Nerve Growth Factor Regulates Sodium But Not Potassium Channel Currents in Sympathetic B Neurons of Adult Bullfrogs

SAOBO LEI, WILLIAM F. DRYDEN, AND PETER A. SMITH
Department of Pharmacology and University Centre for Neuroscience, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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Lei, Saobo, William F. Dryden, and Peter A. Smith. Nerve growth factor regulates sodium but not potassium channel currents in sympathetic B neurons of adult bullfrogs. J Neurophysiol 86: 641–650, 2001. The TTX-sensitive and -resistant components of the voltage-gated Na+ current (TTX-s \( I_{Na} \) and TTX-r \( I_{Na} \)) are increased within 2 wk of cutting the axons of B-cells in bullfrog paravertebral sympathetic ganglia (BFSG). Axotomy also increases the noninactivating, voltage-activated K+ current (M current \( I_{K} \)), whereas delayed rectifier K+ current \( I_{K} \) is reduced. We found that similar effects were produced when BFSG B cells were dissociated from adult bullfrogs and maintained in a defined-medium, neuron-enriched, low-density, serum-free culture. Thus the density of TTX-s \( I_{Na} \), TTX-r \( I_{Na} \), and \( I_{M} \) were transiently increased, whereas \( I_{K} \) density was decreased. Reduction in voltage-sensitive, Ca\(^{2+}\)-dependent K+ current (\( I_{C} \)) was attributed to previously documented decreases in Ca\(^{2+}\) channel current (\( I_{Ca} \)). To test whether axotomy- or culture-induced changes in ion channel function reflect loss of retrograde influence of nerve growth factor (NGF), we examined the effect of murine β-NGF on TTX-s \( I_{Na} \), TTX-r \( I_{Na} \), \( I_{K} \), and \( I_{M} \). Culture of neurons for 15 days in the presence of NGF (200 ng/ml), more than doubled total \( I_{Na} \) density but did not enhance neurite outgrowth. The TTX-r \( I_{Na} \) density was increased about threefold and the TTX-s \( I_{Na} \) density increased 2.4-fold. NGF did not affect the activation or inactivation kinetics of the total Na+ conductance. Effects of NGF were blocked by the transcription inhibitors, corydine (20 μM) and actinomycin D (0.01 μg/ml). \( I_{K} \) and \( I_{M} \) were unaffected by NGF, and although \( I_{C} \) was enhanced, this likely reflected the known effect of NGF on \( I_{C} \) in BFSG neurons. Na+ channel synthesis and/or expression in adult sympathetic neurons is therefore subject to selective regulation by NGF. Despite this, the increase in \( I_{Na} \) and \( I_{M} \) as well as the decrease in \( I_{K} \) seen in BFSG neurons in culture or after axotomy cannot readily be explained in terms of alterations in the availability of target-derived NGF.

INTRODUCTION

Damage to the axon of a peripheral or a central neuron promotes characteristic changes in the morphological, electrophysiological, and biochemical properties of the surviving cell body (Gordon 1983; Titmus and Faber 1990). We have used the B cells of bullfrog sympathetic ganglia (BFSG) to understand the relationship between axotomy-induced changes in specific ion channels and changes in action potential (AP) shape and regeneration (Gordon et al. 1987; Jassar et al. 1993, 1994; Kelly et al. 1986, 1989; Petrov et al. 2001; P. S. Pennefather and P. A. Smith, unpublished data). In these cells, axotomy increases spike width and spike height but decreases the peak amplitude and duration of the afterhyperpolarization (AHP) that follows the AP (Gordon et al. 1987; Kelly et al. 1986). These changes, which take about a week to develop, result from decreases in Ca\(^{2+}\) channel current (\( I_{Ca} \)) and consequent attenuation of Ca\(^{2+}\)-dependent K+ conductances (Jassar et al. 1993, 1994; Petrov et al. 2001; P. S. Pennefather and P. A. Smith, unpublished results). Attenuation of voltage-sensitive Ca\(^{2+}\)-dependent K+ current (\( I_{K} \)) may be secondary to the decrease in \( I_{Ca} \) (Jassar et al. 1994). The delayed rectifier K+ current (\( I_{K} \)) is also reduced by axotomy, whereas the noninactivating K+ current (M current \( I_{M} \)) and the tetrodotoxin-sensitive (TTX-s) and TTX-resistant (TTX-r) components of the Na+ current (\( I_{Na} \)) are increased (Jassar et al. 1993, 1994).

\( I_{Ca} \) is also reduced in vivo following injection of nerve growth factor (NGF) antibodies under the skin of the thighs (Lei et al. 1997). This area of the skin contains the mucous glands that are targets for most B-cell axons (Horn et al. 1988). These results are consistent with the hypothesis that the retrograde influence of NGF is responsible for maintaining the properties of Ca\(^{2+}\) channels in adult BFSG neurons and that the response of Ca\(^{2+}\) channels to axotomy reflects interruption of this retrograde signal. Further corroborative evidence for this idea was recently obtained by reversibly disconnecting BFSG B cells from their targets by means of an injection of 6-hydroxydopamine. Degeneration of peripheral sympathetic terminals was associated with attenuation of cell body \( I_{Ca} \), and only after recovery and reestablishment of target contact did cell body \( I_{Ca} \) recover (Petrov et al. 2001). These results would be expected if \( I_{Ca} \) declined as a consequence of the loss of the retrograde influence of NGF. Its protracted recovery could then be ascribed to the gradual restoration of the retrograde influence of target-derived NGF.

