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Analysis of Mutant Platelet-Derived Growth Factor Receptors Expressed in PC12 Cells Identifies Signals Governing Sodium Channel Induction during Neuronal Differentiation

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The mechanisms governing neuronal differentiation, including the signals underlying the induction of voltage-dependent sodium (Na^+) channel expression by neurotrophic factors, which occurs independent of Ras activity, are not well understood. Therefore, Na^+ channel induction was analyzed in sublines of PC12 cells stably expressing platelet-derived growth factor (PDGF) β receptors with mutations that eliminate activation of specific signaling molecules. Mutations eliminating activation of phosphatidylinositol 3-kinase (PI3K), phospholipase C_γ (PLC_γ), the GTPase-activating protein (GAP), and Syp phosphatase failed to diminish the induction of type II Na^+ channel α-subunit mRNA and functional Na^+ channel expression by PDGF, as determined by RNase protection assays and whole-cell patch clamp recording. However, mutation of juxtamembrane tyrosines that bind members of the Src family of kinases upon receptor activation inhibited the induction of functional Na^+ channels while leaving the induction of type II α-subunit mRNA intact. Mutation of juxtamembrane tyrosines in combination with mutations eliminating activation of PI3K, PLC_γ, GAP, and Syp abolished the induction of type II α-subunit mRNA, suggesting that at least partially redundant signaling mechanisms mediate this induction. The differential effects of the receptor mutations on Na^+ channel expression did not reflect global changes in receptor signaling capabilities, as in all of the mutant receptors analyzed, the induction of c-*fos* and transin mRNAs still occurred. The results reveal an important role for the Src family in the induction of Na^+ channel expression and highlight the multiplicity and combinatorial nature of the signaling mechanisms governing neuronal differentiation.

Efforts to understand the mechanisms controlling the development and maintenance of the nervous system have identified a number of growth factors with profound effects on neurons. These factors include the neurotrophin family of growth factors, which promote the survival and differentiation of distinct yet overlapping populations of neurons (for reviews, see references 42, 52, and 70). Of the neurotrophins, nerve growth factor (NGF) is the most extensively characterized and has served as the prototype for understanding their actions (for a review, see reference 45). However, other factors, including epidermal growth factor and fibroblast growth factor, also appear to play important roles in the nervous system (22, 82, 84; for reviews, see references 29 and 74). In addition, platelet-derived growth factor (PDGF) is expressed in the nervous system and can enhance neuronal survival, neurite outgrowth, and differentiation (14, 55, 64, 68).

The effects of growth factors are often mediated through membrane-spanning receptors that exhibit intrinsic tyrosine kinase activity and undergo ligand-dependent autophosphorylation (for a review, see reference 86). The tyrosine-phosphorylated regions in the cytoplasmic domain of the receptor then serve as binding sites for the Src homology 2 (SH2) domains of numerous signaling proteins (for reviews, see references 36, 58, 59, and 78). For example, among the tyrosines of the TrkA receptor that become phosphorylated upon activation of the receptor by NGF, those at positions 785, 751, and 490 are necessary for association and activation of phospholipase C_γ (PLC_γ), phosphatidylinositol 3-kinase (PI3K), and Shc, respectively (47, 71). Likewise, the PDGF receptor appears to activate many of the same signaling proteins as TrkA (for a review, see reference 8). For example, the tyrosine phosphatase Syp associates with the tyrosine at position 1009 of the PDGF receptor (46), PLC_γ associates with the tyrosines at positions 1009 and 1021 (37, 63), PI3K associates with the tyrosines at positions 740 and 751 (3, 9, 38, 39), and the GTPase-activating protein (GAP) associates with the tyrosine at position 771 (35, 41, 53). Finally, phosphorylation of the tyrosine at position 716 enhances Grb2 binding and p21Ras activation *in vitro* (2), while PDGF receptor association with the Src family of tyrosine kinases, $pp60^{c\text{-}src}$ (Src), $p59^{fyn}$ (Fyn), and $pp62^{c\text{-}yes}$ (Yes), occurs at the tyrosines at positions 581 and 579 (44, 54).

Efforts to determine which tyrosine kinase receptor-mediated signals underlie neuronal differentiation have taken advantage of the well-characterized response of the rat pheochromocytoma (PC12) cell line to NGF. The numerous morphological and biochemical changes that occur include neurite outgrowth, the induction of immediate-early genes such as c-*fos*, and an increase in the expression of neuron-specific genes, including those encoding for the metalloproteinase transin or the pore-forming α subunit of the voltage-dependent sodium (Na^+) channel (for reviews, see references 25 and 26). Of the seven Na^+ channel α-subunit genes known to be expressed in the nervous system (1, 12, 21, 56, 65, 72), wild-type PC12 cells express the PN-1 and type II genes (12, 49). NGF causes a rapidly induced, transient increase in PN-1

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α-subunit mRNA (12, 75) as well as a more slowly developing, sustained induction of type II α-subunit mRNA (18). In contrast to the induction of neurite outgrowth and transin mRNA (11, 73), in PC12 cells the induction of Na^+ channel α-subunit mRNA and induction of Na^+ current density occur independent of Ras activity (11, 17) and as such represent a relatively novel class of NGF responses. Therefore, there is considerable interest in determining the mechanisms underlying Na^+ channel induction, both as a means of identifying pathways governing key aspects of neuronal differentiation and as a means of determining the molecular events involved in a biologically important yet poorly understood mode of NGF action.

Analysis of TrkA and PDGF receptors with mutations that selectively disrupt activation of specific signals has proven effective in identifying signals underlying various biological responses to NGF and PDGF (47, 71, 79, 80). For example, signals important for the NGF-mediated induction of peripherin mRNA and neurite outgrowth were identified when mutated TrkA receptors were expressed in mutant PC12 sublines (nnr cells) that lack detectable amounts of TrkA (47, 71). However, this approach has proven problematic for analysis of Na^+ channel induction, as many of the nnr sublines exhibit constitutively elevated levels of Na^+ channel α-subunit mRNA and Na^+ current density in the absence of NGF or TrkA expression (16). Furthermore, stable expression of the wild-type TrkA receptor in these cells has inconsistent effects on Na^+ channel expression (16).

In this study, problems associated with the nnr cells have been circumvented by adopting an alternative approach that takes advantage of our previous analyses of the PDGF receptor (18, 28, 79). From these studies, it was clear that mutational analysis of the PDGF receptor was advantageous: the receptor is not normally expressed in PC12 cells, yet when introduced, enables PDGF to induce many aspects of PC12 cell differentiation to the same extent as NGF, including persistent activation of mitogen-activated protein kinase (MAPK), cessation of cell division, induction of c-*fos* mRNA, neurite outgrowth, and induction of Na^+ channel expression (18, 28, 79). Furthermore, this approach allowed the endogenous response to NGF to be used as an indication of the capability of the cells to increase Na^+ channel expression and serve as an internal control when one is comparing the effectiveness of PDGF receptor mutants. Finally, while our previous studies indicated that induction of the type II α-subunit gene could account for the observed increase in Na^+ channel α-subunit mRNA and functional Na^+ channel expression at later times (18), they also indicated that the mechanisms regulating these two stages of expression could be distinct and separable (23). Therefore, PC12 cells stably expressing mutated PDGF receptors that lack the ability to activate specific signals were analyzed by using both whole-cell patch clamp recording and RNase protection assays in order to identify cellular events underlying the sustained induction of Na^+ channel mRNA and current density that occurs during neuronal differentiation of these cells. The results highlight the specific combinations of signaling events necessary for the growth factor-mediated induction of neuronal Na^+ channel expression.

