The Activity of cAMP-dependent Protein Kinase Is Required at a Posttranslational Level for Induction of Voltage-dependent Sodium Channels by Peptide Growth Factors in PC12 Cells

David D. Ginty,* Gary R. Fanger,‡§ John A. Wagner,* and Robert A. Maue‡§‖

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and Dana Farber Cancer Institute, Boston, Massachusetts 02115; and Programs in ‡Cellular and Molecular Neurosciences and §Biochemistry, Department of ‖Physiology, Dartmouth Medical School, Hanover, New Hampshire 03755-3833

Abstract. The synthesis and expression of voltage-dependent sodium (Na) channels is a crucial aspect of neuronal differentiation because of the central role these ion channels play in the generation of action potentials and the transfer of information in the nervous system. We have used rat pheochromocytoma (PC12) cell lines deficient in cAMP-dependent protein kinase (PKA) activity to examine the role of PKA in the induction of Na channel expression by nerve growth factor (NGF) and basic FGF (bFGF). In the parental PC12 cell line both NGF and bFGF elicit an increase in the density of functional Na channels, as determined from whole-cell patch clamp recordings. This increase does not occur in two PC12 cell lines deficient in both isozymes of PKA (PKAI and PKAII), and is strongly reduced in a third line deficient in PKAII, but not PKAI. Despite the inability of the neurotrophic factors to induce functional Na channel expression in the PKA-deficient cells, Northern blot hybridization studies and saxitoxin binding assays of intact cells indicate that NGF and bFGF are still capable of eliciting increases in both Na channel mRNA and Na channel protein in the membrane. Thus, PKA activity appears to be necessary at a posttranslational step in the synthesis and expression of functional Na channels, and thereby plays an important role in determining neuronal excitability.

VOLTAGE-dependent sodium (Na) channels play a central role in the production and propagation of action potentials in electrically excitable cells (Hodgkin and Huxley, 1952), and ultimately play a key role in the coding and transfer of information in the nervous system. This makes the synthesis of Na channels and acquisition of electrical excitability a crucial aspect of neuronal differentiation and suggests that an analysis of the mechanisms governing Na channel expression will reveal processes fundamental to the development of the nervous system.

Na channels purified from mammalian brain contain a glycoprotein of ~260 kD, termed the α subunit, as their principal component. Associated with the α subunit of the channels from mammalian brain are a β1 subunit of 36 kD and a β2 subunit of 33 kD (Hartshorne et al., 1982; Hartshorne and Catterall, 1984). Although the β subunits may modify the functional properties of Na channels, injection of *Xenopus* oocytes with mRNA encoding the α subunit alone is sufficient to produce functional Na channels (Noda et al., 1986*b*; Auld et al., 1988; Suzuki et al., 1988). Molecular biological studies have revealed a large family of α subunit genes, including at least four (type I, II, IIa, and III) expressed in the nervous system, each encoded by a distinct mRNA (Noda et al., 1986*a*; Auld et al., 1988; Kayano et al., 1988) and each able to form functional channels when expressed in *Xenopus* oocytes (Noda et al., 1986*b*; Auld et al., 1988; Suzuki et al., 1988).

While a great deal is known about the biochemical and functional properties of Na channels (for reviews see Barchi, 1988; Stephan and Agnew, 1991), comparatively little is known about the variety of mechanisms that appear to regulate their expression. For example, the neuronal-specific expression of the type II α subunit is achieved at least in part via neuronal-specific transcription of the type II α subunit gene (Maue et al., 1990). However, during development of the nervous system the abundance and regional distribution of the different α subunit mRNAs undergo distinct changes (Suzuki et al., 1988; Beckh et al., 1989). Furthermore, translational and posttranslational events, in addition to transcription, appear to be developmentally regulated, rate-limiting steps in the expression of the α subunit of Na channels in the brain and retina (Wollner et al., 1988; Scheinman et al., 1989), and may contribute to the differential pattern of expression observed for the different types of Na channel proteins in the nervous system (Gordon et al., 1987).

David D. Ginty's present address is Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115.

John A. Wagner's present address is Department of Neurology and Neuroscience and Department of Cell Biology and Anatomy, Cornell University Medical Center, New York, New York 10021.

The rat pheochromocytoma (PC12) cell line is a useful model system in which to study the expression of a variety of markers of differentiation, including the formation of neural processes and the development of electrical excitability (Greene and Tischler, 1976; Dichter et al., 1977; Fugita et al., 1989; Halegoua et al., 1991). PC12 cells express the type II α subunit gene, the predominant Na channel gene expressed in the nervous system, and in response to nerve growth factor (NGF)[1] there is an increase in type II α subunit mRNA underlying an increase in the density of functional Na channels (Mandel et al., 1988). Basic FGF (bFGF) also increases the expression of voltage-dependent Na channels in PC12 cells (Pollock et al., 1990).

NGF and bFGF are expressed in the nervous system in developing and adult organisms (Logan et al., 1985; Gospodarowicz et al., 1986; Levi-Montalcini, 1987; Bothwell, 1991). They play a critical role in the development of the sympathetic and sensory nervous systems, as well as an important role in the survival and development of neurons in the central nervous system (for reviews see, Thoenen and Barde, 1980; Wagner, 1991; Wagner and Kostyk, 1991; Gage et al., 1991). Interest in these neurotrophic factors has been enhanced by the realization that they may play a role in the development or therapy of neurodegenerative diseases (Hefti et al., 1989; Stewart and Appel, 1989).

The biochemical mechanisms underlying induction of Na channels by NGF and bFGF are not well understood. In particular, the role of cAMP-dependent protein kinase (PKA) activity has been controversial (Kalman et al., 1990; Pollock et al., 1990). We have taken advantage of a series of PKA-deficient PC12 lines (Van Buskirk et al., 1985; Ginty et al., 1991*b*) to assess the importance of these kinases in the regulation of Na channel expression by NGF and bFGF. Our results indicate that while PKA activity is not required either for the induction of Na channel mRNA, or for the insertion of Na channel proteins into the membrane, PKA activity does appear to be required for the expression of fully functional Na channels. Thus, the level of active PKA expressed in a neuronal cell can have a profound effect on the excitability of the cell and thereby its ability to generate and transfer electrical signals within the nervous system.

Materials and Methods

Cell Culture

Cells were maintained in a humidified CO_2 environment in DME containing 0.45% glucose, 10% FBS, 5% heat inactivated horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). Except where noted, media for the AB-11 and 123.7 cells also contained 500 μg/ml G418 (Gibco Laboratories). Media was changed every other day and cells were passaged once a week. Cells were either grown in tissue culture flasks and plates (Falcon Labware, Becton Dickinson, Lincoln Park, NJ) or in suspension in 100 mm plastic Petri dishes (Fisher Scientific, Springfield, NJ), which prevents neurite outgrowth but leaves other responses to neurotrophic agents intact, including increases in Na channel expression (Greene and Tischler, 1976; Rudy et al., 1982; Mandel et al., 1988). For cells grown in suspension, parallel cultures using the same passage of cells and the same lot and aliquot of growth factor were grown on tissue culture plastic and the neurite outgrowth used as an additional indication that the treatment was successful. Cells were treated with fresh growth factor, either 10 ng/ml bovine bFGF (Collaborative Research, Bedford, MA), 100 ng/ml of 7S NGF (Upstate Biotechnology, Inc., Plattsburg, NY), or purified β-NGF (prepared by the method of Mobley et al. [1976]), every time the media was changed. For the electrophysiological studies, cells grown in suspension were plated on 35-mm tissue culture plates (Falcon) 1-3 h before recording. During this time the cells adhered to the dish, yet remained round and did not extend long neurites that would interfere with the quality of the voltage clamp.