When BFSG neurons are placed in defined-medium culture, the changes in \( I_{Ca} \) resemble those initiated by axotomy in vivo: the current is reduced and inactivation is increased. Inclusion of NGF in the cultures reverses these changes (Lei et al. 1997). Three questions arise from these observations. First, does tissue culture produce an increase TTX-s \( I_{Na} \), TTX-r \( I_{Na} \), and \( I_{M} \) in the same way as axotomy? Is \( I_{K} \) unchanged and \( I_{K} \) attenuated? If this is so, might the culture system be a useful model for studying retrograde influences in vivo?

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for examining the role of target-derived NGF or other growth factors in the maintenance of ion channels in BFGS neurons? Second, is the NGF-induced increase in \( I_{\text{Ca}} \) but one aspect of a general improvement in the “health” of sympathetic neurons that were previously cultured without appropriate neurotrophic support? If this was the case, NGF might be expected to increase all types of ionic currents in culture. Third, because \( I_{\text{Na}} \) and \( I_{\text{M}} \) increase after axotomy when the retrograde influence of NGF should be reduced (Jassar et al. 1993; Petrov et al. 2001), might NGF exert a tonic downregulation of \( I_{\text{Na}} \) and \( I_{\text{M}} \) in the cell bodies of adult BFGS B neurons in vivo? If this was so, NGF might be expected to inhibit rather than potentiate \( I_{\text{Na}} \) and \( I_{\text{M}} \) in BFGS neurons in culture.

To test these possibilities, we examined the effect of NGF on the properties of Na\(^{+}\) and K\(^{+}\) currents in BFGS neurons in defined-medium, serum-free, neuron-enriched, low-density culture (Lei et al. 1997, 1998). This system removes any influences of glial cells (Assouline et al. 1987), adhesion molecules, or injury-induced inflammation that may be active in vivo. Although the changes in currents seen in culture resembled those seen with axotomy, addition of NGF did not relieve \( I_{\text{K}} \) to control levels and did not affect \( I_{\text{Na}} \). Thus changes in K\(^{+}\) currents seen in culture or after axotomy must occur via NGF-independent mechanisms. Since NGF potentiated rather than inhibited the TTX-s and TTX-r components of \( I_{\text{Na}} \), the axotomy-induced increase in these currents must also occur via an NGF-independent mechanism. Some of these findings have been communicated to the Society for Neuroscience (Dryden et al. 1998).

**Methods**

All experimental procedures were reviewed and approved by the University of Alberta Animal Welfare Committee. This committee maintains standards set forth by the Canadian Council for Animal Care.

**Isolation and culture of BFGS neurons**

Adult bullfrogs were killed by rapid decapitation and spinal cord function terminated by pithing. The VIIth, IXth, and Xth paravertebral sympathetic ganglia were removed from both sides of the animal, and the cells were dissociated using trypsin and collagenase as previously described (Selyanko et al. 1990). Neurons were used for acute recording within 2–10 h of plating or were maintained for ≤2 wk in serum-free, neuron-enriched, defined-medium culture (Lei et al. 1997, 1998). The culture medium consisted of diluted L-15 medium (73%) supplemented with 10 mM glucose, 1 mM CaCl\(_2\), 100 units/ml penicillin, and 100 μg/ml streptomycin. The total yield of ganglion cells from each frog was preplated into two or three 35-mm petri dishes (Lei et al. 1997, 1998). This system removes any molecules, or injury-induced inflammation that may be active in vivo. Although the changes in currents seen in culture resembled those seen with axotomy, addition of NGF did not relieve \( I_{\text{K}} \) to control levels and did not affect \( I_{\text{Na}} \). Thus changes in K\(^{+}\) currents seen in culture or after axotomy must occur via NGF-independent mechanisms. Since NGF potentiated rather than inhibited the TTX-s and TTX-r components of \( I_{\text{Na}} \), the axotomy-induced increase in these currents must also occur via an NGF-independent mechanism. Some of these findings have been communicated to the Society for Neuroscience (Dryden et al. 1998).

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**Electrophysiology**

Currents were recorded by whole cell patch-clamp methods using an Axoclamp 2A amplifier in discontinuous, single-electrode voltage-clamp mode. Generally, external solutions were 250 mM Osmol/kg, and internal solutions were 240 mM Osmol/kg. \( I_{\text{Na}} \) was evoked by a series of depolarizing voltage commands from a holding potential of −80 mV (except where otherwise stated). With low-resistance patch electrodes (2–5 MΩ), it was possible to use high switching frequencies >30 kHz with high clamp gains (>8 to <30 mV/nA). The fidelity of the clamp was confirmed by examining the voltage recording. Recordings from cells where the voltage trace was slow to rise or distorted were discarded. For recording \( I_{\text{Na}} \), the solution in the bath (external) contained (in mM) 97.5 NaCl, 20 TEA-Br, 4 MnCl\(_2\), and 2.5 Tris-Cl (pH 7.2), and the solution inside the pipette (internal) contained (in mM) 103 CsCl, 9 NaCl, 5 TEA-Br, 2.5 Cs-HEPES, and 1 Cs-EGTA (pH 7.2) (Jones 1987).