MATERIALS AND METHODS

Cell culture. Cells were maintained in a humidified CO_2 environment in Dulbecco's modified Eagle's medium containing 100 U of penicillin (GIBCO Laboratories, Grand Island, N.Y.) per ml, 100 mg of streptomycin (GIBCO) per ml, 5% plasma-derived fetal bovine serum (Cocalico Biologicals, Reamstown, Pa.), and 5% heat-inactivated plasma-derived horse serum (Cocalico). Media for stably transfected cells also contained 250 μg of G418 (GIBCO) per ml. Cells were plated on 100-mm-diameter (RNA analysis) or 35-mm-diameter (electrophysiology) tissue culture dishes (Falcon Labware, Becton Dickinson, Lincoln Park, N.J.), and some were treated with either 100 ng of 7S NGF per ml or 30 ng of PDGF-BB (Upstate Biotechnology Inc., Lake Placid, N.Y.) per ml every 2 days, when the medium was changed. PC12 sublines stably expressing wild-type and mutant PDGF β receptors were established and initial signaling events were characterized in previous studies (79). Briefly, tyrosine residues in the cytoplasmic portion of the human PDGF β receptor were changed to phenylalanine by using commercially available *in vitro* mutagenesis systems (Promega Corp., Madison, Wis.; Amersham, Arlington Heights, Ill.). Wild-type and mutant receptor genes were cloned into a retroviral expression vector (pLXSN[2]) and transfected into the PA317 packaging cell line, and supernatants from PA317 cultures were used to introduce the receptor genes into PC12 cells by retrovirus-mediated infection. Individual neomycin-resistant PC12 cells were propagated as separate sublines. Sublines expressing similar levels of PDGF β receptors were identified by immunoblotting (79), and only those sublines were used for further study.

Analysis of PDGF receptor kinase activity. PC12 sublines expressing either wild-type or mutant PDGF receptors (79) were either untreated or treated with 30 ng of PDGF per ml for 10 min at 37°C, washed twice with cold phosphate-buffered saline, and harvested with 0.5 ml of lysis buffer (70 mM β-glycerophosphate [pH 7.2], 100 μM Na_3VO_4, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 5 μg of leupeptin per ml, 20 μg of aprotinin per ml, 1 mM dithiothreitol). After samples were centrifuged at 14,000 × *g* for 10 min to remove nuclei and cell debris, PDGF β receptors were immunoprecipitated from the lysates by using a mouse monoclonal antibody (PR7212) that recognizes an extracellular epitope (27). Immunoprecipitates from ~7 × 10^5 cells were subjected to a standard in vitro kinase assay {10 min of incubation at 30°C in 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0)–10 mM $MnCl_2$–20 μg of aprotinin per ml–[γ-^{32}P]ATP} which was modified to include 0.5 μg of a purified glutathione *S*-transferase (GST) fusion protein containing amino acids 550 to 850 of rat PLC_γ as previously described (81). Proteins were resolved by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis, and radiolabeled proteins were detected by autoradiography.

RNA isolation and analysis. Total cellular RNA was isolated by the method of Chirgwin et al. (7). RNase protection assays were performed essentially as described by Hod (33), with modifications as in our previous studies (17, 18). A probe specific for type II Na^+ channel α-subunit mRNA was generated by using [α-^{32}P]UTP (New England Nuclear, Boston, Mass.), a commercially available kit (Promega), and a type II Na^+ channel α-subunit cDNA template developed in a previous study (17). Samples of RNA (20 μg) from untreated or growth factor-treated cells were hybridized with 2.5 × 10^5 cpm of this probe for 14 to 16 h at 46°C. The samples were then digested with 24 μg of RNase A per ml and 160 U of RNase T_1 (Ambion, Austin, Tex.) for 1 h at 30°C before they were separated on a 6% polyacrylamide gel and exposed to Kodak XAR film for 24 h at −80°C. As an internal control for RNA isolation and loading variations, 10^5 cpm of a [α-^{32}P]UTP-labeled probe specific for either cyclophilin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also included in the hybridization mixture. Both cyclophilin mRNA and GAPDH mRNA are constitutively expressed in PC12 cells and unaffected by growth factor treatment (17, 19, 48). Northern blot analysis was performed as previously described (16–18). RNA samples (30 μg) were size fractionated on 0.8% agarose gels containing 2.0 M formaldehyde and transferred to a nylon membrane (Zetabind; Cuno, Inc., Meriden, Conn.), and the membrane was baked at 80°C for 1 h. Random-primed cDNA probes specific for c-*fos* mRNA and transin mRNA, generated by using [α-^{32}P]dCTP (NEN), a commercially available kit (Bethesda Research Laboratories, Grand Island, N.Y.), and full-length cDNAs corresponding to either c-*fos* or transin mRNA, were hybridized with the membrane at 5 × 10^5 cpm/ml. For each PC12 subline, RNA was isolated from at least two separate growth factor treatments and multiple RNase protection and Northern blot experiments were performed on each, with the average fold inductions given in the text (mean ± standard error of the mean [SEM]) and representative examples shown in the figures. Densitometric analysis of the autoradiographic signals representing the various mRNAs was performed by using the NIH Image program. For each experiment, several autoradiographic exposures were analyzed to confirm that the intensity of the signals was within the linear range of the film and that consistent measurements were obtained.

Electrophysiological recording and analysis. Na^+ current density was determined as in previous studies (16–18), using whole-cell patch clamp measurements of peak Na^+ current amplitude and cell membrane capacitance. Prior to recording, the culture medium was replaced with a saline solution (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM HEPES, 6 mM glucose [pH 7.2]). Patch electrodes were pulled from capillary glass (Sutter Instruments, Novato, Calif.) and, when filled with 140 mM CsCl, 10 mM EGTA, and 10 mM HEPES in order to minimize the contribution of other voltage-activated currents during the recordings, had resistances of 3 to 7 mΩ. Whole-cell patch clamp recordings were made at room temperature (20 to 24°C), using a List EPC-7 patch clamp amplifier (Medical Systems Corp., Greenvale, N.Y.). 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. By routinely compensating for 50 to 70% of the series resistance, the estimated series resistance errors were reduced to 5 mV or less. An Atari computer-based system (Instrutech Corp., Elmont, N.Y.) was used to apply voltage commands and record currents. Cells were held at −80 mV and every 3 s prepulsed to −120 mV for 40 ms prior to a 20-ms depolarization to a

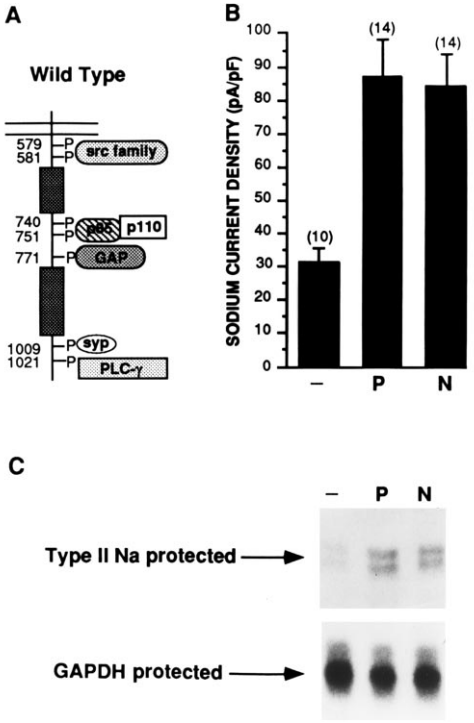

FIG. 1. Na^+ channel expression in PC12 cells expressing wild-type PDGF β receptors. (A) Schematic illustrating the interaction of signal transduction molecules with phosphorylated tyrosine residues in the cytoplasmic domain of the wild-type PDGF β receptor. The Src family members Fyn, Yes, and Src (Src family), the regulatory subunit (p85) of PI3K (p85/p110), GAP, the protein tyrosine phosphatase PTP1D (Syp), and PLC_γ interact with specific phosphorylated tyrosine residues (P) of the receptor, with numbers indicating the positions of the tyrosine residues in the human PDGF β-receptor sequence. In panels B and C, PC12 cells stably expressing these receptors were untreated (−), PDGF treated (P), or NGF treated (N) for 7 days prior to analysis. In panel B, the average Na^+ current density in these cells is shown, with the number of cells in each group in parentheses. Error bars represent the SEM. Cell membrane capacitances (mean ± SEM; in picofarads) were 6.3 ± 0.6 for untreated cells, 9.8 ± 0.4 for PDGF-treated cells, and 11.0 ± 0.7 for NGF-treated cells. In panel C, results of a representative RNase protection assay are shown. Prior to RNase digestion, RNA samples were incubated in a solution containing both a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for the constitutively expressed GAPDH mRNA, with the signal corresponding to GAPDH mRNA serving as an internal control (see Materials and Methods). Signals representing the protected mRNA fragments are shown after a 24-h (type II Na^+ channel α-subunit mRNA) or 1-h (GAPDH) exposure to film.

potential between −60 and +30 mV. Current recordings were digitally filtered at 2 kHz during analysis, and scaled pulse (P/4) routines were used to remove linear leakage currents. The maximum Na^+ current elicited and the cell membrane capacitance were used to calculate Na^+ current density in individual cells. Statistical significance was determined by using a two-tailed Student's *t* test.

