

# K-252a and Staurosporine Selectively Block Autophosphorylation of Neurotrophin Receptors and Neurotrophin-Mediated Responses

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The same receptor tyrosine kinase (RTK) can mediate strikingly different biological responses in a fibroblast as opposed to a neuron. We have compared the rapidly induced tyrosine phosphorylations mediated by various RTKs in both NIH3T3 fibroblasts and in the PC12 neuronal precursor cell line and found that each RTK induces a distinct pattern of protein tyrosine phosphorylations in the two cell types. These findings are consistent with a model in which various cell types present a given RTK with different menus of signal transduction components, allowing the same RTK to elicit fundamentally distinct biological responses. Although there are obvious overlaps in the tyrosine phosphorylations induced by different RTKs in the same cell, there are also clear differences. The attempt to dissect these differences revealed that the kinase inhibitors K-252a and staurosporine inhibit RTK autophosphorylation and thus the biological consequences of receptor/ligand interaction. These inhibitors displayed substantially greater specificity for a subset of RTKs (including the neurotrophin receptors) than for other RTKs and acted as remarkably selective blockers of neurotrophin action in both neuronal and nonneuronal cells. A potential therapeutic application for these inhibitors is discussed.

## INTRODUCTION

A multicellular organism undergoes development, maintains homeostasis, and responds to environmental cues via mechanisms that depend on cell-to-cell communication. A vast array of distinct cellular receptors are responsible for specifically recognizing, and distinguishing between, the numerous molecular moieties that transfer information from one cell to another. One class of receptors are transmembrane proteins, known as receptor tyrosine kinases (RTKs), that use an extracellular domain to bind to their cognate polypeptide factors and an intracytoplasmic tyrosine kinase catalytic domain to initiate the signal transduction process (reviewed in Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990; Aaronson, 1991). Binding of factor to the RTK extracellular domain activates the catalytic domain, resulting in phosphorylation of tyrosine residues within the receptor itself. This autophosphorylation then regulates interaction of the activated receptor with intracellular substrates, such as phospholipase C- $\gamma$ 1, p21<sup>ras</sup>

GTPase-activating protein, and src-like tyrosine kinases (Koch *et al.*, 1991). These substrates, in turn, regulate a variety of signaling pathways inside of the cell. Signaling cascades that initiate with tyrosine phosphorylations eventually also result in the serine/threonine phosphorylation of downstream molecules due to the activation of serine/threonine kinases, such as raf, protein kinase C (PKC), and ribosomal S6 protein kinase (Ullrich and Schlessinger, 1990). Activation of at least one family of kinases, known either as the mitogen-activated protein (MAP) kinases or as the extracellular signal-regulated kinases (ERKs), requires both tyrosine and serine/threonine phosphorylations, suggesting that they may be located at a key transition point between upstream and downstream signal transducing molecules (Boulton *et al.*, 1991). RTK-activated signaling pathways eventually extend into the nucleus and can ultimately elicit phenotypic responses as fundamentally different and apparently incongruous as cell growth and proliferation com-

pared with growth arrest and differentiation (reviewed in Aaronson, 1991).

The ultimate phenotypic response to RTK activation, whether it be growth promoting or growth inhibiting, appears to depend on both the RTK that is being activated as well as the particular cellular context in which it is being activated. It is cellular context that is perhaps most important, because activation of the same RTK in different cells can lead to very different phenotypic effects. One of the most striking examples of this phenomenon involves fibroblast growth factor (FGF), which was discovered based on its ability to induce proliferation of fibroblasts but more recently has been found to mediate growth arrest and differentiation in neuronal cells (Togari *et al.*, 1983, 1985; Wagner, 1991). In contrast to FGF, a number of factors related to nerve growth factor (NGF) were originally discovered because of their ability to promote neuronal survival and differentiation and were not thought to act as proliferative agents (Barde, 1989). These factors, which include brain-derived neurotrophic factor (BDNF) (Leibrock *et al.*, 1989), neurotrophin-3 (NT-3) (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990), and neurotrophin-4/5 (Berkemeier *et al.*, 1991; Hollböök *et al.*, 1991; Ip *et al.*, 1992), are collectively known as the neurotrophins and they recently have been shown to utilize the *trk* family of RTKs (Berkemeier *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Ip *et al.*, 1992). Unexpectedly, it was found that introduction of the *trk* receptors into fibroblasts enables the neurotrophins to elicit FGF-like proliferative responses from these cells (Cordon-Cordo *et al.*, 1991; Glass *et al.*, 1991; Klein *et al.*, 1991; Lamballe *et al.*, 1991). These examples suggest that RTKs do not inherently determine whether the responses they evoke are growth promoting as opposed to growth inhibitory but rather depend on the cellular context in which they are activated.