To study K\(^{+}\) currents, the internal solution consisted of (in mM) 110 KCl, 10 NaCl, 2 MgCl\(_2\), 0.4 CaCl\(_2\), 4.4 EGTA, 5 HEPES, 10 d-glucose, 0.125 cyclic AMP, and 0.1 leupeptin (pH 7.2), and total outward current was initially recorded in response to depolarizing steps from a holding potential of −80 mV in an external solution containing (in mM) 40 KCl, 2 CaCl\(_2\), 40 NMG-Cl, 2.5 Tris-Cl, 94 sucrose, and 10 d-glucose. After replacing the preceding external solution with a solution containing (in mM) 0.1 CaCl\(_2\), 40 KCl, 2 MgCl\(_2\), 40 NMG-Cl, 2.5 Tris-Cl, 94 sucrose, and 10 d-glucose, the voltage-sensitive, Ca\(^{2+}\)-activated K\(^{+}\) conductance (\( I_{\text{K}} \)), which depends on the influx of extracellular Ca\(^{2+}\) (Jassar et al. 1994), was blocked, and the remaining current was attributable to \( I_{\text{K}} \) (delayed rectifier). \( I_{\text{C}} \) was recorded from a holding potential of −40 mV. Outward currents were activated in response to depolarizing voltage commands and recorded in the absence and presence of extracellular Ca\(^{2+}\). \( I_{\text{C}} \) was then derived by taking the difference between the total outward current at each potential and \( I_{\text{K}} \) that persisted after Ca\(^{2+}\) channels were blocked. 40 mM extracellular K\(^{+}\) was used to limit the amplitude of \( I_{\text{C}} \) and \( I_{\text{K}} \) and thereby to facilitate voltage control. For \( I_{\text{C}} \) and \( I_{\text{K}} \), leak subtraction was done by applying 1/4 amplitude hyperpolarizing pulses, multiplying the responses by four and addition.

\( I_{\text{Na}} \) was studied using an external solution containing (in mM) 117 NaCl, 2 KCl, 2 MgCl\(_2\), 2 CaCl\(_2\), 5 HEPES/NaOH (pH 7.2), and 10 d-glucose and an internal solution consisting of (in mM) 110 KCl, 10NaCl, 2 MgCl\(_2\), 0.4 CaCl\(_2\), 4.4 EGTA, 5 HEPES/KOH (pH 7.2), and 10 d-glucose (Selyanko et al. 1990). \( I_{\text{Na}} \) deactivation and activation were studied following a series of hyperpolarizing steps from a holding potential of −30 mV. At this voltage, \( I_{\text{K}} \) was inactivated (Adams et al. 1982) as was much of the N-type Ca\(^{2+}\) conductance (\( g_{\text{Ca},N} \)) that contributes to Ca\(^{2+}\) influx and hence to the activation of \( I_{\text{C}} \). Leak currents were calculated by linear regression of the current-voltage relationship between −110 and −80 mV. These voltages are more negative than the activation range for \( I_{\text{K}} \), and leak current therefore dominates (Adams et al. 1982). Pure \( I_{\text{Na}} \) at the voltages between −70 and −30 mV was obtained by subtracting the leak current (Jassar et al. 1994).

The petri dishes were superfused with external solution at a flow rate of 2 ml/min. This allowed exchange of solutions within 2 min. Input capacitance (\( C_{\text{in}} \)) was calculated by integrating the capacitative transient that accompanied a 10-mV depolarizing command from −80 mV. Since membrane capacitance per unit area is constant, current densities were expressed in terms of current per unit capacitance, i.e., nanoampere per picofarad or for smaller currents, picoampere per picofarad.

All data are presented as means ± SE, and Student’s two-tailed t-test or ANOVA was used to assess statistical significance (\( P < 0.05 \)). In graphs where no error bars are visible, the error bars are smaller than the symbols used to designate the data points. All chemicals were from Sigma, St. Louis, MO, except for NGF (2.5 s)
and anti-NGF antibody, which were from Alomone Laboratories, Jerusalem, Israel, and Leibovitz’s L-15 medium and penicillin-streptomycin antibiotics, which were from Gibco BRL.

RESULTS

Increase of \( \text{Na}^+ \) currents in culture and enhancement of this effect by NGF

Figure 1 illustrates typical recordings of total \( I_{\text{Na}} \) from an acutely dissociated cell (A), a cell maintained in defined medium for 15 days (B), and a cell maintained in defined medium supplemented with 200 ng/ml NGF for 15 days (C). Even in the absence of NGF, total \( I_{\text{Na}} \) increased after 15 days in culture. A further and pronounced increase in current occurred when neurons were cultured in the presence of NGF for 15 days. Currents were elicited by a series of 12-ms depolarizing voltage commands from a holding potential of \(-80\) mV in 10-mV steps. \( I_{\text{Na}} \) and the underlying conductance (\( g_{\text{Na}} \)) activated quickly and had almost completely inactivated at the end of the voltage command in acute cells and in cells cultured for 15 days with or without NGF. Under all three conditions, \( I_{\text{Na}} \) began to activate near \(-20\) mV, and the peak currents generally occurred at \(+10\) mV. Figure 1D shows the current-voltage relationship for total \( I_{\text{Na}} \) from 23 acutely dissociated cells, 20 cultured cells in the absence of NGF for 15 days, and 20 cultured cells in the presence of NGF for 15 days. This again illustrates that \( I_{\text{Na}} \) recorded after 15 days in the absence of NGF was significantly greater than that in acutely isolated cells and that treatment of the cells with NGF for 15 days produced a further increase in \( I_{\text{Na}} \). Differences were apparent at all test potentials and maximum total \( I_{\text{Na}} \) at \(+10\) mV recorded in the
presence of NGF was significantly greater than the current recorded in its absence (P < 0.01).

