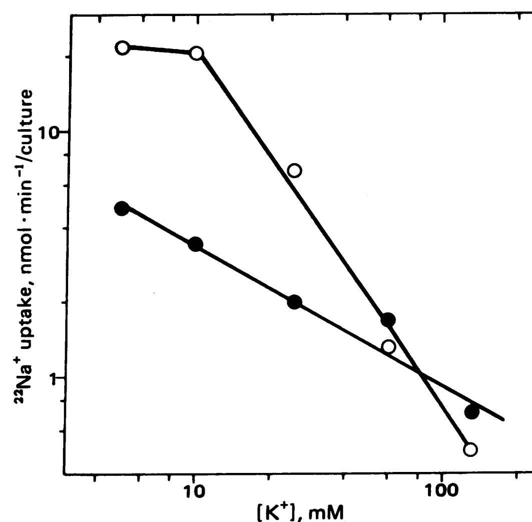

FIG. 5. Voltage dependence of scorpion toxin action in LV10 and N18 cells. N18 (O) or LV10 (●) cells were incubated for 30 min in medium with the indicated K^+ concentrations and either 0.5 nM (N18) or 25 nM (LV10) scorpion toxin. Initial rates of $^{22}Na^+$ uptake were measured in medium containing 5.4 mM K^+ and 200 μM veratridine. Choline chloride concentrations in the incubation media were varied so that $[K^+] + [\text{choline}^+] = 135.4$ mM. The concentrations of scorpion toxin were selected to be near the apparent K_d for each cell line. The increment over veratridine-stimulated uptake caused by scorpion toxin is plotted. Data are presented in log-log format because membrane potential depends approximately logarithmically on $[K^+]_{out}$ in N18 cells (18) and the K_d for scorpion toxin depends logarithmically on membrane potential (16).

DISCUSSION

Somatic cell genetic methods have been used previously to analyze the expression of differentiated neuronal functions in neuroblastoma cells. Breakefield and Nirenberg (20) developed an effective method to select neuroblastoma clones capable of synthesis of monoamine neurotransmitters. Minna *et al.* (21), Greene *et al.* (22), and Peacock *et al.* (23) used somatic cell hybridization techniques to develop clonal lines of neuroblastoma hybrids expressing different combinations of neuronal properties. Among the hybrid cell lines derived from those studies were clones that lacked voltage-sensitive sodium channels (21, 23). These clones have been particularly valuable in analyzing the mechanism of interaction of neurotoxins with sodium channels (16, 24). Methods to rationally select for neuroblastoma or hybrid clones with particular defects in sodium channel function were not developed in those studies, however. Such methods would be valuable in analyzing the molecular basis of electrical excitability.

The goal of the experiments described in this report is to develop methods to select such variant cell clones and to analyze their properties. Our results demonstrate the utility of neurotoxins that activate sodium channels as selective agents to isolate variant neuroblastoma cells with missing or altered sodium channels. The toxins are highly effective cytotoxic agents, reducing the plating efficiency of N18 cells to 0.004% of the control. Eight out of the 10 resistant clones isolated had missing or altered sodium channels. The phenotype of the isolated clones has remained stable for over 100 generations, indicating that the new properties of these cells result from an inheritable genetic change. Seven of the isolated clones have no detectable sodium channels. These clones may have arisen from mutational events that cause a completely nonfunctional sodium channel or prevent expression of the gene(s) for sodium channels, or they may have arisen from a nonmutational event leading to inhi-

bition of expression of the gene(s) for sodium channels. One clone, LV10, has sodium channels with an altered K_d and voltage dependence for scorpion toxin binding. Because these cells have sodium channels with altered properties, it seems likely that this clone arose from a mutational event. These results suggest that the selection procedure described will be useful in mutational analysis of sodium channel function.

The properties of clone LV10 are consistent with earlier experiments suggesting that the three toxin receptor sites associated with sodium channels are located on functionally separate components (5, 6, 8). Thus, in this clone, the affinity for scorpion toxin was altered 40-fold and the voltage dependence of scorpion toxin binding changed significantly without detectable alterations in apparent K_d for batrachotoxin or tetrodotoxin. Analysis of a number of independently selected scorpion toxin-resistant clones should allow us to determine whether the different toxin receptor sites are the products of the same or different genes.

Although cells of clone LV10 have a specific alteration in K_d at the scorpion toxin receptor site, the maximum rate of $^{22}Na^+$ uptake stimulated by batrachotoxin (Fig. 2) or by scorpion toxin plus veratridine (Fig. 3) was reduced approximately 80%. This reduction in maximum $^{22}Na^+$ uptake may be due to a reduction in the steady-state density of functional sodium channels in the surface membrane of LV10 cells or to a reduction in the maximum rate of ion transport by an individual active sodium channel. Either of these possibilities could arise as a secondary consequence of mutation at the scorpion toxin receptor site.

Comparisons of the voltage dependence of activation and inactivation of sodium channels determined by voltage clamp methods with the voltage dependence of scorpion toxin binding reveal a close correspondence between voltage-dependent activation of sodium channels and voltage-dependent inhibition of scorpion toxin binding in both mouse neuroblastoma cells and frog skeletal muscle (25). These results suggest that the change of state leading to activation of the sodium channel results in a conformational change at the scorpion toxin receptor site which causes reduced affinity for scorpion toxin. Changes in the voltage dependence of scorpion toxin binding should be reflected in changes in the voltage dependence of activation of sodium channels. We would therefore expect sodium channels in clone LV10 to have altered voltage dependence of activation. Electrophysiologic experiments are required to test this possibility.

The experiments described here demonstrate the utility of neurotoxin selection for isolation of variant cell clones with missing or altered sodium channels. However, most of the isolated clones lack functional sodium channels rather than have altered sodium channels. Because cells with altered sodium channels are more useful in analyzing sodium channel function, current experiments in our laboratory are directed toward increasing the frequency of isolation of such clones through mutagenesis and use of multistep selection regimes. Biochemical and physiological analysis of the properties of a collection of clones with altered sodium channels should provide important new insight into sodium channel function.

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