Although cellular context apparently plays a crucial role in determining the type of response a given RTK will elicit, different RTKs can display distinct signaling capabilities when activated within the same cell. Thus, although NGF, FGF, and epidermal growth factor (EGF) all elicit similar biochemical responses from the PC12 pheochromocytoma cell line, only NGF and FGF receptor activation result in growth arrest and neuronal differentiation of these cells (reviewed in Fujita *et al.*, 1989). Furthermore, although the differentiative effects of NGF and FGF on PC12 cells appear similar, their signaling pathways can be distinguished using the closely related protein kinase inhibitors K-252a and staurosporine (Doherty *et al.*, 1988; Koizumi *et al.*, 1988; Hashimoto and Hagino, 1989; Lazarovici *et al.*, 1989; Maher, 1989; Altin *et al.*, 1991; Miyasaka *et al.*, 1991). K-252a and staurosporine were initially characterized as potent PKC and cyclic nucleotide-dependent kinase inhibitors *in vitro* (Kase *et al.*, 1987) but are now known

to have broader actions that include inhibition of tyrosine-specific protein kinases (Fujita-Yamaguchi and Kathuria, 1988; O'Brian and Ward, 1990). Although these inhibitors do not seem to diminish FGF or EGF responses in PC12 cells, they are able to block even the earliest detectable signaling processes induced by NGF, including NGF-induced tyrosine phosphorylation, at concentrations that do not effect PKC activity (Doherty *et al.*, 1988; Koizumi *et al.*, 1988; Hashimoto and Hagino, 1989; Lazarovici *et al.*, 1989; Maher, 1989; Altin *et al.*, 1991; Miyasaka *et al.*, 1991). Thus, these inhibitor studies predict the existence of at least one critical kinase component that is specific to, and located at an early point in, the NGF activated signaling pathway in PC12 cells.

To determine whether the influences of cellular context, as well as the inherent differences between the signaling capabilities of individual RTKs, are apparent early in RTK signaling pathways, we compared rapidly tyrosine phosphorylated targets of individual RTKs in both a neuronal (PC12) and a fibroblast (NIH3T3) cell line. We also addressed the mechanisms by which K-252a and staurosporine specifically inhibit neurotrophin responses.

## MATERIALS AND METHODS

### *Factors and Inhibitors*

The preparation and purification of recombinant human BDNF and NT-3, produced in chinese hamster ovary cells, have been described previously (Squinto *et al.*, 1991). 2.5S NGF was purified to homogeneity from the submaxillary gland of the adult mouse. Bovine brain bFGF was purchased from RD Systems (Minneapolis, MN). Recombinant human EGF was purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant human platelet-derived growth factor (PDGF)-BB was purchased from Upstate Biotechnology (Lake Placid, NY). K-252a and staurosporine were purchased from Kamiya Biomedical (Thousand Oaks, CA). O-phospho-DL-tyrosine was purchased from Sigma Chemical (St. Louis, MO).

### *Cells, Cell Culture, and DNA Transfections*

NIH3T3 cells and *trkB* expressing NIH3T3 cells were maintained as described (Glass *et al.*, 1991). NIH3T3 expressing *trkA* were constructed by transfecting the full-length rat *trkA* cDNA (a kind gift of S.O. Meakin and E.M. Shooter, Stanford University) subcloned into pCDM8 (Aruffo and Seed, 1987), whereas NIH3T3 cells expressing *trkC* were constructed by transfecting a full-length rat *trkC* cDNA (Valenzuela, Maisonpierre, and Yancopoulos, unpublished data) subcloned in the pVCO57 vector (Stitt, Glass, and Yancopoulos, unpublished data). NIH3T3 cells stably expressing the human EGF receptor (EGFR) were generated by using a retroviral vector expressing the full-length human EGFR cDNA (Hantzopoulos and Yancopoulos, unpublished data). Transfection and selection were carried out as previously described (Glass *et al.*, 1991). PC12 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% horse serum, 6% calf serum, 1% penicillin, 1% streptomycin, and 2 mM glutamine.

### *Cell Survival and Proliferation Assay*

Survival and proliferation assays were performed as described (Glass *et al.*, 1991) on NIH3T3 cells cultured in serum-free defined media (a

3:1 mixture of DMEM and Ham's F12 containing 20  $\mu\text{g}/\text{ml}$  insulin, 0.1  $\mu\text{M}$  selenous acid [sodium salt], 5  $\mu\text{g}/\text{ml}$  transferrin, and 500  $\mu\text{g}/\text{ml}$  bovine serum albumin in the absence or presence of either growth factors (FGF 50 pM, PDGF 500 pM, and EGF 2 nM) or neurotrophins (NGF, BDNF, and NT-3, 500 pM) with or without the addition of K-252a (50 or 100 nM). Briefly,  $2.5 \times 10^3$  cells were plated per well in the 96-well format. After 48 h the viability of cells was determined by using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay (procedure carried out as specified by Promega, Madison, WI). With this assay, the optical density measured at 570–650 nm is directly proportional to the number of surviving cells.

### Antibody Preparation

The polyclonal antibody used to immunoprecipitate both trkA and trkB was made in rabbits using a peptide corresponding to the carboxy-terminal region of trkA (ALAQAPPVDVLG, as per Klein *et al.*, 1991) and then purified by ammonium sulfate precipitation and protein A chromatography. The polyclonal antibody used to immunoprecipitate the ERKs was raised by injecting rabbits with recombinant rat ERK2. The monoclonal antibody used to immunoblot both ERK1 and ERK2 was obtained from Zymed Laboratories (S. San Francisco, CA), whereas monoclonal antibodies to the human EGF receptor and polyclonal antibodies to the human PDGF receptor (type A/B) were obtained from Upstate Biotechnology.

