The Tyrosine Kinase Inhibitor Tyrphostin Blocks the Cellular Actions of Nerve Growth Factor

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ABSTRACT: A series of the synthetic protein kinase inhibitors known as tyrphostins were examined for their effects on the tyrosine autophosphorylation of the pp140$^{c\text{-}trk}$, nerve growth factor (NGF) receptor. One of the tyrphostins, AG879, inhibited NGF-dependent pp140$^{c\text{-}trk}$ tyrosine phosphorylation, but did not affect tyrosine phosphorylation of epidermal growth factor or platelet-derived growth factor receptors. In addition, the tyrosine phosphorylation of the receptor-associated protein pp38 was also attenuated by the tyrphostin. This effect was time- and dose-dependent, although inhibition of pp38 phosphorylation occurred earlier and at lower concentrations of the compound. AG879 also inhibited NGF-induced PLC-γ1 phosphorylation, phosphatidylinositol-3 (PI3) kinase activation, the association of the tyrosine-phosphorylated proteins pp100 and pp110 with the p85 subunit of PI-3 kinase, mitogen activated protein and *raf*-1 kinases, and c-*fos* induction. In addition, AG879 inhibited NGF-induced neurite outgrowth in PC12 cells. These data indicate that tyrosine kinase activity of the pp140$^{c\text{-}trk}$ NGF receptor is essential for the cellular actions of this growth factor.

Nerve growth factor (NGF)[1] promotes the survival, growth, and development of sympathetic and sensory neurons. Although the precise biochemical events involved in NGF action remain unknown, numerous studies have indicated that protein phosphorylation plays a central role in the actions of this factor. NGF increases the phosphorylation of several cellular proteins (Halegoua et al., 1980; Nakanishi et al., 1985; Landreth et al., 1985) and is known to stimulate the activities of several serine/threonine kinases, including protein kinase C (Heasley et al., 1989a; Hama et al., 1986), mitogen-activated protein (MAP) kinase (Miyasaka et al., 1990), ribosomal S6 kinase (Mathuda et al., 1987), *raf*-1 kinase (Ohmichi et al., 1992b), and others (Lee et al., 1985; Rawland et al., 1987; Vulliet et al., 1989; Aletta et al., 1988; Heasley et al., 1989b). Recent evidence (Kaplan et al., 1991a; Klein et al., 1991; Ohmichi et al., 1992b) indicates that these phosphorylation events are initiated by the direct binding of NGF to its receptor, the pp140 *trk* protooncogene. This receptor is a protein tyrosine kinase, and both its activity and tyrosine autophosphorylation are increased by NGF (Kaplan et al., 1991b; Ohmichi et al., 1991b).

A number of studies have demonstrated that certain protein kinase inhibitors can potently inhibit some of the cellular actions of NGF. Staurosporine and related compounds block the stimulation by NGF of neurite outgrowth (Koizumi et al., 1988), induction of the c-*fos* protooncogene (Chan et al., 1989), and MAP kinase activity (Miyasaka et al., 1990). These inhibitory effects were due to the selective attenuation of NGF-stimulated tyrosine phosphorylation (Miyasaka et al., 1991), resulting from the direct inhibition of the tyrosine kinase activity of the pp140$^{c\text{-}trk}$ NGF receptor (Ohmichi et al., 1992a; Berg et al., 1992; Tapley et al., 1992).

Tyrphostins are a family of protein tyrosine kinase blockers that selectively inhibit receptor autophosphorylation (Lyall et al., 1989). Certain of these compounds can discriminate between receptor and nonreceptor tyrosine kinases, also selectively blocking postreceptor effects of growth factors (Margolis et al., 1989; Posner et al., 1989; Lyall et al., 1989; Yaish et al., 1988). In this report, we have evaluated a series of tyrphostins for inhibition of the tyrosine kinase activity of the NGF receptor and subsequent inhibition of NGF action.

EXPERIMENTAL PROCEDURES

Materials. ^{125}I-Labeled anti-mouse Ig (3000 Ci/mmol), ^{125}I-NGF (2000 Ci/mmol), and [α-^{32}P]dATP (6000 Ci/mmol) were from Amersham (Arlington Heights, IL). [γ-^{32}P]-ATP (3000 Ci/mmol) was from NEN (Bannockburn, IL). [^{32}P]Orthophosphate (285 Ci/mg) and Dulbecco's modified Eagle's medium without phosphate and glutamine were from ICN (Irvine, CA). NGF 2.5S was from Bioproducts for Science (Indianapolis, IN). Receptor grade EGF was from Collaborative Research (Lexington, MA). PDGF was from Bachem (Torrance, CA). Anti-phosphotyrosine and anti-p85 subunit of PI-3 kinase antisera were from Upstate Biotechnology (Lake Placid, NY). Anti-MAP kinase antiserum was from Zymed Laboratories (South San Francisco, CA). All other reagents were purchased from Sigma (St. Louis, MO) and were the highest quality available. Anti-*trk* antiserum (Ohmichi et al., 1991a,b), anti-p75 antiserum (Ohmichi et al., 1991c), and anti-EGF receptor antiserum (Decker, 1984) were developed as described previously. Anti-PDGF receptor antiserum was raised by immunizing rabbits with a trpE–PDGF receptor bacterial fusion protein containing residues 2179–2729 of human PDGF receptor (Gronwald et al., 1988).

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[1] Abbreviations: PLC, phospholipase C; GAP, GTPase activating protein of *ras*; PI, phosphatidylinositol; NGF, nerve growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; pp, phosphoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Anti-Phosphotyrosine Immunoblots. For analysis of protein tyrosine phosphorylation in whole cell lysates, PC12 cells were grown in 60-mm dishes. After treatment, cells were washed once with ice-cold phosphate-buffered saline (PBS), followed by the addition of 100 μL of Laemmli SDS sample buffer (Laemmli, 1970). Samples were heated at 95 °C for 5 min, and 30-μg protein aliquots were loaded onto 8% SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose paper and immunoblotted with anti-phosphotyrosine antiserum as described (Decker et al., 1990). In some experiments, lysates were immunoprecipitated with antiserum raised against *trk*, EGF receptor, or PDGF receptor. PC12 or swiss 3T3 cells were grown in 150-mm dishes prior to treatment. Cells were washed once with ice-cold PBS and lysed in 1 mL of 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton-X 100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 30 mM *p*-nitrophenyl phosphate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride (HNTG buffer) according to Margolis et al. (1990). Lysates were centrifuged for 10 min at 10000*g*, and supernatants were incubated for 60 min with anti-*trk* antiserum, anti-EGF receptor antiserum, or anti-PDGF receptor antiserum. Immunoprecipitates were mixed for 30 min with protein A–Sepharose beads, and immune complexes bound to the beads were washed 3 times with 1 mL of the same lysis buffer. Immune complexes were solubilized in 25 μL of Laemmli sample buffer and loaded onto 8% polyacrylamide gels.

