Inhibition of the Cellular Actions of Nerve Growth Factor by Staurosporine and K252A Results from the Attenuation of the Activity of the *trk* Tyrosine Kinase†

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ABSTRACT: The protein kinase inhibitors staurosporine and K252A inhibit some of the cellular actions of nerve growth factor (NGF). To explore the molecular mechanisms involved, we test the ability of these agents to block one of the earliest cellular responses to NGF, protein tyrosine phosphorylation. Concentrations of 10–100 nM staurosporine and K252A inhibit NGF-dependent tyrosine phosphorylation in PC12 cells and inhibit *trk* oncogene-dependent tyrosine phosphorylation in *trk*-transformed NIH3T3 (*trk*-3T3 cells). In contrast, these compounds are without effect on epidermal growth factor (EGF)-stimulated tyrosine phosphorylation in PC12 cells. NGF-stimulated tyrosine phosphorylation of the pp140$^{c\text{-}trk}$ NGF receptor and tyrosine phosphorylation of pp70^{trk} are also inhibited by similar concentrations of staurosporine and K252A, whereas tyrosine phosphorylation of the EGF receptor, insulin receptor, and v-src is not affected. Both staurosporine and K252A inhibit the autophosphorylation of pp70^{trk} on tyrosine residues in an in vitro immune complex kinase reaction. Incubation of *trk*-3T3 cells with 10 nM staurosporine causes rounded transformed cells to revert to a normal flattened phenotype, whereas *src*-transformed cells are unaffected by this agent. These data suggest that staurosporine and K252A specifically inhibit the *trk* tyrosine kinase activity through a direct mechanism, probably accounting for the attenuation by these agents of the cellular actions of NGF.

Although the mechanisms by which nerve growth factor (NGF)[1] supports the survival and differentiation of sympathetic and sensory neurons remain elusive, changes in protein phosphorylation play an important role in the cellular actions of the growth factor. A number of serine/threonine kinases have been identified that are activated in response to NGF (Lee et al., 1985; Hama et al., 1986; Matsuda & Guroff, 1987; Rawland et al., 1987; Heasley & Johnson, 1989a,b; Vulliet et al., 1989; Miyasaka et al., 1990a,b). One of these kinases, the mitogen-activated protein (MAP) kinase, is itself thought to be phosphorylated on tyrosine residues (Rossomando et al., 1989). Indeed, both NGF and epidermal growth factor (EGF) cause the tyrosine phosphorylation of several proteins in PC12 cells, including two of molecular mass 40 and 42 kDa (Miyasaka et al., 1991). The NGF-sensitive protein tyrosine kinase in PC12 cells was recently identified as the 140-kDa *trk* protooncogene. Several studies have indicated that pp140$^{c\text{-}trk}$ is directly activated by binding of NGF, and in fact constitutes a separate NGF receptor (Kaplan et al., 1991a,b; Klein et al., 1991; Hempstead et al., 1991; Ohmichi et al., 1991b). Interestingly, both NGF and EGF stimulate the tyrosine phosphorylation of PLC-γ (Kim et al., 1991), and we have recently observed that NGF stimulates the association of the pp140$^{c\text{-}trk}$ with the *src* homology domain of PLC-γ (Ohmichi et al., 1991b). Additionally, the oncogenic pp70^{trk} exhibited a similar association with PLC-γ (Ohmichi et al., 1991a).

A number of studies have demonstrated that certain protein kinase inhibitors can potently inhibit some of the cellular actions of NGF. Two structurally related compounds, staurosporine and K252A, which inhibit the activity of protein kinase C, can block the stimulation by NGF of neurite outgrowth (Koizumi et al., 1988) and induction of the *c-fos* protooncogene (Chan et al., 1989). In previous studies, we demonstrated that both MAP kinase (Miyasaka et al., 1990a) and tyrosine phosphorylation (Miyasaka et al., 1991) stimulated by NGF, but not EGF, were potently attenuated by staurosporine and K252A. These observations led us to explore the selectivity of these compounds as tyrosine kinase inhibitors.

Experimental Procedures

Materials. ^{125}I-Labeled protein A (30 mCi/mg) was from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN (Bannockburn, IL). NGF 2.5S was from Bioproducts for Science (Indianapolis, IN). Receptor-grade EGF was from Collaborative Research (Lexington, MA). Insulin was from Eli Lilly (Indianapolis, IN). Staurosporine, K252A, and H7 were obtained from Calbiochem (La Jolla, CA) and freshly prepared to use as a 1000-fold-concentrated stock solution in dimethyl sulfoxide. All other

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[1] Abbreviations: SSP, staurosporine; PLC-γ1, phospholipase C-γ1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; NGF, nerve growth factor; Ins.R, insulin receptor; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PP, phosphoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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reagents were purchased from Sigma (St. Louis, MO) and were the highest quality available.