### Immunoprecipitations, Immunoblotting, and Tyrosine Phosphorylation Studies

To prepare protein extracts from NIH3T3 cells,  $\sim 3 \times 10^6$  cells were starved for 60 min in serum-free defined media (containing insulin, see above) and then treated with factor for 5 min and lysed at 4°C in RIPA lysis buffer (phosphate-buffered saline containing 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 0.14 U/ml aprotinin). Extracts from PC12 cells were prepared in the same way except that  $1 \times 10^7$  cells were used, and these cells were not serum starved. Because prolonged starvation of either of these cells leads to marked phenotypic changes, including cell death, induced tyrosine phosphorylations were examined under the conditions described and also in the media lacking all nutrients; the different methods of starvation did not appreciably effect the induced tyrosine phosphorylation patterns (see Figure 2). A portion of the total protein extracts were resolved by gel electrophoresis and immunoblotted with the indicated antibodies. The remaining lysates were immunoprecipitated with the indicated antibodies, either directly conjugated to agarose (anti-phosphotyrosine monoclonal antibody; Upstate Biotechnologies) or in combination with a goat anti-rabbit or anti-mouse secondary antibodies conjugated to agarose (Sigma Chemical). Autophosphorylation of immunoprecipitated proteins was detected by immunoblotting with anti-phosphotyrosine antibodies. The specificity of the anti-phosphotyrosine antibodies for detecting tyrosine phosphorylated proteins was determined by simultaneously incubating an immunoblot with 2.5 mM O-phospho-DL-tyrosine in addition to the anti-phosphotyrosine antibody (see RESULTS).

## RESULTS

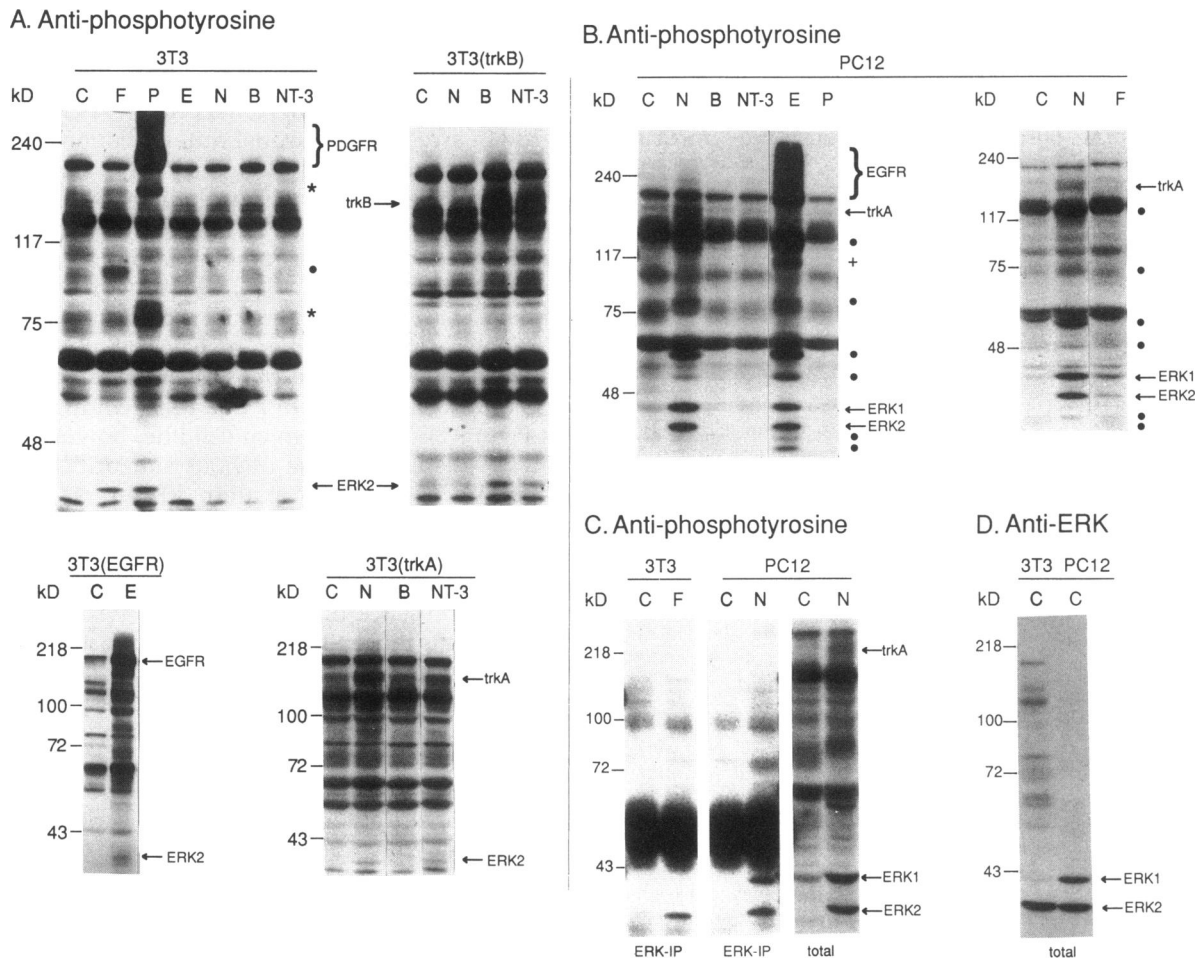
### RTK Mediated Phosphorylation Patterns Depend on Both Cellular Context as well as the Particular RTK Activated

