J. Membrane Biol. 145, 187–192 (1995)

The Journal of
Membrane
Biology

Sodium Channels in Cultured Neuroblastoma Cells Grown in High Glucose or L-Fucose

R.E. Wachtel,[1,2] **S.A. Kraske,**[1] **M.A. Yorek**[1,3]

[1]Department of Veterans Affairs Medical Center, Iowa City, IA 52246
[2]Department of Anesthesia, University of Iowa, Iowa City, IA, 52242
[3]Department of Internal Medicine and Diabetes Endocrinology Research Center, University of Iowa, Iowa City, IA 52242

Received: 8 September 1994/Revised: 1 February 1995

Abstract. Patch clamp techniques were used to record whole cell and single channel Na^+ currents from NB41A3 neuroblastoma cells grown in culture. Cells were grown for two weeks in control medium or medium supplemented with 30 mM D-glucose of 30 mM L-fucose.

Cells exposed to glucose or L-fucose had smaller whole cell Na^+ currents than cells grown in unsupplemented medium, consistent with earlier studies (Yorek, Stefani & Wachtel, 1994). Whole cell macroscopic currents showed no change in activation or inactivation kinetics. Single channel current properties and opening probability were also unchanged.

The number of [^{3}H]saxitoxin binding sites, and therefore the total number of Na^+ channels, was not reduced in cells grown in glucose or L-fucose (Yorek et al., 1994). Therefore, we conclude that some of the channels must have been rendered nonfunctional by the conditioning media. The finding that single channel properties are not altered suggests that channels become nonfunctional in an all-or-none manner.

Key words: Sodium channels — Glucose — L-fucose — Diabetes — Neuropathy — Neuroblastoma cells

Introduction

Peripheral neuropathy is one of the most frequent long-term complications associated with insulin-dependent diabetes mellitus (*see* review by Ross, 1993). Patients may exhibit both sensory and motor dysfunction that is usually more severe in distal portions of the limb. These neuropathies may be secondary to vasculopathies and/or to derangements of metabolic function in the nerve.

Neuroblastoma cells in culture have been used as a model system to examine some of the metabolic changes associated with chronic exposure to the high carbohydrate levels found in diabetes. When grown in the presence of high glucose, neuroblastoma cells exhibit numerous abnormalities, including a noncompetitive inhibition of the *myo*-inositol transporter, a decrease in *myo*-inositol accumulation and incorporation into phospholipids, a depletion of intracellular *myo*-inositol, a decrease in Na^+/K^+/ATPase pump activity, and a reduction in resting membrane potential (Yorek & Dunlap, 1989, 1991; Yorek, Dunlap & Ginsberg, 1987, 1988*a*). Depletion of intracellular *myo*-inositol is accompanied by accumulation of sorbitol, a product of glucose metabolism (Yorek et al., 1987; Yorek, Dunlap & Ginsberg, 1988*b*). These changes in metabolism are similar to those seen in animal models of diabetic neuropathy (Clements & Stockard, 1980; Gillon, Hawthorne & Tomlinson, 1983; Greene et al., 1984; Green, Lattimer & Sima, 1987; Tomlinson, Moriarty & Mayer, 1984).

L-fucose is another sugar elevated in diabetes that is normally found in low concentrations in serum (Sirakor, 1971; Radhakrishnamurthy et al., 1976). Like glucose, L-fucose also decreases *myo*-inositol accumulation and decreases Na^+/K^+/ATPase activity in neuroblastoma cells (Yorek et al., 1992). L-fucose is not metabolized by the aldose reductase pathway, however, and does not cause polyol accumulation (Yorek et al., 1992, 1993). Comparisons between the effects of glucose and L-fucose can be used to help elucidate specific metabolic alterations that underlie changes in cellular function.