Figure 1E shows the time course of the increase in total $I_{\text{Na}}$ as cells were maintained in culture. There was a clear increase in current after 1 day in culture. $I_{\text{Na}}$ reached a peak of four times the amplitude recorded from acutely isolated cells within the first 2 days of culture (P < 0.01). Afterward, the current declined gradually, but it was still 2.3 times larger that of the acutely dissociated cells on the 15th day of culture (P < 0.01; Fig. 1E, ○). NGF promoted further increases in $I_{\text{Na}}$, but its onset of action was quite slow. Only after 6 days was there a significant difference between the amplitude of $I_{\text{Na}}$ in cells cultured in the presence and absence of NGF. All currents subsequently recorded in the presence of NGF were significantly larger than those recorded in its absence. By the 15th day, the current in the cells cultured with NGF was 2.4 times larger than that of the cells cultured in the absence of NGF (P < 0.01; Fig. 1E, □).

We have previously observed an increase in the size of BFSG B neurons, as measured from their $C_{\text{m}}$, as they are maintained in culture with or without NGF (Lei et al. 1997). Figure 1F shows the time course of the increase in $C_{\text{m}}$ for the cells used in the present study. $C_{\text{m}}$ did not change within the first day of culture (P > 0.05). This contrasted with $I_{\text{Na}}$, which more than doubled on the first day (Fig. 1E). On the second day of culture, $C_{\text{m}}$ increased by only 39% in contrast to the fourfold increase in the current (Fig. 1E). The increase in $I_{\text{Na}}$ therefore precedes the increase in $C_{\text{m}}$. At day 15, $C_{\text{m}}$ was roughly doubled but NGF failed to promote any further increase in cell size (Fig. 1F).

The effects of NGF on $\text{Na}^+$ channels were further studied by analysis of changes in current density. Figure 1G (○) shows that the initial, transient peak of $I_{\text{Na}}$ seen in the absence of NGF was more conspicuous when current density was used as an indicator because on the second day of culture, there is far greater increase in $I_{\text{Na}}$ than in $C_{\text{m}}$. The $I_{\text{Na}}$ density therefore reached a maximum at this time but then declined until after 9 days; there was no statistical difference between the current density of the cultured cells and that of acutely dissociated cells. Addition of NGF to the culture medium did not change the current density of the cultured cells and it prevented the current density from declining with time after the initial peak (Fig. 1G, □). By 15 days, current density in NGF-treated cells was 2.5 times larger than in untreated cells.

Although we used neuron-enriched, Ara-C-treated cultures, there is evidence that neurotrophic factors can be produced by PC12 cells (Gill et al. 1998) and by sympathetic neurons (Korschning 1993). We therefore wondered whether the initial transient increase in $I_{\text{Na}}$ density seen after 2 days in culture in the absence of exogenous NGF (Fig. 1G) may result from the presence of endogenous NGF or some other neurotrophin in our system. To test for the involvement of NGF, a serum containing NGF antibody was included in the culture medium. The control group was treated with serum containing a control Ig-G antibody raised against nonneuronal protein. Anti-NGF (0.5 μg/ml) inhibited only 34% of the enhanced current density on the second day of culture (P < 0.05; Table 1). The current density of the cells cultured with anti-NGF for 2 days was therefore still 71% higher than that of the acutely dissociated cells (P < 0.05). The antibodies therefore exerted only a very weak effect on the initial, transient enhancement of $I_{\text{Na}}$ that was seen when BFSG neurons were placed in culture.

**NGF increases both TTX-sensitive and -insensitive components of $I_{\text{Na}}$**

$I_{\text{Na}}$ in BFSG B neurons consists of a larger TTX-s component and a much smaller TTX-r component (Jassar et al. 1993; Jones 1987). NGF induces TTX-r $I_{\text{Na}}$ in PC12 cells (Rudy et al. 1982b) and increases expression of $\alpha$-SNS mRNA that codes for a TTX-r $\text{Na}^+$ channel in DRG neurons (Black et al. 1997; Dib-Hajj et al. 1998). By contrast, NGF decreases mRNA for one type of TTX-s $I_{\text{Na}}$ (sodium channel III; $\alpha$-III) in sensory neurons (Black et al. 1997). We therefore examined the effects of NGF on both TTX-s $I_{\text{Na}}$ and TTX-r $I_{\text{Na}}$ in BFSG neurons.

Total $I_{\text{Na}}$ was recorded in the normal external solution, and TTX-r currents were recorded in 1 μM TTX. The TTX-s component of $I_{\text{Na}}$ was then derived by subtraction. Peak TTX-s $I_{\text{Na}}$ density recorded after 15 days in NGF was about 2.4 times greater than after 15 days in culture alone (P < 0.01; Fig. 2A). A slightly stronger effect was seen on TTX-r current which was increased about 3 fold (P < 0.01; Fig. 2B). Even though NGF preferentially enhanced TTX-r current, the contribution of this component to the total $I_{\text{Na}}$ was little changed; 15.8% of the total current was TTX-insensitive in the absence of NGF compared with 17.9% in its presence.