Immunoprecipitation of Cross-Linked NGF–Receptor Complexes. PC12 cells were harvested, pelleted, and resuspended in ice-cold binding buffer (137 mM NaCl, 4.7 mM KCl, 25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mg/mL glucose, 1 mg/mL bovine serum albumin, and 10 mM HEPES, pH 7.0) containing 1 mM phenylmethanesulfonyl fluoride. After the addition of unlabeled NGF or 100 μM AG879 to the cells, ^{125}I-NGF was added at a concentration of 1 nM, and the cells were incubated on ice for 2 h. Disuccinimidyl suberate (DSS) in dimethyl sulfoxide was added at a final concentration of 150 μM, and the cells were further incubated for 30 min at room temperature. Unreacted reagents were quenched by washing the cells twice in 10 mM Tris–saline, pH 7.4. Cells were lysed in 500 μL of HNTG buffer. Lysates were centrifuged for 10 min at 10000*g*, and supernatants were incubated for 60 min with anti-*trk* antiserum or anti-p75 antiserum. Protein A–Sepharose beads were added for 30 min with mixing, and immune complexes bound to the beads were washed 3 times with 1 mL of HNTG buffer. Immune complexes were solubilized in 25 μL of Laemmli sample buffer and electrophoresed on 8% SDS–polyacrylamide gels, followed by autoradiography.

In Vitro Kinase Assays. PC12 cells were grown in 150-mm dishes. After treatment, cells were washed once with ice-cold PBS and lysed in 1 mL of HNTG buffer. Lysates were cooled to 0 °C for 5 min, followed by the addition of 40 μL of rabbit IgG–agarose. After incubation at 4 °C for 15 min, samples were centrifuged at 10000*g* for 10 min. Anti-*trk* antiserum was added to the collected supernatants, followed by incubation for 60 min. Immune complexes were then precipitated with protein A–Sepharose and washed 3 times with the same lysis buffer, followed by one wash with reaction buffer (20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, and 5 mM $MgCl_2$). Immunoprecipitates were then resuspended in 20 μL of reaction buffer, and the indicated concentration of tyrphostin was added just before the addition of 2 μL of 20 μM [γ-^{32}P]ATP (10 μCi). After incubation for 5 min at 24 °C, reactions were stopped with Laemmli sample buffer, and equal amounts of protein were electrophoresed on 8% SDS–polyacrylamide gels.

Assay of Mitogen-Activated Protein (MAP) Kinase Activity. This activity was assayed as described (Miyasaka et al., 1990). Briefly, 10-μL aliquots of cell lysates were incubated with bovine brain microtubule associated protein-2 (MAP-2) at 0.2 mg/mL for 10 min at 30 °C in a final volume of 25 μL containing 50 mM Tris-HCl (pH 7.4), 2 mM EGTA, 10 mM $MgCl_2$, and 40 μM [γ-^{32}P]ATP (1 μCi). The reaction was stopped by the addition of Laemmli SDS sample buffer, and phosphorylated MAP-2 was resolved by 7.2% SDS–PAGE. Commassie blue-stained phospho-MAP-2 was excised from the gels, and incorporated radioactivity was measured by Cerenkov counting.

^{32}P Labeling of MAP Kinase. PC12 cells were grown in 60-mm dishes. Cells were washed once with phosphate-free DMEM medium and incubated for 2 h in phosphate-free DMEM with 1 mCi of [^{32}P]orthophosphate. After treatment, cells were washed once with ice-cold PBS before the addition of 100 μL of boiling 1% SDS, and 10 mM Tris-HCl, pH 7.4. Lysates were heated for 5 min at 100 °C and diluted 1:10 with ice-cold HNTG buffer. Lysates were cooled to 0 °C for 5 min, followed by the addition of 40 μL of rabbit IgG–agarose. After incubation at 4 °C for 15 min, samples were centrifuged at 10000*g* for 10 min. The supernatant was then incubated at 4 °C for 60 min with anti-MAP kinase antiserum. Immunocomplexes were precipitated with protein G/protein A–agarose and washed 3 times with HNTG buffer. Isolated proteins were analyzed by electrophoresis on 8% SDS–PAGE. Phosphoamino acid analysis was performed as previously described (Decker, 1984).

Northern Blot Analysis. Total cellular RNA was isolated from PC12 cells by guanidinium isothiocyanate treatment followed by centrifugation in a cushion of CsCl as described (Glisin et al., 1977). Ten micrograms of RNA was electrophoresed in 1% agarose containing 2.2 M formaldehyde. After being transferred to a nitrocellulose filter (Thomas, 1980), RNA was hybridized to radioactive v-*fos* probe.

Chemical Synthesis of Inhibitors. The compounds are numbered according to their notation in Table I and are arranged in ascending order. Stock solutions were prepared in DMSO and were directly added. Workup means adding water to the reaction mixture and extracting it with $CHCl_3$ (or ethyl acetate for polar compounds), washing the organic phase to neutrality, drying on $MgSO_4$, filtering, and evaporating the phase to dryness. All compounds were fully characterized spectroscopically (^{1}H NMR and MS). Chemical shifts are in ppm relative to a TMS internal standard.

Tyrphostin tyrosine kinase inhibitors *AG17*, *AG18*, *AG126*, *AG528*, and *AG561* were synthesized as described previously (Gazit et al., 1989, 1991).

AG1024 was prepared as described previously (Birchall and Harney, 1978): NMR ($CDCl_3$) 7.93 (1 H, d, J = 2.3 Hz), 7.91 (1 H, d, J = 2.3 Hz), 7.60 (1 H, s, vinyl), 6.55 (1 H, s, OH), 1.42 (9 H, s); MS *m/e* 306, 304 (M^+, 34, 36), 291, 289 ($M - CH_3$, 90, 88), 210 ($M - Br - CH_3$, 100), 195 ($M - Br - 2CH_3$, 45).

3,4-Dihydroxy-5-methoxybenzaldehyde. 5-Iodovanillin (1.4 g, 5 mmol) and 1 g of $CuSO_4 \cdot 5H_2O$ in 30 mL of 4 N aqueous NaOH were refluxed for 16 h. Workup (HCl, EtOAc) and chromatography gave 0.36 g (42% yield) of a white solid: mp 128 °C; NMR (acetone-d_6) δ 9.77 (1 H, s, CHO), 7.11, 7.09 (2 H, 2d, J = 1.8 Hz), 3.91 (3 H, s, OCH_3);

MS *m/e* 168 (M^+, 100), 167 (83), 139 (M – CHO, 13), 125 (36), 97 (45).

AG34 was prepared from the aldehyde as described in Gazit et al. (1989) in 73% yield.

AG879. 3,5-Di-*tert*-butyl-4-hydroxybenzaldehyde (0.47 g, 2 mmol), 0.2 g (2.4 mmol) of thiocyanoacetamide and 30 mg of β-alanine in 40 mL of EtOH were refluxed for 6 h. Water and HCl were added, and the reaction mixture was extracted with EtOAc. Evaporation gave 0.34 g (54% yield) of a white solid: mp 210 °C; NMR (acetone-d_6) δ 8.47 (1 H, s, vinyl), 8.02 (2 H, s), 1.48 (18 H, s); MS *m/e* 316 (M^+, 100), 303 (35), 301 (99), 268 (16), 260 (M – $(CH_3)_2$=C, 45), 245 (17), 228 (22), 219 (52), 203 (10), 143 (11), 129 (11).