Electrophysiological Recording and Analysis

Recording conditions and procedures were similar to those used in previous studies (Mandel et al., 1988; Garber et al., 1989; Ifune and Steinbach, 1990; Kalman et al., 1990; Pollock et al., 1990). Just before recording, the culture medium was replaced with a saline solution (151 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.6 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 6 mM Hepes, 5.5 mM glucose). Whole-cell patch-clamp recordings (Hamill et al., 1981) were made at room temperature (20–24°C) using a List EPC-7 patch-clamp amplifier (Medical Systems Corp.). Patch electrodes were pulled from capillary glass (WPI, Inc.) and had resistances of 3–7 MΩ when filled with an internal solution designed to minimize the contribution of other voltage-activated currents (140 mM CsCl, 10 mM EGTA, 10 mM Hepes). Electrode tips were coated with Sylgaard (Dow Corning) to minimize electrode capacitance. Electronic compensation was used to reduce the effective series resistance and the time constant of membrane charging, and provided measurements of access resistance and cell membrane capacitance. Series resistance compensation of 50–70% was routinely used. Data were analyzed from cells with estimated series resistance errors of 5 mV or less. Voltage commands were applied and measurements of currents were made using an Atari computer-based acquisition system and software (Instrutech Corp., Elmont, NY). The voltage clamp pulse protocol involved holding the cells at −80 mV and then once every 3 s stepping the membrane voltage through a 40-ms prepulse to −120 mV, followed by a 20-ms depolarizing test pulse. The depolarizing test pulse was −60 mV for the first prepulse/pulse pair, and was incremented by 10 mV in each subsequent pair until it reached +30 mV. Each prepulse/pulse in the series was immediately followed by a proportionally smaller pair of P/4 pulses of identical duration from a holding potential of −120 mV. In some experiments (see Results), a holding potential of −120 mV and prepulse to −160 mV was used. The current signal was low pass filtered at 10 kHz, digitized at 20 kHz for storage, and digitally filtered at 2 kHz during analysis. Linear leakage currents and capacity transients were subtracted with scaled pulse (P/4) routines. For each test voltage, the peak inward current and time-to-peak current were measured. For each cell, the current vs. voltage relationship and the kinetic information were used as additional indications of the quality of the voltage clamp. The maximum Na current amplitude elicited from a cell, along with the measure of cell capacitance, was used to calculate Na current density. For each experimental condition, several platings of cells and different lots of growth factor were used without any difference in results.

RNA Isolation and Northern Blot Analysis

For RNA isolation, cells were plated in 150-mm tissue culture dishes at $\sim 10^7$ cells per dish, or maintained in suspension in plastic Petri dishes (Fisher) at $\sim 10^6$ cells per dish. Total cellular RNA was isolated by the method of Chirgwin et al. (1979) and 20-μg samples were size-fractionated on 1.0% agarose gels containing 2.0 M formaldehyde. After electrophoresis, the RNA was transferred to a nitrocellulose filter overnight, the filter baked at 80°C for 2 h, and then prehybridized for 4–5 h at 65°C in 50% formamide/0.1% SDS/0.1% ficoll/0.1% polyvinylpyrolidine/0.1% BSA/6 ×SSC (150 mM NaCl, 15 mM Na citrate, pH 7)/20 mM EDTA/100 μg/ml denatured salmon sperm DNA. A complementary RNA probe was generated using a commercially available kit (Promega Biotec, Madison, WI) and ^{32}P-labeled UTP. The template was a cDNA encoding a portion of the rat brain type II α subunit corresponding to amino acids 1528–1912 of the published sequence (Noda et al., 1986*a*). This probe has been used in previous studies to detect the ~9.5-kb Na channel transcripts (Mandel et al., 1988; Beckh et al., 1989). The specific activity of the probe was 0.5–2.0 $\times$ 10^9 dpm/μg, and was added directly to the prehybridization solution at a final activity of 10^6 dpm/ml. After a 12–18-h hybridization at 65°C, the filter was washed three times (30 min each) at room temperature in 0.5× SSC/0.5% SDS, twice (30 min each) at 64°C in 0.2× SSC/0.5% SDS, and

1. *Abbreviations used in this paper*: bFGF, basic FGF; NGF, nerve growth factor; PKA, cAMP-dependent protein kinase; PKAI, type I cAMP-dependent protein kinase; PKAII, type II cAMP-dependent protein kinase; RI, regulatory subunit of PKAI; STX, saxitoxin.

then autoradiographed with preflashed film. In some cases the internal control for RNA loading artifacts was the ethidium bromide staining, photography, and quantitation of the ribosomal RNA bands, while in others it was the densitometric analysis of the autoradiographic densities representing the 1-kb cyclophilin mRNA, which is constitutively expressed in PC12 cells and unaffected by growth factor treatment (Machida et al., 1989).

Saxitoxin Binding Assays

Saxitoxin (STX) binding was measured using previously developed procedures (Catterall and Morrow, 1978; Rudy et al., 1982). Cells were maintained in 35-mm tissue culture plates (Falcon). Culture medium was gently removed and replaced with 1 ml of a solution containing 130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgCl_2$, 5.5 mM glucose, 50 mM Tris/Hepes, pH 7.4, and, based on the apparent K_d for STX (~2 nM), a saturating concentration (20 nM) of [^{3}H]STX (Amersham Corp., Arlington Heights, IL). Parallel cultures incubated in the same solution plus 2 μM unlabeled tetrodotoxin (TTX) (Calbiochem Behring Corp., LaJolla, CA) were used in determining nonspecific binding. Cells were incubated with continuous gentle shaking at 4°C for 45 min in order to achieve equilibrium, and then unbound [^{3}H]STX was removed by quickly washing three times (3 s each) at 0°C with 3 ml of a solution containing 163 mM choline chloride, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5 mM Tris/Hepes, pH 7.4. Cells were then dissolved in 1 ml of 0.5% Triton X-100 and the extracts counted by scintillation counting. Cell number was determined from parallel plates cultured under identical conditions and quantified using a hemocytometer. In each experiment, assays were done in triplicate.