**Culture of neurons with or without NGF did not change the activation kinetics of total $g_{\text{Na}}$**

To study the effects of establishing cultures and the effects of NGF on $g_{\text{Na}}$ activation kinetics, the absolute currents evoked by a series of voltage steps were converted to conductances by the equation $g_{\text{Na}} = I_{\text{Na}}/(V_m - E_{\text{Na}})$, where $V_m$ is the command potential and $E_{\text{Na}}$ is the $\text{Na}^+$ equilibrium potential (+60 mV for $[\text{Na}^+]_0 = 97.5$ mM and $[\text{Na}^+]_o = 9$ mM). Conductance values were normalized and fitted to a Boltzmann equation of the form $g/g_{\text{max}} = [1 + \exp(-(V_m - V_a)/(k_a))]^{-1}$, where $g$ is the conductance; $g_{\text{max}}$, the maximal conductance; $V_m$, the command potential; $V_a$, voltage at which half activation is achieved; $k_a$, slope factor of activation curve. Table 2 summarizes the results fitted from 23 acutely dissociated cells, 20 cells cultured without NGF for 15 days, and 20 cells cultured with NGF for 15 days. There were no statistically significant differences among the values of $V_a$ for the acutely dissociated cells, cells cultured with and without NGF for 15 days, suggesting the voltage dependence of activation for those cells was

| TABLE 1. Effect of NGF antibodies on transient increase in $I_{\text{Na}}$ density seen after 2 days in culture |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | 2 Days in Culture | 2 Days in Culture + Anti-NGF | Acutely Isolated | 2 Days in Culture + Control Antibody |
| $I_{\text{Na}}$ density at −10 mV, nA/pF | 0.43 ± 0.03 | 0.29 ± 0.04* | 0.17 ± 0.02 | 0.41 ± 0.03 |

* P < 0.05 compared to 2 days in culture. Values are means ± SE. NGF, nerve growth factor.

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not changed. Although the slope factor ($k_a$) for acutely dissociated cells was statistically larger than that of the cells cultured without NGF for 15 days ($0.01 < P < 0.05$) and that of the cells cultured with NGF for 15 days ($P < 0.01$), there was no significant difference between the $k_a$ value of the NGF-treated cells and that of the cells cultured alone ($P > 0.05$). These differences however had little influence on the overall Boltzmann fit to the pooled data as the activation curves for acutely isolated cells and cultured cells with or without NGF were almost superimposed (Fig. 2C).

**Culture of neurons with or without NGF did not change $g_{Na}$ inactivation kinetics**

The voltage dependence of steady-state inactivation ("$h_{in}$ plot"; Fig. 2D) was obtained by clamping the neurons to a series prepulse potentials (from $-70$ to $+10$ mV) for 20 ms to allow inactivation to develop prior to the application of a 12-ms test pulse to $+10$ mV. Currents recorded in response to the test pulse were normalized to the maximal current and then fitted by Boltzmann equation $I/I_{max} = [1 + \exp((V - V_{1/2})/k_1)]^{-1}$ (where $I$ is current; $I_{max}$ is maximal current; $V$ is prepulse potential; $V_{1/2}$ is voltage for half-maximal inactivation reached, and $k_1$ is slope factor of inactivation curve). Values of $V_{1/2}$ and $k_1$ for each cell were obtained by curve fitting. The results are also summarized in Table 2. There were no significant differences for the values of $V_{1/2}$ and $k_1$ among the

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**TABLE 2. Boltzmann parameters for activation and inactivation of total $g_{Na}$**

<table>
<thead>
<tr>
<th></th>
<th>$V_{1/2}$</th>
<th>$k_a$</th>
<th>$V_{1/2}$</th>
<th>$k_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>$0.82 \pm 1.29$</td>
<td>$7.29 \pm 0.53$</td>
<td>$-19.32 \pm 2.42$</td>
<td>$7.70 \pm 0.37$</td>
</tr>
<tr>
<td>Culture</td>
<td>$-2.14 \pm 0.66$</td>
<td>$5.44 \pm 0.48^*$</td>
<td>$-17.46 \pm 2.59$</td>
<td>$7.74 \pm 0.40$</td>
</tr>
<tr>
<td>NGF</td>
<td>$-2.40 \pm 0.93$</td>
<td>$5.31 \pm 0.21^{**}$</td>
<td>$-21.39 \pm 2.19$</td>
<td>$7.22 \pm 0.29$</td>
</tr>
</tbody>
</table>

Values are means ± SD. * $0.05 > P > 0.01$ compared with acutely dissociated cells. ** $P < 0.01$ compared with acutely dissociated cells.  

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FIG. 2. Effects of NGF on TTX-sensitive and -resistant components of $I_{Na}$ and effects on the kinetics of the total $g_{Na}$. A: plots of density of TTX-sensitive $I_{Na}$ vs. voltage for cells cultured for 15 days with (○) or without (□) NGF. B: plots of density of TTX-resistant $I_{Na}$ vs. voltage for cells cultured for 15 days with (○) or without (□) NGF. Note difference in scale between A and B: TTX-resistant current roughly one-quarter of the amplitude of the TTX-sensitive current. C: activation curves for normalized, total $g_{Na}$ obtained from (●) acutely dissociated cells (○) cells cultured for 15 days without NGF and (□) cells cultured for 15 days with NGF. Currents activated by a series of voltage commands from $V_h = -80$ mV were converted to conductance, and Boltzmann fitting was applied to the means. D: steady-state inactivation ($h_{in}$) plots for total, normalized $g_{Na}$ calculated from currents recorded at $+10$ mV following a series of 20-ms prepulses to different potentials as indicated on the abscissa. Data from (●) acutely dissociated cells (○, n = 30) cells cultured for 15 days without NGF (□, n = 26), and cells cultured for 15 days with NGF (■, n = 33). Boltzmann fitting was applied to the means. E: voltage dependence of rate of inactivation for total $g_{Na}$. Time constants of inactivation ($\tau$) were obtained by single-exponential fitting of the decay of the total current. The reciprocals of the time constants were defined as the inactivation rate. There is no difference between the inactivation rates observed in acutely dissociated cells (●), cells cultured for 15 days without NGF (○), and cells cultured for 15 days with NGF (■).
acutely dissociated cells, cultured with and without NGF for 15 days ($P > 0.05$ for all the groups), suggesting that the voltage dependence of inactivation has not been changed by culture with or without NGF.