We previously reported that cultured neuroblastoma cells grown in the presence of elevated levels of glucose or L-fucose had smaller peak inward sodium currents (Yorek, Stefani & Wachtel, 1994). Veratridine-stimulated $^{22}Na^+$ uptake was also inhibited by about 50% in glucose or 30% in L-fucose. In contrast, [^{3}H]saxitoxin binding was not reduced, indicating that the number of sodium channels was not affected. These earlier results

Correspondence to: R.E. Wachtel

suggested that the reduction in sodium current was not due simply to a decrease in the number or expression of sodium channels, but resulted from an alteration in function of existing channels.

The present studies were undertaken to determine whether channel properties, including gating kinetics and channel opening probability, had been altered by growth conditions. Results demonstrate that channel properties are unchanged, suggesting that the observed decrease in peak current is simply due to a decrease in the number of functional channels available for activation.

Materials and Methods

NB41A3 mouse neuroblastoma cells were cultured in Hams F10 medium containing 5.6 mM glucose. The medium was supplemented with 15% horse serum, 2.5% heat-inactivated bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 294 μg/ml glutamine. The medium was replaced every other day and cells were passed weekly 1:20. Cultures were used for a maximum of 18 weeks. The medium was supplemented for 2 weeks prior to study with 30 mM D-glucose or 30 mM L-fucose, concentrations which previously have been shown to produce maximal effects (Yorek et al., 1987, 1988*b*). When confluent, cells were seeded onto cover slips for electrophysiological experiments.

Patch clamp techniques were used to record whole cell and single channel voltage-gated sodium currents. Currents were recorded with a Dagan 3100 integrating patch clamp, low-pass filtered at 4 kHz, digitized at 200 μsec per point, and stored on the hard disk of an IBM PC-AT. Pulse protocols and data collection were performed by pCLAMP software package (Axon Instruments). Experiments were performed at room temperature.

For recording of whole cell currents, cells were bathed in a solution containing (in mM): 145 NaCl, 4 KCl, 1.8 $CaCl_2$, 0.4 $MgCl_2$, 1 $CoCl_2$, 5 glucose, and 5 HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), pH 7.4. Electrodes had tip diameters of 4–8 μm and resistances of 2–4 Mohm, and were filled with a solution containing (in mM): 12 NaCl, 120 CsCl, 20 TEACl (tetraethylammonium chloride), 2 EGTA (ethylene glycol-bis(β-aminoethylether-N,N,N′,N′-tetraacetic acid) and 5 HEPES, pH 7.1. To generate *I-V* plots for steady state activation studies, cells were held at a resting potential of −110 mV and depolarized by a series of 8 msec voltage clamp pulses to potentials ranging from −50 to +80 mV in 10 mV increments. The interval between pulses was 5 sec. For steady state inactivation experiments, cells were held for 3 sec at conditioning potentials from −110 to −40 mV, then depolarized by an 8 msec test pulse to −10 mV. To determine the time course of inactivation, cells were depolarized to −10 mV for 2 sec to induce inactivation, then held at potentials from −110 to −60 mV for durations ranging from 1.25 to 2000 msec to permit recovery. The extent of recovery was assessed by measuring the peak current at a test potential of −10 mV.

For single channel experiments, the bathing solution contained (in mM): 140 KCl (to "zero" membrane potential), 5 NaCl, 0.8 $MgCl_2$, 1.5 $CoCl_2$, 5 glucose, and 5 HEPES, pH 7.4, while electrodes were filled with 145 NaCl, 5 KCl, 0.4 $MgCl_2$, 2.8 $CoCl_2$, 5 glucose, and 5 HEPES, pH 7.4. Cell attached patches were hyperpolarized to −110 mV (pipette potential +110 mV), then depolarized by a series of 8 msec pulses to −15 mV (pipet potential +15 mV) at 1 Hz. Single channel currents were analyzed to determine single channel amplitude, mean channel open time, and probability of channel opening. Single channel amplitude was determined from the mean amplitude of events greater than 0.5 msec in duration. Mean channel open time was determined according to the method of maximum likelihood and was corrected for missed events less than 0.1 msec in duration (Colquhoun & Sigworth, 1983; Wachtel, 1991). Empty traces not containing opening events were used to subtract capacitive transients. All sweeps were averaged, and opening probability was calculated from the ratio of current amplitude at the peak of the average to the amplitude that would be expected if the channel opened during every depolarizing pulse. Only patches containing a single channel were used to calculate opening probability.