Results

Effects of PKA Deficiency on the NGF- and bFGF-mediated Increases in Functional Na Channel Density in PC12 Cells

Using the whole-cell recording configuration of the patch clamp technique (Hamill et al., 1981), we could easily detect the increase in Na channel expression that occurs in the parental PC12 cell line upon exposure to NGF or bFGF (Fig. 1, *a* and *c*) (Mandel et al., 1988). In these experiments, cells were grown in suspension under conditions that allow the induction of Na channel expression in response to neurotrophic agents, while avoiding the problems obtaining adequate voltage-clamp in cells with extensive neurite outgrowth (Mandel et al., 1988). Macroscopic Na currents elicited by brief depolarizing pulses between −50 to +30 mV were measured and the peak Na current normalized to the area of cell membrane, the latter estimated from cell capacitance measurements. This allowed changes in Na current density to be distinguished from changes in Na current amplitude simply resulting from changes in cell size. When assayed under these conditions, only a small number (4/20) of untreated PC12 cells had appreciable Na currents (100–240 pA maximum peak Na current), consistent with previous studies (Mandel et al., 1988; Garber et al., 1989; Pollock et al., 1990). In contrast, in cells grown in the presence of NGF for 7 d, there was a dramatic increase in both the fraction of cells (18/22) with large Na currents (>100 pA) and the average Na current density (Fig. 1 *c*). The average Na current density in the NGF-treated cells was significantly greater ($P < 0.01$) than in untreated PC12 cells, despite an accompanying increase in cell capacitance (Fig. 1) that presumably reflects the hypertrophy of the cells in response to NGF (Greene and Tischler, 1976. Thus, the increase in current per cell was even larger than the increase in current density. Similar changes were observed when cells were treated with bFGF for 7 d (Fig. 1 *c*). Large Na currents could be elicited from 81% of the cells (21/26). The average Na current density was significantly greater ($P < 0.01$) than in untreated cells, despite an increase in cell capacitance. The properties of the Na currents were similar before and after treatment with NGF or bFGF: Na currents could first be detected between −40 to −50 mV, reached their maximum between −10 to −20 mV, and, at the most depolarized potentials, peaked within 1 ms of the onset of depolarization.

To determine if PKA plays a role in the induction of voltage-dependent Na channels by NGF and bFGF, the effect of these neurotrophic factors on functional Na channel expression was determined in two PKA-deficient PC12 lines (AB-11 and 123.7) which were constructed by incorporating a gene encoding either one of two mutant regulatory subunits (RI) of PKA (Woodford et al., 1989) into parental PC12 cells (Ginty et al., 1991*b*). In both cell lines the overexpression of the mutant RI subunits represses cAMP-dependent activation of both the type I isozyme (PKAI) and the type II isozyme (PKAII) of PKA by ~90% in both the presence and absence of NGF (Ginty et al., 1991*b*). While responses to cAMP are repressed, both cell lines still respond to NGF, as evidenced by increases in cell size, neurite outgrowth, protein phosphorylation, and the expression of egr-1, fos, GAP-43, and ornithine decarboxylase (Ginty et al., 1991*a*,*b*). However, when compared to the parental PC12 cell line, there are striking differences in Na channel expression in both PKA-deficient lines (Fig. 1, *b* and *c*). In untreated AB-11 cells, none of the cells (0/27) had appreciable Na currents (>50 pA), and the average Na current density (Fig. 1 *c*) was significantly less than that detected in untreated PC12 cells ($P < 0.05$). Even after exposure to NGF for 7 d, there were no AB-11 cells exhibiting appreciable Na currents (0/20), nor was there any change in the average Na current density (Fig. 1 *c*), despite a slight increase in cell capacitance that suggested the cells responded to the treatment. Similarly, exposure to bFGF did not produce any cells with appreciable currents (0/23), nor did it result in an increase in average Na current density (Fig. 1 *c*).

The results obtained with the 123.7 cells were equally dramatic (Fig. 1, *b* and *c*). None of the untreated 123.7 cells (0/24) had appreciable Na currents and the Na current density (Fig. 1*c*) was less than in untreated PC12 cells. After treatment with NGF or bFGF for 7 d, there was an increase in cell capacitance, yet none of the cells had large Na currents (0/40) and the average current density actually declined slightly (Fig. 1 *c*). The same results were obtained when AB-11 and 123.7 cells were cultured in the absence of neomycin (data not shown). In addition, there was no detectable change in Na channel expression in AB-11 cells that were plated on tissue culture plastic throughout NGF treatment ($n = 6$), nor any effect on NGF-mediated Na channel induction when PC12 cells stably expressed an unrelated fusion gene (RSV-CAT) (data not shown). Altered inactivation properties of the Na channels did not appear to account for the absence of large Na currents in the PKA-deficient cells treated with NGF, as a holding potential of −120 mV and a prepulse of −160 mV failed to unmask any Na currents (data not shown). Finally, the failure of NGF to induce Na channel expression was not due to a failure of these cells to respond to NGF, because in addition to the increases in cell capacitance (and presumably cell size) both of these lines extended neurites in response to NGF and bFGF when parallel cul-