To begin to explore the mechanisms by which cellular context modulates interpretation of RTK activation and to distinguish between the signaling capabilities of individual RTKs, we compared the protein tyrosine phosphorylations that occur immediately after activation of

a variety of endogenous or introduced RTKs in both a neuronal line and a fibroblast line. Activation of FGF receptors (FGFR), NGF (trkA), or BDNF (trkB), but not EGFR, in the pheochromocytoma cell line, PC12, results in growth arrest and neuronal differentiation (Fujita *et al.*, 1989; Squinto *et al.*, 1991). In contrast, activation of all of these receptors, as well as endogenous PDGF receptors (PDGFR), in NIH3T3 fibroblasts leads to proliferative responses (Zhan and Goldfarb, 1986; Cordon-Cardo *et al.*, 1991; Glass *et al.*, 1991; Klein *et al.*, 1991; Lamballe *et al.*, 1991). Immunoblot analysis using anti-phosphotyrosine antibodies revealed that each RTK elicited different tyrosine phosphorylation patterns in NIH3T3 as compared with PC12 cells (compare A and B in Figure 1). Within a given cell line, however, all of the RTKs displayed some obvious similarities in their tyrosine phosphorylation patterns (Figure 1). The detected patterns represent true tyrosine phosphorylated proteins because challenging an immunoblot of total protein lysates with anti-phosphotyrosine antibodies, but in the presence of excess phosphotyrosine, effectively prevents the detection of phosphorylated proteins (compare left to right panels in Figure 2). These results demonstrate that the distinct biological responses elicited by activation of the same RTK in different cell types are preceded by distinct early signaling events in the different cells. Furthermore, within a given cell type, there are significant overlaps in the early signaling events mediated by different RTKs.

The observation that cellular context can influence RTK phosphorylation patterns was most obvious when focusing on a defined set of substrates—the ERKs. Particular tyrosine phosphorylated proteins could unequivocally be identified as ERK1 or ERK2 based on direct immunoprecipitations using anti-ERK antibodies (Figure 1C). In PC12 cells, both ERK1 and ERK2 were tyrosine phosphorylated after activation of any of the RTKs examined (Figure 1B). In NIH3T3 fibroblasts, however, only ERK2 was tyrosine phosphorylated when signaling pathways for two endogenous receptors (FGFR and PDGFR) as well as three introduced receptors (EGFR, trkA, and trkB) were stimulated (Figure 1A). To explore the possibility that activation of the same RTK in different cells results in distinct patterns of tyrosine phosphorylation because of a different availability of downstream substrates, we directly compared the intracellular levels of ERK1 and ERK2 in PC12 as opposed to NIH3T3 cells. A direct comparison of PC12 and NIH3T3 cell lysates immunoblotted with anti-ERK antibodies demonstrates that ERK1 and ERK2 are present at similar levels in PC12 cells, whereas only ERK2 is prominently expressed in NIH3T3 cells (Figure 1D).

Although the cellular environment seems to play a major role in determining the phosphorylations that result from RTK activation, we also find that the individual RTKs themselves can display qualitative differences in their tyrosine phosphorylation patterns. For example,

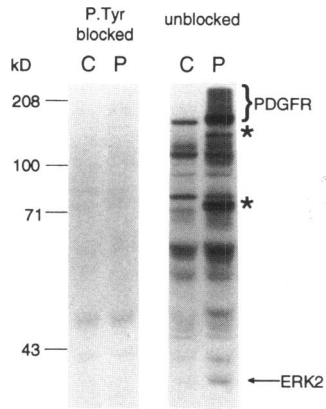


**Figure 1.** RTK-mediated induction of protein tyrosine phosphorylation in NIH3T3 or PC12 cells. Untreated cells (C) were compared with those treated with either FGF (F), PDGF (P), EGF (E), NGF (N), NT-3 (NT-3), or BDNF (B). Brackets or arrows indicate the presumed position of phosphorylated forms of the EGFR, PDGFR, trkA, trkB, ERK1, and ERK2, and the molecular weights of protein standards are indicated on the left of each panel. (A) Anti-phosphotyrosine immunoblot of total protein lysates prepared from NIH3T3 cells (3T3) or NIH3T3 cells expressing introduced trkA [3T3(trkA)], trkB [3T3(trkB)], or EGFR [3T3(EGFR)] treated with various factors. \*, phosphorylated proteins unique to PDGFR activation (Morrison *et al.*, 1989). (B) Anti-phosphotyrosine immunoblot of total protein lysates prepared from PC12 cells treated with various factors as in A. \*, shared NGF- and EGF-induced protein phosphorylations; +, highlights the single additional protein tyrosine phosphorylation induced by EGF. The only detectable phosphorylations induced by FGF are those of ERK1 and ERK2. (C) The assignment of ERK1 and ERK2 in A and B was verified by immunoblotting total lysates from PC12 cells (total) adjacent to ERK proteins specifically precipitated (ERK-IP) from NIH3T3 and PC12 cell lysates using polyclonal anti-ERK antibodies. (D) Anti-ERK immunoblot of total protein extracts prepared from untreated NIH3T3 (3T3) or PC12 cells.

NGF treatment of PC12 cells results in tyrosine phosphorylation of most, but not all, of the proteins that are phosphorylated in response to EGF; FGF treatment of PC12 cells results in tyrosine phosphorylation of a smaller subset of these proteins (Figure 1B). In NIH3T3 cells, activation of FGFR, PDGFR, trkA, or trkB results in relatively similar patterns of tyrosine phosphorylation, in which ERK2 is one of the few nonreceptor proteins whose tyrosine phosphorylation is appreciably increased (Figure 1A); FGF treatment results in the phosphorylation of one additional protein (~90 kDa), whereas PDGF treatment results in two additional protein phosphorylations (~140 and

80 kDa), consistent with previous findings (Molloy *et al.*, 1989; Morrison *et al.*, 1989). As with PC12 cells, activation of the EGFR in NIH3T3 cells results in the tyrosine phosphorylation of a broad set of cellular proteins (Figure 1A).