(3-Methoxy-4-hydroxy-5-iodobenzylidene)malononitrile (AG963). To 1.4 g (5 mmol) of 5-iodovanillin and 0.4 g (6 mmol) of malononitrile in 25 mL of ethanol was added 3 drops of piperidine, and the reaction mixture was refluxed for 4 h. Workup gave 0.8 g (49% yield) of a yellow solid: mp 188 °C; NMR ($CDCl_3$) δ 7.76 (1 H, *J* = 1.8 Hz, H_6), 7.65 (1 H, d, *J* = 1.8 Hz, H_2), 7.56 (1 H, s, vinyl), 6.85 (1 H, s, OH), 3.99 (3 H, s, OCH_3); MS *m/e* 327 (13), 326 (M^+, 100), 283 (18), 128 (35), 101 (22).

AG974. AG963 (0.65 g, 2 mmol) and 0.6 mL (6 mmol) of BBr_3 in 40 mL of CH_2Cl_2 were stirred under argon for 1 h at room temperature. Water was added, and the reaction mixture was extracted with EtOAc to give 0.46 g (73% yield) of a light-red solid (yellow in solution): mp 105 °C; NMR (acetone-d_6) δ 8.03 (1 H, s, vinyl), 7.88 (1 H, d, *J* = 2.3 Hz, H_2), 7.72 (1 H, d, *J* = 2.3 Hz, H_6); MS *m/e* 312 (M^+, 38), 254 (74), 185 (M – I, 27), 158 (M – I – HCN, 11), 157 (64), 130 (19), 129 (23), 127 (100).

N-(Cyanoacetyl)thiobenzamide. N-(Cyanoacetyl)benzamide (1.05 g, 6 mmol) and 2.5 g of Lawson reagent in 40 mL of toluene were refluxed for 3 h under N_2. Evaporation and chromatography gave 0.52 g (45% yield) of a white solid: mp 87 °C; NMR ($CDCl_3$) δ 7.37 (5 H, m), 4.85 (2 H, d, *J* = 7.0 Hz), 3.96 (2 H, s, CH_2CN); MS *m/e* 191 (24), 190 (M^+, 100).

AG1007. The preceding thio amide (0.26 g, 1.4 mmol) 0.19 g (1.4 mM) of 3,4-dihydroxybenzaldehyde, and 15 mg of β-alanine in 30 mL of ethanol were refluxed for 4 h. Workup with ethyl acetate gave an oily solid. Trituration with CH_2Cl_2/C_6H_6 gave 0.27 g (64% yield) of a yellow solid: mp 195 °C; NMR (acetone-d_6) δ 8.24 (1 H, s, vinyl), 7.69 (1 H, d, *J* = 2.2 Hz, H_2), 7.45–7.28 (6 H, m), 6.93 (1 H, d, *J* = 8.3 Hz, H_5), 5.06 (2 H, s, CH_2N); MS *m/e* 310 (M^+, 25), 293 (M – OH, 35), 172 (M – SH – $NHCH_2C_6H_5$, 15), 123 (15), 106 (55), 91 (100).

3-tert-Butyl-4-hydroxybenzaldehyde. To 50 g of 2-*tert*-butylphenol and 60 g of NaOH in 60 mL of H_2O and 25 mL of methanol was added 50 mL of chloroform in small portions for 40 min. The color changed to green and then to red, and the reaction was exothermic. The reaction mixture was stirred for 1.5 h at 50 °C, acidified with HCl, and extracted with CH_2Cl_2. Workup gave a red oil, which was chromatographed to give a clear oil. Crystallization with benzene/hexane gave 5.5 g (9% yield) of a pink-white solid: mp 135 °C (lit. mp 142 °C; Birchall & Harney, 1978; Katsumi et al., 1986); NMR ($CDCl_3$) δ 9.85 (H, s, CHO), 7.85 (1 H, d, *J* = 2.0 Hz, H_2), 7.65 (1 H, dd, *J* = 8.2, 2.0 Hz, H_6), 6.85 (1 H, d, *J* = 8.2 Hz, H_5), 6.43 (1 H, s, OH), 1.44 (9H, s).

3-tert-Butyl-4-hydroxy-5-iodobenzaldehyde. 3-*tert*-Butyl-4-hydroxybenzylaldehyde (1.1 g, 6.1 mmol) and 0.4 g of NaOH in 20 mL of H_2O and 20 mL of ethanol were stirred at room temperature. Four grams of I_2 and 4 g of KI were added. After 0.5 h excess iodine was reduced with sodium thiosulfate. Workup with CH_2Cl_2 gave a reddish solid, mainly product as determined by NMR. Chromatography gave 0.9 g, (48% yield) of a pinkish white solid: mp 115 °C (lit. mp 124 °C; Birchall & Harney, 1978); NMR ($CDCl_3$) δ 9.79 (1 H, s, CHO), 8.08 (1 H, d, *J* = 1.9 Hz, H_2), 7.79 (1 H, d, *J* = 1.9 Hz, H_6), 6.10 (1 H, s, OH), 1.42 (9 H, s).

3-tert-Butyl-4,5-dihydroxybenzaldehyde. 3-*tert*-Butyl-4-hydroxy-5-iodobenzaldehyde (0.7 g, 2.3 mM), 0.5 g of $CuSO_4$, and 2.4 g of NaOH in 15 mL of H_2O were refluxed for 14 h under argon. HCl was added, and the reaction was worked up with EtOAc. Evaporation gave 0.2 g (45% yield) of a gray-white solid: mp 189 °C; NMR (acetone-d_6) δ 9.77 (1 H, s, CHO), 7.41 (1 H, d, *J* = 1.9 Hz), 7.30 (1 H, d, *J* = 1.9 Hz), 1.44 (9 H, s); MS *m/e* 194 (M^+, 47), 180 (47), 151 (59).

AG1034. To 95 mg (0.5 mmol) of AG1033 and 50 mg (0.75 mmol) of malononitrile in ethanol was added 1 drop of piperidine, and the reaction mixture was refluxed for 4 h. Workup (HCl, EtOAc) gave, after flash chromatography, 70 mg (62% yield) of a yellow-brown solid: mp 195 °C; NMR (acetone-d_6) δ 8.04 (1 H, s, vinyl) 7.60 (1 H, d, *J* = 2.1 Hz), 7.55 (1 H, d, *J* = 2.1 Hz), 1.43 (9 H, s); MS *m/e* 242 (M^+, 65), 227 (M – CH_3, 100), 199 (M – CH_3 – CO, 35), 171 (50).