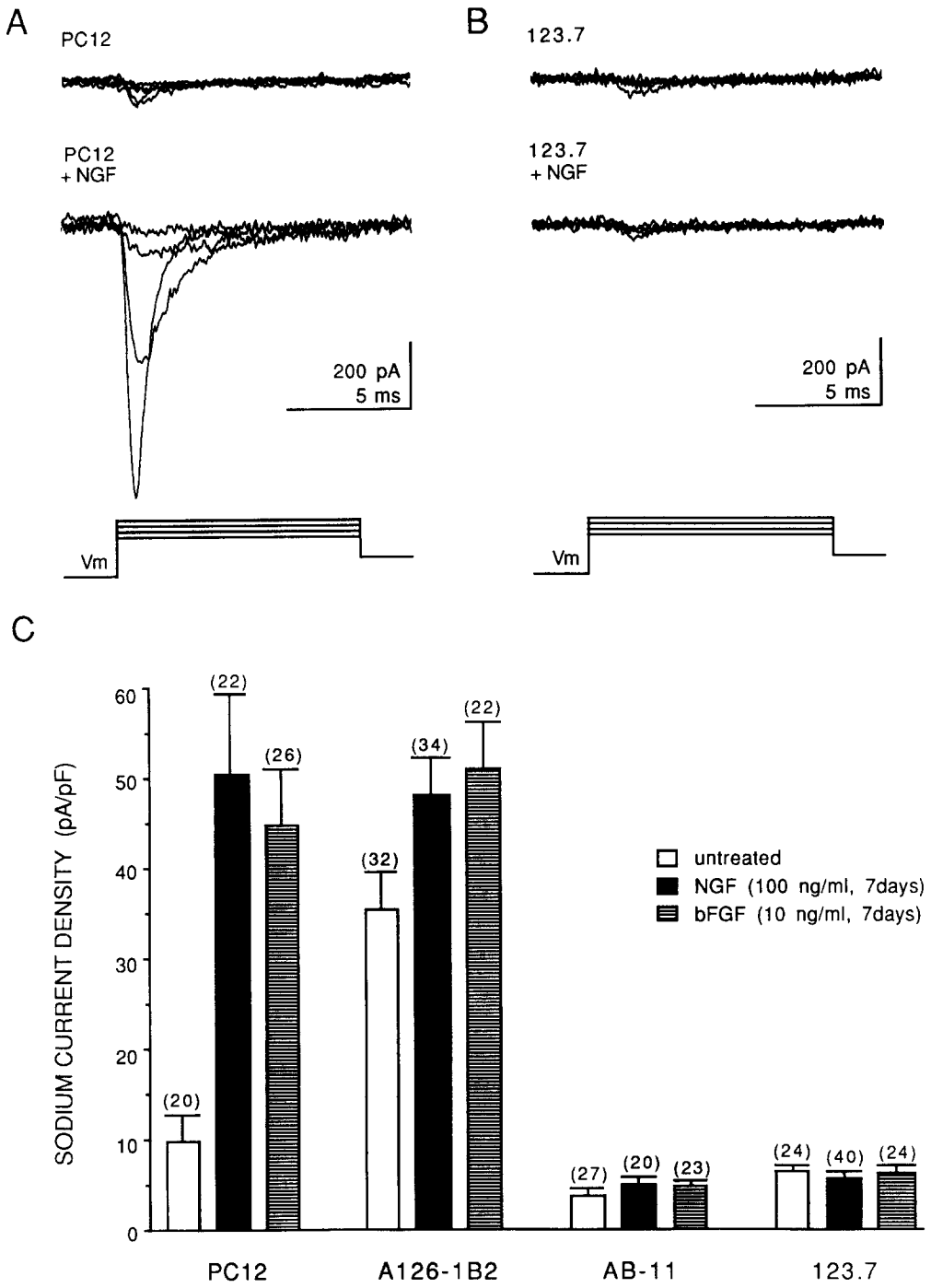

Figure 1. Macroscopic Na currents in PC12 and PKA-deficient PC12 cells cultured in either the presence or absence of peptide growth factors. (*A*) Voltage-dependent Na currents in untreated and NGF-treated PC12 cells (*B*). Voltage-dependent Na currents in untreated and NGF-treated 123.7 cells. In both *A* and *B*, cells were held at −80 mV, and the superimposed current records shown were elicited by depolarizing pulses to −50, −40, −30, and −20 mV after a 40-ms prepulse to −120 mV. Capacitative and leakage currents were subtracted from the records using scaled pulse (-P/4) routines. (*C*) Average Na current density in PC12 and PKA-deficient PC12 cells in response to NGF and bFGF. The number of cells in each group is indicated in parentheses above the bars. The SEM is shown as an error bar. Average cell capacitance (mean ± SEM) was also calculated for PC12 cells (7 ± 0.3 pF, n = 20), NGF-treated PC12 cells (10 ± 0.8 pF, n = 22), bFGF-treated PC12 cells (9.6 ± 0.6 pF, n = 26), A126 cells (11.1 ± 0.5 pF, n = 32), NGF-treated A126 cells (14 ± 1.1 pF, n = 34), bFGF-treated A126 cells (12.7 ± 0.7 pF, n = 22), AB-11 cells (7.2 ± 0.5 pF, n = 27), NGF-treated AB-11 cells (8.3 ± 0.6 pF, n = 20), bFGF-treated AB-11 cells (8.0 ± 0.4 pF, n = 23), 123.7 cells (6.9 ± 0.3 pF, n = 24), NGF-treated 123.7 cells (8.9 ± 0.4 pF, n = 40), and bFGF-treated 123.7 cells (10.5 ± 0.9 pF, n = 24). The statistical significance of differences between groups was determined by a nonparametric analysis of variance (H test) (Freund, 1973).

tures were plated on tissue culture plastic (data not shown). Thus, in the absence of active PKA, NGF and bFGF are apparently not capable of evoking an increase in the expression of functional Na channels in PC12 cells.

We also determined the effect of neurotrophic agents on functional Na channel expression in A126-1B2, a PC12 line that has reduced PKA activity. These cells, which were isolated after nitrosoguanidine mutagenesis, have reduced activity of PKAII, yet normal activity of PKAI, which constitutes ~20% of the total PKA activity (Van Buskirk et al., 1985). These cells exhibit many of the expected responses to NGF and bFGF, and have been used to show that while PKA does not have an essential role in the regulation of a number of events by NGF or FGF, including rapid changes in gene expression and ornithine decarboxylase activity, as well as later changes in neurite outgrowth and transin gene expression (Van Buskirk et al., 1985; Damon et al., 1990; Machida et al., 1991), it is important for the down regulation of calmodulin-dependent protein kinase (Brady et al., 1990) and the induction of early response genes in the absence of protein kinase C (Damon et al., 1990). Surprisingly, a majority of untreated A126-1B2 cells (22/32) exhibited large Na currents. The Na currents appeared qualitatively similar to those in PC12 cells, with the maximum current occurring in response to depolarizations to between −10 to −20 mV and the peak current occurring ~1 ms after the onset of de-

