Nerve growth factor and basic fibroblast growth factor bind to and activate receptor tyrosine kinases, causing sequential signaling via the p21ras/extracellular signal-regulated kinase pathway. The necessity and sufficiency of this signaling pathway in transducing neuronal differentiation have been tested in the PC12 cell model. Although necessary for morphological changes, the sufficiency of p21ras-mediated signaling in these events has come into question. We report that growth factor induction of voltage-gated calcium channels, a hallmark of physiological differentiation, also requires p21ras-mediated signaling, but cannot be driven by p21ras activation alone. Thus, constitutive expression of the dominant negative N17ras mutant blocks growth factor-induced increases in ω-conotoxin GVIA-sensitive, nimodipine-sensitive, and ω-conotoxin GVIA/nimodipine-resistant calcium currents, but it does not block sodium current induction. However, manipulations that produce sustained activation of the p21ras signaling pathway and the neurite extension characteristic of morphological differentiation fail to increase calcium channel current densities. These results indicate the existence of distinct signaling requirements for morphological and physiological differentiation and further emphasize the importance of p21ras-independent signaling pathways in growth factor-mediated neuronal development.

Key features of the developing neuronal phenotype include process extension and ion channel expression, whereby cells establish the synaptic contacts and the electrophysiological repertoire necessary for intercellular signaling. Coordination of morphological and physiological development essential to the proper wiring of the nervous system is controlled in part by growth factors such as NGF and bFGF. An excellent model system for studying NGF- and bFGF-induced neuronal differentiation is the well characterized PC12 pheochromocytoma cell line. In response to NGF or bFGF, PC12 cells carry out a developmental program representative of a maturing neuron, in which they cease cell division, extend neurites, and become electrically excitable (1–4). The acquisition of electrical excitability is due to an increase in the number of voltage-gated sodium and calcium channels (5–13). Although the bFGF- and NGF-activated signaling pathways responsible for morphological differentiation are beginning to be understood, little is known about the growth factor-activated pathways that govern physiological differentiation. Indeed, a key question remains as to whether or not the pathways identified with morphological changes are necessary and sufficient for physiological differentiation, particularly ion channel expression.

The signal cascade responsible for growth factor-induced morphological differentiation in PC12 cells is the same ubiquitous pathway activated by a number of mitogenic and differentiating growth factors. Thus, NGF and bFGF bind to and activate receptor tyrosine kinases, which in turn cause the sequential activation of the membrane-associated GTP-binding protein p21ras, followed by B-Raf kinase, Mek-1 kinase, and the extracellular signal-regulated kinases ERK1 and ERK2 (14–21). In PC12 cells, sustained activation of the p21ras/ERK pathway by NGF and bFGF is suggested to be necessary for cessation of cell division and commitment to morphological differentiation (21–24); however, the sufficiency of this pathway in driving morphological differentiation is now in question (25). In contrast, EGF stimulation of proliferation, rather than differentiation, is thought to result from more transient activation of the same p21ras/ERK pathway by the EGF receptor tyrosine kinase (22, 26, 27). Only when the EGF receptor is overexpressed in PC12 cells does EGF stimulation result in morphological differentiation that is accompanied by the sustained activation of the p21ras/ERK pathway (23). Even though p21ras/ERK stimulation is required in both proliferative and differentiating scenarios, it seems inadequate to explain all aspects of the mature neuronal phenotype. For instance, NGF- and bFGF-induced expression of sodium channels, essential for action potential initiation, is independent of p21ras (28, 29). And although NGF- and bFGF-induced calcium channel expression shifts PC12 cells to a more ω-CotX-sensitive (i.e. mature neuron-like) secretory phenotype (12, 30), the necessity or sufficiency of p21ras/ERK in this process remains unresolved.