Cell Culture. The *trk*-transformed NIH3T3 cell line (*trk*-3T3) is a third-cycle NIH3T3 transformant derived from a human colon carcinoma DNA that carries the *trk* oncogene, and was the generous gift of Dr. E. Santos and Dr. D. Martin-Zanca (Martin-Zanca et al., 1986). *trk*-3T3 and Rous sarcoma virus-transformed 3Y1 cells were grown in DMEM containing 4500 mg/L D-glucose and 10% fetal bovine serum. PC12 cells were grown on collagen-coated plastic tissue culture dishes in RPMI medium with 10% fetal bovine serum and 5% horse serum. Rat fibroblasts expressing roughly 300 000 normal human insulin receptors (308 hIR), which were the generous gift of Dr. Donald A. McClain (Maegana et al., 1988; McClain et al., 1988), were grown in DME/F12 medium with 10% fetal bovine serum and 100 nM methotrexate.

Immunoblotting. For analysis of protein tyrosine phosphorylation in whole cell lysates, PC12 cells and *trk*-transformed NIH3T3 cells were grown in 100-mm dishes. After treatment, cells were washed once with 10 mL of phosphate-buffered saline, 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 subjected to immunoprecipitation with antisera raised against *trk*, PLC-γ1, EGFR, pp60^{src}, or the insulin receptor. Cells were grown in 150-mm dishes prior to treatment. Cells were washed twice with 12 mL of 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 EGTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 30 mM *p*-nitrophenol phosphate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride (HY buffer) according to Margolis et al. (1990). Lysates were centrifuged for 10 min at 10000*g* and supernatants incubated for 60 min with anti-*trk* antibody (Ohmichi et al., 1991a,b), anti-PLC-γ1 antibody (Ohmichi et al., 1991a), anti-EGF receptor antibody made against the human EGF receptor which recognized the kinase domain of the EGF receptor (Decker, 1984), anti-pp60^{src} antibody made against a trpE-pp60^{src} fusion protein (Ohmichi et al., 1991c), or monoclonal anti-human insulin receptor antibody (α1R-1) which was the generous gift of Dr. Steven Jacobs (Kull et al., 1983). Immunoprecipitates were mixed for 30 min with protein A–Sepharose beads for polyclonal antibody or with protein G–protein A–agarose for monoclonal antibody, 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 on 8% gels.

In Vitro Kinase Assay. *trk*-3T3 cells were grown in 100-mm dishes. Cells were washed twice with 10 mL of ice-cold PBS and lysed in 1 mL of HY 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. Four microliters of anti-*trk* antiserum was added to the collected supernatants, followed by incubation for 1 h. Immune complexes were then precipitated with protein A–Sepharose and washed 3 times with the same lysis buffer, followed by one wash with 20 mM HEPES, 1 mM $MnCl_2$, and 5 mM $MgCl_2$, pH 7.4. Immune precipitates were then resuspended in 20 μL of the HEPES, $MnCl_2$, and $MgCl_2$ buffer with or without the indicated concentrations of staurosporine and K252A before the addition of 2 μL of [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–PAGE, followed by autoradiography on Kodak XAR-5 film.

Results

Staurosporine and K252A Selectively Attenuate Protein Tyrosine Phosphorylation. The effects of staurosporine and K252A on NGF- and EGF-stimulated protein tyrosine phosphorylation were evaluated in intact PC12 cells by immunoblotting with anti-phosphotyrosine antibodies. Both growth factors caused the tyrosine phosphorylation of a number of proteins, including two predominant proteins of molecular mass 40 and 42 kDa, as well as a band at 55 kDa (Figure 1A). NGF-stimulated tyrosine phosphorylation was completely attenuated by pretreatment of cells with 1 μM staurosporine or K252A. Pretreatment of cells with another protein kinase C inhibitor, H7, or by a 15-min or 24-h incubation with phorbol myristate acetate had no effect on NGF-stimulated tyrosine phosphorylation (data not shown). Staurosporine and K252A also inhibited tyrosine phosphorylation detected by immunoblotting in intact *trk* oncogene transformed cells (Figure 1B). Tyrosine phosphorylation of proteins of molecular mass 46, 62, 70, 85, and 130 kDa in these cells was dose-dependently-inhibited by staurosporine and K252A (Figure 1B). Concentrations as low as 10 nM for both compounds almost completely blocked these phosphorylation events. Exposure of cells to phorbol myristate acetate did not compromise tyrosine phosphorylation.

These data suggested a direct inhibitory effect of staurosporine and K252A on the kinase activity of pp140^{trk} and of the pp70 *trk* oncogene. The addition of staurosporine and K252A to intact PC12 cells dose-dependently-inhibited the NGF-dependent tyrosine phosphorylation of pp140$^{c\text{-}trk}$ (Figure 2A). Exposure of cells to either compound at concentrations of 10 nM resulted in greater than 50% inhibition of phosphorylation, and 100 nM concentrations were totally inhibitory. The protein kinase inhibitor H7 (Figure 2A, lane 9) or down-regulation of protein kinase C by incubation with phorbol myristate acetate for 24 h (not shown) had no effect. Interestingly, tyrosine phosphorylation of pp70^{trk} in intact *trk*-transfected 3T3 cells was even more sensitive to staurosporine and K252A. Exposure of these cells to either compound at concentrations of 10 nM almost completely inhibited tyrosine phosphorylation of p70^{trk}, whereas H7 and phorbol myristate acetate had no effect (Figure 2B).