A patch was assumed to contain only a single channel if no double openings were observed in 250 sweeps. Assuming the channels in a patch are homogeneous and open independently, then the number of channels in a patch will obey a binomial distribution (Horn, 1991). A z distribution may be invoked to determine statistically the minimum number of sweeps needed to assume that only a single channel is present in the patch:

$$\frac{y/n - P_o}{\frac{P_o\,(1 - P_o)}{n}} \geqslant z(\alpha)$$

where y is the number of double openings observed (O), n is the number of sweeps, P_o is open probability, and $z(\alpha)$ refers to the standard normal distribution function at tail probability α. With an observed open probability of about 0.26, there could conceivably be two channels present, each with an open probability of 0.13. The probability of observing two simultaneous openings is then 0.13^2, or $P_o = 0.017$. With $y = 0$ and $\alpha = 0.05$, about $n = 250$ sweeps without a double opening are required to assume that only a single channel is present in the patch.

Results

As previously reported (Yorek et al., 1994), average peak inward sodium currents were reduced in cells exposed to glucose or L-fucose for two weeks (Fig. 1*B*). Glucose reduced peak inward sodium current about 35%, while L-fucose reduced the current about 70%. Inward currents were maximum at −10 mV and were 816 ± 69 pA (n = 39 ± SEM) in control, 523 ± 77 pA (n = 27) for glucose cells, and 247 ± 45 pA (n = 34) for L-fucose cells.

Although peak sodium currents were smaller in cells grown in glucose or L-fucose, current properties were not otherwise affected. Figure 2 shows that the voltage-dependence of steady state activation and inactivation was not altered. To determine steady state activation, normalized conductance was plotted as a function of depolarizing potential. Data points were fit by Boltzmann functions giving half-maximal activation at −19 mV in control, −21 mV for glucose, and −22 mV for L-fucose. To determine steady state inactivation, relative current was plotted as a function of the size of the depolarizing prepulse. Half-maximal inactivation occurred at −76 mV in control, −78 mV for glucose, and −81 mV for L-fucose.

The time course and voltage dependence of recovery from inactivation were also studied. Cells were depolarized to −10 mV for 2 sec to induce inactivation. They were then allowed to recover during a conditioning prepulse, and the extent of recovery was assessed by a test