Taken together, our findings suggest that one reason the same receptor can elicit strikingly different responses in different cell types is because the receptor is presented with distinct sets of signal transduction components in the different cell types. Furthermore, the reason many RTKs elicit similar responses from the same cell is because they are constrained by the pathway components available in that cell. On the other hand, the distinct



**Figure 2.** Patterns detected using anti-phosphotyrosine antibodies represent true protein tyrosine phosphorylations. Total protein lysates obtained from NIH3T3 cells that were untreated (C) or treated with 50 ng/ml of PDGF (P) were immunoblotted with anti-phosphotyrosine antibodies either in the absence (unblocked) or presence (P-Tyr blocked) of 2.5 mM O-phospho-DL-tyrosine. Shown are lysates starved for 60 min in serum/nutrient-free DMEM before factor treatment; similarity to phosphotyrosine patterns seen in Figures 1 and 3 (in which cells were starved in defined media containing insulin and other nutrients) indicates that starvation conditions do not appreciably alter constitutive and induced tyrosine phosphorylations detected by the anti-phosphotyrosine antibody used in these studies. For reference to the phosphorylation patterns shown in Figure 1, ERK2 and the PDGF receptor are noted, as are two proteins specifically induced on treatment of cells with PDGF (denoted by asterisks).

signaling capabilities of individual RTKs within the same cell may result from their unique abilities to interact with at least some cellular components.

#### ***K-252a and Staurosporine Block the Tyrosine Phosphorylations Induced by Activation of Some, but not All, RTKs***

Presumably due to improved detection capabilities, our studies have revealed that NGF, EGF, and FGF induce more complex patterns of protein tyrosine phosphorylation in PC12 cells than previously appreciated (compare Figure 1B with results described by Maher, 1989; Miyasaka *et al.*, 1991); the NGF- and FGF-induced patterns in PC12 cells tend to be more complex than those induced by the same factors in NIH3T3 fibroblasts. Our ability to better define receptor- and context-specific tyrosine phosphorylations compelled us to reinvestigate the target of K-252a and staurosporine inhibition specific to the NGF pathway and whether this target was present in both neuronal and nonneuronal cells. In both PC12 and NIH3T3 cells, all NGF-induced protein tyrosine phosphorylations were blocked with both inhibitors, whereas the EGF and FGF pathways remained intact (Figure 3, A and B); the characteristic ERK phosphorylations seen after RTK activation in NIH3T3 and PC12 cells provided convenient markers of pathway inhibition. BDNF-induced ERK2 phosphorylations were sim-

ilar to those of NGF in that they were completely blocked by both K-252a and staurosporine (Figure 3, A and B), as were PDGF-induced ERK2 phosphorylations (Figure 3A).

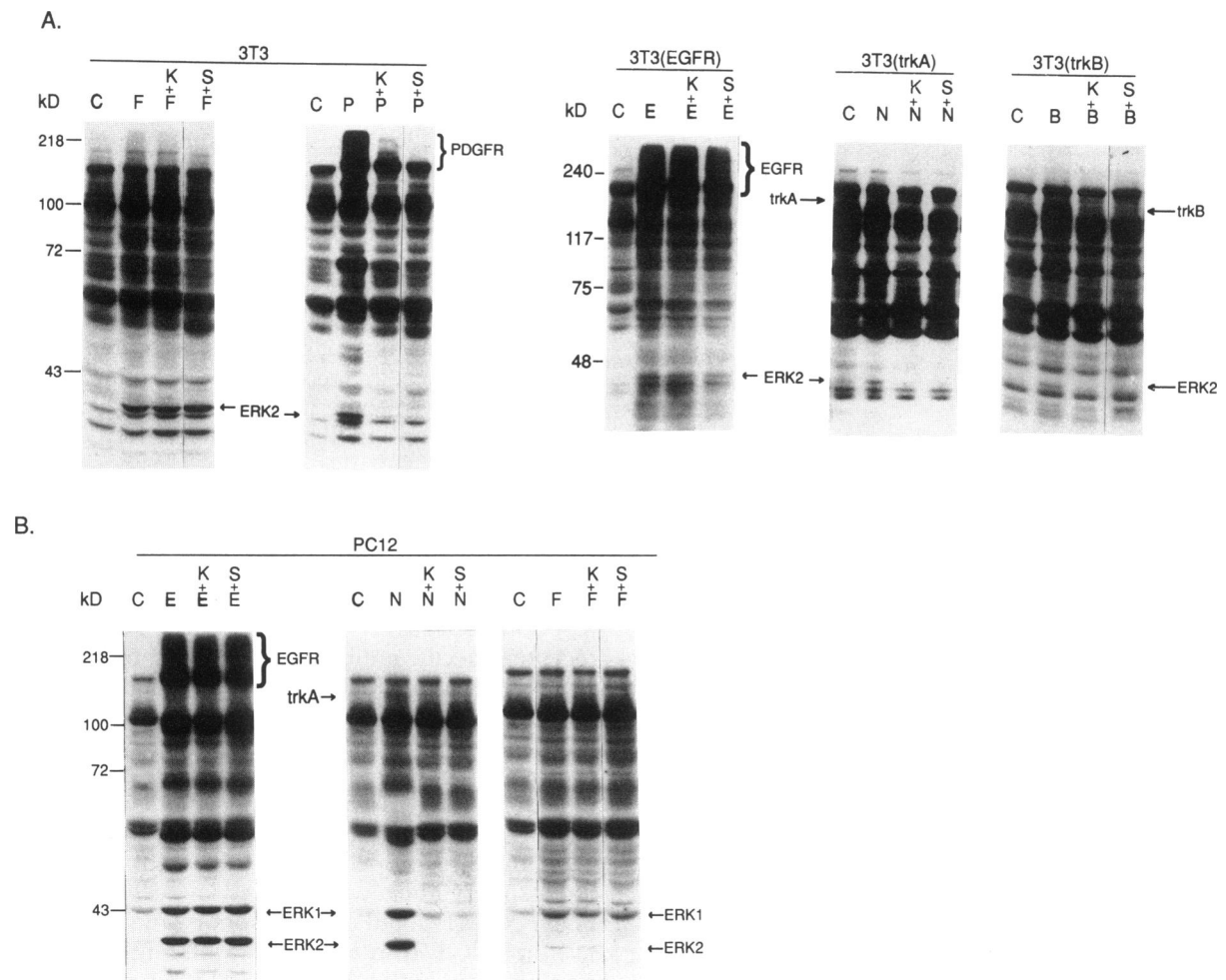
Surprisingly, the tyrosine phosphorylations blocked by K-252a and staurosporine included those of high molecular weight proteins that presumably represent the activated RTKs. This potential inhibition of receptor autophosphorylation was particularly obvious in NIH3T3 cells expressing large amounts of endogenous or introduced RTKs (e.g., PDGFR, trkA, trkB; see Figure 3A). These findings suggested that K-252a and staurosporine may act in a receptor-specific manner to block the initial step of some RTK-mediated signaling pathways by preventing ligand-induced RTK autophosphorylation.