RESULTS

The kinase activity of the PDGF receptor is required for induction of Na^+ channel expression. To provide a basis for comparison and confirm that activation of the human PDGF receptor could induce neuronal Na^+ channel expression, PC12 sublines stably transfected with a cDNA encoding the β form of the human PDGF receptor were analyzed. Initial biochemical analysis of these sublines (79) indicated that the Src family of tyrosine kinases, as well as PI3K, GAP, PLC_γ, and Syp, associated with the receptor in a PDGF-dependent manner (Fig. 1A), consistent with the results obtained for other cell lines (for reviews, see references 8 and 31). Furthermore, treatment of the cells with PDGF elicited neurite outgrowth comparable to that induced by NGF, in accord with our previous analysis of the murine PDGF β receptor (18, 28). To determine if activation of the human PDGF receptor could induce functional Na^+ channel expression, Na^+ currents in untreated and growth factor-treated cells were compared by using whole-cell patch clamp recording. By using previously established procedures (see Materials and Methods), cells were held at −80 mV and briefly hyperpolarized to −120 mV, and then Na^+ currents were assayed in response to depolarizations between −60 to +30 mV. Measurements of the peak inward Na^+ current and the cell membrane capacitance (as an estimate of cell size) were used to estimate the Na^+ current density in individual cells. As expected, treatment of the cells with NGF for 7 days caused a significant increase ($P < 0.01$) in both average peak Na^+ current and average Na^+ current density (Fig. 1B). Treatment of the cells with PDGF for 7 days also caused significant increases ($P < 0.01$) in average peak Na^+ current and average Na^+ current density, with the level of induction comparable to that elicited by NGF (Fig. 1B). Similar results were obtained from an additional PC12 subline expressing wild-type PDGF receptors, with the PDGF-induced levels of Na^+ current density (78.0 ± 9.4 pA/pF; $n = 11$) again comparable to that induced by NGF (93.8 ± 6.7 pA/pF; $n = 7$). The results from both of these sublines were also consistent with our previous analysis of a number of PC12 sublines expressing the murine PDGF receptor (18). In fact, in five independent sublines of PC12 cells expressing wild-type PDGF receptors, the increase in Na^+ current density elicited by PDGF was virtually identical to that mediated by NGF, indicating that when the capabilities of the exogenous PDGF receptors were evaluated relative to the endogenous responses to NGF, the results for a given PDGF receptor were consistent and differences due to subclonal variation were negligible.

To confirm that there were concomitant increases in type II Na^+ channel α-subunit mRNA in these cells after growth factor stimulation, a ^{32}P-labeled probe specific for type II α-subunit mRNA was used in RNase protection assays of total cellular RNA from cells that were either untreated, NGF treated, or PDGF treated for 7 days. A probe specific for GAPDH mRNA was also included in the hybridization as an internal control for variations in harvesting and handling the RNA samples during analysis (see Materials and Methods). Similar to wild-type PC12 cells (18, 49), in the sublines expressing wild-type human PDGF receptors, there were low levels of type II α-subunit mRNA in the absence of growth factor treatment and easily discernible increases (3.4 ± 0.43-fold; $n = 3$) in response to NGF (Fig. 1C). As expected, PDGF elicited a comparable increase (3.2 ± 0.34-fold; $n = 3$) in type II α subunit mRNA (Fig. 1C). The NGF- and PDGF-mediated inductions of type II Na^+ channel mRNA were also comparable in another PC12 subline expressing human wild-type PDGF receptors (data not shown), and the results from both of these sublines were consistent with the results that we obtained from other PC12 sublines expressing murine wild-type PDGF receptors (18).

To demonstrate that the tyrosine kinase activity of the PDGF β receptor was necessary for the PDGF-mediated induction of Na^+ channel expression, we analyzed clonal PC12 sublines stably expressing mutated PDGF β receptors that have been rendered kinase inactive by changing the lysine in the ATP binding region (at position 634) to arginine. Since this receptor (K634R) lacks kinase activity, it is unable to autophosphorylate and bind the Src family of tyrosine kinases, PI3K, GAP, Syp, and PLC_γ (79), as illustrated in Fig. 2A. As expected, while NGF could elicit neurite outgrowth in this subline of PC12 cells, PDGF was ineffective. Patch clamp anal-

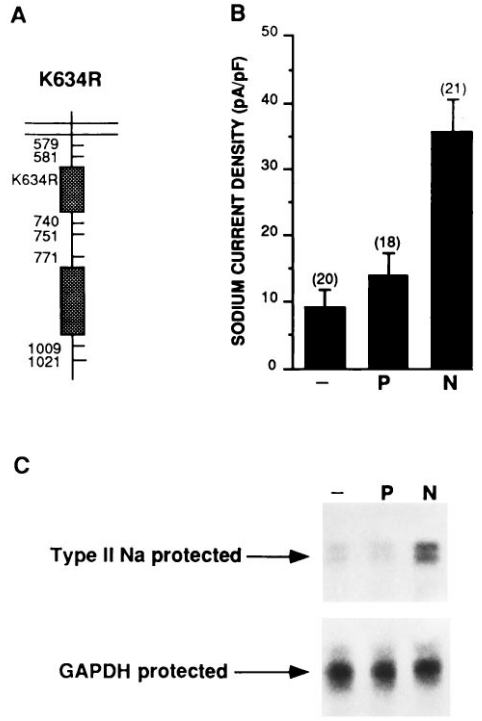

FIG. 2. Na^+ channel expression in PC12 cells expressing kinase-inactive PDGF receptors. (A) Schematic illustrating the cytoplasmic domain of a human PDGF β receptor in which the lysine residue at position 634 has been changed to an arginine residue (K634R), rendering the receptor kinase inactive and resulting in a lack of PDGF-dependent signal activation. Numbers indicate positions of the tyrosine residues in the human PDGF β-receptor sequence. In panels B and C, PC12 cells stably expressing these receptors were untreated (−), PDGF treated (P), or NGF treated (N) for 7 days prior to analysis. In panel B, the average Na^+ current density in these cells is shown, with the number of cells in each group in parentheses. Error bars represent the SEM. Cell membrane capacitances (mean ± SEM; in picofarads) were 7.8 ± 0.4 for untreated cells, 7.1 ± 0.3 for PDGF-treated cells, and 12.2 ± 0.6 for NGF-treated cells. In panel C, results of a representative RNase protection assay are shown. Prior to RNase digestion, RNA samples were incubated in a solution containing both a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for GAPDH mRNA, with the signal corresponding to GAPDH mRNA serving as an internal control (see Materials and Methods). Signals representing the protected mRNA fragments are shown after a 24-h (type II Na^+ channel α-subunit mRNA) or 1-h (GAPDH) exposure to film.

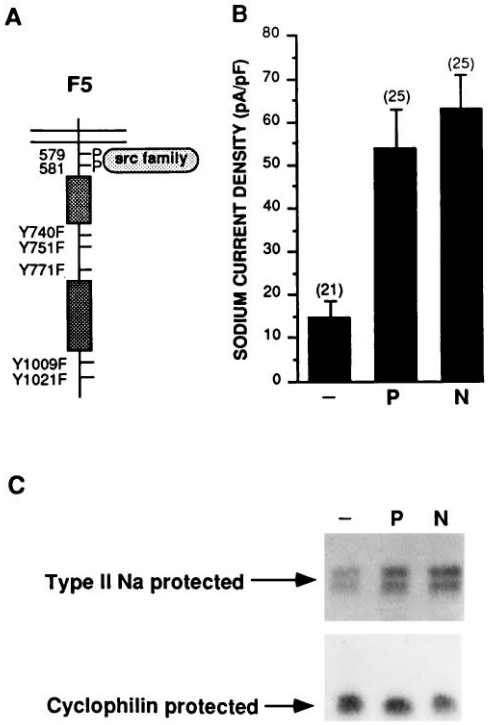

FIG. 3. Na^+ channel expression in PC12 cells expressing F5 mutant PDGF receptors. (A) Schematic of the cytoplasmic domain of the F5/115 PDGF β receptor, in which the tyrosines at positions 740, 751, 771, 1009, and 1021 of the human PDGF β receptor were changed to phenylalanine, thereby eliminating the PDGF-dependent association of PI3K (p85/p110), GAP, Syp, and PLC_γ with the receptor, while leaving the interactions at tyrosines 579 and 581 intact, including the association with members of the Src family of tyrosine kinases. In panels B and C, PC12 cells stably expressing these receptors were untreated (−), PDGF treated (P), or NGF treated (N) for 7 days prior to analysis. In panel B, the average Na^+ current density in these cells is shown, with the number of cells in each group in parentheses. Error bars represent the SEM. Cell membrane capacitances (mean ± SEM; in picofarads) were 7.1 ± 0.3 for untreated cells, 11.3 ± 0.4 for PDGF-treated cells, and 12.6 ± 0.5 for NGF-treated cells. In panel C, results of a representative RNase protection assay are shown. Prior to RNase digestion, RNA samples were incubated with a solution containing both a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for the constitutively expressed cyclophilin mRNA, with signals representing cyclophilin mRNA serving as an internal control (see Materials and Methods). Signals representing the protected mRNA fragments are shown after a 24-h (type II Na^+ channel α-subunit mRNA) or 1-h (cyclophilin) exposure to film.