Growth factor-induced PC12 differentiation is an applicable model for synaptic maturation in the developing nervous system, and therefore, it is important to specify the signals responsible for driving both the morphological and physiological aspects of this process. To understand the coordination and divergence of these processes, we are examining the relationships between the growth factor-activated pathways governing neurite outgrowth as well as expression of calcium and sodium channels. We report here that NGF or bFGF induction of calcium channels, particularly those sensitive to ω-CotX, requires p21ras as a signal transducer. However, activation of p21ras (and by implication, ERKs) alone is not sufficient to explain growth factor-induced functional expression of calcium channels. Thus, in the context of neuronal differentiation, there appears to be a continuum of signaling requirements for...
growth factor-induced morphological and physiological responses. On one hand, p21ras/ERK signaling is required and may or may not be sufficient for morphological differentiation, while sodium channel expression is independent of p21ras. Between these two different requirements for p21ras is calcium channel induction, which we show to be dependent on p21ras, but for which p21ras is not sufficient. Therefore, although activation of the p21ras/ERK pathway is critical to morphological neuronal differentiation, it is clear that other signals are required for complete development of the mature physiological phenotype.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells constitutively expressing the neomycin resistance gene (Z-1) or the neomycin resistance gene and the dominant negative N17ras mutant (17-2) were generous gifts of Dr. Geoff Cooper (Dana Farber Cancer Institute, Boston). v-Crk PC12 and GSRas1 cells were generous gifts of Dr. Raymond Birge (Rockefeller University, New York) and Dr. Simon H alegoua (State University of New York, Stony Brook, NY), respectively. PC12 cells were cultured as described previously (12). Briefly, cells were plated onto rat tail collagen-coated plastic Petri dishes and grown in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum, 10% heat-inactivated horse serum, 25 units/ml penicillin, 25 µg/ml streptomycin, and G418 (400 µg/ml) where needed. Cells were passaged weekly, and medium was replenished every other day. NGF (100 ng/ml), bFGF (40 ng/ml), or EGF (25 ng/ml) was applied for a period of 5–8 days to cells grown in Dulbecco’s modified Eagle’s medium with 1% horse serum. Fresh medium and growth factor were applied every other day. 1 µM dexamethasone in 0.01% dimethyl sulfoxide (final concentration) or 0.01% dimethyl sulfoxide vehicle alone was added to Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 10% heat-inactivated horse serum, 25 units/ml penicillin, and 25 µg/ml streptomycin.

Electrophysiology—Whole cell patch clamp recordings were obtained with either an EPC-7 (List Electronics, Greeneville, NY) or an Axopatch 200A (Axon Instruments, Inc., Foster City, CA) patch clamp amplifier, and patch pipettes pulled from Corning 7740 glass (Corning Inc., Corning, NY; resistances of 1–4 MΩ). Current signals from the patch clamp amplifier were filtered at 5 kHz (8-pole Bessel at −3 db) and saved directly to a disk. All current records were leak-subtracted with a standard P/N leak subtraction procedure. Data acquisition and analysis were done with Macintosh Centris 650 computers running Pulse and PulseFit software (Instrutech Corp., Great Neck, NY). To record voltage-gated calcium channel currents, the bath solution consisted of 135 mM tetraethylammonium chloride, 4 mM KCl, 10 mM BaCl2, 1 mM MgCl2, 5 mM glucose, 10 mM HEPES, and 1 MµM tetrodotoxin, pH 7.2. To record sodium currents, the bath solution consisted of 138 mM NaCl, 9 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM tetraethylammonium chloride, 200 µM CdCl2, and 10 mM HEPES, pH 7.2. The patch pipette solution contained 150 mM CsCl, 2 mM MgATP, 0.5 mM GTP, 2 mM BaATP, and 10 mM HEPES, pH 7.2. Whole cell capacitative transients, elicited by 20-mV depolarizing steps, were compensated with the analog compensation circuitry of the patch clamp amplifier. The whole cell capacitance, a measure of membrane area, was read directly from the compensation control dial of the amplifier. Whole cell current amplitudes were normalized to this indirect measure of membrane area and are expressed as picoamperes/picofarad.