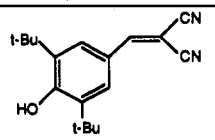
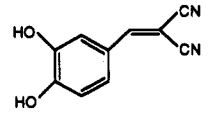
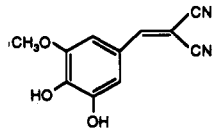
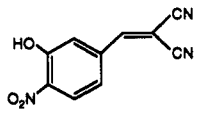
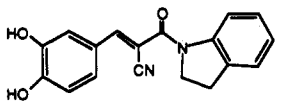
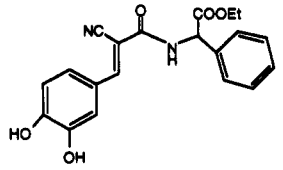
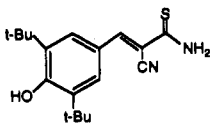
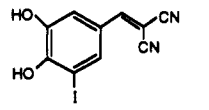
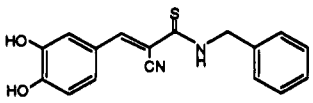
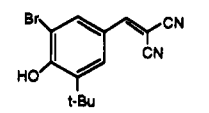
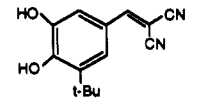
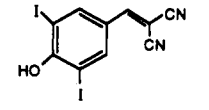
3,5-Diiodo-4-hydroxybenzaldehyde. 4-Hydroxybenzaldehyde (1.8 g, 1.5 mmol), 1 g of NaOH, 10 g of iodine, and 6.6 g of KI in 40 mL of H_2O were heated at 60 °C for 3 h. Workup ($Na_2S_2O_4$, HCl, and CH_2Cl_2) and chromatography gave two compounds. (a) 3,5-Diiodo-4-hydroxybenzaldehyde, 1.03 g (19% yield), a white solid: mp 197 °C; NMR ($CDCl_3$) δ 9.74 (1 H, s, CHO), 8.20 (2 H, s), 6.28 (s, OH); MS *m/e* 374 (M^+, 100), 373 (93), 218 (M – I – CHO, 42), 127 (30), 119 (M – H 2I, 80), 92 (75), 91 (97). (b) 3-Iodo-4-hydroxybenzaldehyde, 0.55 g (16% yield), a white solid: mp 98 °C; NMR ($CDCl_3$) δ 9.80 (1 H, s, CHO), 8.22 (1 H, d, *J* = 2.2 Hz, H_2), 7.80 (1 H, dd, *J* = 8.2, 2.2 Hz, H_6), 7.11 (1 H, d, *J* = 8.2 Hz, H_5), 6.30 (1 H, s, OH); MS *m/e* 248 (M^+, 100), 247 (95), 219 (215), 92 (22).

AG1049. To 165 mg (0.5 mmol) of 3,5-diiodo-4-hydroxybenzaldehyde and 70 mg (1.06 mmol) of malononitrile in 30 mL of ethanol was added 1 drop of piperidine, and the reaction mixture was refluxed for 3 h. Workup (HCl, CH_2Cl_2) gave 165 mg (89% yield) of a yellow solid: mp 195 °C; NMR ($CDCl_3$) δ 8.26 (2 H, s), 7.50 (1 H, s, vinyl); MS *m/e* 422 (M^+, 100), 295 (M – I, 18), 168 (M – 2I, 50), 140 (27), 113 (20).

RESULTS

Tyrphostin AG879 Inhibits Autophosphorylation of the NGF Receptor and NGF-Dependent Cellular Tyrosine Phosphorylation. Tyrphostins selectively inhibit the tyrosine kinase activity of growth factor receptors in vitro (Yaish et al., 1988). To evaluate the sensitivity of the $pp140^{c\text{-}trk}$ NGF receptor to these compounds, PC12 cells were pretreated with a number of tyrphostins at a concentration of 100 μM. Twelve compounds, AG17, AG18, AG34, AG126, AG528, AG561, AG879, AG974, AG1007, AG1024, AG1034, and AG1049, shown in Table I, were evaluated. Each compound was added to cells for 10 min prior to the addition of 100 nM NGF for 1 min. Following the treatment, lysates were precipitated with anti-*trk* antiserum (Figure 1A). Tyrosine-phosphorylated proteins were detected by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antiserum. In confirmation of previous findings (Ohmichi et al., 1991b, 1992c), NGF stimulated the tyrosine phosphorylation of $pp140^{c\text{-}trk}$ and the receptor-associated 38-kDa protein. Two

Table I

AG no.	structure	mol form	MW
17		$C_{18}H_{22}N_2O$	282
18		$C_{10}H_6N_2O_2$	186
34		$C_{11}H_8N_2O_3$	216
126		$C_{10}H_5N_3O_3$	215
528		$C_{18}H_{14}N_2O_3$	306
561		$C_{21}H_{20}N_2O_5$	380
879		$C_{18}H_{24}N_2SO$	316
974		$C_{10}H_5IN_2O_2$	312
1007		$C_{17}H_{14}N_2SO_2$	310
1024		$C_{14}H_{13}N_2BrO$	305
1034		$C_{14}H_{14}N_2O_2$	232
1049		$C_{10}H_4N_2I_2O$	422

of the tyrphostins, AG528 and AG561, partially attenuated the tyrosine phosphorylation of pp38, but had no significant effect on the receptor. Only one of the tyrphostins, AG879, significantly inhibited the NGF-stimulated tyrosine phosphorylation of both pp140$^{c\text{-}trk}$ and pp38. In contrast, addition of 100 μM AG879 to PC12 cells (Figure 1B) or swiss3T3 cells (Figure 1C) had no effect on the EGF-dependent tyrosine phosphorylation of the EGF receptor or the PDGF-dependent tyrosine phosphorylation of the PDGF receptor.

To examine the inhibition by AG879 of NGF-induced tyrosine phosphorylation of pp140$^{c\text{-}trk}$ and pp38 in more detail, the time course of kinase inhibition was examined (Figure 2). Some reduction in receptor phosphorylation was observed after a 10-min incubation, although pretreatment of cells with 100 μM AG879 for 90 min completely inhibited NGF-induced tyrosine phosphorylation of pp140$^{c\text{-}trk}$. Interestingly, the attenuation of pp38 phosphorylation was observed after only 10 min of pretreatment.

To evaluate the dose dependence of this effect, PG12 cells were pretreated with increasing concentrations of AG879 for 90 min before the addition of NGF, followed by lysis and evaluation of tyrosine-phosphorylated pp140$^{c\text{-}trk}$ and pp38, as described above (Figure 3). Inhibition of the tyrosine phosphorylation of pp140$^{c\text{-}trk}$ and pp38 by this compound was dose dependent, with an EC_{50} of approximately 10 μM for inhibition of receptor phosphorylation. Receptor phosphorylation was almost completely blocked by 50 μM AG879. As described above for the time course, pp38 phosphorylation was more sensitive to the inhibitor than was that of the receptor. Tyrosine phosphorylation of this protein was not detectable after incubation with as little as 5 μM AG879.