ysis indicated that PDGF was also unable to stimulate an increase in functional Na^+ channel expression in these cells, even though NGF caused a significant increase ($P < 0.01$) in average Na^+ current density (Fig. 2B). Furthermore, RNase protection assays revealed that PDGF failed to increase type II α-subunit mRNA expression in these cells (1.1 ± 0.12-fold; $n = 4$), while there was a clear induction of type II α subunit mRNA in response to NGF (2.9 ± 0.27-fold; $n = 4$), as shown in Fig. 2C. Similar results were obtained from another PC12 subline expressing the K634R receptor, with an increase in type II mRNA (2.9-fold) and Na^+ current density in response to NGF (from 1.5 ± 0.8 pA/pF [$n = 5$] to 20.6 ± 4.8 pA/pF [$n = 14$]) but not in response to PDGF (1.8 ± 0.8 pA/pF; $n = 8$). Therefore, activation of the human PDGF β receptor induces Na^+ channel expression in PC12 cells in a manner dependent on the kinase activity of the receptor, indicating that specific tyrosine phosphorylations of the receptor and the cellular signaling events associated with them are among the initial events underlying the increase in Na^+ channel expression.

Analysis of Na^+ channel regulation by the F5 mutant PDGF receptor. To begin identifying signals necessary for the PDGF-mediated induction of Na^+ channel expression in PC12 cells, Na^+ channel expression was analyzed in PC12 sublines stably expressing human PDGF β receptors in which the tyrosines at positions 740, 751, 771, 1009, and 1021 had been changed to phenylalanine (see Materials and Methods). In other cell types, this mutant receptor (which we refer to as F5) lacks the ability to associate with PI3K, GAP, Syp, and PLC_γ in a PDGF-dependent manner (79). Likewise, the activation of these signaling proteins is also compromised in PC12 cells (79), as illustrated in Fig. 3A. Despite the inability to activate these signals, activation of the F5 receptor by PDGF still elicited neurite outgrowth in PC12 cells that was indistinguishable from that elicited by NGF (19, 79). Furthermore, patch clamp analysis of these cells revealed no significant differences between the abilities of NGF and PDGF to increase the proportion of cells with appreciable (>50 pA) Na^+ current (from 11/21 to 25/25 and 23/25, respectively) or to cause a significant increase ($P < 0.01$) in average peak Na^+ current and average

Na^+ current density (Fig. 3B). RNase protection assays consistently showed that in PC12 cells expressing the F5 receptor, PDGF caused an increase in type II Na^+ channel α-subunit mRNA (2.2 ± 0.11-fold; $n = 3$) that was comparable to the increase (2.5 ± 0.21-fold; $n = 3$) elicited by NGF (Fig. 3C). Therefore, despite the inability to associate with either PI3K, GAP, Syp, or PLC_γ, activation of the F5 receptor can induce type II Na^+ channel α-subunit mRNA and Na^+ current density to levels comparable to that elicited by NGF.

Analysis of Na^+ channel regulation by the F579/581 mutant PDGF receptor. Previous studies have shown that signaling proteins, including members of the Src family of nonreceptor tyrosine kinases, associate with the phosphorylated juxtamembrane tyrosines at positions 579 and 581 of the human PDGF β receptor (44, 54; for a review, see reference 8). There is also evidence to suggest that Src plays an important role in the differentiation of PC12 cells (11, 43). Therefore, as part of the effort to uncover signals important for the induction of Na^+ channel expression, PC12 sublines stably expressing PDGF receptors with the tyrosines at positions 579 and 581 mutated to phenylalanine were assayed for Na^+ channel expression. In PC12 cells, this receptor (F579/581), unlike the wild-type receptor, lacks the ability to associate with and activate the Src family of kinases (79), as illustrated in Fig. 4A. Despite this, treatment of cells expressing this receptor with PDGF was sufficient to induce neurite outgrowth comparable to that caused by NGF (19, 79), indicating that this neuron-specific late event was normal, even though the receptor has lost its interaction with the Src family of kinases. In cells stably expressing the F579/581 receptor, NGF treatment increased the proportion of cells with appreciable (>50 pA) Na^+ current (from 2/15 to 24/24) and caused a significant increase ($P < 0.01$) in the average Na^+ current and average Na^+ current density (Fig. 4B). Treatment of these cells with PDGF also resulted in an increase in the proportion of cells with appreciable (>50 pA) Na^+ current (from 2/15 to 21/22). However, the increase in average peak Na^+ current and average Na^+ current density in response to PDGF was clearly reduced and was significantly less ($P < 0.01$) than that elicited by NGF (Fig. 4B). Similar results were obtained upon analysis of an additional subline expressing the F579/581 receptor, with the Na^+ current density after PDGF treatment (27.2 ± 7.0 pA/pF; $n = 12$) greater than in untreated cells (14.0 ± 6.9 pA/pF; $n = 11$) but significantly less ($P < 0.01$) than in NGF-treated cells (69.8 ± 14.1 pA/pF; $n = 10$). To determine if this could be accounted for by changes in type II α-subunit mRNA levels, RNase protection assays were used to analyze RNA from cells that were untreated, PDGF treated, or NGF treated for 7 days (Fig. 4C). In contrast to the induction of functional Na^+ channel expression, the induction of type II α-subunit mRNA elicited by PDGF (2.8 ± 0.25-fold; $n = 3$) was comparable to the induction observed in response to NGF (2.2 ± 0.38-fold; $n = 3$). This finding was confirmed when Northern blot analysis was used as an independent means to detect changes in Na^+ channel mRNA expression. When a ^{32}P-labeled Na^+ channel probe that recognizes multiple types of Na^+ channel α-subunit mRNA was used, the increase in α-subunit mRNA in response to NGF (2.8-fold) was comparable to the increase observed in response to PDGF (2.9-fold). Therefore, while mutation of tyrosines 579 and 581 did not dramatically influence the PDGF-mediated induction of type II α-subunit mRNA, it did have a significant effect on the increase in Na^+ current density.