Nimodipine (Research Biochemicals Inc., Natick, MA) was prepared fresh daily as a 5 mM stock solution in ethanol and diluted in bath solution immediately prior to use. α-CaTX (Bachem Biosciences, Philadelphia) was prepared as a 0.5 mM stock solution in distilled water, and aliquots were stored at −30 °C. A fresh aliquot was diluted in the bath solution for each experiment. Drugs were pressure-applied from blunt-tipped, fire-polished micropipettes positioned 5–10 mm from the cell.

RESULTS

Expression of N17ras Inhibits Increased Functional Expression of Calcium Channels Induced by Neurophins—To determine whether or not p21ras is necessary for induction of calcium channels by growth factors, we have looked at the effects of NGF and bFGF on calcium channel current density in a PC12 cell line (17-2) coexpressing the dominant negative p21ras mutant, N17ras, along with the neomycin resistance marker. We first determined that untreated 17-2 cells, as well as the PC12 cell line (Z-1) expressing only the neomycin resistance marker, express calcium channel currents identical to those in untreated wild-type cells. This was done by examining the biophysical and pharmacological properties of both the composite whole cell current and its component currents. The current-voltage relationships show that for cells held at −90 mV, the voltage threshold for activation of the composite calcium channel current is approximately −25 mV, maximal current is observed at 10–20 mV, and the apparent reversal po-
Wild-type, Z-1, and 17-2 PC12 cells similarly express at least three pharmacologically distinct calcium channel currents. Pharmacologically distinct calcium channel currents were distinguished by sequential application of 5 μM ω-CoTX and 5 μM nimodipine. The remaining current was defined as ω-CoTX/nimodipine-resistant current. To facilitate comparison of records, all currents are scaled to similar size (note scale bar). Single exponential fits to current decays (Simplex optimization algorithm with user defined start parameters) were used to obtain inactivation time constants (τ). Growth factor treatment did not affect current kinetics in any of these three cell lines, although there were increases in the relative densities of all current components in wild-type and Z-1 cells (Fig. 3). Recording procedures were identical to those described for Fig. 1, except that current records were refiltered at 2 kHz for display.

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To kinetically describe individual calcium channel current components, we have applied Ω-CoTX and then digitally subtracted the remainder current from the initial current to obtain "pure" Ω-CoTX-resistant and Ω-CoTX-sensitive component currents. Inactivation time constants were then determined for each component (Fig. 2). In wild-type cells, inactivation kinetics of the Ω-CoTX-sensitive and Ω-CoTX-resistant currents observed for untreated cells (mean $\tau$ = 122 and 913 ms, $n$ = four cells) are essentially unchanged by growth factor treatment ($\tau$ = 153 and 950 ms, $n$ = four cells), suggesting that there is an increase in channel numbers, but no change in channel types (12). This result was confirmed for Z-1 and 17-2 cells, and among all three lines, inactivation time constants for these two current types are comparable both with and without growth factor treatment. The inactivation time constants in untreated Z-1 and 17-2 cells for Ω-CoTX-sensitive ($\tau$ = 125 and 135 ms, $n$ = three to eight cells throughout) and Ω-CoTX-resistant ($\tau$ = 890 and 879 ms) currents are unaffected by either NGF ($\tau$ = 150 and 167 ms for Z-1 and 17-2 Ω-CoTX-sensitive currents; $\tau$ = 914 and 811 ms for Ω-CoTX-resistant currents) or bFGF ($\tau$ = 156 and 139 ms and $\tau$ = 799 and 907 ms). Thus, each cell type under all treatment conditions has similar current components, and in each, the Ω-CoTX-sensitive component inactivates much faster than the Ω-CoTX-insensitive components. In this regard, our results are consistent with other studies on wild-type PC12 cells (6, 10, 11, 32). However, unlike Garber et al. (6), and in agreement with others (10, 11, 32), we did not observe the presence of a low threshold or "T"-type calcium channel current under any condition.