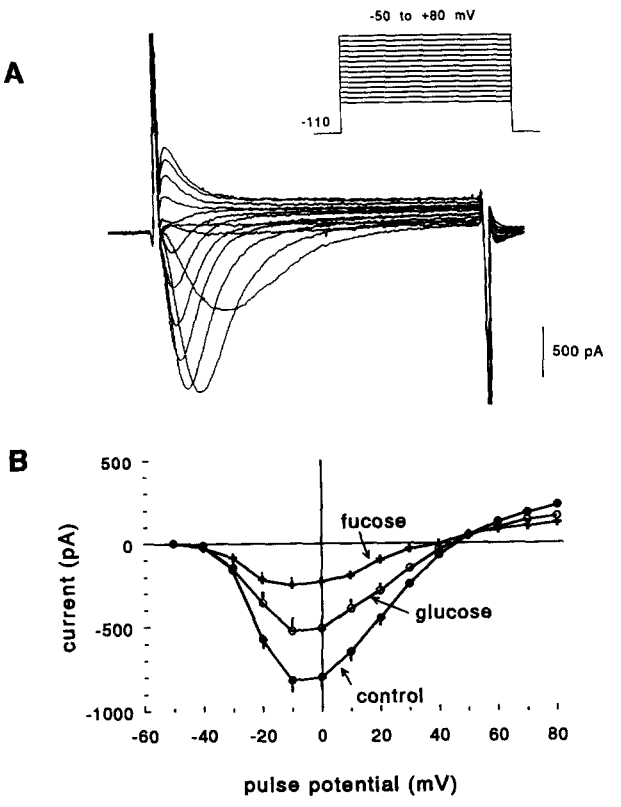

Fig. 1. *(A)* A representative family of voltage clamp currents recorded from a control cell. The pulse protocol is illustrated in the upper right; cells were held at −110 mV and depolarized by a series of 14 8-msec voltage clamp pulses to various potentials. Leakage currents and capacitive transients have not been subtracted. *(B)* Averaged current-voltage relationships. Peak current is plotted as a function of pulse potential. Inward currents were maximum at −10 mV and were 816 ± 69 pA (n = 39 ± SEM) in control, 523 ± 77 pA (n = 27) for glucose, and 247 ± 45 pA (n = 34) for L-fucose. Reversal potentials were +45 mV in control, +43 mV for glucose, and +41 mV for L-fucose.

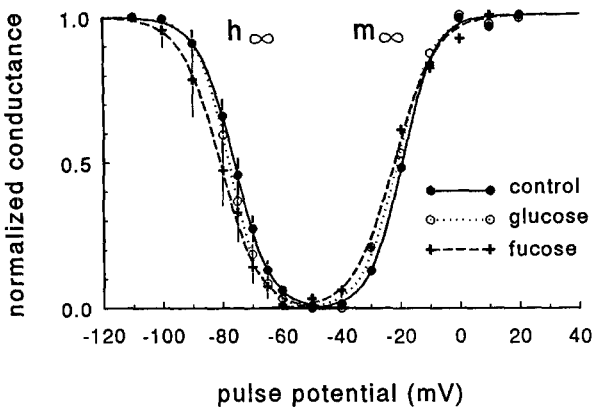

Fig. 2. Steady state activation (m_∞) and inactivation curves (h_∞). (m_∞) Cells were held at a resting potential of −110 mV and depolarized by a series of 8 msec voltage clamp pulses to potentials ranging from −50 to +80 mV in 10 mV increments. m^3 is relative conductance, determined from the ratio of peak current to driving force, $I/(V_m - V_{rev})$, where I is peak current, V_m is the step potential, and V_{rev} is the reversal potential for the currents. Smooth curves are Boltzmann functions $1/(1 + \exp((V_m - V_{1/2}) / b))$, where half-maximal activation occurred at $V_{1/2}$ = −19 mV in control, −21 mV for glucose, and −22 mV for L-fucose. b = 6.0 mV in control, 5.3 mV for glucose, and 6.5 mV for L-fucose. (h_∞) Cells were held for 3 sec at conditioning potentials from −110 to −40 mV, then depolarized by 8 msec test pulses to −10 mV. Current was measured at each prepulse potential relative to the current obtained at a conditioning potential of −110 mV. Smooth curves are Boltzmann functions $1/(1 + \exp((V_{1/2} - V_m)/b))$, where half-maximal inactivation occurred at $V_{1/2}$ = −76 mV in control, −78 mV for glucose, and −81 mV for L-fucose. b = 5.6 mV in control, 6.0 mV for glucose, and 7.0 mV for L-fucose.

pulse to −10 mV. Figure 3*A* shows an example of the time course of recovery from inactivation for prepulses to −80 mV. Recovery curves were fit by two exponential components. At all potentials tested the fast component always constituted at least 80% of the total recovery, and Fig. 3*B* shows how the faster time constant varies with prepulse potential. Although the time constants appear to differ slightly at the more depolarized potentials, this apparent difference may simply arise from the difficulty in measuring slow macroscopic currents smaller than 100 pA.