To explore the specificity of tyrosine kinase inhibition by these compounds, we examined their effects on the tyrosine phosphorylation of the EGF receptor, pp60$^{v\text{-}src}$, or the insulin receptor by anti-phosphotyrosine immunoblotting of immunoprecipitates of these proteins (Figure 3). Addition of up to 1 μM staurosporine or K252A to PC12 cells had no effect on the EGF-dependent tyrosine phosphorylation of the EGF receptor or of receptor-associated proteins migrating at 120 and 62 kDa (Figure 3A). Similarly, addition of 1 μM staurosporine or K252A to chicken embryo fibroblasts transformed with Rous sarcoma virus had no effect on the tyrosine phosphorylation of pp60$^{v\text{-}src}$ or its associated 110- and 130-kDa phosphoproteins (Figure 3B). These inhibitors were also without effect on the insulin-dependent tyrosine phosphorylation of the insulin receptor in rat-1 fibroblasts transfected with the wild-type human insulin receptor (Figure 3C).

Staurosporine and K252A Block the trk-Dependent Tyrosine Phosphorylation of Phospholipase Cγ-1. Both pp70^{trk}

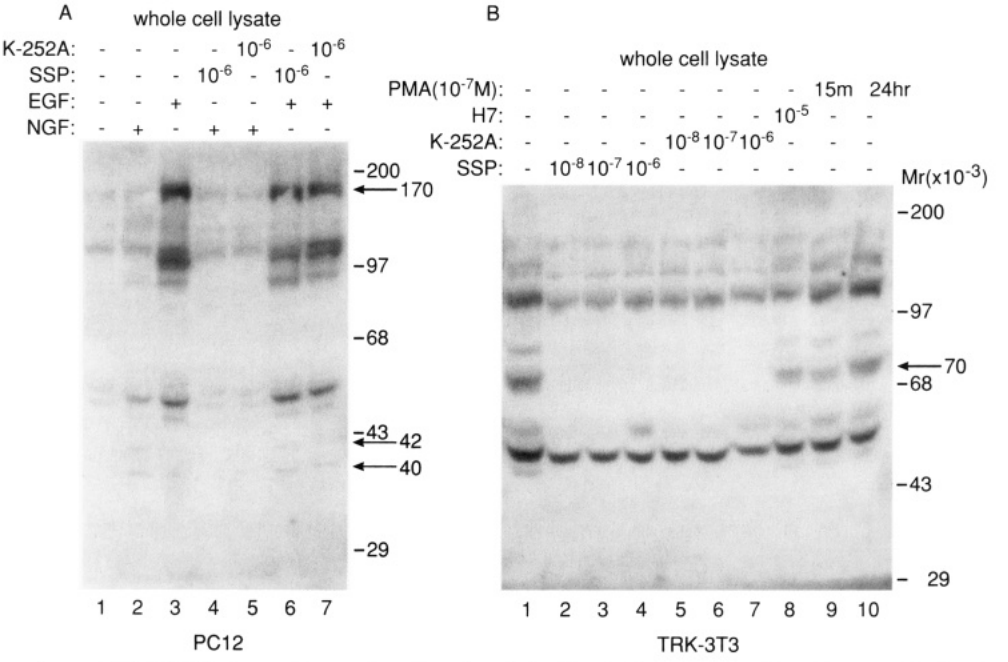

FIGURE 1: Staurosporine and K252A block protein tyrosine phosphorylation in whole cell lysates from PC12 and *trk*-3T3 cells. (A) After preincubation with or without staurosporine (1 μM) or K252A (1 μM) for 10 min, PC12 cells were treated for 1 min with 10 nM NGF (lanes 2, 4, 5) or 10 nM EGF (lanes 3, 6, 7). Thirty micrograms of protein from each whole cell lysate was analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody as described under Experimental Procedures. (B) *trk*-transformed 3T3 cells were treated with or without the indicated concentration of staurosporine (lanes 2–4) or K252A (lanes 5–7) for 10 min, with H7 (10 μM, lane 8) for 10 min, and with phorbol myristate acetate (100 nM) for 15 min (lane 9) or 24 h (lane 10). Thirty micrograms of protein from each whole cell lysate was analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody.