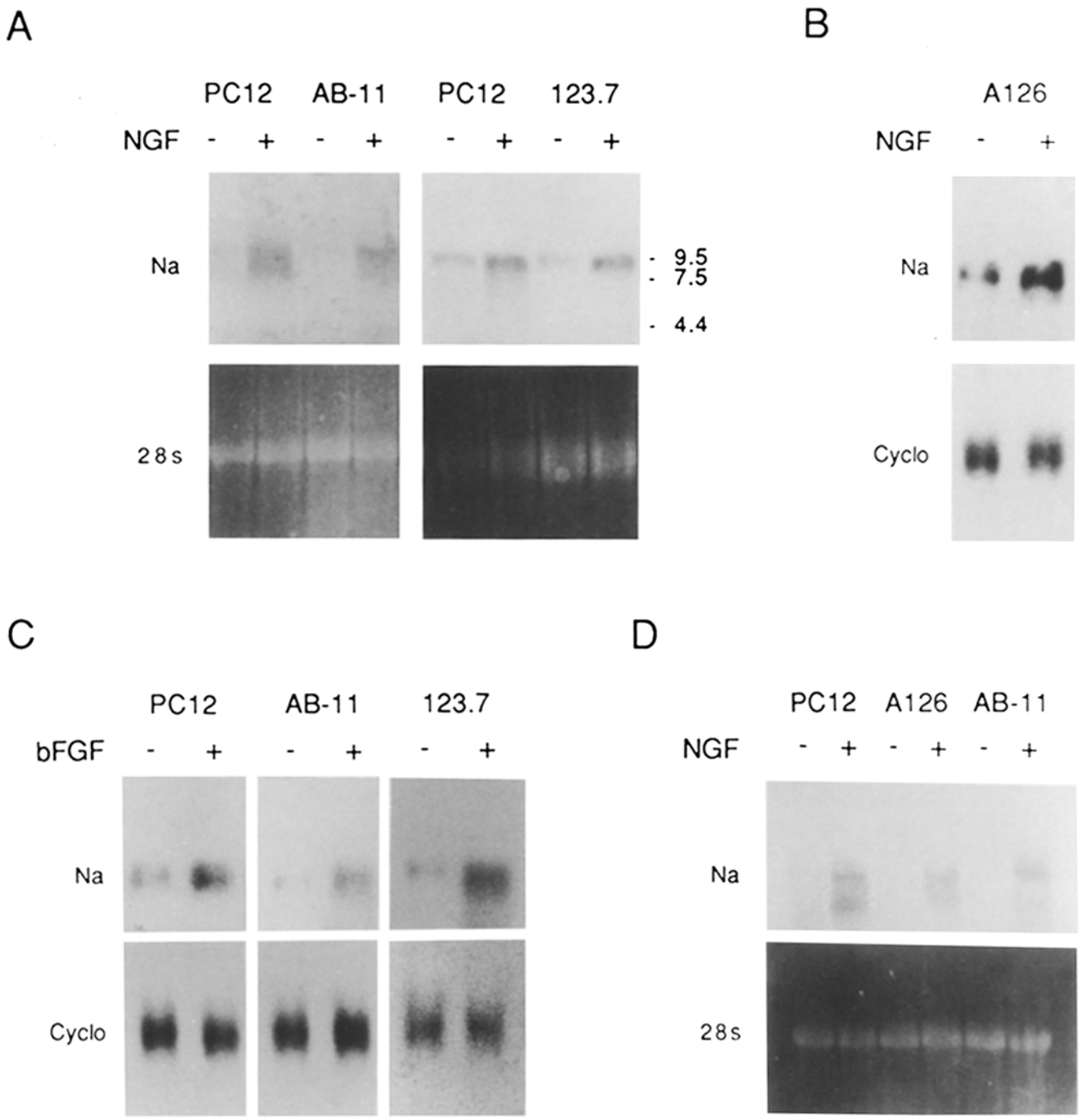

Figure 2. Northern blot hybridization analysis of Na channel mRNA expression in PC12 and PKA-deficient PC12 cells in response to growth factor treatment. (*A*) Na channel mRNA (*Na*) in PC12, 123.7, and AB-11 cells. Untreated (−) or NGF-treated (+) cells were maintained on tissue culture plastic for 5 d. (*B*) Na channel mRNA (*Na*) in A126-1B2 cells. Untreated (−) or NGF-treated (+) cells were maintained on tissue culture plastic for 7 d. (*C*) Na channel mRNA (*Na*) in untreated (−) and bFGF-treated (+) PC12, AB-11, and 123.7 cells maintained on tissue culture plastic for 7 d. (*D*) Na channel mRNA (*Na*) in PC12, A126-1B2, and AB-11 cells. Untreated (−) and NGF-treated (+) cells were maintained in suspension for 7 d. The position and molecular sizes of a commercially available RNA ladder (*BRL*) is shown in *A*. The ethidium bromide staining of the 28s ribosomal bands (*28s*) before transfer (*A* and *D*) or the 1-kb cyclophilin band (*Cyclo*) for each lane (*B* and *C*) is shown below and were used as an internal control to insure that equivalent amounts of RNA were loaded in each lane.

polarization. Even though A126-1B2 cells are larger than PC12 cells, as noted visually and by the estimates of cell capacitance, the average Na current density in the untreated A126-1B2 cells was still significantly ($P < 0.01$) higher than in untreated PC12 cells (Fig. 1 *c*). After exposure to either NGF or bFGF for 7 d, there appeared to be a slight increase in the Na current density (Fig. 1 *c*) as well as an increase in the proportion of cells with large Na currents (28/34 and 19/22, respectively) and an increase in the average cell capacitance. However, the increase in Na current density was

not statistically significant ($P > 0.05$). Thus, A126-1B2 cells, like AB-11 cells and 123.7 cells, appear to be resistant to growth factor–induced increases in functional Na channel expression.

Effects of PKA Deficiency on the NGF- and bFGF-mediated Increases in Na Channel mRNA Levels in PC12 Cells

To help define the step in the expression of functional Na channels at which the activity of PKA was necessary, the expression of Na channel mRNA was measured using Northern blot hybridization analysis. In untreated PC12 cells there was a detectable amount of Na channel mRNA (Fig. 2 *a*), consistent with previous results (Mandel et al., 1988). Exposure to NGF caused a marked increase in the steady-state levels of Na channel mRNA (Fig. 2 *a*), as did exposure to bFGF (Fig. 2 *c*). The increase in Na channel mRNA in response to growth factors was observed whether cells were plated on tissue culture plastic (Fig. 2 *a*) or grown in suspension (Fig. 2 *d*).

Na channel mRNA levels also increased in both AB-11 and 123.7 cells in response to NGF or bFGF (Fig. 2), despite the fact that the density of functional Na channels in these cells did not increase (see above). In untreated 123.7 cells the levels of Na channel mRNA appeared to be slightly lower than found in untreated PC12 cells (Fig. 2 *a*). In response to NGF treatment there was a four- to fivefold increase in Na channel mRNA, comparable to that seen in the parental PC12 cells (Fig. 2 *a*). Increases in Na channel mRNA levels in 123.7 cells also occurred in cells treated with NGF while grown in suspension (data not shown) and in cells grown in the presence of bFGF (Fig. 2 *c*). Similarly, NGF caused an increase in the level of Na channel mRNA in AB-11 cells, whether grown on tissue culture plastic (Fig. 2 *a*) or in suspension (Fig. 2 *d*). AB-11 cells treated with bFGF also had higher levels of Na channel mRNA than their untreated counterparts (Fig. 2 *c*).

In A126-1B2 cells, there was also an increase in the steady-state level of Na channel mRNA in response to NGF treatment, irrespective of whether the cells were plated on tissue culture plastic (Fig. 2 *b*) or grown in suspension (Fig. 2 *d*). Similar increases in Na channel mRNA levels occurred in A126-1B2 cells treated with bFGF (data not shown). Thus, bFGF, like NGF, causes an increase in Na channel mRNA in PC12 cells, and the increase in Na channel mRNA levels caused by these two growth factors apparently occurs via mechanisms that are independent of PKA activity. Apparently, PKA activity must be required at some posttranscriptional step in the growth factor–mediated induction of functional Na channels in PC12 cells.

Effects of PKA Deficiency on the NGF- and bFGF-mediated Increases in Saxitoxin Binding to PC12 Cells

To determine whether the accumulation of Na channel mRNA in the PKA-deficient cells resulted in an increase in the density of α subunit proteins in the cell membrane, [^{3}H]saxitoxin ([^{3}H]STX) binding to Na channel α subunit was measured using previously developed procedures for intact cells (Catterall and Morrow, 1978; Rudy et al., 1982). In untreated PC12 cells there was a small amount of specific [^{3}H]STX binding (Fig. 3), consistent with the results of the patch clamp recordings and Northern blot hybridization analysis presented here (see above), as well as with the results of previous studies (Rudy et al., 1982). Upon exposure to NGF for 7 d, there was a significant increase ($P < 0.01$) in the amount of binding (Fig. 3). The magnitude of the increase was comparable to results obtained in earlier studies (Rudy et al., 1982), given the differences in the length of NGF treatment (7 d vs 14 d). There were significant increases ($P < 0.01$) in [^{3}H]STX binding to 123.7 cells (Fig. 3) and AB-11 cells (data not shown) upon exposure of the cells to NGF. The magnitude of the increase in the PKA-deficient cells was similar to that observed for the parental PC12 cells (Fig. 3). Based on the results of the [^{3}H]STX binding assays, it appears that the growth factor–mediated increases in the level of Na channel α subunit in the membrane occur via PKA-independent mechanisms. However,