To check whether AG879 inhibits NGF binding, PC12 cells were exposed to ^{125}I-NGF in the presence of unlabeled NGF or 100 μM AG879, followed by cross-linking and immunoprecipitation with anti-*trk* antiserum or anti-p75 antiserum (Figure 4). As previously reported (Ohmichi et al., 1991b), the specific displaceable binding of ^{125}I-NGF to pp140$^{c\text{-}trk}$ and p75 is detected on SDS–PAGE, with a labeled band migrating at approximately 160 kDa, reflecting the 140-kDa receptor and the 22-kDa ligand (Figure 4A), or 97 kDa, reflecting the 75-kDa receptor and the 22-kDa ligand (Figure 4B). Under these conditions, 100 μM AG879 had no effect on the binding of ^{125}I-NGF to pp140$^{c\text{-}trk}$ or p75. In addition, Scatchard analysis of ^{125}I-NGF binding to 3T3 cells expressing pp140$^{c\text{-}trk}$ (Ohmichi et al., 1992b) revealed no effect of AG879 on binding affinity or capacity (not shown).

To examine the effect of AG879 on cellular tyrosine phosphorylation induced by NGF and EGF, whole cell lysates from treated PC12 cells were analyzed by SDS–PAGE followed by immunoblotting with antiphosphotyrosine antiserum (Figure 5). As described previously (Miyasaka et al., 1991), NGF caused the tyrosine phosphorylation of a number of proteins, including two predominant proteins of molecular mass 40 and 42 kDa, as well as bands at 140 and 55 kDa (Figure 5, lane 2). Preincubation of cells with AG879 for only 90 min inhibited these tyrosine phosphorylations induced by NGF (Figure 5, lane 3). Although EGF also caused the tyrosine phosphorylation of a number of proteins, including two predominant proteins of molecular mass 40 and 42 kDa, as well as bands at 170 and 55 kDa (Figure 5, lane 5), AG879 was without an inhibitory effect (Figure 5, lane 6).

AG879 Inhibits the Stimulation of MAP Kinase Activity by NGF. One of the most rapid and well characterized responses to NGF in PC12 cells is the stimulation of MAP kinase activity (Miyasaka et al., 1990), resulting from the phosphorylation of the protein on tyrosine and serine or threonine residues due to the stimulation of an activating tyrosine/threonine kinase (Kosako et al., 1992). The effect of AG879 on NGF-induced MAP kinase activity was evaluated in PC12 cells and 3T3 cells expressing the *trk* protooncogene (Ohmichi et al., 1992b) (Figure 6). Exposure of PC12 cells to 100 μM compound alone caused a 50% increase in MAP kinase activity assayed in cell lysates. This increase in activity was not observed with other tyrphostins in other cell types. Moreover, direct addition of AG879 to the lysate had no effect on MAP kinase activity (not shown). Addition of EGF to PC12 cells in the presence of vehicle alone caused a 2-fold

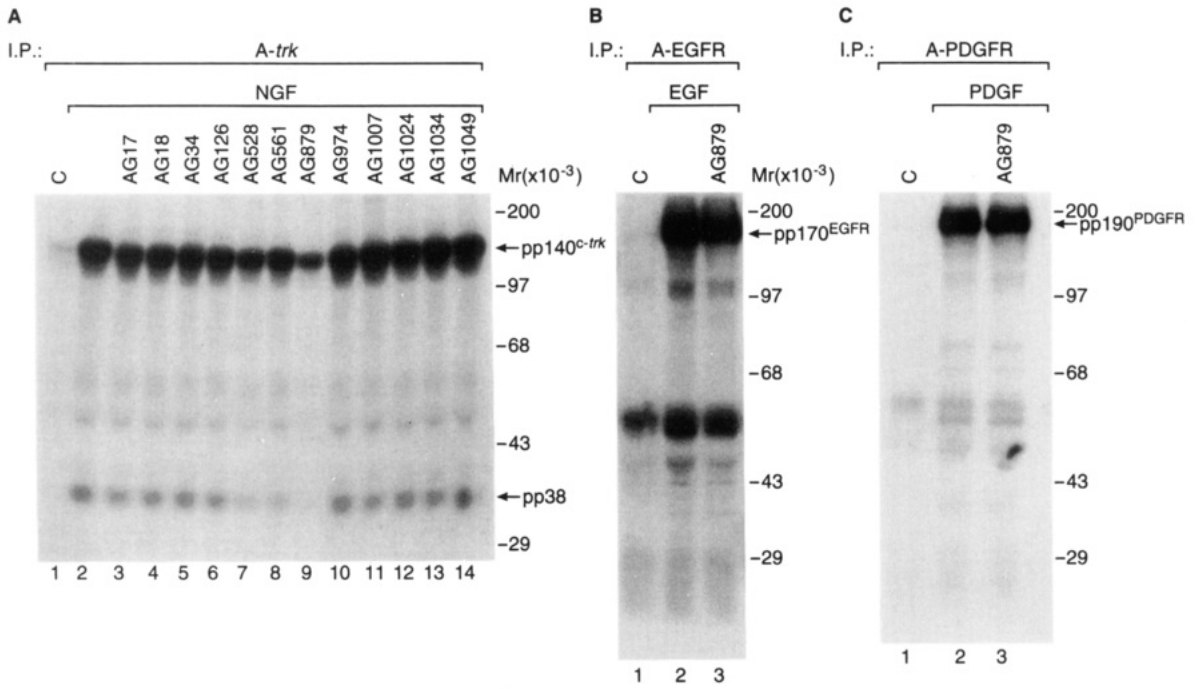


FIGURE 1: AG879 inhibition of the NGF-dependent tyrosine phosphorylation of pp140$^{c\text{-}trk}$ and pp38 in PC12 cells. PC12 cells were grown in 150-mm dishes. (A) After preincubation without tyrphostins (lane 2) or with 100 μM tyrphostin AG17, AG18, AG34, AG126, AG528, AG561, AG879, AG974, AG1007, AG1024, AG1034, or AG1049 (lanes 3–14) for 10 min, PC12 cells were treated for 1 min with 100 nM NGF (lanes 2–9). Lysates from PC12 cells were immunoprecipitated with anti-*trk* antiserum, and immunoprecipitates were subjected to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antiserum as described in Experimental Procedures. (B and C) After preincubation with (lane 3) or without (lanes 1 and 2) 100 μM AG879 for 10 min, PC12 (B) or swiss3T3 cells (C) were treated for 1 min with 10 nM EGF (B) or 10 nM PDGF (C) (lanes 2 and 3). Lysates from cells were immunoprecipitated with anti-EGF receptor antiserum (B) or anti-PDGF receptor antiserum (B), and immunoprecipitates were subjected to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antiserum.

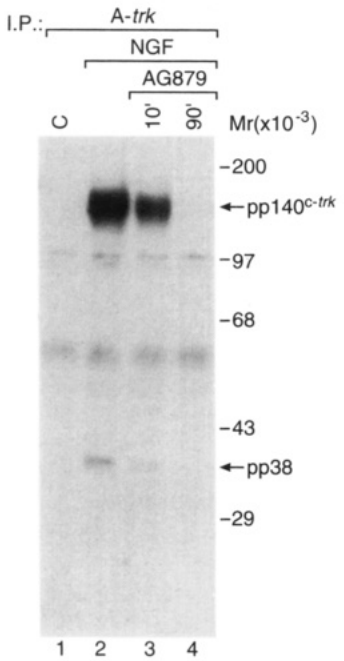


FIGURE 2: Time course of inhibition of NGF-stimulated pp140$^{c\text{-}trk}$ tyrosine phosphorylation by AG879. PC12 cells were grown in 150-mm dishes. After preincubation without AG879 (lanes 1 and 2) or with 100 μM AG879 for 10 min (lane 3) or 90 min (lane 4), PC12 cells were treated for 1 min with 100 nM NGF (lanes 2–4). Lysates from PC12 cells were immunoprecipitated with anti-*trk* antiserum, and immunoprecipitates were subjected to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antiserum as described in Figure 1.