Expression of N17ras Does Not Inhibit Sodium Channel Induction by Neurotrophins—We show above that in untreated PC12 cells, constitutive N17ras expression has no effect on the type or amount of calcium channels expressed. However, it is possible that N17ras may inhibit growth factor induction of calcium channels (and neurite outgrowth) by down-regulating NGF and bFGF receptors. If this is the case, then there should also be inhibition of other key features of bFGF- and NGF-induced differentiation, such as expression of Type I and II sodium channels (5, 7, 8, 13). To test this possibility, we assayed sodium channel current density in 17-2 (and Z-1) cells after 5–8 days of treatment with NGF or bFGF. In agreement with Fanger et al. (29), we found that constitutive N17ras expression does not inhibit growth factor-induced increases in sodium channel expression (Fig. 4). Although the absolute amount of sodium current induction differs between Z-1 and 17-2 cells, the 5-fold relative increase in response to NGF is the same for both lines and is comparable to the increase that we observed for wild-type PC12 cells (increase in mean current density from 3.1 ± 0.1 pA/pF ($n$ = 25) to 26.1 ± 2.7 pA/pF ($n$ = 22)). These results indicate that in cells expressing N17ras, NGF and bFGF receptors are present and competent to drive p21ras-independent processes such as sodium channel expression. Also, these results support the interpretation that the failure to observe calcium channel induction in these cells is not due to a deficit in growth factor receptor function, but is instead due to inhibition of a specific p21ras-dependent signaling pathway downstream of the receptors.

Constitutive Expression of Activated p21ras or Hyperstimulation of Endogenous p21ras Is Not Sufficient to Induce Calcium Channel Functional Expression—If activation of p21ras and its downstream signaling effectors alone is sufficient to mediate growth factor induction of calcium channels, then it should be possible to up-regulate calcium channels by activating p21ras in the absence of either NGF or bFGF receptor stimulation. We took two approaches to activating p21ras to assess its sufi-
of p21ras, under the control of the inducible murine mammary tumor virus promoter (GSRas1) (28), were treated with 1 μM dexamethasone for 3 days. Whole cell calcium channel current densities were then recorded from those cells with neurites longer than two-cell diameters, i.e., cells that showed clear morphological differentiation, and these densities were compared with those from cells that were chronically treated with 0.01% dimethyl sulfoxide vehicle alone. Expression of exogenous ras transcript occurs within hours after start of dexamethasone treatment (28), and robust neurite outgrowth is observed within the first day of treatment. However, the densities of the α,β,γ, and δ subunits, and α8β,γ, and δ subunits, were not significantly affected by growth in 1 μM dexamethasone (6.4 ± 0.8 pA/pF, n = 15; 3.9 ± 0.7 pA/pF, n = 5; and 3.6 ± 0.9 pA/pF, n = 5, respectively). This is reflected by the lack of change in total calcium current density after dexamethasone treatment (Fig. 5). In wild-type PC12 cells, NGF application activates endogenous p21ras within minutes (27). In one experiment with wild-type PC12 cells, we found that after 24 h of NGF treatment, mean total calcium current density was unchanged (19.2 ± 3.4 pA/pF (n = 3) compared with 17.3 pA/pF (n = 9)), but after 48 h of treatment, current density had increased to 36.2 ± 7.5 pA/pF (n = 7) (in cells with at least one neurite of length greater than one-cell diameter). Although this change in current density is still statistically insignificant, it suggests that growth factor induction of calcium channels is well underway within 48 h. Thus, it seems reasonable to conclude that had induction of activated p21ras been sufficient to induce calcium channels, then increased current density would have been observed at 3 days after start of dexamethasone treatment, especially as p21ras is overexpressed when driven by the murine rat progesterone receptor (GSRas1) (28), and robust neurite outgrowth is expected from the untreated condition assessed by Student’s t test at p < 0.05.