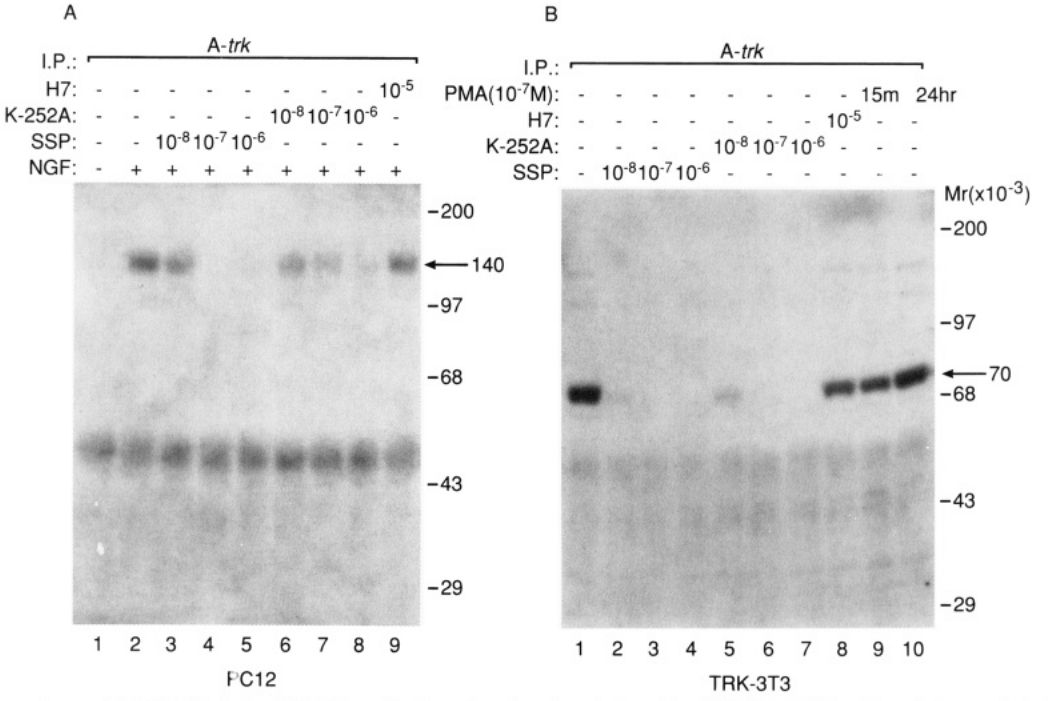

FIGURE 2: Staurosporine and K252A block the NGF-dependent tyrosine phosphorylation of pp140$^{c\text{-}trk}$ in PC12 cells and the tyrosine phosphorylation of pp70^{trk} in *trk*-3T3 cells. (A) After preincubation with or without the indicated concentrations of staurosporine (lanes 3–5), K252A (lanes 6–8), or H7 (10 μM, lane 9) for 10 min, PC12 cells were treated for 1 min with 10 nM NGF (lanes 2–9). Lysates from PC12 cells were immunoprecipitated with anti-*trk* antisera, and immunoprecipitates were subject to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody as described in Figure 1. (B) *trk*-transformed 3T3 cells were treated with or without the indicated concentrations of staurosporine (lanes 2–4), K252A (lanes 5–7), or H7 (10 μM, lane 8) for 10 min or with phorbol myristate acetate (100 nM) for 15 min (lane 9) or 24 h (lane 10). Whole cell lysates were immunoprecipitated with anti-*trk* antibody prior to analysis by immunoblotting with anti-phosphotyrosine antibody as described in Figure 1.

(Ohmichi et al., 1991a) and the pp140$^{c\text{-}trk}$ NGF receptor (Ohmichi et al., 1991b) specifically bind to and tyrosine-phosphorylate PLCγ-1. To determine whether these inhibitors will block this action of the *trk* proteins, cells were treated with staurosporine and K252A. PLCγ-1 was then immunoprecipitated from NGF-treated PC12 cells or *trk*-3T3 cells, followed by immunoblotting with anti-phosphotyrosine antisera (Figure 4). Incubation of cells with 1 μM staurosporine or K252A completely prevented the detection of tyrosine-phosphorylated pp140$^{c\text{-}trk}$ in anti-PLCγ-1 immunoprecipitates, and also