Inactivation time constants during 12-ms depolarizing voltage pulses were obtained by single-exponential fitting to the decay of the current. Inactivation rate (reciprocals of the time constants) is plotted against voltage in Fig. 2E. Between $-10$ and $+40$ mV there is no significant difference in the inactivation rate among the acutely dissociated cells, cultured cells without NGF for 15 days, and cultured cells with NGF for 15 days ($P > 0.05$).

**Transcription dependence of the effect of NGF on Na$^+$ channels**

The transcriptional inhibitors actinomycin D and cordycepin were used to test whether NGF-induced increases in $I_{\text{Na}}$ involved changes in gene expression.

Inclusion of cordycepin (20 $\mu$M) or actinomycin D (0.01 $\mu$g/ml) did not significantly change the control $I_{\text{Na}}$, density recorded after 12–15 days in culture. The inhibitors did, however, completely block the augmentation of current density induced by NGF. The results, which are shown in Fig. 3A, suggest that the effects of NGF on Na$^+$ channels are dependent on gene induction.

**Effects of NGF in vivo**

The preceding results pose a problem. If a retrograde influence of target-derived NGF is responsible for maintaining $I_{\text{Na}}$, the current would be predicted to decrease when cells lose target contact as they are placed in culture. Instead the opposite happens, $I_{\text{Na}}$ density transiently increases as neurons are cultured (Fig. 1G) and similar changes are seen with axotomy in vivo (Jassar et al. 1993). In an attempt to resolve this apparent contradiction, we also examined the effect of NGF on intact Gtured (Fig. 1F). Between $-10$ and $+40$ mV there is no significant difference in the inactivation rate among the acutely dissociated cells, cultured cells without NGF for 15 days, and cultured cells with NGF for 15 days ($P > 0.05$).

**Effects of NGF on voltage-dependent Ca$^{2+}$-activated K$^+$-channel current ($I_C$)**

To examine whether NGF affects the properties of $I_C$, the current was elicited by 50-ms pulses from a holding potential of $-40$ mV to minimize contamination by $I_K$ because, at this voltage, $I_K$ is largely inactivated (Adams et al. 1982). To obtain adequate voltage control, $[K^+]_o$, was raised to 40 mM ($E_K = -23$ mV).

Figure 4, A and B, illustrates $I_C$ traces obtained by digitally subtracting the Cd$^{2+}$-resistant current from the total current for a cell cultured without NGF for 15 days and a cell cultured with NGF for 15 days, respectively. The current in the NGF-treated cell is much greater. Figure 4C shows the current-voltage relationship of $I_C$ from all cells studied. $I_C$ began to activate when the command potential was $-30$ mV and reached a maximum at $+30$ mV. At more positive voltages, the current declined gradually because of the decreased influx of Ca$^{2+}$ via Ca$^{2+}$ channels (Jassar et al. 1994). $I_C$ of the cells cultured with NGF was significantly greater than that of the cells cultured without NGF for 15 days (the differences between the values of the cultured cells without NGF and those of the cells cultured with NGF were statistically significant for all voltages positive to $-20$ mV, $P < 0.01$). Figure 4D shows the time course of the change in $I_C$. In the absence of NGF, total $I_C$ decreased slightly throughout the 15 days experimental period (Fig. 4D, ). Inclusion of NGF in the culture medium more than doubled the current seen in the absence of NGF (Fig. 4D, ).

Figure 4E shows the effect of culture with or without NGF on $I_C$ density. This decreased significantly after 3 days of culture because $C_{in}$ increased at this time (Fig. 1F). Inclusion of NGF prevented the decrease in $I_C$ density.
requires influx of Ca\textsuperscript{2+} via voltage-gated Ca\textsuperscript{2+} channels, it is possible that effects of NGF on I\textsubscript{C} reflect its action on Ca\textsuperscript{2+} channels rather than on I\textsubscript{C} channels per se. We therefore plotted the means of the Ca\textsuperscript{2+} current from cells cultured with NGF for 1–15 days (Ba\textsuperscript{2+} was used as the charge carrier) (data from Lei et al. 1997) against the corresponding I\textsubscript{C} amplitudes. I\textsubscript{C} amplitudes were linearly proportional to their corresponding Ca\textsuperscript{2+} channel current amplitudes (Fig. 4F, P < 0.01 for a correlation coefficient of 0.829). These results are consistent with the hypothesis that the effects of NGF on I\textsubscript{C} are secondary to its effect on Ca\textsuperscript{2+} channels.