#### ***K-252a and Staurosporine Directly and Selectively Block RTK Autophosphorylation***

To unambiguously demonstrate that K-252a and staurosporine selectively block RTK autophosphorylation, receptor-specific antibodies were used to immunoprecipitate the individual RTKs from ligand-treated cells. Immunoblotting of the precipitates with anti-phosphotyrosine antibodies confirmed that K-252a and staurosporine completely abolish autophosphorylation of all members of the trk family of receptors, whereas unrelated receptor tyrosine kinases were less sensitive to these inhibitors. Specifically, trkA, trkB, and trkC autophosphorylation was blocked in response to NGF, BDNF, or NT-3, but autophosphorylation of the EGFR was not blocked in response to EGF (Figure 4). Concentrations of both inhibitors (100 nM for staurosporine and 200 nM for K-252a) that completely abolished autophosphorylation of the trk receptors only partially blocked PDGFR autophosphorylation (Figure 4), although these concentrations were apparently sufficient to prevent PDGF-induced signaling pathways, as evidenced by complete inhibition of ERK2 phosphorylation (Figure 3A). Higher concentrations of these inhibitors almost completely blocked PDGFR autophosphorylation (Figure 4) but did not affect FGF- or EGF-induced tyrosine phosphorylation. Thus, K-252a and staurosporine directly block RTK autophosphorylation and their subsequent signaling events while acting in a receptor-specific manner that may allow them to be particularly effective blockers of the trk family of RTKs.

#### ***K-252a Specifically Blocks Biological Responses that Depend on Activation of the Trks and PDGFR but not FGFR or EGFR***

Inhibitors that can selectively block the action of a subset of RTKs would be invaluable tools for dissecting the roles of these RTKs in normal and diseased states. However, the utility of such inhibitors for research and therapeutic applications would depend on how they



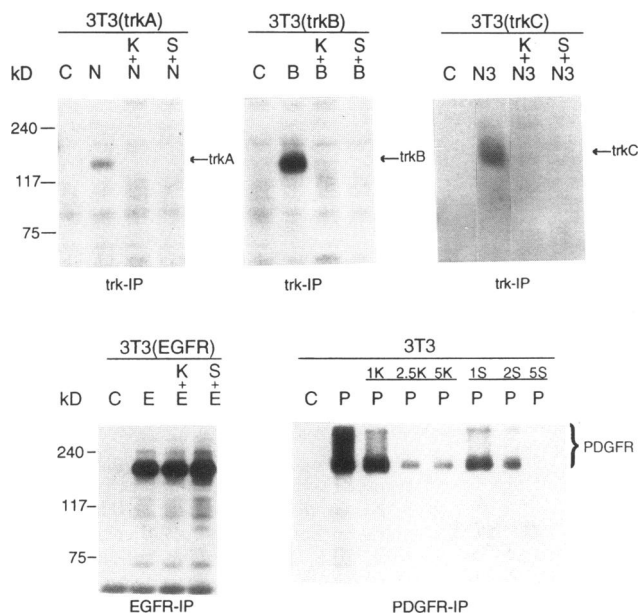
**Figure 3.** Effect of K-252a and staurosporine on RTK-mediated induction of protein tyrosine phosphorylation in NIH3T3 (A) or PC12 cells (B). Cells were untreated (C) or treated with factors as described in Figure 1. In some cases, cells were first pretreated with either 200 nM K-252a (K) or 100 nM staurosporine (S). Brackets or arrows indicate the positions of phosphorylated forms of the EGFR, PDGFR, trkA, trkB, ERK1, and ERK2.

effect the myriad of other protein kinases normally required for cellular growth, differentiation, and survival. To evaluate the potential utility of K-252a and staurosporine for such purposes, we used a biological assay system in which the survival and proliferation of NIH3T3 cells is completely dependent on the presence of the cognate ligand for a variety of endogenous (i.e., FGFR and PDGFR) or introduced (i.e., trkA, trkB, trkC, and EGFR) RTKs (Zhan and Goldfarb, 1986; Glass *et al.*, 1991; Ip *et al.*, 1992). We found that the survival and proliferation of NIH3T3 cells using the neurotrophin/trk and PDGF/PDGFR pathways were sensitive to concentrations of K-252a similar to those that specifically blocked trk and PDGFR mediated tyrosine phosphorylations, whereas cells dependent on the FGF/FGFR or EGF/EGFR pathway were relatively resistant to these concentrations of K-252a (Figure 5). These biological specificities of K-252a action were strikingly

consistent with its ability to directly inhibit the particular RTK being used to support ligand-dependent cell survival and proliferation. Thus, these results highlight the remarkable specificity of K-252a action on living cells—it blocks the initial step (i.e., RTK autophosphorylation) in some, but not all, RTK-dependent signal transduction pathways without compromising components of other pathways essential for survival and proliferation.