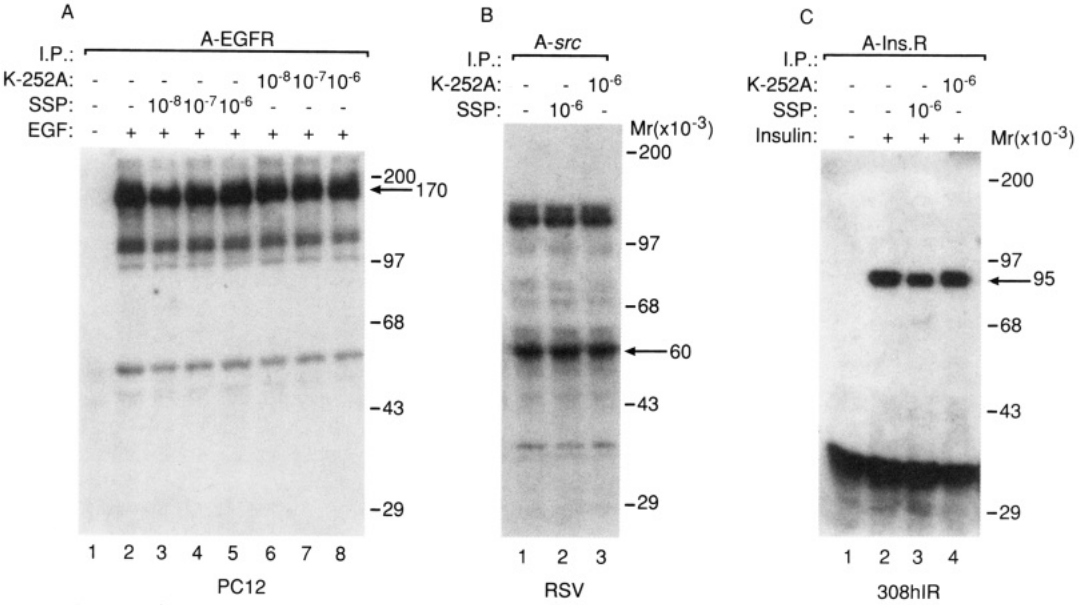

FIGURE 3: Staurosporine and K252A do not inhibit EGF-dependent tyrosine phosphorylation of the EGF receptor, tyrosine phosphorylation of pp60^{src}, or the insulin-dependent tyrosine phosphorylation of the insulin receptor. (A) After preincubation with or without the indicated concentration of staurosporine (lanes 3–5) or K252A (lanes 6–8) for 10 min, PC12 cells were treated for 1 min with 10 nM EGF (lanes 2–8). Lysates were immunoprecipitated with anti-EGFR antisera followed by SDS–PAGE and immunoblotting with anti-phosphotyrosine antibody as described in Figure 1. (B) Rous sarcoma virus-transformed 3Y1 cells were treated with or without staurosporine (1 μM) or K252A (1 μM) for 10 min. Lysates were immunoprecipitated with anti-*src* antisera before analysis by immunoblotting with anti-phosphotyrosine antibody. (C) After preincubation with or without staurosporine (1 μM) or K252A (1 μM) for 10 min, rat fibroblasts expressing roughly 300 000 normal human insulin receptors (308 hIR) were treated for 5 min with 100 nM insulin (lanes 2–4). Lysates from 308 hIR cells were immunoprecipitated with a monoclonal anti-insulin receptor antibody (αIRh1) before analysis by immunoblotting with anti-phosphotyrosine antibody.

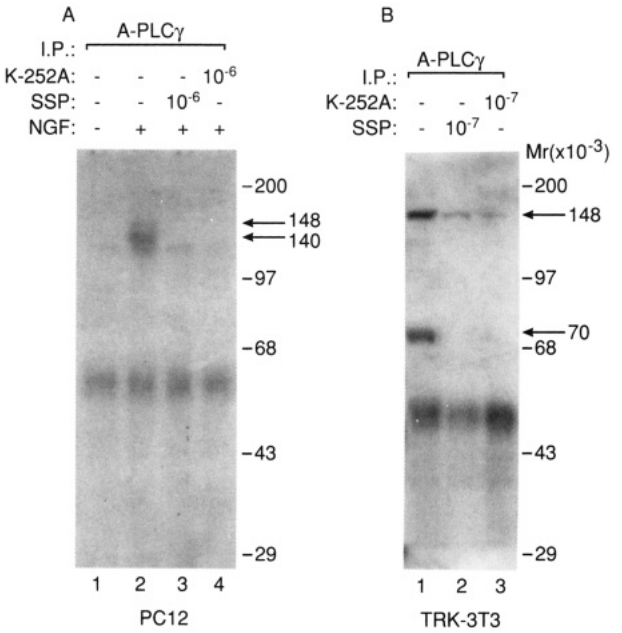

FIGURE 4: Staurosporine and K252A inhibit the NGF-dependent tyrosine phosphorylation of PLC-γ1 in PC12 cells and the tyrosine phosphorylation of PLC-γ1 in *trk*-3T3 cells. (A) After preincubation with or without staurosporine (1 μM) or K252A (1 μM) for 10 min, PC12 cells were treated for 1 min with 10 nM NGF (lanes 2–4). Lysates were immunoprecipitated with anti-PLC-γ1 antisera, and immunoprecipitates were subject to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody as described in Figure 1. (B) *trk*-transformed 3T3 cells were treated with or without staurosporine (1 μM) or K252A (1 μM) for 10 min. Lysates from *trk*-transformed 3T3 cells were immunoprecipitated with anti-PLC-γ1 antibody before analysis by immunoblotting with anti-phosphotyrosine antibody.