Analysis of Na^+ channel regulation by the F5/579 mutant PDGF receptor. Analysis of neurite outgrowth in PC12 cells suggests that activation of redundant signaling pathways underlies this response to growth factors (71, 79). Given that the activation of either the F5 or F579/581 receptor was sufficient to induce type II Na^+ channel α-subunit mRNA, it was possible that similar mechanisms governed α-subunit mRNA expression. Therefore, we analyzed PC12 sublines stably expressing a cDNA encoding human PDGF β receptors with phenylalanine substitutions for tyrosine 579 in combination with phenylalanine substitutions at tyrosines 740, 751, 771, 1009, and 1021. Characterization of this receptor (F5/579) showed that it was unable to associate with the Src family of kinases, PI3K, GAP, Syp, and PLC_γ, upon activation by PDGF (79), as illustrated in Fig. 5A. Furthermore, PDGF was unable to stimulate neurite outgrowth in these cells, even though NGF was able to do so (19, 79). Patch clamp analysis of these cells indicated that both NGF and PDGF could increase the proportion of these cells with appreciable (>50 pA) Na^+ current (from 13/19 to 26/27 and 27/29, respectively). However, the PDGF-mediated increase in average peak Na^+ current and average Na^+ current density was significantly less ($P < 0.01$) than that elicited by NGF (Fig. 5B), similar to the results from the F579/581 receptor (Fig. 4). Results consistent with these were also obtained for another subline expressing F5/579 re-

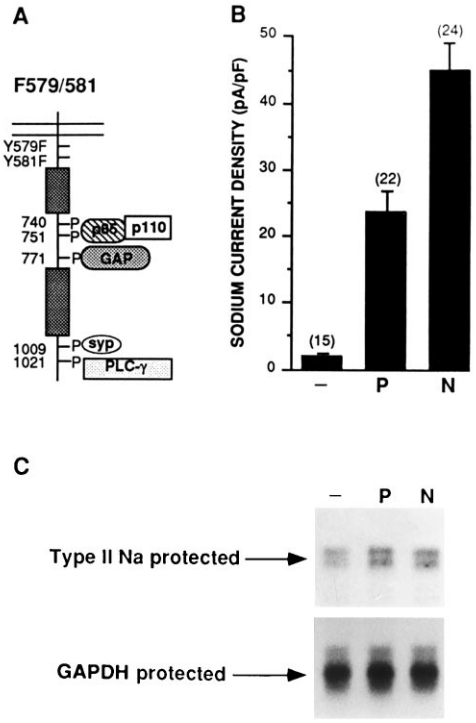

FIG. 4. Na^+ channel expression in PC12 cells expressing F579/581 mutant PDGF receptors. (A) Schematic of the cytoplasmic domain of the F579/581 PDGF β receptor, in which the tyrosines at positions 579 and 581 were changed to phenylalanine, thereby eliminating associations normally occurring at these sites, including interactions with members of the Src family of tyrosine kinases, while leaving intact the PDGF-dependent association of PI3K (p85/p110), GAP, Syp, and PLC_γ with the receptor. In panels B and C, PC12 cells stably expressing these receptors were untreated (−), PDGF treated (P), or NGF treated (N) for 7 days prior to analysis. In panel B, the average Na^+ current density in these cells is shown, with the number of cells in each group in parentheses. Error bars represent the SEM. Cell membrane capacitances (mean ± SEM; in picofarads) were 7.4 ± 0.4 for untreated cells, 13.6 ± 0.8 for PDGF-treated cells, and 12.9 ± 0.4 for NGF-treated cells. In panel C, results of a representative RNase protection assay are shown. Prior to RNase digestion, RNA samples were incubated in a solution containing both a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for GAPDH mRNA, with the signal corresponding to GAPDH mRNA serving as an internal control (see Materials and Methods). Signals representing the protected mRNA fragments are shown after a 24-h (type II Na^+ channel α-subunit mRNA) or 1-h (GAPDH) exposure to film.

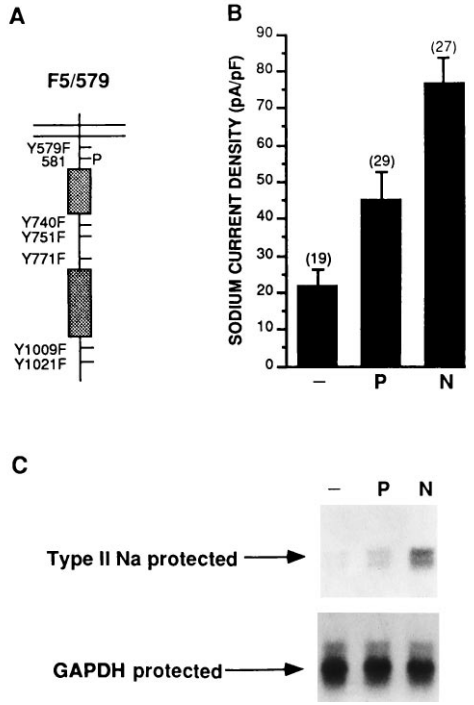

FIG. 5. Na^+ channel expression in PC12 cells expressing F5/579 mutant PDGF receptors. (A) Schematic of the cytoplasmic domain of the F5/579 PDGF β receptor, in which tyrosines at positions 579, 740, 751, 771, 1009, and 1021 were changed to phenylalanine, thereby eliminating the PDGF-dependent association of this receptor with members of the Src family of tyrosine kinases, PI3K, GAP, Syp, and PLC_γ. In panels B and C, PC12 cells stably expressing these receptors were untreated (−), PDGF treated (P), or NGF treated (N) for 7 days prior to analysis. In panel B, the average Na^+ current density in these cells is shown, with the number of cells in each group in parentheses. Error bars represent the SEM. Cell membrane capacitances (mean ± SEM; in picofarads) were 6.0 ± 0.3 for untreated cells, 9.0 ± 0.6 for PDGF-treated cells, and 10.7 ± 0.5 for NGF-treated cells. In panel C, results of a representative RNase protection assay are shown. Prior to RNase digestion, RNA samples were incubated in a solution containing both a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for GAPDH mRNA, with the signal corresponding to GAPDH mRNA serving as an internal control (see Materials and Methods). Signals representing the protected mRNA fragments are shown after a 24-h (type II Na^+ channel α-subunit mRNA) or 1-h (GAPDH) exposure to film.

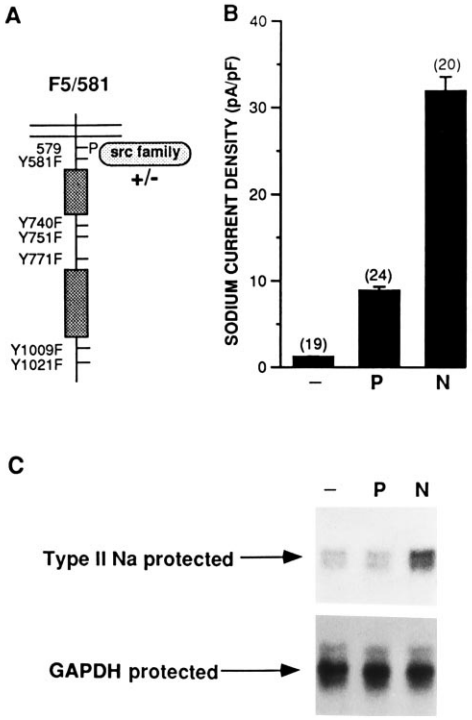

FIG. 6. Na^+ channel expression in PC12 cells expressing F5/581 mutant PDGF receptors. (A) Schematic of the cytoplasmic domain of the F5/581 PDGF β receptor, in which the tyrosines at positions 581, 740, 751, 771, 1009, and 1021 were changed to phenylalanine, thereby eliminating the association of the receptor with PI3K, GAP, Syp, and PLC_γ and reducing the PDGF-dependent association with the Src family of kinases. In panels B and C, PC12 cells stably expressing these receptors were untreated (−), PDGF treated (P), or NGF treated (N) for 7 days prior to analysis. In panel B, the average Na^+ current density in these cells is shown, with the number of cells in each group in parentheses. Error bars represent the SEM. Cell membrane capacitances (mean ± SEM; in picofarads) were 7.1 ± 0.4 for untreated cells, 10.9 ± 0.5 for PDGF-treated cells, and 10.3 ± 0.6 for NGF-treated cells. In panel C, results of a representative RNase protection assay are shown. Prior to RNase digestion, RNA samples were incubated with a solution containing both a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for the constitutively expressed GAPDH mRNA, with signals representing GAPDH mRNA serving as an internal control (see Materials and Methods). Signals representing the protected mRNA fragments are shown after a 24-h (type II Na^+ channel α-subunit mRNA) or 1-h (GAPDH) exposure to film.

ceptors, with the Na^+ current density in PDGF-treated cells (24.6 ± 3.7 pA/pF; $n = 11$) greater than in untreated cells (9.2 ± 3.98 pA/pF; $n = 8$) but significantly less ($P < 0.01$) than in NGF-treated cells (52.9 ± 6.4 pA/pF; $n = 14$). Interestingly, RNase protection assays indicated that activation of the F5/579 receptor by PDGF failed to increase type II Na^+ channel α-subunit mRNA levels in these cells (1.2 ± 0.14-fold; $n = 4$), whereas treatment with NGF elicited obvious increases (3.3 ± 0.35-fold; $n = 4$). Furthermore, Northern blot analysis with a ^{32}P-labeled Na^+ channel probe that recognizes multiple types of Na^+ channel α-subunit mRNA also failed to detect an increase in α-subunit mRNA following treatment of these cells with PDGF, whereas NGF caused a 2.9-fold induction (data not shown). The results provide further evidence that signals associated with phosphorylation of the tyrosine at position 579 are important for full induction of Na^+ current density and also suggest that redundant signals underlie the induction of type II α-subunit mRNA, with either signals associated with the phosphorylation of tyrosine at 579 or signals associated with phosphorylation of tyrosine at 740, 751, 771, 1009, or 1021 necessary for the induction to occur.