**Lack of effect of NGF on delayed-rectifier K\textsuperscript{+} current (I\textsubscript{K})**

I\textsubscript{K} was recorded from a holding potential of −80 mV in the presence of 100 μM Cd\textsuperscript{2+} to block I\textsubscript{C}. I\textsubscript{K} started to activate at −30 mV and increased with increasing depolarization. During a relatively brief, 50-ms depolarization voltage command, the current did not inactivate. Figure 5, A and B, illustrates typical recordings of I\textsubscript{K} from a cultured cell without NGF and a cultured cell with NGF for 15 days, respectively. The current amplitudes are quite similar. Figure 5C shows that I\textsubscript{K} density (at −80 mV) decreased with time in culture and that this decrease was not prevented by NGF. The observed I\textsubscript{K} density 1.05 ± 0.06 nA/pF seen after 1 day in culture is similar to the value seen in control, freshly dissociated neurons (B. S. Jassar and P. A. Smith, unpublished observations). Figure 5D shows that the I\textsubscript{K}-voltage relationship was not changed by NGF.

**Lack of effect of NGF on M current (I\textsubscript{M})**

Hyperpolarizing command pulses from a holding potential of −30 mV promote g\textsubscript{M} deactivation. The conductance then reactivates on repolarization to −30 mV (Adams et al. 1982). Figure 6, A–C, illustrates representative current responses evoked from an acutely isolated cell, a cell cultured without NGF for 15 days, and a cultured cell with NGF for 15 days. Although there was an obvious increase in I\textsubscript{M} in cells that were maintained in culture, the presence of NGF had no additional effect on the amplitude of I\textsubscript{M} relaxations over the 15-day experimental period. Figure 6D shows that the I\textsubscript{M}-voltage relationship was not changed by NGF.
There were three main findings from these experiments. First, the electrophysiological changes seen when BFSG neurons are maintained in defined-medium culture in the absence of NGF resemble those produced by axotomy in vivo. Thus TTX-s $I_{Na}$, TTX-τ $I_{Na}$, and $I_M$ are increased, whereas $I_K$ is decreased (Jassar et al. 1993, 1994). Both axotomy and culture produced an increase in $I_{Na}$ that was not accompanied by alterations in channel kinetics. Moreover, culture-induced changes in the Ca²⁺-dependent $K^+$ conductance, $I_C$, are likely secondary to previously documented changes in $I_{Ca}$ (Lei et al. 1997).

Second, the effects of NGF were selective for certain types of currents; TTX-s $I_{Na}$ and TTX-τ $I_{Na}$ were increased, whereas the K⁺ currents, $I_M$ and $I_K$, were unaffected. Again, increases in $I_C$ likely reflected NGF-induced increases in $I_{Ca}$ (Jassar et al. 1993; Lei et al. 1997). The overall implication is that NGF-induced increases in $I_{Na}$ and $I_{Ca}$ cannot be ascribed to a generalized improvement in the “health” of cultured sympathetic neurons that had previously been denied appropriate neurotrophic support.

Third, there was no evidence for down-regulation of channel function by NGF. Thus increases in $I_{Na}$ or $I_M$ (Jassar et al. 1993, 1994) that are seen in culture or that are induced by axotomy do not reflect removal of a tonic, retrograde inhibitory effect of NGF on ion channel function. Because axotomy, culture, and NGF application all increased $I_{Ca}$, NGF-dependent and -independent mechanisms must control expression of Na⁺ channels on the cell bodies of BFSG neurons.

A broad range of concentrations (from 2 to 300 ng/ml) have been used to maintain or examine responses of neuronal cultures to NGF (Hilborn et al. 1998; Levine et al. 1995; Mandelzys et al. 1995; Riccio et al. 1997; Sjogreen et al. 2000; Tsui-Pierchala et al. 2000). In one study, 2 ng/ml NGF was used to maintain cultures prior to testing for physiological responses with 200 ng/ml (Riccio et al. 1997). The concentration of NGF (200 ng/ml) that we used is thus toward the upper end of the range used by other investigators. Despite this, it does not promote a growth response yet serves to distinguish positive effects on Na⁺ and Ca²⁺ channels (Lei et al. 1997) from a lack of effect on K⁺ channels. It would be useful to know whether a selective effect could be observed with lower concentrations of NGF.

### Changes in $I_{Na}$

The increase in $I_{Na}$ that followed either the inclusion of NGF in cultures (Fig. 1) or the injection of NGF under the skin of the thighs (Fig. 3B) is consistent with the long-standing hypothesis that the availability of target-derived NGF is necessary for the maintenance of Na⁺ channel function in sympathetic neurons (Rudy et al. 1982a; Toledo-Aral et al. 1995). NGF may also fulfill this function in small, nociceptive sensory neurons (Fjell et al. 1999).

If NGF is responsible for maintaining $I_{Na}$, why does the current increase after axotomy (Jassar et al. 1993) or in NGF-free culture (Fig. 1) when access to target-derived NGF is restricted or denied? The implication is that two opposing processes control $I_{Na}$ expression: one is NGF and the other is an NGF-independent phenomenon that is associated with the loss of an axon. One possibility is that axotomy, or the establishment of NGF-free cultures, causes the accumulation in the soma of Na⁺ channels destined for the axon. This idea is supported by the observation that chronic constriction of sensory nerves leads to accumulation of Na⁺ channels in the plasma membrane at the site of injury (Novakovic et al. 1998). Another possibility is that the increase in $I_{Na}$ reflects a change in the rate of insertion or degradation of channels that have already been synthesized. Further information about the mechanism of action of NGF may be obtained by examining the time course of effects of the transcription inhibitors, cordycepin and actinomycin D. These experiments remain to be done.