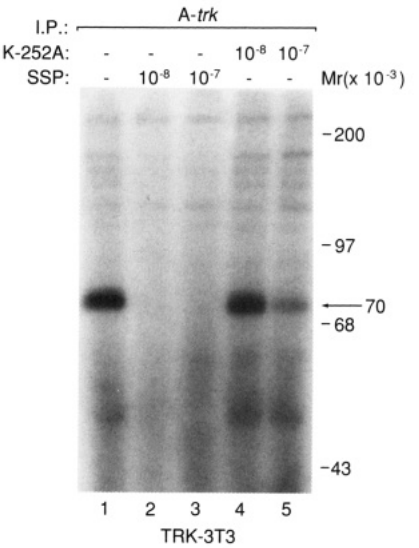

FIGURE 5: Staurosporine and K252A directly inhibit the tyrosine kinase activity of pp70^{trk}. *trk*-3T3 cells were lysed in 1 mL of HY buffer and subjected to immunoprecipitation with anti-*trk* antibody as described. Immune precipitates were then resuspended in 20 μL of the HEPES, $mNCl_2$, and $MgCl_2$ buffer with or without the indicated concentration of staurosporine (lanes 2, 3) or K252A (lanes 4, 5) for 10 min at 4 °C, before the addition of 2 μL 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.

blocked the NGF-dependent tyrosine phosphorylation of both proteins (Figure 4A). Similarly, 100 nM staurosporine or K252A completely abolished the detection of tyrosine-phosphorylated pp70^{trk} in anti-PLCγ-1 immunoprecipitates in *trk*-3T3 cells, completely blocked the tyrosine phosphorylation of pp70^{trk}, and caused an over 90% inhibition of PLCγ-1 tyrosine phosphorylation (Figure 4B).

Staurosporine and K252A Directly Inhibit the Autophosphorylation of pp70^{trk}. To definitively demonstrate the inhibition of the tyrosine autophosphorylation of the *trk* protein kinase activity by staurosporine and K252A, we examined the effect of these compounds in an in vitro phosphorylation reaction in anti-pp70^{trk} immunoprecipitates from *trk*-3T3 cells

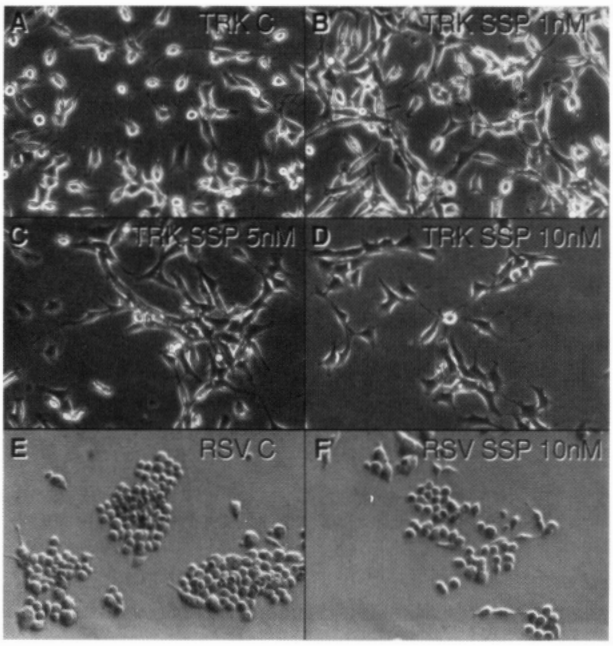

FIGURE 6: Staurosporine reverts the transformed phenotype of *trk*- but not *src*-transformed cells. *trk*-3T3 cells (A–D) or Rous sarcoma virus-transformed 3Y1 cells (E, F) were incubated in the presence of the indicated concentrations of staurosporine for 24 h. Cells were photographed using a phase-contrast light microscope.

(Figure 5). The addition of 10 nM staurosporine or 100 nM K252A completely inhibited the direct tyrosine autophosphorylation of pp70^{trk} presumably by blocking ATP binding. Phosphoamino acid analysis of the in vitro pp70^{trk}-phosphorylated protein yielded exclusively phosphotyrosine, with no detectable phosphoserine or phosphothreonine (data not shown).

Staurosporine Specifically Reverts the Transformed Phenotype of trk-Transfected but Not src-Transfected Cells. To explore the functional significance of *trk* protein tyrosine kinase inhibition, we evaluated morphological changes induced by the inhibitors in cells transfected with the oncogene (Figure 6). Addition of 1, 5, and 10 nM staurosporine to *trk*-3T3 cells caused a dose-dependent reversion of the transformed phenotype of most cells, causing rounded cells to flatten (Figure 6A–D), whereas untransfected 3T3 cells were unaffected by this concentration of the agent. Addition of 10 nM K252A induced a similar reversion (not shown). In contrast, Rous sarcoma virus-transformed cells were unaffected by concentrations of staurosporine (10 nM) that altered the morphology of *trk*-3T3 cells (Figure 6E, F). K252A was similarly ineffective (not shown). In addition, 10 nM staurosporine or K252A had no effect on EGF-dependent growth of NIH3T3 cells expressing the human EGF receptor (not shown).