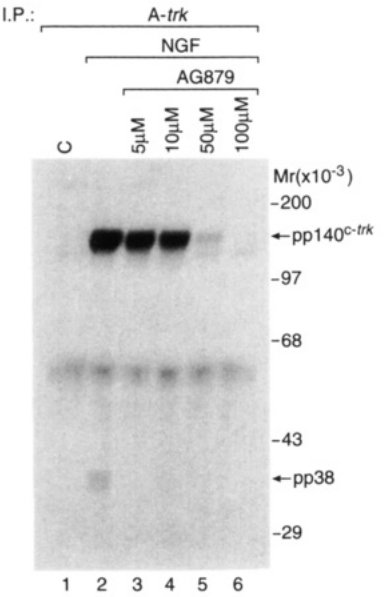


FIGURE 3: Dose dependence of inhibition of NGF-stimulated pp140$^{c\text{-}trk}$ tyrosine phosphorylation by AG879. PC12 cells were grown in 150-mm dishes. After preincubation without AG879 (lanes 1 and 2) or with the indicated concentrations of AG879 (lanes 3–6) for 90 min, PC12 cells were treated for 1 min with 100 nM NGF (lanes 2–6). Lysates from PC12 cells were immunoprecipitated with anti-*trk* antiserum, and immunoprecipitates were subjected to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antiserum as described in Figure 1.

increase in activity, while pretreatment with AG879 had no effect on stimulation by EGF. In contrast, the stimulation observed with NGF was totally blocked by only a 10-min preincubation with the inhibitor. In 3T3-c-*trk* cells, AG879 had no effect on MAP kinase when added alone (Figure 6B). EGF was also ineffective in these cells, which do not express EGF receptors (Ohmichi et al., 1992b). In contrast, pretreatment with 100 μM AG879 for 10 min completely

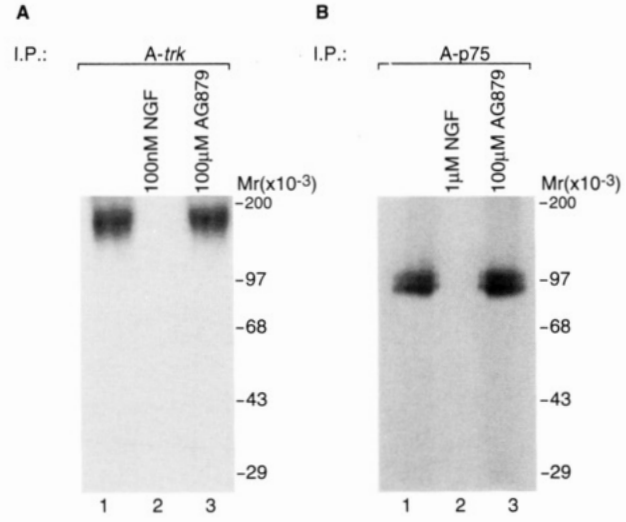


FIGURE 4: Lack of AG879 inhibition of NGF binding. PC12 cells were harvested, pelleted, and resuspended in ice-cold binding buffer. Cells were incubated with 100 nM (A, lane 2) or 1 μM (B, lane 2) unlabeled NGF or 100 μM AG879 (A and B, lane 3), followed by addition of 1 nM ^{125}I-NGF. Receptors were cross-linked with DSS, as described in Experimental Procedures. Following the cross-linking reaction, cells were lysed and precipitated with anti-*trk* antiserum (A) or anti-p75 antiserum (B). The resulting immunoprecipitates were electrophoresed on 8% polyacrylamide gels, followed by autoradiography.

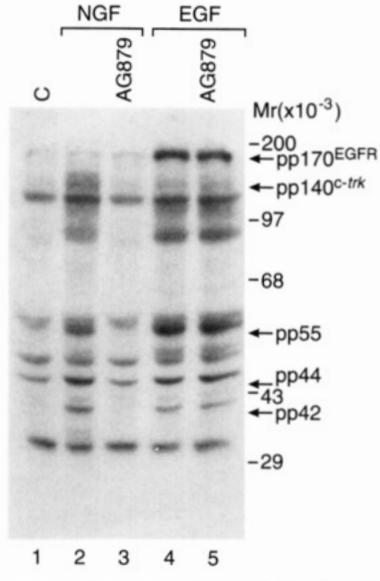


FIGURE 5: AG879 inhibition of NGF-stimulated protein tyrosine phosphorylation in whole cell lysates from PC12 cells. PC-12 cells were grown in 60-mm dishes. After preincubation without AG879 (lanes 1, 2, and 4) or with 100 μM AG879 for 90 min (lanes 3 and 5), PC12 cells were treated for 1 min with 100 nM NGF (lanes 2 and 3) or 10 nM EGF (lanes 4 and 5). Thirty micrograms of protein from each whole cell lysate was analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antiserum as described in Figure 1.

abolished the stimulation of MAP kinase caused by NGF in 3T3-c-*trk* cells. These results indicate that other kinases in the MAP kinase cascade, also stimulated by EGF, are not directly inhibited by this tyrphostin. Moreover, in separate experiments we observed that 100 μM AG879 completely abolished the NGF-dependent activation of the *raf*-1 kinase without inhibiting stimulation of the kinase by EGF (data not shown). These data indicate that attenuation of MAP and *raf*-1 kinase activation by NGF reflects inhibition of the receptor tyrosine kinase.

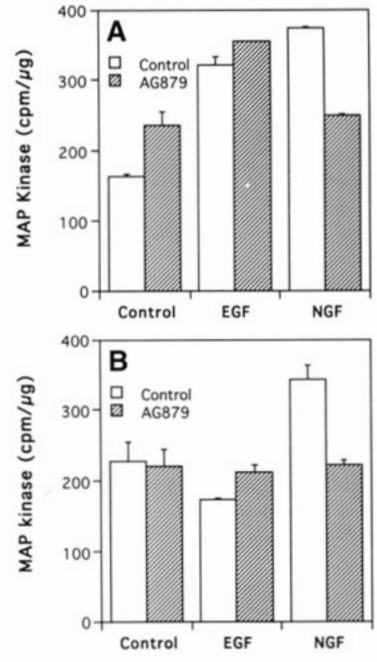


FIGURE 6: AG879 inhibition of the stimulation of MAP kinase activity by NGF but not by EGF. PC12 (A) and 3T3-c-*trk* (B) cells were grown in 60-mm dishes and incubated in serum-free media for 1 h. After preincubation with or without 100 μM AG879 for 10 min, cells were treated for 5 min with 10 nM NGF or 10 nM EGF. Lysates were prepared and assayed for phosphorylation of microtubule associated protein-2, as detailed in Experimental Procedures. Results are the means of triplicate determinations and are expressed as cpm ^{32}P incorporated into MAP-2/μg lysate protein ± SD. Identical results were obtained in six separate experiments.