The initial transient increase in $I_{Na}$ seen in cultured BFSG neurons is too rapid to reflect a response to NGF released from the cultured neurons themselves (Gill et al. 1998). The increase is apparent even after 1 day in culture (Fig. 1E), whereas the response to exogenously applied NGF takes at least 3 days to develop, and it is 6 days before the difference attains statistical significance (Fig. 1E). If the initial, transient increase in $I_{Na}$ reflects NGF-independent inappropriate insertion of Na⁺ channels into the soma, it remains to be explained why this effect is attenuated, albeit slightly, by NGF antibodies (Table 1).

Our results with transcription inhibitors suggest that induc-
tion of gene expression in response to NGF was involved in the regulation of Na\(^+\) channels. We do not yet know whether the gene or genes that were induced encode Na\(^+\) channel proteins per se or whether they encode subsidiary proteins that are required for normal Na\(^+\) channel function. In the light of studies on PC12 cells; however, we favor a direct effect on Na\(^+\) channel genes. In that system, NGF induces brain type II/IIA Na\(^+\) channel mRNA (Fanger et al. 1993, 1995a,b) as well as the expression of peripheral nerve type I (PN1) mRNA (D’Arcangelo et al. 1993; Toledo-Aral et al. 1997). These both code for TTX-s channels. Although the types of TTX-s Na\(^+\) channel genes that may be induced by NGF in adult BFSG neurons remain to be determined, the α-subunits of type III Na\(^+\) channels are unlikely to be involved these are downregulated by NGF in adult rat sensory neurons (Black et al. 1997).

The finding that TTX-r \(I_{\text{Na}}\) was potentiated by NGF (Fig. 6) goes along with the observation that α-SNS mRNA is upregulated (Black et al. 1997) and TTX-r channels in axotomized sensory neurons are “rescued” by NGF (Dib-Hajj et al. 1998). In BFSG, however, this may reflect an action of NGF on some other mRNA that codes for a TTX-r channel as α-SNS is apparently not expressed in sympathetic neurons (Akopian et al. 1996). These conclusions are in accord with the results from PC12 cells where NGF increases functional Na\(^+\) channels and induces TTX-r currents (Rudy et al. 1982b).

**General lack of effect of NGF on K\(^+\) channels**

\(I_{\text{M}}\) in BFSG increases in culture or after axotomy (Jassar et al. 1994), but since it is insensitive to NGF (Fig. 6), this increase is likely to reflect an NGF-independent mechanism, i.e., NGF neither maintains nor tonically inhibits the current. The mechanisms underlying \(g_{\text{M}}\) regulation therefore require further investigation. \(I_{\text{K}}\) was not affected by NGF (Fig. 5), and of the three K\(^+\) channel types examined, only voltage-dependent, Ca\(^{2+}\)-activated K\(^+\) channels \((I_{\text{C}})\) appeared to be affected by NGF (Fig. 4). Because \(I_{\text{C}}\) channels are dependent on Ca\(^{2+}\) influx for their activation (Lancaster and Pennefather 1987; Pennefather et al. 1985), the observed enhancement of \(I_{\text{C}}\) could reflect the documented effect of NGF on voltage-gated Ca\(^{2+}\) channels (Lei et al. 1997). This is possible because the \(I_{\text{C}}\) amplitude correlated well with that of the Ca\(^{2+}\) channel currents in NGF-treated cells (Fig. 4F). Despite this correlation, we cannot discount a possible additional direct effect of NGF on \(I_{\text{C}}\) channels per se. Such an effect has been seen in NIH3T3 and C3H10T1/2 cell lines where NGF increases the expression of Ca\(^{2+}\)-activated K\(^+\) channels (Huang and Rane 1994).

**Selectivity of action of NGF**

In this and our previous study (Lei et al. 1997), we showed that treatment of cultured BFSG neurons with NGF selectively increases \(I_{\text{Na}}\) and \(I_{\text{Ca}}\), whereas \(I_{\text{K}}\) and \(I_{\text{M}}\) are unaffected. Because its effects are selective, we have argued that NGF-induced alterations in ion channel function cannot be ascribed to a general improvement in the health of cultured neurons on exposure to neurotrophin. This idea is supported by the observation that injection of NGF into the target field of BFSG B neurons increases \(I_{\text{Na}}\) in vivo (Fig. 3B). Moreover, the selective effects of NGF on Na\(^+\) and not on K\(^+\) channels in culture is not part of a generalized “growth response”, as under the conditions employed, NGF does not increase \(C_{\text{in}}\) (Fig. 1F). It also does not stimulate neurite outgrowth (Lei et al. 1997). Similar neurotrophin modulation of ion channels without the initiation of a growth response has been described in cultured basal forebrain neurons (Levine et al. 1995). These findings therefore reinforce the emerging concept that the complement of ion channel types seen in a given neuronal type relate specifically to the trophic agents that are at that neuron’s disposal. It is possible that each ion channel type is maintained by one or more specific target-derived trophic substance(s). A particularly clear example of this idea has been provided in sensory neurons. Here it was shown that TTX-r \(I_{\text{Na}}\) was regulated by NGF but not by brain-derived neurotrophic factor.
(BDNF), whereas GABA_A channels were regulated by BDNF and not by NGF (Oyelese et al. 1997).

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