Discussion

Staurosporine and K252A are structurally related microbial alkaloid protein kinase inhibitors that potently inhibit protein kinase C by competitively blocking the binding of ATP (Koizumi et al., 1990). Although these compounds were thought to preferentially inhibit protein kinase C, they also attenuate the activities of other serine/threonine protein kinases (Ruegg & Burgess, 1989), and have been reported to inhibit certain tyrosine kinases (Nakano et al., 1987). In PC12 cells, both staurosporine and K252A selectively inhibit some of the actions of NGF (Hashimoto, 1988; Koizumi et al., 1988). For example, staurosporine blocked the NGF-dependent induction of the *c-fos* protooncogene (Chan et al., 1989), and K252A blocked NGF-induced neurite outgrowth (Koizumi et al., 1988). Although it was suspected that the attenuation of these actions of NGF reflected inhibition of protein kinase C activity, subsequent studies demonstrated that the kinase C-independent activation of MAP kinase (Miyasaka et al., 1990a) and tyrosine phosphorylation (Miyasaka et al., 1991) were also inhibited by these two compounds, suggesting that they interfered with NGF action at an earlier step. This possibility has been confirmed by the data presented in this study, showing that staurosporine and K252A dose-dependently-inhibit the NGF-dependent pp140$^{c\text{-}trk}$ and pp70^{trk} tyrosine phosphorylation, and totally block the tyrosine kinase activity of pp70^{trk} assayed in vitro. These effects occur at nanomolar concentrations, several log lower than those necessary for nonspecific inhibition of serine/threonine protein kinases such as cAMP-dependent protein kinase and calmodulin-dependent protein kinases. Moreover, the lack of an inhibitory effect of the kinase C inhibitor H7, or similarly by down-regulation of cellular protein kinase C due to prolonged incubation with phorbol esters, further suggests that the observed effects of staurosporine and K252A are not mediated by inhibition of protein kinase C.

What is the nature of the observed specificity in the inhibition of the *trk* protein tyrosine kinase activity by staurosporine and K252A? Although the actual mechanism by which these compounds inhibit protein kinase activities remains unclear, they function as competitive inhibitors of ATP binding to the catalytic domain of the kinase (Koizumi et al., 1990). Comparison of the amino acid sequences for the putative ATP binding sites for pp70^{trk}, pp140$^{c\text{-}trk}$, insulin receptor, pp60$^{v\text{-}src}$, EGF receptor, PDGF receptor, and protein kinase C family members reveals no striking homologies outside the consensus LGXGXXG sequence (thought to be responsible for ATP binding) that might explain the relative sensitivities to these inhibitors. Thus, the molecular mechanisms underlying inhibition of the *trk* kinase activities may require further analysis of the structures of these proteins.

Registry No. SSP, 62996-74-1; NGF, 9061-61-4; K252A, 99533-80-9; Tyr, 60-18-4; tyrosine kinase, 80449-02-1.

References

Aletta, T. M., Lewis, S. A., Cowan, N. J., & Greene, L. A. (1988) *J. Cell Biol. 106*, 1573–1581.

Chan, B. L., Chao, M. V., & Saltiel, A. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1756–1760.

Decker, S. J. (1984) *Mol. Cell. Biol. 4*, 571–575.

Decker, S. J., Ellis, C., Pawson, T., & Velu, T. (1990) *J. Biol. Chem. 265*, 7009–7015.

Hama, T., Huang, K. P., & Guroff, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2353–2357.

Hashimoto, S. (1988) *J. Cell Biol. 107*, 1531–1539.

Heasley, L. E., & Johnson, G. L. (1989a) *J. Biol. Chem. 264*, 8646–8652.

Heasley, L. E., & Johnson, G. L. (1989b) *Mol. Pharmacol. 35*, 331–338.

Hempstead, B. L., Martin-Zanca, D., Kaplan, D. R., Parada, L. F., & Chao, M. V. (1991) *Nature 350*, 678–683.

Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., & Parada, L. F. (1991a) *Science 252*, 554–557.

Kaplan, D. R., Martin-Zanca, D., & Parada, L. F. (1991b) *Nature 350*, 158–160.

Kim, U. H., Fink, D., Kim, H. S., Park, D. J., Contreras, M. L., Guroff, G., & Rhee, S. G. (1991) *J. Biol. Chem. 266*, 1359–1362.

Klein, R., Jing, S., Nanduri, K. V., O'Rourke, E., & Barbacid, M. (1991) *Cell 65*, 189–197.