## DISCUSSION

Our results are consistent with a model in which distinct cell types present RTKs with different menus of signal transduction components, which in turn determine the ultimate biological responses the RTKs can elicit from that cell. These components presumably include “primary” substrates that directly interact with the RTKs, as well as “secondary” substrates that are further



**Figure 4.** Selective disruption of RTK autophosphorylation by K-252a and staurosporine. NIH3T3 cells alone and those expressing either EGFR, trkA, trkB, or trkC were pretreated with either K-252a or staurosporine and then stimulated with growth factors or neurotrophins as described in Figure 3. RTKs were immunoprecipitated with receptor antibodies specific to either EGFR, the trks, or PDGFR and immunoblotted with anti-phosphotyrosine antibodies as described in MATERIALS AND METHODS. K refers to 200 nM of K-252a, whereas S refers to 100 nM staurosporine. Higher concentrations of these inhibitors used to block the PDGFR are denoted as multiples of K and S. The positions of autophosphorylated EGFR, trkA, trkB, trkC, and PDGFR are indicated along with the positions of molecular weight markers.

downstream in the signaling pathway. Thus, the same RTK induces a distinct pattern of protein tyrosine phosphorylation (of which the ERKs are just 1 example) in a fibroblast cell as compared with a neuronal cell. These differences early in the signal transduction process are presumably further amplified, ultimately resulting in the fundamentally different phenotypic responses to RTK activation displayed by a fibroblast (i.e., proliferation) compared with a neuronal-like cell (i.e., growth arrest and neuronal differentiation). Just as cellular context dictates that the same receptor can have differing effects on distinct cell types, cellular context (e.g., that of an NIH3T3 cell) can apparently also constrain different RTKs (e.g., FGFR, EGFR, PDGFR, and the trk family of receptors) to elicit similar biological responses (e.g., survival and proliferation) by limiting the transduction components (e.g., presence of ERK2 but not ERK1) available in that cell.

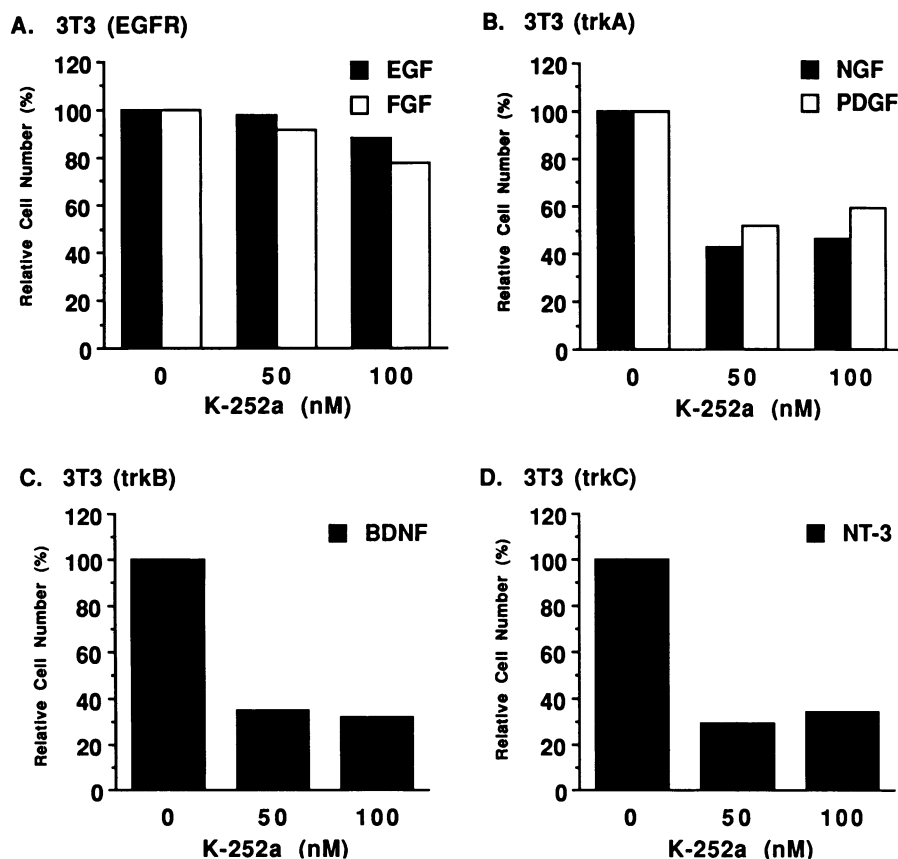
Although RTKs often elicit similar responses when activated within the same cellular environment, it is clear that different RTKs can display distinct signaling capabilities. Thus, not all RTKs induce proliferation in fibroblasts (Zhan and Goldfarb, 1986) or neuronal dif-

ferentiation of PC12 cells (reviewed in Fujita *et al.*, 1989). Our data reveal that differences between RTK signaling capabilities are apparent at the level of the tyrosine phosphorylations they induce (e.g., although NGF, FGF, and EGF all stimulate phosphorylation of ERK1 and ERK2 in PC12 cells, there are other notable differences in the tyrosine phosphorylations they induce in these cells). Furthermore, the RTKs display dramatically different susceptibilities to K-252a and staurosporine inhibition, revealing intrinsic differences within the RTK catalytic domains that may provide a basis for their distinct signaling capabilities.