**FIG. 4.** Increases in sodium channel current densities due to chronic growth factor treatment of PC12 cells are not inhibited by the dominant negative N17 ras mutant. Cells were grown with NGF or bFGF) or without (untreated) growth factors for 5–8 days. At the top are shown individual sample records for whole cell sodium channel currents in PC12 cells constitutively expressing either the neomycin resistance marker (Z-1) or the neomycin resistance marker and the dominant negative N17 ras mutant (17-2). Cells were held at −90 mV, and command steps to the indicated voltages were given sequentially at 1.5-s intervals. Current records were filtered at 5 kHz and leak-subtracted with a standard P/N procedure. Cumulative data for whole cell currents normalized to cell membrane area are shown in the column graph at the bottom. Each column represents the mean ± S.E. of current densities taken from 10 to 15 cells. *, significant difference from the untreated condition assessed by Student’s t test at p < 0.05.

**FIG. 5.** Induced expression of activated p21ras (GSRas1 + dexamethasone) or sustained stimulation of endogenous p21ras (v-Crk + EGF) fails to increase calcium channel current densities in PC12 cells. Cumulative data are shown for calcium channel current densities in two different PC12 cell lines. One expresses activated p21ras under the control of the murine mammary tumor virus promoter (GSRas1), and the other expresses v-Crk, an oncogenic adaptor protein that allows sustained p21ras/ERK activation and morphological differentiation in response to EGF receptor stimulation. A, 3-day application of 1 μM dexamethasone (Dex) to GSRas1 cells (recordings from 29 cells) produced sustained activation of the p21ras/ERK signaling pathway (28), but failed to increase calcium channel current density relative to 0.01% dimethyl sulfoxide-treated vehicle controls (n = 38 cells). Exposure of these cells to NGF (100 ng/ml for 6 days) and dexamethasone produced normal increases in calcium channel current density (n = 8 cells), indicating that dexamethasone itself does not suppress calcium channel induction. B, 5–7-day application of EGF (25 ng/ml) to v-Crk cells also did not significantly increase calcium current density (n = 18 cells) relative to water-treated vehicle controls (n = 11 cells). However, these cells responded to treatment with NGF (100 ng/ml for 6 days) with significant increases in calcium channel current densities (n = five cells). Whole cell calcium channel current recordings and normalization to whole cell capacitance were performed as described for Figs. 1 and 2. Each column represents the mean ± S.E. of current densities. Activation of p21ras by dexamethasone (A) or EGF (B) produced no significant differences in calcium current densities (Student’s t test at p < 0.05).
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mammary tumor virus promoter. The failure of p21ras induction to up-regulate calcium channel density in GSrar1 cells could not be attributed to some suppressive action of the dexamethasone treatment itself since in GSrar1 cells treated for 6 days with NGF and dexamethasone, calcium channel density increased to a level (72.5 ± 8.0 pA/pF, n = 8) comparable to that observed with NGF treatment alone.

We used a second approach to further rule out the possibility that dexamethasone suppressed the induction of calcium channels, as has been observed for some other NGF-inducible events (33), and to see if p21ras stimulation alone was sufficient to induce calcium channel up-regulation. Application of EGF to PC12 cells stably transfected with v-Crk, an oncogenic adaptor protein similar in function to the p21ras guanine nucleotide exchange factor-docking protein Grb2, results in sustained activation of endogenous p21ras and the downstream mitogen-activated protein kinase pathway (34–36). As a result, in cells expressing v-Crk, EGF induces morphological differentiation. PC12 cells expressing v-Crk were grown in the presence of EGF for 5–7 days, sufficient time to produce robust neurite outgrowth. In cells with at least one neurite greater than two-cell diameters in length, there was a small but statistically insignificant increase in calcium channel current density compared with v-Crk-expressing cells grown in the absence of EGF (Fig. 5). As a positive control, these cells were treated with NGF (for 6 days), and they responded with a significant increase in calcium channel current density (64.0 ± 20 pA/pF, n = 5). Therefore, this experiment and the experiment with expression of activated p21ras suggest that p21ras signaling alone, even if it is sustained, is insufficient to increase calcium channel current density in PC12 cells.