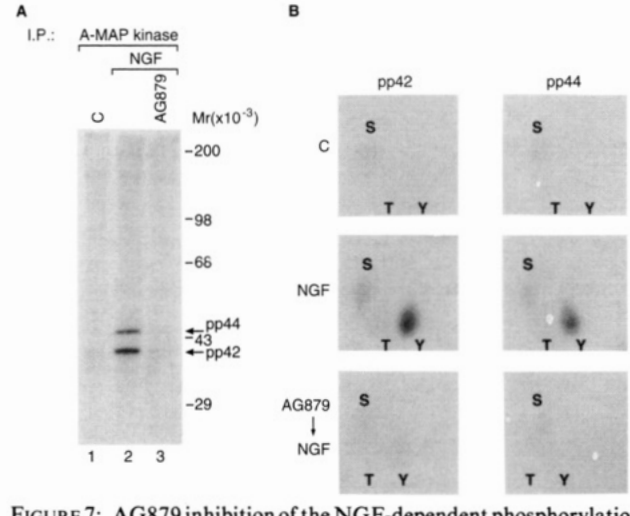


FIGURE 7: AG879 inhibition of the NGF-dependent phosphorylation of MAP kinase. (A) PC12 cells were grown in 60-mm dishes. Cells were labeled with 1 mCi of [^{32}P]orthophosphate for 2 h. After preincubation without AG879 (lanes 1 and 2) or with 100 μM AG879 (lane 3) for 10 min, PC12 cells were treated for 1 min with 100 nM NGF (lanes 2 and 3). Lysates from PC12 cells were subsequently immunoprecipitated with anti-MAP kinase antiserum and subjected to SDS–PAGE followed by autoradiography. (B) The phosphorylated 42- and 44-kDa bands were excised and subjected to phosphoamino acid analysis as described in Experimental Procedures.

To further explore the effect of AG879 on the NGF-dependent phosphorylation of MAP kinase, PC12 cells were labeled with [^{32}P]orthophosphate for 2 h prior to treatment (Figure 7A). Cells were subsequently immunoprecipitated with anti-MAP kinase antiserum and subjected to SDS–PAGE followed by autoradiography. NGF caused the rapid phosphorylation of the 42- and 44-kDa MAP kinase proteins. Preincubation of cells with AG879 blocked this effect.

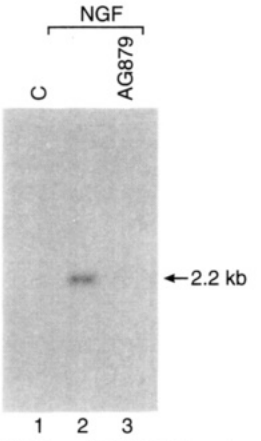


FIGURE 8: AG879 inhibition of NGF-induced expression of the c-*fos* protooncogene in PC12 cells. After preincubation without AG879 (lanes 1 and 2) or with 100 μM AG879 (lane 3) for 10 min, PC12 cells were treated for 30 min with 100 nM NGF (lanes 2 and 3). Ten micrograms of total cellular RNA was extracted and electrophoresed in 2.2 M formaldehyde/1% agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled v-*fos* probe.

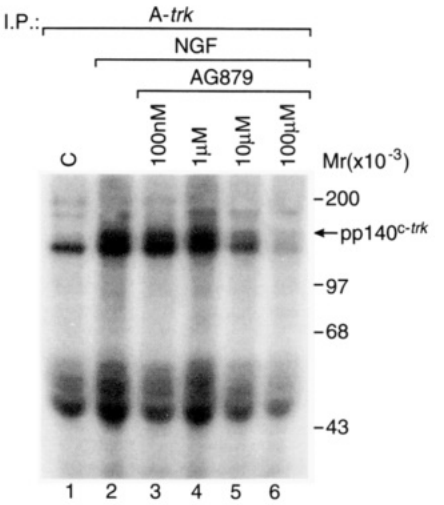


FIGURE 9: Direct inhibition of the tyrosine kinase activity of pp140$^{c\text{-}trk}$ by AG879. PC12 cells were grown in 150-mm dishes. Cells were incubated with (lanes 2–6) or without (lane 1) NGF for 1 min, lysed in 1 mL of HNTG buffer, and subjected to immunoprecipitation with anti-*trk* antiserum as described in Experimental Procedures. Immunoprecipitates were then resuspended in 20 μL of buffer (HEPES, $MnCl_2$, and $MgCl_2$) without AG879 (lanes 1 and 2) or with the indicated concentrations of AG879 (lanes 3–6), before the addition of 20 μM of [γ-^{32}P]ATP (10 μCi). After incubation for 5 min at 24 °C, reactions were stopped, and phosphoproteins were analyzed on 8% SDS–PAGE, followed by autoradiography.

Phosphoamino acid analysis of the excised band phosphorylated in response to NGF revealed the presence of phosphotyrosine, phosphoserine, and a trace of phosphothreonine (Figure 7B). Preincubation of cells with AG879 inhibited the NGF-induced phosphorylation of all three residues.

We have previously reported that NGF binding to the pp140$^{c\text{-}trk}$ receptor stimulates its association with the SH2 domains of PLC-γ1 (Ohmichi et al., 1991b). Incubation of both PC12 and 3T3-c-*trk* cells with 100 μM AG879 completely prevented the tyrosine phosphorylation of PLC-γ1 and its subsequent association with the NGF receptor and pp38 (data not shown). Although the NGF receptor does not form a stable complex with the GTPase activating protein of *ras* (Ohmichi et al., 1991b), the growth factor can stimulate PI-3 kinase activity and tyrosine phosphorylation of two proteins (pp100 and pp110) that specifically bind to the p85 subunit of PI-3 kinase (Ohmichi et al., 1992d). Preincubation of cells with 100 μM AG879 totally prevented the NGF-induced increase in PI-3 kinase activity and association of pp100 and pp110 with the p85 subunit of PI-3 kinase (data not shown).

AG879 Blocks Induction of the c-fos Protooncogene by NGF. One of the earliest responses to NGF treatment in PC12 cells is the transient induction of the c-*fos* protooncogene (Greenberg et al., 1985; Curran et al., 1985; Kruijer et al., 1985; Milbrandt, 1986). We examined the effect of AG879 on NGF-induced c-*fos* expression. PC12 cells were treated with 100 μM AG879 for 10 min prior to addition of 100 nM NGF for 30 min. Total RNA was prepared, and steady-state levels of c-*fos* mRNA were examined by Northern blot analysis (Figure 8). As previously described (Chan et al., 1989), the 2.2-kb c-*fos* mRNA was detectable after a 30-min incubation with NGF (Figure 8, lane 2). The addition of 100 μM AG879 to cells completely blocked NGF-induced c-*fos* expression (Figure 8, lane 3).