Analysis of Na^+ channel regulation by the F5/581 mutant PDGF receptor. To further confirm the importance of the signals associated with the tyrosines in the juxtamembrane region of the receptor, Na^+ channel expression was analyzed in PC12 sublines stably expressing a PDGF receptor that contains phenylalanines instead of tyrosines at positions 581, 740, 751, 771, 1009, and 1021. As illustrated in Fig. 6A, the mutations in this receptor (F5/581) effectively block association and activation of PI3K, GAP, Syp, and PLC_γ and dramatically reduce the association and activation of the Src family of kinases (79). Despite this, PDGF treatment of the sublines stably expressing the F5/581 receptor still resulted in some neurite outgrowth (19, 79), presumably due to the low residual level of Src activation mediated by the F5/581 receptor (79). Patch clamp analysis of these cells indicated that both NGF and PDGF could increase the proportion of cells with appreciable (>50 pA) Na^+ current (from 0/19 to 20/20 and 18/24, respectively). However, similar to the results from the F579/581 and F5/579 receptors, the PDGF-mediated induction of average peak Na^+ current and average Na^+ current density in the cells expressing the F5/581 receptor was significantly less ($P < 0.01$) than that

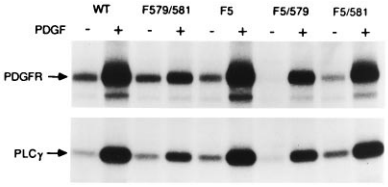

FIG. 7. Kinase activities of wild-type and mutant PDGF receptors expressed in PC12 cells. Representative results from an *in vitro* kinase assay show the levels of autophosphorylation of wild-type and mutant PDGF receptors (PDGFR) immunoprecipitated from lysates of PC12 sublines that were either untreated (−) or treated with 30 ng of PDGF per ml for 10 min (+) (top) and the levels of phosphorylation of a GST-PLC_γ fusion protein (PLC_γ) that was added to the assays as an exogenous substrate (bottom). Quantitation of the signals by PhosphorImager analysis showed that the fold increases in autophosphorylation in response to PDGF were highest for the wild-type (7.0) and F5 (6.7) receptors, slightly lower for the F5/579 (5.5) and F5/581 (4.6) receptors, and lowest for the F579/581 receptor (1.6). The fold increase in phosphorylation of the PLC_γ fusion protein followed a similar pattern, with the largest increase for the wild-type (10) and F5 (6.1) receptors, a slightly smaller increase for the F5/579 (4.6) and F5/581 (5.2) receptors, and the smallest increase for the F579/581 receptor (2.1). Signals representing the PDGF receptors and the PLC_γ fusion protein are shown after a 5-h exposure to film.

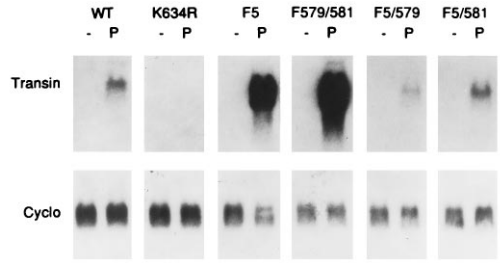

FIG. 8. Induction of transin mRNA in PC12 cells expressing either wild-type or mutant PDGF receptors. Shown is a representative Northern blot of transin (Transin) and cyclophilin (Cyclo) mRNAs in 20-μg samples of total cellular RNA isolated from untreated (−) or PDGF-treated (P) PC12 cells stably expressing either wild-type or mutant PDGF receptors. Signals are shown after a 72-h (transin mRNA) or 1-h (cyclophilin mRNA) exposure to film.

elicited by NGF (Fig. 6B). These results were confirmed upon analysis of a separate subline expressing the F5/581 receptor, with the Na^+ current density in NGF-treated cells (41.1 ± 7.5 pA/pF; $n = 11$) significantly greater ($P < 0.01$) than in cells that were untreated (6.1 ± 4.3 pA/pF; $n = 7$) or treated with PDGF (15.2 ± 3.0 pA/pF; $n = 15$). From RNase protection analysis, it was clear that activation of the F5/581 receptor with PDGF did not induce type II α-subunit mRNA (1.0 ± 0.1-fold; $n = 3$), while NGF elicited a severalfold increase (3.3 ± 0.26-fold; $n = 3$) in these cells (Fig. 6C). As anticipated, Northern blot analysis with a Na^+ channel probe that recognizes multiple types of Na^+ channel α-subunit mRNA confirmed that activation of the F5/581 receptor was unable to induce α-subunit mRNA, whereas NGF elicited a 3.5-fold induction (data not shown). The results are consistent with those obtained from analysis of the F5/579 receptor and demonstrate that the mutations in the F5/581 and F5/579 PDGF receptors are sufficient for eliminating the signals underlying the PDGF-mediated induction of type II α-subunit mRNA in PC12 cells. Furthermore, as with the F5/579 receptor, the results indicate that the signals associated with the phosphorylation of tyrosines at 579 and 581 are critical for the induction of functional Na^+ channels in response to PDGF.

PDGF receptor mutants with reduced kinase activity that are ineffective in regulating Na^+ channel expression still elicit changes in transin and c-*fos* mRNAs. In the initial characterization of these PC12 sublines, we showed that mutation of the tyrosines at positions 579 and 581 of the PDGF receptor resulted in a loss of Src family regulation and a reduction in receptor kinase and autophosphorylation activity (79). These differences in the intrinsic kinase activity of the wild-type and mutant PDGF receptors were also evident in the cells used in the experiments described here, as indicated by the levels of receptor autophosphorylation and phosphorylation of exogenous substrate that were detected in *in vitro* kinase assays (Fig. 7). As in our previous studies (79), the F5 receptor did not appear to have significant alterations in autophosphorylation activity or ability to phosphorylate a GST-PLC_γ fusion protein compared to the wild-type receptor, while autophosphorylation activity and kinase activity were reduced in the F5/579 and F5/581 receptors, and the lowest levels of autophosphorylation activity and kinase activity were exhibited by the F579/581 receptor (Fig. 7). As expected, there was no autophosphorylation or kinase activity detected for the K634R kinase-inactive receptor (data not shown). The similarity of these results to those of our previous study (79) suggests that the cells were responding as originally characterized and confirms there is a reduction in the kinase activity of the F5/579, F5/581, and F579/581 receptors in these cells.

To show that the inability of the F579/581, F5/579, and F5/581 receptors to induce Na^+ channel expression was not simply due to a general reduction in kinase activity that interfered with all PDGF-mediated responses, the growth factor-mediated inductions of transin and c-*fos* mRNAs were assayed by Northern blot analysis. Transin mRNA expression is similar to the expression of Na^+ channel α-subunit mRNA in that it is neuron specific and in PC12 cells is a relatively late response to NGF (48). Treatment with PDGF induced transin mRNA expression in PC12 cells expressing the wild-type PDGF receptor but not in cells expressing the kinase-inactive (K634R) form of the receptor (Fig. 8). More importantly, PDGF elicited an increase in transin mRNA in all of the PC12 sublines expressing mutant PDGF receptors, with the induction of transin mRNA in PC12 sublines expressing the F5 and F579/581 receptors greater than that induced by activation of the wild-type receptor (Fig. 8). In fact, activation of the F579/581 receptor resulted in the largest induction of transin mRNA (Fig. 8), even though this receptor has the lowest intrinsic kinase activity (as judged by either receptor autophosphorylation or *in vitro* phosphorylation of the GST-PLC_γ fusion protein) of the mutant PDGF receptors examined (Fig. 7). Therefore, the level of intrinsic kinase activity of the different mutant PDGF receptors does not determine the extent of transin mRNA induction, supporting the contention that the differential regulation of Na^+ channel expression by the various mutant PDGF receptors is not due to differences in their intrinsic kinase activity and instead is due to the lack of specific signaling events.