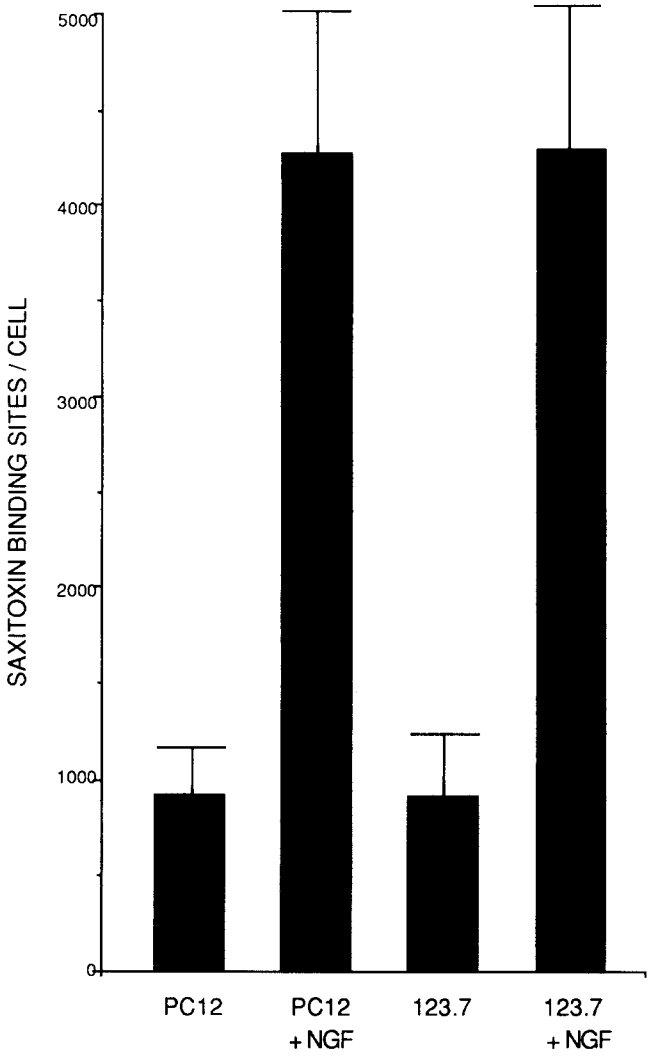

Figure 3. [^{3}H]Saxitoxin binding to PC12 and PKA-deficient cells exposed to peptide growth factors. The amount of specific [^{3}H]-STX binding to PC12 and 123.7 cells in response to NGF is shown. Untreated and NGF-treated (*+NGF*) cells were maintained on tissue culture plastic for 7 d. For each treatment, the bars represent the average of four to five experiments, each performed in triplicate. Values presented are the mean and corresponding SEM. The amount of binding under different conditions was compared using unpaired *t* tests (Freund, 1973).

based on the electrophysiological data, it appears that these Na channels are not fully functional in PKA-deficient cells. Thus, active PKA is presumably required at a posttranslational level in order for these channels to function normally.

Discussion

We have studied regulation of voltage-dependent Na channel expression by NGF and bFGF at three levels: the level of mRNA expression, the level of insertion of the α subunit of the channel into the membrane, and at the level of the expression of the functional channel. These studies have used a series of PKA-deficient cell lines to define an important step in Na channel expression that is controlled by PKA. Consistent with previous studies (Mandel et al., 1988), there is a four- to fivefold increase in Na channel mRNA levels in response to treatment with NGF; we have now demonstrated a similar increase occurs in response to bFGF. Both NGF and bFGF also elicit a similar four- to fivefold increase in Na channel mRNA in three PKA-deficient lines, AB-11, 123.7, and A126-1B2 cells, demonstrating for the first time that increases in Na channel mRNA in response to these growth factors can occur via PKA-independent mechanisms. These observations are reminiscent of experiments that show PKA-independent increases in mRNA for other "late" genes whose expression is associated with growth factor-mediated neuronal differentiation, including those encoding GAP-43 and transin (Ginty et al., 1991; Machida et al., 1991). Likewise, there are a number of early genes whose induction is not absolutely dependent on functional PKA (Ginty et al., 1991*a*,*b*), although PKA may be necessary for the induction of some genes in the absence of functional protein kinase C (Damon et al., 1990).

The increase in Na channel mRNA expression in the parental PC12 cells as well as in the PKA-deficient mutants was accompanied by an increase in the expression of Na channels as measured by [^{3}H]STX binding. The changes in Na channel expression in both the mutant and parental PC12 cells were in agreement with the previously documented changes in [^{3}H]STX binding to the parental PC12 cell line upon treatment with NGF (Rudy et al., 1982). Because of the specificity of STX binding, the results indicate that there was an increase in the density of Na channel proteins in the membrane of the PKA-deficient PC12 cells in response to growth factor treatment and for the first time provide evidence that the synthesis and expression of the Na channel proteins in the cell membrane can occur via PKA-independent mechanisms.

Despite a minor effect, if any, of PKA deficiency on the induction of Na channel mRNA and the expression of the protein at the membrane, there is a profound effect of the PKA deficiency on the formation of functional channels in response to both NGF and bFGF. Initial experiments in PC12 cells demonstrated five- to sixfold increases in the density of functional Na channels in response to both NGF and bFGF. The magnitude of this increase was similar to that observed in previous studies (Garber, et al., 1989; Ifune and Steinbach, 1990; Pollock et al., 1990), as were the measurements of cell capacitance, peak Na current amplitude, and Na current density, when assayed under similar conditions. The average Na current density reported by Pollock et al. (1990) was larger than the values reported here, presumably due to the result of longer growth factor treatments (7 vs 10 d) and the presence of extensive neurite outgrowth, the latter not required for Na channel expression, but resulting in slightly higher levels of Na channel expression (Rudy et al., 1982; Pollock et al., 1990). Although all PKA-deficient cell lines responded to the growth factors, as evidenced by neurite outgrowth in parallel cultures and increases in cell capacitance, the expression of functional Na channels in response to NGF or bFGF was eliminated or strongly reduced in these lines. In the AB-11 and 123.7 lines there was no measurable increase in functional channels, while in the A126-1B2 line there was no significant increase in channel expression. Intriguingly, the A126-1B2 cells differed from the other cell lines primarily in the high basal level of expression of functional Na channels, not in the level of induction after treatment with peptide growth factors. The reason for this is unknown. It is possible that a minimum of PKA activity is required in order for elevated Na current density, and that the basal activity in the A126-1B2 cells is sufficient, while the lower levels in the other cell lines are not. The selective loss of only one of the isoforms of PKA in the A126-1B2 cells also raises the possibility that PKAI and PKAII have distinct roles in the regulation of Na channel expression. Alternatively, the results may reflect a variation between A126-1B2 cells and the other cell lines that is not linked to the known changes in PKA expression (for discussion see Ginty et al., 1991*b*). Although the potential ambiguities of the A126-1B2 line further emphasize the advantage of the defined mutations and alterations in the AB-11 and 123.7 cells, the fact that the change in the expression of functional Na channels in the A126-1B2 cells was smaller than might be expected from the magnitude of the induction of Na channel mRNA supports the results obtained in the other mutant cell lines that indicate that PKA activity is necessary for the NGF-mediated increase in functional Na channels. This is consistent with work of Kalman et al. (1990) and extends their findings in two ways: we have shown that induction of the channel by both NGF and bFGF requires PKA, and, more importantly, we have shown that this requirement for PKA occurs posttranscriptionally and posttranslationally.