AG879 Directly Inhibits the Autophosphorylation of pp140$^{c\text{-}trk}$. To definitively demonstrate the inhibition of the tyrosine autophosphorylation of the pp140$^{c\text{-}trk}$ kinase activity by AG879, we examined the effect of AG879 in an in vitro phosphorylation reaction in anti-pp140$^{c\text{-}trk}$ immunoprecipitates from PC12 cells (Figure 9). PC12 cells were treated with NGF, and receptors were immunoprecipitated with anti-*trk* antiserum prior to incubation with [γ-^{32}P]ATP, in the presence of increasing concentrations of AG879. AG879 dose-dependently inhibited the NGF-dependent tyrosine autophosphorylation of pp140$^{c\text{-}trk}$, with an IC_{50} of approximately 10 μM.

AG879 Inhibits NGF-Induced Neurite Outgrowth. Inhibition of the tyrosine kinase activity of *trk* proteins by staurosporine and related compounds (Ohmichi et al., 1992a) can block NGF-dependent neurite outgrowth in PC12 cells (Koizumi et al., 1988). To evaluate the effect of AG879 on NGF-induced neurite outgrowth, PC12 cells were treated with 1 or 10 nM AG879 for 10 min before the addition of NGF for 3 days (Figure 10). As little as 10 nM AG879 almost completely inhibited NGF-induced neurite outgrowth, without significantly altering the viability of cells. Although the differentiative effects of NGF occur over a longer time course than the early phosphorylation events, NGF-induced neurite outgrowth was more sensitive to inhibition by AG879 than the receptor tyrosine kinase.

DISCUSSION

Tyrphostins are a series of protein tyrosine kinase inhibitors that were originally modeled after the microbial inhibitor erbstatin (Yaish et al., 1988). These compounds were derived from the benzylidene malononitrile nucleus, resembling the phenolic group of tyrosine, with additional substituents directed to increase biological activity. Some of these substitutions resulted in significant discrimination among tyrosine kinases (Levitzki et al., 1992), even within a subfamily of normal and transforming oncogenic tyrosine kinases (Anafi et al., 1992). These reported differences in specificity led us to explore the inhibition by selected tyrphostins of the NGF receptor tyrosine kinase. We have surveyed a variety of compounds representing a range of structures. One particular tyrphostin, AG879, proved to be most effective in blocking the *trk* tyrosine autophosphorylation and subsequent actions of NGF. Interestingly, this compound appeared to be more effective in blocking the tyrosine phosphorylation of the receptor-associated protein pp38 than the autophosphorylation of the receptor itself. Moreover, phosphorylation of PLC-γ1, MAP kinase, and *raf*-1 kinase and activation of PI-3 kinase were

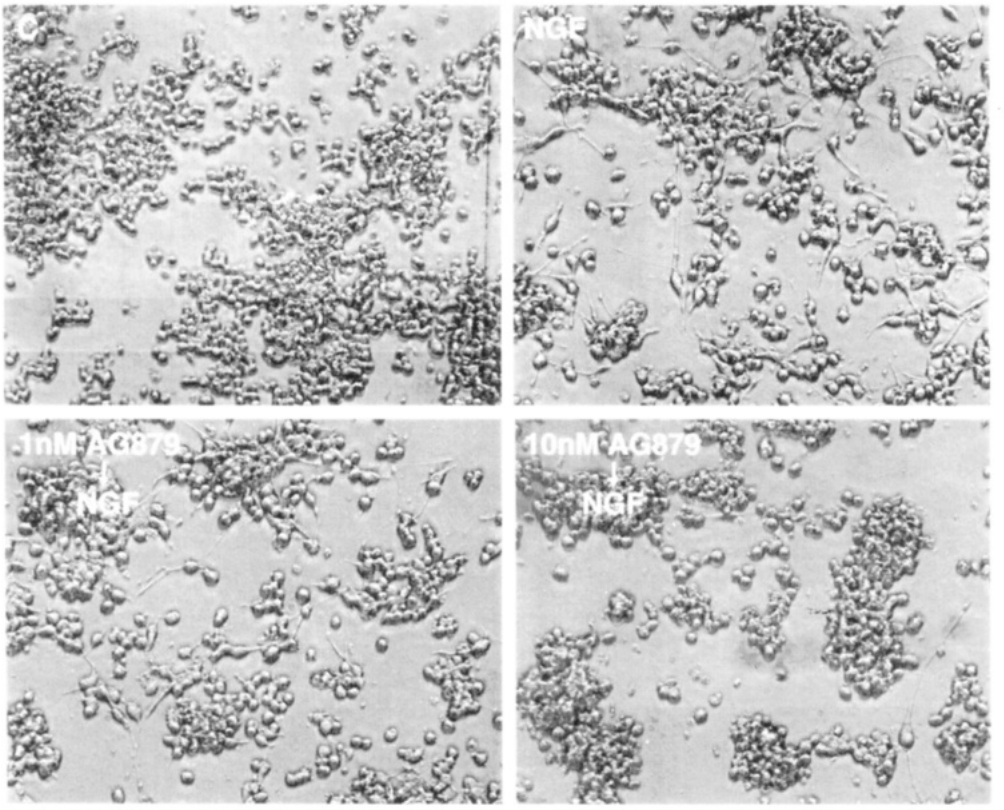


FIGURE 10: AG879 inhibition of NGF-induced neurite outgrowth. PC12 cells were treated with 1 nM (C) or 10 nM (D) AG879 for 10 min before the addition of 5 nM NGF (B–D) for 3 days. Cells were photographed using a phase-contrast light microscope.

also completely inhibited after a 10-min preincubation, whereas significant inhibition of the receptor autophosphorylation was only observed after a 90-min incubation. The increased sensitivity of postreceptor events to inhibition by tyrphostin suggests that AG879 can indeed compete for the substrate binding site. Moreover, assays in the same cells revealed that AG879 had no effect on the kinase activity or autophosphorylation of EGF receptor, even when used at concentrations that almost completely blocked NGF receptor function. Although the molecular basis for this specificity is unclear, discrimination among tyrosine kinases has been observed for other tyrphostins (Yaish et al., 1988; Gazit et al., 1989, 1991; Anafi et al., 1992).

The data presented here add to an increasing body of evidence that the protein tyrosine kinase activity of the pp140$^{c\text{-}trk}$ NGF receptor is necessary for all of the cellular actions of the growth factor. Indeed, the selective blockade of this kinase correlates with the alteration of a wide diversity of responses, including MAP and *raf* kinases, PI-3 kinase, c-*fos* induction, and morphological changes induced by NGF. These inhibitory effects can be produced by two different kinds of kinase inhibitors: (1) the specific tyrphostin described here, which was designed to mimic tyrosyl-containing substrates and does not inhibit ATP binding (Yaish et al., 1988), and (2) staurosporine and related compounds, which are less selective for tyrosine kinases by virtue of their specific competition for the ATP binding of the kinases (Ohmichi et al., 1992a). The further evaluation of these and other selective protein tyrosine kinase inhibitors may help to dissect the precise signaling mechanisms that discriminate between differentiative and mitogenic effects of growth factors in neuronal cells.

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