The ability of K-252a and staurosporine to block autophosphorylation of the three known members of the trk family in NIH3T3 fibroblasts is consistent with the notion that these inhibitors act directly on the highly related trk catalytic domains without relying on accessory molecules specific to neuronal cells, such as the low-affinity NGF receptor, to mediate their effects. The inhibitory actions of K-252a and staurosporine are not strictly limited to the trk RTKs; although they do not inhibit FGF or EGF receptors, they do block PDGF-induced autophosphorylation and proliferation, although higher concentrations are required. Despite the fact that K-252a and staurosporine were initially characterized as exceptionally potent PKC and cyclic nucleotide-dependent kinase inhibitors (Kase *et al.*, 1987), they block trk-mediated biological responses at concentrations where they do not effect these serine/threonine kinases (Koizumi *et al.*, 1988; Lazarovici *et al.*, 1989). Furthermore, these concentrations of K-252a and staurosporine are not obviously detrimental to trk-independent metabolic processes, because they do not interfere with FGF-induced differentiation of neuronal cells (Koizumi *et al.*, 1988; Matsuda and Fukuda, 1988) or FGF- and EGF-mediated proliferation of fibroblasts, as demonstrated here.

The remarkable specificity displayed by K-252a and staurosporine suggests great promise for these class of compounds for both research and therapeutic applications. The only other class of kinase inhibitors capable of selectively blocking RTK actions are the tyrphostins, some of which have been shown to be selective blockers of the EGFR (Yaish *et al.*, 1988; Lyall *et al.*, 1989; Levitzki and Gilon, 1991). In contrast to K-252a and staurosporine, which are thought to act by competitively blocking the kinase nucleotide binding site, the tyrphostins apparently act by disrupting interaction of the RTK with its substrates. Thus, K-252a and staurosporine appear to be more effective than the tyrphostins at blocking the first step in RTK signaling pathways (receptor autophosphorylation) and may therefore allow these molecules to more effectively prevent all subsequent signaling events. In any case, K-252a and staurosporine represent a second class of molecules that can act as selective RTK inhibitors. Their related chemical structures and their selective biological activity should





**Figure 5.** Effect of K-252a on biological responses that depend on activation of RTKs. In defined medium, NIH3T3 cells alone or those expressing either the EGFR, trkA, trkB, or trkC were simultaneously treated with the appropriate survival molecule in the presence of K-252a. Cell viability was determined after 48–72 h using an MTT dye assay as described in MATERIALS AND METHODS. The relative cell number is expressed as a percentage of the cells surviving factor plus inhibitor treatment to those grown in the presence of factor alone. Data represent the average of at least triplicate values; standard deviations were consistently <5% of the average experimental value.

provide a basis for the rational design of additional RTK inhibitors with even greater receptor specificity. The development of such RTK-specific inhibitors would allow research and therapeutic approaches to take advantage of some of the same strategies nature uses to restrict the actions of generally available ligands by allowing for actions only on cells expressing the appropriate receptor.

RTK-specific inhibitors could be used to define the role of individual RTKs in particular biological processes. A variety of disease states, particularly those that result in the abnormal growth of cells, have been shown to result, in part, from the inappropriate activation of RTKs (reviewed in Aaronson, 1991). Hence, small molecule inhibitors of RTKs could prove to be more precise, and presumably less toxic, chemotherapeutic agents than currently available drugs that tend to target general processes, such as nucleic acid metabolism or cytoskeletal architecture. Specific examples of abnormal cell growth triggered by the activation of RTKs is perhaps best observed in some tumor cells that use autocrine loops to drive cell proliferation. For example, melanomas and some astrocytomas use an FGF/FGFR autocrine loop to promote unregulated cell proliferation (Becker *et al.*, 1989; Morrison, 1991). We recently have used antisense approaches to demonstrate that the neurotro-

phins are involved in autocrine loops that are required not only for proliferation but also for survival of neuroblastoma tumor cells (Squinto, Nye, Glass, Fandl, Gies, Israel, Furth, and Yancopoulos, unpublished data). Disruption of the neurotrophin-dependent autocrine loop in these tumor cells with either antisense oligonucleotides directed against the neurotrophic factor, or with K-252a that blocks receptor autophosphorylation, results in rapid cell death. However, the potential clinical application of antisense oligonucleotides awaits the resolution of several technical barriers (Chrisey and Hawkins, 1991). Therefore, small molecule inhibitors of trk autophosphorylation, such as K-252a or structurally related analogues, might offer the most immediate hope for a highly selective and easily deliverable treatment for neuroblastoma or other tumor cells that use a neurotrophin/trk autocrine survival loop.

**Note added in proof.** Subsequent to the completion of our studies, similar results concerning the ability of K252a to inhibit trkA autophosphorylation in PC12 cells were reported by M.M. Berg, D.W. Sternberg, L.F. Parada, and M.V. Chao (J. Biol. Chem. 267, 13–16 [1992]), and similar results with K252a inhibition of all trk family members were reported by P. Tapley, F. Lambelle, and M. Barbacid (Oncogene 7, 371–381 [1992]).

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