The induction of functional Na channels by NGF and bFGF is a "late" response in the cascade of changes caused by these growth factors and the functional expression of this multisubunit protein complex in the cell membrane probably results from the convergence of many cellular processes. Although we do not know the exact point at which PKA activity is required, the expression of Na channel proteins in the membrane, as indicated by [^{3}H]STX binding, without an increase in functional Na channels suggests that PKA activity is required at a posttranslational level. Among the possibilities include a requirement during the posttranslational subunit assembly and expression of the channel in the membrane or a phosphorylation of the channel protein itself. There are a number of precedents for the involvement of PKA in these aspects of ion channel expression. For example, Ross et al. (1991) have shown that agents that increase cAMP increase subunit assembly and surface expression of the acetylcholine receptor channel. Analysis of Na channel biosynthesis in the developing brain (Schmidt et al., 1985; Schmidt and Catterall, 1986; Scheinman et al., 1989) and retina (Wollner et al., 1988), have led to the suggestion that there is a developmentally regulated translational or post-

translational step in neuronal Na channel expression. In particular, the temporal correlation between the covalent attachment of the B2 subunit to the α subunit and the appearance of the complex on the cell surface has been used to suggest that the $\beta 2$ subunit is important for assembly and/or insertion and that this posttranslational association is a regulated and rate limiting step in the formation of functional Na channels at the cell surface during development. In the studies presented here, the large increase in the number of [^{3}H]STX binding sites in the PKA-deficient cells suggests that large numbers of α subunits are being expressed in the surface membrane. In addition, although coinjection of small molecular weight mRNAs may increase the level of Na channel α subunit expression in *Xenopus* oocytes (Auld et al., 1988), the α subunit by itself can form functional channels (Noda et al., 1986*b*; Auld et al., 1988; Suzuki et al., 1988). As a result, it does not seem likely that the results in the PKA-deficient PC12 cells were due to alterations in subunit assembly that prevented Na channel expression to occur. Another possibility is that PKA is necessary for the phosphorylation of the α subunit of the Na channel protein. The α subunit of the Na channel is a substrate for PKA in vitro and in situ (Costa et al., 1982; Rossie and Catterall, 1987). In all of the α subunits cloned from mammalian brain, there is an intracellular segment of the channel protein that contains a concentration of putative PKA phosphorylation sites (Rossie et al., 1987; for discussion see Trimmer and Agnew, 1989). In preliminary experiments where mRNA from a cloned α subunit gene is expressed in *Xenopus* oocytes, measures that presumably decrease the PKA-mediated phosphorylation of the expressed Na channel protein, including the injection of phosphatases or the mutation of putative PKA phosphorylation sites of the protein, dramatically decrease the amplitude of the Na currents (Smith, R. D., and A. L. Goldin. 1991. *Neurosci Abstr.* 17:953). An effect of PKA at this late point in the synthesis and expression of Na channels might explain the apparent lack of function of the channels induced by NGF and bFGF in the AB-11 and 123.7 cell lines. If a limited degree of channel phosphorylation can result in some functional activity, then the basal PKA activity in the A126-1B2 cells may be sufficient to explain the presence of functional Na channels in this cell line.

In summary, while an initial analysis of events occurring earlier in the response to NGF suggested PKA activity did not play a central role in NGF mediated neuronal differentiation (Ginty et al., 1991*a*), the results presented here demonstrate that at least one critical component of that process, the functional expression of voltage-dependent Na channels, requires the activity of PKA. These experiments support the idea that it is the combination of a variety of signalling pathways that underlies neuronal differentiation. In particular, these experiments suggest that the activity of PKA can modify the expression of the voltage-dependent Na channel, and thereby influence electrical excitability. More provocatively, the results suggest that compartmentalized differences in the level of PKA activity could lead to local modifications of electrical excitability in discrete domains of the cell membrane.

We thank Leslie Henderson and Paul Gardner for helpful discussions during this project and Dottie Philips for expert technical assistance.

This research was supported by National Institutes of Health grants CA40929 (to J. A. Wagner), NS08764 (NRSA to D. D. Ginty), and NS28767 (to R. A. Maue), as well as grants from the American Cancer Society (IRG-157F), the Hitchcock Foundation, and the Alfred P. Sloan Foundation (to R. A. Maue).

Received for publication 13 November 1991.

References

Auld, V. G., A. L. Goldin, D. S. Krafte, J. Marshall, J. M. Dunn, W. A. Catterall, H. A. Lester, N. Davidson, and R. J. Dunn. 1988. A rat brain Na channel alpha subunit with novel gating properties. *Neuron.* 1:449–461.

Barchi, R. L. 1988. Probing the molecular structure of the voltage-dependent sodium channel. *Annu. Rev. Neurosci.* 11:455–495.

Beckh, S., M. Noda, H. Lubbert, and S. Numa. 1989. Differential regulation of three messenger RNAs in the rat central nervous system during development. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3611–3616.

Bothwell, M. 1991. Tissue localization of nerve growth factor and nerve growth factor receptor. *Curr. Top. Micro. Immunol.* 165:55–70.

Brady, M. J., A. C. Nairn, J. A. Wagner, and H. C. Palfrey. 1990. Nerve growth factor-induced down regulation of calmodulin-dependent protein kinase III in PC12 cells involves cyclic AMP-dependent protein kinase. *J. Neurochem.* 54:1034–1039.

Catterall, W., and S. C. Morrow. 1978. Binding of saxitoxin to electrically excitable neuroblastoma cells. *Proc. Natl. Acad. Sci. USA.* 75:1782–1786.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acids from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Costa, M. R. C., J. E. Casnellie, and W. A. Catterall. 1982. Selective phosphorylation of the α subunit of the sodium channel by cAMP-dependent protein kinase. *J. Biol. Chem.* 257:7918–7921.

Damon, D., P. A. D'Amore, and J. A. Wagner. 1990. Nerve growth factor and fibroblast growth factor regulate neurite outgrowth and gene expression in PC12 cells via both protein kinase C- and cAMP-independent mechanisms. *J. Cell Biol.* 110:1333–1339.

Dichter, M. A., A. S. Tischler, and L. A. Greene. 1977. Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line Nature (Lond.). 268:501–504.

Freund, J. E. 1973. Modern Elementary Statistics. Prentice Hall, Inc. Englewood Cliffs, New Jersey. 563 pp.

Fujita, K., P. Lazarovici, and G. Guroff. 1989. Regulation of the differentiation of PC12 Pheochromocytoma cells. *Environ. Health Perspectives.* 80:127–142.

Gage, F. H., M. H. Tuszynski, K. S. Chen, A. M. Fagan, and G. A. Higgins. 1991. Nerve growth factor function in the central nervous system. *Curr. Top. Micro. Immunol.* 165:71–93.

Garber, S. S., T. Hoshi, and R. W. Aldrich. 1989. Regulation of ionic currents in pheochromocytoma cells by nerve growth factor and dexamethasone. *J. Neurosci.* 9:3976–3987.

Ginty, D. D., D. Glowacka, D. S. Bader, H. Hidaka, and J. A. Wagner. 1991*a*. Induction of immediate early genes by Ca^{++} influx requires cAMP-dependent protein kinase in PC12 cells. *J. Biol. Chem.* 266:17454–17458.

Ginty, D. D., D. Glowacka, C. DeFranco, and J. A. Wagner. 1991*b*. Nerve growth factor-induced neuronal differentiation after dominant repression of both type I and type II cAMP-dependent protein kinase activities. *J. Biol. Chem.* 266:15325–15333.