As another independent assay of the abilities of the mutant PDGF receptors, Northern blot analysis was used to measure the PDGF-mediated induction of c-*fos* mRNA in the PC12 sublines. As in our earlier study (18), PDGF induced c-*fos* mRNA expression in PC12 cells stably expressing the wild-type PDGF receptor (Fig. 9). Furthermore, PDGF increased c-*fos* mRNA in all of the PC12 sublines stably expressing mutant PDGF receptors (Fig. 9) except those expressing the kinase-inactive (K634R) receptor (data not shown). In the sublines expressing the F579/581 and F5/579 receptors, the induction of c-*fos* mRNA was more extensive than that elicited by NGF, whereas in the cells expressing the F5 and F5/581 receptors, the inductions were somewhat reduced (Fig. 9). Thus, the

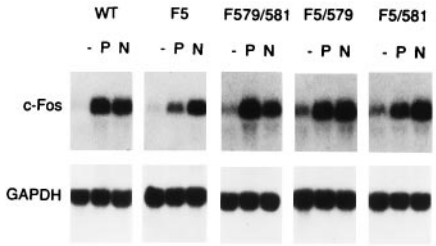

FIG. 9. Induction of c-*fos* mRNA in PC12 cells stably expressing either wild-type or mutant PDGF receptors. Shown is a representative Northern blot of c-*fos* and GAPDH mRNAs in 10-μg samples of total cellular RNA isolated from untreated (−), PDGF-treated (P), or NGF-treated (N) PC12 cells expressing either wild-type or mutant PDGF receptors. Signals are shown after a 24-h (c-*fos* mRNA) or 1-h (GAPDH mRNA) exposure to film.

extent to which c-*fos* mRNA was induced did not correlate with the intrinsic kinase activity of the mutant receptors and instead resulted in a third distinct pattern compared to the transin and Na^+ channel mRNA inductions. Cumulatively, the results indicate that the lack of Na^+ channel α-subunit mRNA induction by the F5/579 and F5/581 PDGF receptors is not simply due to decreases in the intrinsic kinase activity of these receptors and instead results from the loss of specific signaling pathways normally recruited by the PDGF receptor, with the loss of signals involving the tyrosines at 579 and 581, such as Src family activation, particularly important.

DISCUSSION

The cellular mechanisms underlying the influence of growth factors on neuronal differentiation are not yet understood, despite considerable interest and effort in determining how they differ from growth factor-mediated signals governing cell growth and proliferation (for reviews and discussions, see references 6, 36, 51, and 78). From recent work, differences in the extent and duration of commonly observed growth factor responses have been proposed as the basis for this difference, with the sustained rather than transient activation of MAPK thought to be sufficient for neuronal differentiation in PC12 cells (10, 13, 76, 77; for a review, see reference 51). From other studies, it is thought that selective activation of specific signaling proteins and/or differences in the combination of signals that are generated promote differentiation rather than proliferation (for a review, see references 36 and 78). For example, studies using PC12 cells suggest that control of neuron-specific responses can be dissected into discrete components involving multiple signaling mechanisms, with Ras activation required for the induction of transcripts encoding transin, SCG10, and VGF (11, 17, 73), Ras and/or PLC_γ activation required for neurite outgrowth (71), PLC_γ activation required for the induction of peripherin neurofilament mRNA and protein (47), and relatively undefined, Ras-independent mechanisms needed for Thy-1 expression and the induction of Na^+ channel mRNA and functional Na^+ channels (11, 17). It is this last, poorly understood category of responses that has served as the focus for the present study, and as part of our interest in defining signals underlying neuronal differentiation, we have concentrated on identifying signals important for the sustained, long-term induction of Na^+ channel expression. As discussed below, our results suggest that among MAPK, PLC_γ, and the Src family of kinases, the activation of at least two of these signals is needed for the growth factor-mediated induction of type II Na^+ channel α-subunit mRNA in PC12 cells, while for full induction of functional Na^+ channel expression, the activation of the Src family of kinases appears necessary. These results provide insight into the mechanisms governing neuronal Na^+ channel expression and emphasize the multiplicity and combinatorial nature of the signaling mechanisms governing neuronal differentiation.

Previous studies have overexpressed receptors endogenous to PC12 cells, such as those for NGF, epidermal growth factor, and insulin (13, 32, 77), in order to gain insight into the mechanisms important for neuronal differentiation. Here, we have analyzed the actions of a series of PDGF receptors in these cells in an effort to identify signals important for Na^+ channel expression, a crucial aspect of neuronal differentiation. While PDGF receptors are expressed in many populations of neurons (14, 55, 64, 68, 69), they are not normally expressed in wild-type PC12 cells. Therefore, in this study, we took advantage of previously generated PC12 sublines that stably express PDGF receptors (79). Although there are no published estimates of the number of PDGF receptors in primary neurons for comparison, the levels of PDGF receptors in these PC12 sublines are such that PDGF elicits in them many of the same responses that it does in primary neurons, including increases in survival, c-*fos* induction, neurite outgrowth, and the induction of enzymes involved in neurotransmitter synthesis (55, 68, 69). Furthermore, in these sublines, PDGF mimics the actions of NGF in all responses examined so far, including the induction of Na^+ channel expression and the activation of signaling molecules such as Ras, B-Raf, Raf-1, and MAPK to levels comparable to those observed in response to NGF (18, 79). Thus, there appear to be physiologically relevant levels of signaling in these sublines in response to PDGF, further supporting their use in identifying signals governing Na^+ channel expression.

Our analysis of these sublines reveals that while the growth factor induction of type II Na^+ channel α-subunit mRNA and Na^+ current density depends on the kinase activity of the receptor, it is the ability to generate specific signals, not simply the level of intrinsic kinase activity (as indicated by either autophosphorylation activity or the phosphorylation of a GST-PLC_γ fusion protein in *in vitro* kinase assays), that is important. For example, all of the mutant receptors analyzed could induce the expression of the immediate-early gene c-*fos* and the neuron-specific transin gene, yet the level of induction of these genes did not correlate with the level of intrinsic kinase activity of the receptor. Furthermore, even the mutant receptors with the lowest level of intrinsic kinase activity were sufficient to induce Na^+ channel expression and neurite outgrowth, another late aspect of PC12 cell differentiation. Finally, phosphorylation of the tyrosine at position 857 of the PDGF receptor is important for stabilizing the kinase activity of the receptor, as mutation of this residue dramatically reduces the PDGF-stimulated kinase activity (20, 40). When PC12 cells stably expressing this mutant receptor were analyzed by patch clamp recording, we found that the NGF- and PDGF-mediated inductions of Na^+ channel expression were similar (19). Thus, the rapidly occurring peak in the level of receptor kinase activity does not appear to be a predominant determinant in regulating Na^+ channel expression; instead, specific receptor tyrosine phosphorylations appear to be critical.

The difference in the abilities of the various mutant PDGF receptors to induce Na^+ channel expression also suggests a reduced role for certain signaling mechanisms in PC12 differentiation. The fact that all of the kinase-active mutants stimulate a sustained activation of Ras and MAPK (79), yet not all of them can induce Na^+ channel expression, suggests that sustained MAPK activity is not sufficient for all aspects of neuronal differentiation in PC12 cells. This possibility is consistent with previous indications that Ras activity is neither