The slower time constant for recovery from inactivation became more prominent at more hyperpolarized potentials. It was slowest at −90 to −100 mV, with a time constant of about 200 msec. No consistent differences in slow recovery were found between control, glucose, and L-fucose cells.

For cells exposed to glucose or L-fucose, sodium channel activation and inactivation profiles appear unchanged even though peak currents are reduced. Therefore the observed decrease in sodium current does not result from a shift in the voltage-dependence of channel gating. The reduction must instead be due to a decrease in the number of functional sodium channels, a decrease in single channel current or open time, or a decrease in channel opening probability.

Single channel experiments were therefore designed to measure single channel properties and opening probability directly. Figure 4 shows examples of single channel currents recorded from cell attached patches in response to depolarizing pulses from −110 mV to −15 mV (changes in pipette potential from +110 mV to +15 mV). Ensemble averages of 250 sweeps each are shown below the single channel currents. Both single channel current amplitude and mean channel open time were unaffected by growth in glucose or L-fucose (Fig. 5). Channel opening probability measured at the peak of the ensemble averages was also unchanged (Fig. 6*A*). These results suggest that the observed decrease in peak current must be due to a reduction in the number of functional channels in the membrane. This possibility is supported by the finding that a greater number of patches from glucose and L-fucose cells did not contain any channels and were

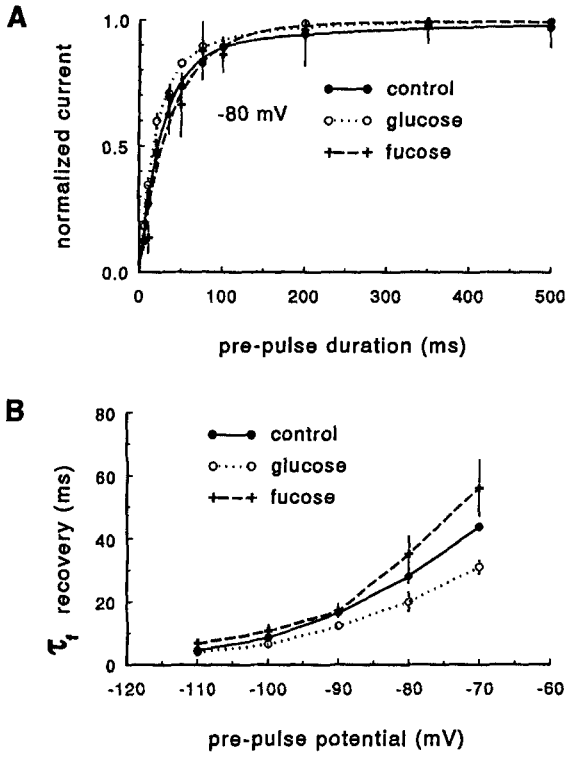

Fig. 3. Voltage-dependence of the time course of recovery from inactivation. Cells were depolarized to −10 mV for 2 sec to produce complete inactivation, then held at conditioning potentials of −110 to −60 mV for durations ranging from 1.25 to 2000 msec to permit recovery from inactivation. (*A*) The time course of recovery at −80 mV. The relative current in response to a test pulse to −10 mV is plotted as a function of the duration of recovery. The smooth curves are the sums of two exponential components with time constants 28 msec and 240 msec and relative amplitude 0.87 for the faster component in control, time constants 20 msec and 100 msec and faster component amplitude 0.80 with glucose, and time constants 35 msec and 80 msec and faster component amplitude 0.80 with L-fucose. Error bars are standard deviations. (*B*) Time constants of the faster component of recovery curves as a function of recovery potential. Recovery curves were described as the sum of two exponential in the range −110 to −80 mV, but only a single component was required at −70 mV. Error bars are asymptotic standard deviations derived from the nonlinear regressions.

classified as empty (Fig. 6*B*). While all patches from control cells showed channel activity, and invariably contained more than a single channel, 5 out of 19 patches from glucose cells and 13 out of 23 patches from L-fucose cells showed no activity even after 500 sweeps.