Gordon, D., D. Merrick, V. G. Auld, R. Dunn, A. L. Goldin, N. Davidson, and W. A. Catterall. 1987. Tissue-specific expression of the R_I and R_{II} sodium channel subtypes. *Proc. Natl. Acad. Sci. USA.* 84:8682–8686.

Gospodarowicz, D., G. Neufeld, and L. Schweigerer. 1986. Fibroblast growth factor. *Mol. Cell. Endocrinol.* 46:187–204.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.

Halegoua, S., R. C. Armstrong, and N. E. Kremer. 1991. Dissecting the mode of action of a neuronal growth factor. *Curr. Top Micro. Immunol.* 165:95–118.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.

Hartshorne, R. P., and W. A. Catterall. 1984. The sodium channel from rat brain. Purification and subunit composition. *J. Biol. Chem.* 259:1667–1675.

Hartshorne, R. P., D. J. Messner, J. C. Coppersmith, W. A. Catterall. 1982. The saxitoxin receptor of the sodium channel from rat brain. Evidence for two nonidentical β subunits. *J. Biol. Chem.* 257:13888–13891.

Hefti, F., J. Hartikka, and B. Knusel. 1989. Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. *Neurobiol. Aging.* 10:515–533.

Hodgkin, A. L., and A. F. Huxley. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.).* 117:500–544.

Ifune, C. K., and J. H. Steinbach. 1990. Regulation of sodium currents and acetylcholine responses in PC12 cells. *Brain Res.* 506:243–248.

Kalman, D., B. Wong, A. E. Horvai, M. J. Cline, and P. H. O'Lague. 1990. Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. *Neuron.* 2:355–366.

Kayano, T., M. Noda, V. Flockerzi, H. Takehashi, and S. Numa. 1988. Primary structure of rat brain sodium channel III deduced from the cDNA sequence *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 228:187–194.

Levi-Montalcini, R. 1987. The nerve growth factor: thirty-five years later. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1145–1154.

Logan, A., M. Berry, G. H. Thomas, N. A. Gregson, and S. D. Logan. 1985. Identification and partial purification of fibroblast growth factor from brains of developing rats and leucodystrophic mutant mice. *Neuroscience.* 15:1239–1246.

Machida, C. M., K. D. Rodland, L. Matrisian, B. E. Magun, and G. Ciment. 1989. NGF induction of the gene encoding the protease transin accompanies neuronal differentiation. *Neuron.* 2:1587–1596.

Machida, C. M., J. D. Scott, and G. Ciment. 1991. NGF-induction of the metalloproteinase transin/stromelysin in PC12 cells: involvement of multiple protein kinases. *J. Cell Biol.* 114:1037–1048.

Mandel, G., S. S. Cooperman, R. A. Maue, R. H. Goodman, and P. Brehm. 1988. Selective induction of brain type II Na channels by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 85:924–928.

Maue, R. A., S. D. Kraner, R. H. Goodman, and G. Mandel. 1990. Neuron-specific expression of the rat brain type II sodium channel gene is directed by upstream regulatory elements. *Neuron.* 4:223–231.

Mobley, W. C., A. Schenker, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry.* 15:5543–5551.

Noda, M., T. Ikeda, T. Kayano, H. Suzuki, H. Takeshima, M. Kurasaki, T. Takahashi, and S. Numa. 1986*a*. Existence of distinct sodium channel messenger RNAs in rat brain. *Nature (Lond.).* 320:188–192.

Noda, M., T. Ikeda, H. Suzuki, H. Takeshima, T. Takahashi, M. Kuno, and S. Numa. 1986*b*. Expression of functional sodium channels from cloned cDNA. *Nature (Lond.).* 322:826–828.

Pollock, J. D., M. Krempin, and B. Rudy. 1990. Differential effects of NGF, FGF, EGF, cAMP, and dexamethasone on neurite outgrowth and sodium channel expression in PC12 cells. *J. Neurosci.* 10:2626–2637.

Ross, A. F., W. N. Green, D. S. Hartman, and T. Claudio. 1991. Efficiency of acetylcholine receptor subunit assembly and its regulation by cAMP. *J. Cell Biol.* 113:623–636.

Rossie, S., and W. A. Catterall. 1987. Cyclic AMP-dependent phosphorylation of voltage-sensitive sodium channels in primary cultures of rat brain neurons. *J. Biol. Chem.* 262:12735–12744.

Rossie, S., D. Gordon, and W. A. Catterall. 1987. Identification of an intracellular domain of the sodium channel having multiple cAMP-dependent phosphorylation sites. *J. Biol. Chem.* 262:17530–17535.

Rudy, B., B. Kirschenbaum, and L. A. Greene. 1982. Nerve growth factor-induced increase in saxitoxin binding to rat PC12 pheochromocytoma cells. *J. Neurosci.* 2:1405–1411.

Scheinman, R. I., V. J. Auld, A. L. Goldin, N. Davidson, R. J. Dunn, and W. J. Catterall. 1989. Developmental regulation of sodium channel expression in the rat forebrain. *J. Biol. Chem.* 264:10660–10666.

Schmidt, J. W., and W. A. Catterall. 1986. Biosynthesis and processing of the α subunit of the voltage-sensitive sodium channel in rat brain neurons. *Cell.* 46:437–445.

Schmidt, J. W., S. Rossie, and W. A. Catterall. 1985. A large intracellular pool of inactive sodium channel α subunits in developing rat brain. *Proc. Natl. Acad. Sci. USA.* 82:4847–4851.

Stephan, M., and W. S. Agnew. 1991. Voltage-sensitive Na^+ channels: motifs, modes, and modulation. *Current Opin. Cell Biol.* 3:676–684.

Stewart, S. S., and S. H. Appel. 1989. Trophic factors in neurologic disease. *Annu. Rev. Med.* 39:193–201.

Suzuki, H., S. Beckh, H. Kubo, N. Yahagi, H. Ishida, T. Kayano, M. Noda, and S. Numa. 1988. Functional expression of cloned cDNA encoding sodium channel III. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 228:195–200.

Thoenen, H., and Y. A. Barde. 1980. Physiology of nerve growth factor. *Physiol. Rev.* 60:1284–1334.

Trimmer, J. S., and W. S. Agnew. 1989. Molecular diversity of voltage-sensitive Na channels. *Annu. Rev. Physiol.* 51:401–418.

Van Buskirk, R., T. Corcoran, and J. A. Wagner. 1985. Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ornithine decarboxylase in response to nerve growth factor but not adenosine agonists. *Mol. Cell. Biol.* 5:1984–1992.

Wagner, J. A. 1991. The FGFs as neuronotrophic factors. *Curr. Top. Micro. Immunol.* 165:5–118.

Wagner, J. A., and S. K. Kostyk. 1991. Regulation of neural survival and differentiation by peptide growth factors. *Curr. Opin. in Cell Biol.* 2:1050–1057.

Wollner, D. A., R. Scheinman, and W. A. Catterall. 1988. Sodium channel expression and assembly during development of retinal ganglion cells. *Neuron.* 1:727–737.

Woodford, T. A., L. A. Corell, G. S. McKnight, and J. D. Corbin. 1989. Expression and characterization of mutant forms of the Type I regulatory subunit of cAMP-dependent protein kinase. *J. Biol. Chem.* 264:13321–13328.