DISCUSSION

Our results show that NGF or bFGF induction of a 3-fold increase in total calcium channel current density in PC12 cells is dependent on signaling via p21ras. Growth factor treatment induces p21ras-dependent differential increases in each of three pharmacologically distinct calcium current components. There is a 5-fold increase in the Ω-CoTX-sensitive current, a 1.5-fold increase in the DHP-sensitive current, and a 4-5-fold increase in the Ω-CoTX/DHP-resistant current, yet all of these increases are blocked in two PC12 cell lines expressing dominant negative N17ras (17-2 and 17-26). Constitutive expression of N17ras does not appear to affect the types or properties of calcium channels expressed in PC12 cells either in the absence or presence of NGF or bFGF treatment. Also, in agreement with Fanger et al. (29), that increases in sodium channel density are unaffected in N17ras-expressing cells suggest that the dominant negative mutant block of calcium channel induction is specific, i.e., other signaling events driven by NGF and bFGF receptors remain intact in cells expressing dominant negative p21ras. It could be argued that functional expression of calcium channels is dependent on their insertion into actively growing membrane and that N17ras inhibition of calcium channels is secondary to its inhibition of neurite outgrowth. However, this is unlikely since NGF induction of calcium channels is unaffected by growing cells in suspension, a condition that prevents neurite extension (11).

Although we have shown that p21ras is necessary for induction of calcium channels by growth factors, it appears that p21ras signaling alone, even if it is sustained, is not sufficient to mediate this action. Induction of oncogenic p21ras with dexamethasone and EGF stimulation of PC12 cells expressing the oncogenic adaptor protein v-Crk both produce sustained activation of the p21ras/ERK pathway as well as neurite extension (28, 36). However, we do not observe calcium channel induction in either case. These results and those described above suggest that the functional expression of calcium channels in response to NGF and bFGF requires activation of the p21ras signaling pathway, but that other p21ras-independent signaling events are also necessary.

The identity of the p21ras-independent pathways required for calcium channel induction may lie in those p21ras-independent pathways implicated in morphological differentiation of PC12 cells. Trk receptor activation and subsequent autophosphorylation on critical tyrosines is required for the association and activation of phospholipase C-γ, phosphatidylinositol 3-kinase, and SHC (37–39), and phospholipase C-γ and SHC appear to be necessary for growth factor-induced morphological differentiation, while phosphatidylinositol 3-kinase is not (38, 39). In addition, non-receptor tyrosine kinases such as c-Src, c-Yes, and Fyn may be activated by growth factors that promote neuronal differentiation (25, 40). Infection of PC12 cells with v-src seems to be sufficient for induction of calcium channels (41), but not sodium channels (28), but this does not preclude the necessity of Src family members for sodium channel induction. Subsequent studies using mutant Trk receptors (39), mutant platelet-derived growth factor receptors expressed in PC12 cells (25), and antisense application (42) may elucidate whether or not phospholipase C-γ, SHC, phosphatidylinositol 3-kinase, or one of the members of the Src non-receptor tyrosine kinase family mediates calcium and sodium channel induction.

p21ras/ERK-mediated signaling appears to be necessary for growth factor-induced morphological differentiation, but different experimental paradigms have produced conflicting results regarding the sufficiency of p21ras/ERK signaling for neurite outgrowth (24, 25). Similarly, we have shown that p21ras signaling alone is insufficient to produce an important component of physiological differentiation, functional expression of a sympathetic neuron-like calcium channel phenotype. Thus, the observations that process extension, calcium channel induction, and sodium channel induction (43) depend to varying degrees on p21ras lend credence to the idea that distinct signal pathways mediate different aspects of neuronal differentiation. Furthermore, it suggests that sustained activation of the p21ras/ERK pathway may not explain all aspects of neuronal differentiation produced by NGF or bFGF. The ability of cells to independently regulate sodium channel induction, calcium channel induction, and neurite outgrowth suggests that process extension can occur in the absence of events dependent on electrical activity. Additional molecules in the extracellular matrix, such as L1 and neuronal cell adhesion molecule, may activate the outgrowth of axons (44), which, upon reaching their target neurons, are exposed to cytokines and growth factors that cause increased expression of sodium and calcium channels. The magnitude and timing of these events could then be the critical determinants of synaptic efficacy and ultimately survival.

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