Discussion

Previous studies have shown that cultured neuroblastoma cells grown in the presence of 30 mM glucose or 30 mM L-fucose showed a decrease in peak inward Na^+ currents (Yorek et al., 1994). Binding of [^{3}H]saxitoxin was not reduced, however, indicating that the total number of Na^+ channels was not altered.

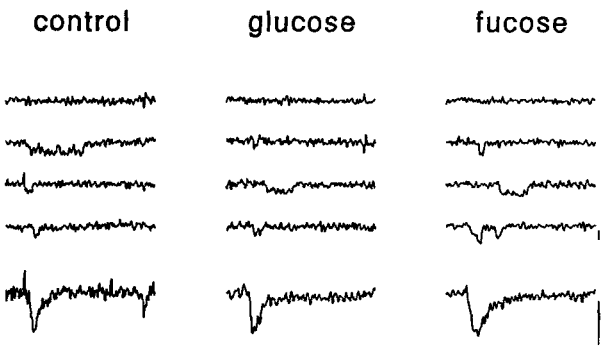

Fig. 4. Representative single channel currents recorded from cell-attached patches in response to depolarizing pulses that changed membrane potential from −110 mV to −15 mV. Cells were bathed in high K^+ solution to zero resting membrane potentials. The first trace in each group is an empty sweep in which no openings occurred. Capacitive transients have been subtracted. Ensemble averages of 250 sweeps are shown below the single channel currents. Calibration marks are 1 pA for the single channel currents and 0.25 pA for the averages.

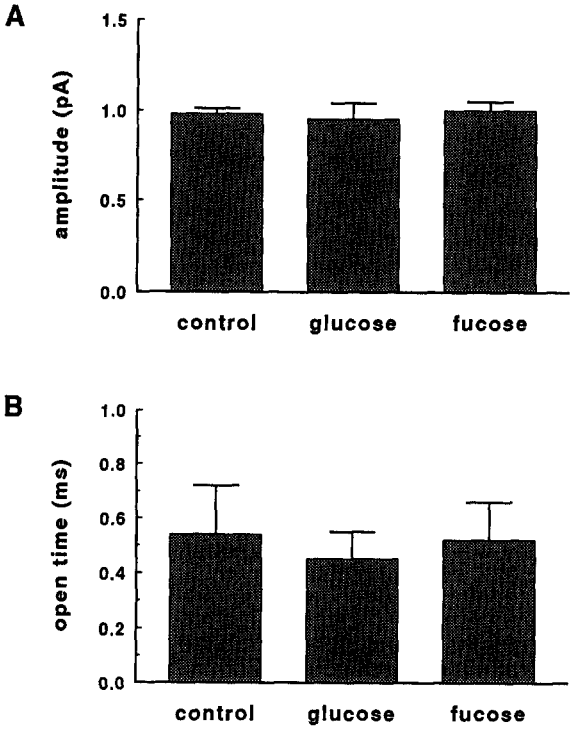

Fig. 5. (*A*) Single channel current amplitude and mean channel open time were not altered by growth conditions. Single channel current at a membrane potential of −15 mV was 0.98 ± 0.03 pA (n = 4 cells ± SD) in control, 0.95 ± 0.09 pA (n = 6) for glucose, and 1.00 ± 0.05 pA (n = 4) for cells grown in L-fucose. (*B*) Mean channel open time was not affected by growth conditions. Channel open time at −15 mV was 0.54 ± 0.18 pA (n = 4) in control, 0.45 ± 0.10 msec (n = 6) for glucose, and 0.52 ± 0.14 msec (n = 4) for cells grown in L-fucose.