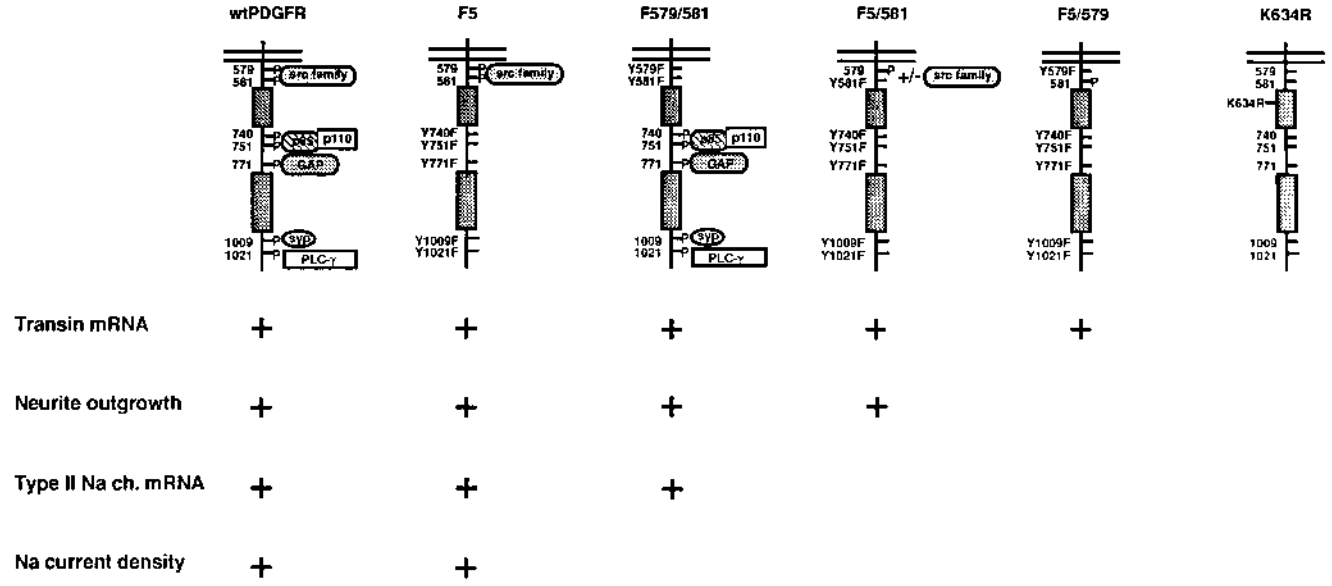

FIG. 10. Summary of neuron-specific responses induced by PDGF in PC12 cells expressing mutant PDGF β receptors. Each of the PDGF receptors analyzed is represented schematically, with the wild-type receptor (wtPDGFR) on the far left and the kinase-inactive receptor (K634R) on the far right. Details regarding the mutations and their effects on signaling by the receptors are described in the text, in previous figures, and in reference 79. Below each receptor diagram, a (+) indicates whether activation of the receptor in PC12 cells by PDGF elicited increases in transin mRNA, neurite outgrowth, type II Na^+ channel α-subunit mRNA, and Na^+ current density to at least the same extent as NGF.

sufficient nor necessary for the induction of Na^+ channel mRNA and functional channels (12, 17). Together, these findings also suggest that the signals associated with the tyrosine at position 716, which have only recently been shown to include Grb2 binding and the activation of Ras (2), are probably not major determinants of Na^+ channel expression in response to PDGF receptor activation.

Our mutational analysis of the PDGF receptor (summarized in Fig. 10) indicates that there are at least partially redundant signals underlying the induction of type II Na^+ channel α-subunit mRNA. Mutations that inhibit the association and activation of either the Src family of kinases (F579/581) or the activation of PI3K, GAP, PLC_γ, and Syp (F5) do not compromise the induction of type II α-subunit mRNA, whereas combining these mutations (F5/579 and F5/581) completely abrogated this induction. This is similar to the induction of neurite outgrowth in PC12 cells by activated TrkA or PDGF receptors, where in both cases at least partially redundant signaling mechanisms are thought to be involved (57, 71, 79). However, for both receptors, the Ras/MAPK pathway, either in combination with or in lieu of either PLC_γ or the Src family of kinases, is thought to underlie neurite outgrowth (71, 79). This contrasts with studies indicating that the activity of the Ras/MAPK pathway is neither necessary nor sufficient for the induction of Na^+ channel α-subunit mRNA (12, 17). Instead, the present results and the results of previous studies (11, 12, 17) together suggest that among MAPK, PLC_γ, and the Src family of kinases, the activation of at least two of these signals is needed in order for the induction of type II Na^+ channel α-subunit mRNA to occur in PC12 cells.

Given the effects of the PDGF receptor mutations on the induction of type II Na^+ channel mRNA, it was of interest to determine whether there were changes in the induction of Na^+ current density. In PC12 cells, there is a severalfold increase in Na^+ current density upon differentiation. Although for a given subclone of PC12 cells the magnitude of this induction is consistent and reproducible, there can be subclonal variation in the extent of the induction and the level of Na^+ channel expression. This may arise from variations in translation, glycosylation, subunit assembly, or other posttranscriptional events. To minimize the influence of this variation when we compared the effects of the PDGF receptor mutations, we evaluated multiple sublines for each receptor mutation in order to confirm the effect of the mutation in different PC12 backgrounds and to help ensure that any effects were not subline specific. In addition, efforts were made to evaluate the effects of a PDGF receptor mutation relative to the effects of NGF *within* the same subline, with the response to NGF effectively serving as an internal standard and indication of the potential for Na^+ channel induction in that particular subline. Analysis of the sublines expressing mutant PDGF receptors revealed that mutation of the tyrosines at positions 579 and 581, either alone (F579/581) or in combination with mutations that eliminate PI3K, GAP, PLC_γ, and Syp activation (F5/579 and F5/581), caused a significant decrease in the induction of Na^+ current density by PDGF. This finding suggests that activation of the Src family of kinases, in addition to playing a role in the induction of type II Na^+ channel mRNA, is necessary for full induction of functional Na^+ channels in PC12 cells. While the basis for the partial increase in Na^+ current density that still occurs upon activation of these receptors (F579/581, F5/579, and F5/581) is unknown, among the possibilities is the residual expression of PN-1 α subunits from the prior expression of the PN-1 gene, which appears to be regulated by mechanisms distinct from those regulating the type II α-subunit gene (11). However, we have also observed a blunted induction of Na^+ current density upon growth factor treatment of primary neurons that do not express PN-1 but lack specific members of the Src family of tyrosine kinases (15), and so the small induction may instead result from mechanisms that can only partially compensate for the loss of the Src family members. For example, even if type II α-subunit mRNA is not induced, the sustained activation of the Ras/MAPK pathway by the mutant receptors may be sufficient to cause an increase in

Na^+ current density, perhaps by influencing Na^+ channel expression at a translational or posttranslational level. Consistent with this possibility, expression of an activated form of Raf-1 (30) in PC12 cells results in sustained activation of MAPK and a minor, though significant, increase in Na^+ current density, without concomitant increases in α-subunit mRNA levels (15). Translational and posttranslational events appear to be developmentally regulated, rate-limiting steps in the expression of α subunits in the brain and retina (66, 85), and efficient expression of functional Na^+ channels appears to require subunit assembly, glycosylation, and phosphorylation (23, 67, 83; for a review, see reference 5). If activation of the Src family of kinases normally influences one of these events, the loss of this signal may decrease the induction of functional Na^+ channel expression without affecting the induction of type II α-subunit mRNA. This may explain the results from the F579/581 receptor, where the PDGF-mediated induction of type II Na^+ α-subunit mRNA occurs without a full increase in Na^+ current density, and is in accord with previous studies of PC12 cells, where in the absence of specific signals, the growth factor induction of Na^+ current density failed to occur despite an increase in type II Na^+ channel α-subunit mRNA (23).

Among the members of the Src family of tyrosine kinases known to interact with the juxtamembrane tyrosines at position 579 and 581 of the PDGF receptor are Src, Fyn, and Yes (44). These kinases are all preferentially expressed in neuronal cells, and while little is known about the role of Yes in neuronal development and differentiation, Fyn and Src appear to have biological roles, particularly in neurite outgrowth (24, 34, 43; for a review, see reference 50). In PC12 cells, v-Src activity mimics many of the responses to NGF, including the induction of Thy-1, SCG10, transin, VGF, and NGFI-A gene expression (11). However, it is not sufficient for the induction of Na^+ channel α-subunit mRNA or the expression of saxitoxin binding sites (and presumably functional Na^+ channels) in the membrane of PC12 cells (11, 62). The present study provides the first indication that activity of the Src family of kinases is necessary for the induction of Na^+ channel expression. The results are intriguing, given recent evidence that juxtamembrane sequences of the TrkA receptor are important for several Ras-independent responses, including the phosphorylation of the SNT protein (60), which can occur in response to v-Src in a Ras-independent manner (61). The results are also interesting given preliminary indication that the Yes protein may interact with the TrkA receptor (4). By indicating that activation of the Src family of kinases is necessary for full induction of neuronal Na^+ channel expression, the results invite further analysis of the individual roles of Src, Fyn, and Yes and set the stage for further analysis of the mechanisms regulating neuronal Na^+ channel expression.

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