Koizumi, S., Contreras, M. L., Matsuda, Y., Hama, T., Lazarovici, P., & Guroff, G. (1988) *J. Neurosci. 8*, 715–721.
Koizumi, S., Mutoh, T., Ryazanov, A., Rudkin, B. B., & Guroff, G. (1990) *Trophic Factors and the Nervous System* (Horrocks, L. A., et al., Eds.) pp 915–202, Raven Press, New York.
Kull, F. C., Jr., Jacobs, S., Su, Y.-F., Svoboda, M. E., Van Wyk, J. J., & Cuatrecasas, P. (1983) *J. Biol. Chem. 258*, 6561–6566.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lee, K. Y., Seeley, P. J., Muller, T. H., Helmer-Matyjek, E., Sabban, E., Goldstein, M., & Greene, L. A. (1985) *Mol. Pharmacol. 28*, 220–228.
Maegana, H., McClain, D. A., Freidenberg, G., Olefsky, J. M., Napier, M., Lipari, T., Dull, T. J., Lee, J., & Ullrich, A. (1988) *J. Biol. Chem. 263*, 8912–8917.
Margolis, B., Bellot, F., Honegger, A. M., Ullrich, A., Schlesinger, J., & Zilberstein, A. (1990) *Mol. Cell. Biol. 4*, 571–575.
Martin-Zanca, D., Hughes, S. H., & Barbacid, M. (1986) *Nature 319*, 743–748.
Martin-Zanca, D., Barbacid, M., & Parada, L. F. (1990) *Genes Dev. 4*, 683–694.
Matsuda, Y., & Guroff, G. (1987) *J. Biol. Chem. 262*, 2832–2844.
McClain, D. A., Maegana, H., Levy, J., Hueckstead, T., Dull, T. J., Lee, J., Ullrich, A., & Olefsky, J. M. (1988) *J. Biol. Chem. 263*, 8904–8911.
Miyasaka, T., Chao, M. V., Sherline, P., & Saltiel, A. R. (1990a) *J. Biol. Chem. 265*, 4730–4735.
Miyasaka, T., Miyasaka, J., & Saltiel, A. R. (1990b) *Biochem. Biophys. Res. Commun. 168*, 1237–1243.
Miyasaka, T., Sternberg, D. W., Miyasaka, J., Sherline, P., & Saltiel, A. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2653–2657.
Nakano, H., Kobayashi, E., Takahashi, I., Tamacki, T., Kuzuu, Y., & Iba, H. (1987) *J. Antibiot. 40*, 706–708.
Ohmichi, M., Decker, S. J., Pang, L., & Saltiel, A. R. (1991a) *J. Biol. Chem. 266*, 14858–14861.
Ohmichi, M., Decker, S. J., Pang, L., & Saltiel, A. R. (1991b) *Biochem. Biophys. Res. Commun. 179*, 217–223.
Ohmichi, M., Decker, S. J., & Saltiel, A. R. (1991c) *Cell Regul. 2*, 691–697.
Rawland, E. A., Muller, T. H., Goldstein, M., & Greene, L. A. (1987) *J. Biol. Chem. 262*, 7504–7513.
Rossomando, A. J., Rayne, D. M., Weber, M. J., & Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6940–6943.
Ruegg, U. T., & Burgess, G. M. (1989) *Trends Pharmacol. Sci. 10*, 218–220.
Vulliet, P. R., Hall, F. L., Mitchell, J. P., & Hardie, D. G. (1989) *J. Biol. Chem. 264*, 16292–16298.

Low-Affinity Binding Sites for 1,4-Dihydropyridines in Skeletal Muscle Transverse Tubule Membranes Revealed by Changes in the Fluorescence of Felodipine†

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ABSTRACT: The fluorescence changes accompanying the binding of the fluorescent calcium channel antagonist, felodipine, to transverse tubule membranes from rabbit skeletal muscle have been used to characterize low-affinity binding sites for 1,4-dihydropyridine derivatives in these preparations. In competition experiments, felodipine inhibited the high-affinity binding of (+)-[^{3}H]PN200-110 to transverse tubule membranes with an apparent K_i of 5 ± 2 nM. Binding of felodipine to additional low-affinity sites resulted in a large, saturable (K_d = 6 ± 2 μM) increase in its fluorescence which could be excited either directly (380 nm) or indirectly via energy transfer from membrane protein (290 nm). The observed fluorescence enhancement was competitively inhibited by other 1,4-dihydropyridines with inhibition constants of 3–21 μM but was unaffected by the structurally unrelated calcium channel antagonists, diltiazem and verapamil, or by Ca^{2+}, Cd^{2+}, and La^{3+}. Both high- and low-affinity binding sites appear to be localized in the transverse tubular system, since the magnitude of the observed fluorescence enhancement was higher in these membranes than in microsomal preparations and was directly proportional to the density of high-affinity sites for (+)-[^{3}H]PN200-110. Furthermore, both high- and low-affinity sites appear to be conformationally coupled since, over the same concentration range that the fluorescence changes were observed, felodipine accelerated the rate of dissociation of [^{3}H]PN200-110 previously bound to its high-affinity sites. Similar behavior has previously been reported for other 1,4-dihydropyridines [Dunn, S. M. J., & Bladen, C. (1991) *Biochemistry 30*, 5716–5721]. These results suggest that skeletal muscle transverse tubule membranes carry both high- and low-affinity binding sites for 1,4-dihydropyridines and that these sites may be important for regulation of calcium channel activity.

The 1,4-dihydropyridine (DHP)[1] class of calcium channel activators and antagonists have been widely exploited as tools in the study of the structure and function of calcium channel proteins. Radiolabeled DHPs have been shown to bind specifically and with high affinity (nanomolar K_ds) to membrane preparations from a variety of excitable tissues including smooth muscle, cardiac muscle, skeletal muscle, and brain [reviewed by Hosey and Lazdunski (1988)]. Transverse tubule

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[1] Abbreviations: DHP, 1,4-dihydropyridine; DMSO, dimethyl sulfoxide; felodipine, 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid 3-ethyl 5-methyl ester; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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