The effect was not simply an osmotic one, since 30 mM D-fructose, a sugar that does not alter *myo*-inositol metabolism, had no effect on Na^+ currents (Yorek et al., 1994). In addition, 30 mM glucose or galactose did not

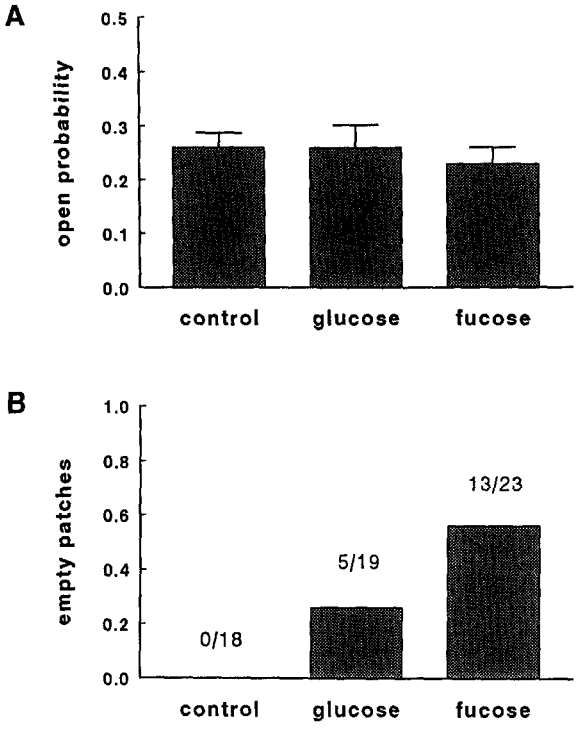

Fig. 6. (*A*) Channel opening probability was not altered by growth conditions. Opening probability was calculated from the ratio of current amplitude at the peak of the average to the amplitude that would be expected if the channel opened with every sweep. Only patches containing a single channel were used to calculate opening probability, which was 0.26 ± 0.03 (n = 3 cells ± SD) in control, 0.26 ± 0.04 (n = 5) for glucose, and 0.23 ± 0.03 (n = 4) for cells grown in L-fucose. (*B*) In cells treated with glucose or L-fucose, some patches were empty and did not appear to contain any channels, while all control patches tested contained at least one channel. A patch was considered not to contain any channels if no openings were observed after at least 500 sweeps.

alter cell shape or size or affect intracellular space (Yorek & Dunlap, 1991).

Results of the present study demonstrate that channels from cells grown in high glucose or L-fucose have normal properties. Macroscopic currents do not show any alterations in the voltage-dependence of channel activation or inactivation or the time course or voltage-dependence of recovery from inactivation. Single channel currents do not show any changes in amplitudes, open times, or opening probability. Thus the channels that do open appear to be functioning normally.

These results suggest that the observed decrease in peak current is due to a reduction in the number of functional channels. Since [^{3}H]saxitoxin binding is not altered, there must be channels still present in the membrane that are nonfunctional and are not available for opening. Channels are rendered nonfunctional in an all-or-none fashion, however, since those channels that can open appear to have normal properties.

A reduction in the number of functional channels may conceivably arise from disturbances in any of several aspects of cell metabolism. For example, phosphorylation by cAMP-dependent protein kinase has been shown to alter the function of neuronal Na^+ channels (Rossie & Catterall, 1987; Dascal & Lotan, 1991; Numann, Catterall & Scheuer, 1991; Li et al., 1992), and channels that are not in the proper state of phosphorylation may be unavailable for opening. Godoy and Cukierman (1994) have reported that activators of protein kinase C modulate the behavior of Na^+ channels by at least three distinct mechanisms. Increased nonenzymatic glycation of the Na^+ channel may also be a factor, since glycation is important in the maintenance of channel activity (Waechter, Schmidt & Catterall, 1983; Zona, Eusebi & Miledi, 1990). Additional experiments will attempt to explore the mechanism by which growth in high glucose or L-fucose renders Na^+ channels nonfunctional.

This work was supported by Merit Review Awards to M.A. Yorek and R.E. Wachtel from the Department of Veterans Affairs and by National Institutes of Health grant DK45453 to M.A. Yorek.

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