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The Staurosporine-Like Compound L-753,000 (NB-506) Potentiates the Neurotrophic Effects of Neurotrophin-3 by Acting Selectively at the TrkA Receptor

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ABSTRACT

K-252b, a member of the staurosporine family of protein kinase inhibitors, selectively potentiates the activation of the nerve growth factor receptor, TrkA, by a nonpreferred ligand, neurotrophin-3 (NT-3), in a variety of cell types. At higher (micromolar) concentrations of K-252b, an inhibitory effect occurs because of the inhibitory action of K-252b on the Trk kinase. By examining analogs of K-252b, we identified the compound L-753,000 (NB-506), which potentiates the action of NT-3 on TrkA but is devoid of the inhibitory action of K-252b. L-753,000 was effective at nanomolar concentrations in a Chinese hamster ovary cell line that expressed TrkA but was devoid of p75, the low-affinity neurotrophin receptor. L-753,000 also potentiated the activation of mitogen-activating protein kinase signaling (downstream from Trk activation) by NT-3 in this cell line. Although L-753,000, like K-252b, had a negligible effect in the absence of NT-3, the compound was found to potentiate NT-3-induced survival in both rat and chick primary cultures of dissociated dorsal root ganglia (DRG) and on neurite outgrowth of chick DRG explants. Unlike K-252b, which at micromolar concentrations inhibits the survival response of NT-3 in dissociated rat DRG, L-753,000 continued to potentiate the actions of NT-3 up to a concentration of 10 μM. Furthermore, the compound, unlike K-252b, did not inhibit an unrelated protein kinase, protein kinase C, at concentrations up to 10 μM. Because L-753,000 selectively potentiates the NT-3-induced stimulation of TrkA without inhibiting Trks and other protein kinases, it represents a novel class of selective modifiers of neurotrophin actions.

Neurotrophins (NTs) are proteins that have a well established role in the maintenance of neuronal populations of cells both during development and in the adult life of the neuron. Preclinical and clinical data suggest that subcutaneous or intravenous administration of NTs may be an effective treatment for peripheral neurodegenerative disorders (McMahon and Priestly, 1995; Rask and Escandon, 1998). Treatment of central nervous system diseases with NTs is also conceivable because of the robust effects of NTs on the affected neurons both in vitro and in animal models (Barde, 1998; Connor and Dragunow, 1998). However, because of their poor pharmacokinetic behavior and bioavailability at the desired targets, proteins are not ideal drug candidates. Therefore, much effort has been made in the search for small-molecule NT mimetics that act like NTs to elicit desired neuroregenerative responses (Swain et al., 1998).

Analogs of NTs may be designed to mimic the conformation of the natural protein ligand and interact with the same binding site on the NT receptor. It may also be possible to find compounds that affect NT receptor function by binding to regions different from those interacting with the NTs, as allosteric regulators of receptor activation. This mechanism would be analogous to the benzodiazepine modification of γ-aminobutyric acid A receptor function. Such compounds could act on their own or could potentiate the action of endogenous NTs.

The receptors for NTs are members of a family of transmembrane tyrosine receptor kinases (TrkA, TrkB, and TrkC). Each NT binds to a preferred receptor in the family: nerve growth factor (NGF) binds mainly TrkA, brain-derived neurotrophic factor (BDNF) and NT-4 bind TrkB, and NT-3 binds TrkC. However, this specificity is not strict and the NTs have measurable affinity (at least in vitro) for the noncognate receptors (Urfer et al., 1995). The binding of an NT to its Trk receptor triggers an array of cellular signaling responses that produce neurotrophic effects, such as cell survival and neurite outgrowth. First, NT-binding induces receptor homodimerization, which is followed by *trans-*

ABBREVIATIONS: NT, neurotrophin; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; MAP, mitogen-activating protein; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; PMSF, phenylmethylsulfonyl fluoride; GAP, growth-associated protein; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbant assay; CHO, chinese hamster ovary; DMSO, dimethyl sulfoxide; PKC, protein kinase C.

phosphorylation of the intracellular region of the receptor by its kinase domains (Greene and Kaplan, 1995). The phosphorylation of particular tyrosine residues on this intracellular region follows a precise sequence of events as the receptor itself is activated. Signaling molecules on the phosphatidylinositol-3-kinase and Ras/mitogen-activating protein (MAP) kinase pathways (e.g., shc), as well as phospholipase C-γ and the suc-associated, neurotrophic factor-induced, tyrosine-phosphorylated target, bind to specific phosphotyrosine-containing recognition sequences and themselves become phosphorylated through the tyrosine kinase activity of the Trk receptor (Greene and Kaplan, 1995). The activated signaling molecules then trigger effects on gene transcription and regulation of cytoskeletal machinery, leading to cell survival and differentiation responses.

The K-252 class of compounds are related indolocarbazoles of microbial origin that have been found to inhibit a range of protein kinases, including Trk, by binding competitively to the ATP binding site of the kinase domain (Berg et al., 1992; Nye et al., 1992; Ohmichi and Decker, 1992). Because of these inhibitory effects, K-252a and K-252b inhibit the actions of NTs in a range of models (Koizumi et al., 1988; Matsuda and Fukuda, 1988; Berg et al., 1992; Knüsel and Hefti, 1992; Knüsel et al., 1992). In addition to inhibiting Trk phosphorylation, K-252b, at lower concentrations than those producing measurable inhibitory effects, potentiates the stimulatory effect of NT-3 on the noncognate receptor, TrkA. This leads to neurotrophic effects that include cholinergic neuron differentiation, peripheral sensory neuron survival, and neurite outgrowth in pheochromocytoma PC12 cells (Knüsel et al., 1992). The NT-3 response in the presence of K-252b is equivalent to an optimal NGF response and leads to the full signaling cascade observed with TrkA activation by NGF (Knüsel et al., 1992; Isono et al., 1994). Interestingly, although the NTs and their receptors are highly homologous, the potentiation effect of K-252b observed in Trk-expressing cell lines was specific for NT-3 acting on TrkA. No effect was observed with the other NTs (NGF, BDNF, or NT-4/5) or on the other Trk receptors (TrkB and TrkC) (Maroney et al., 1997).

Although the potentiation of NT action on the NGF receptor may be potentially useful therapeutically (for the treatment of peripheral neuropathy, for example), the K-252 compounds have non-Trk-related effects that make them undesirable as neurotrophic agents. In addition to its nonspecific kinase inhibitory activity, K-252a was found to enhance MAP kinase activity (Wu and Zhang, 1993; Isono et al., 1994) and focal adhesion kinase activity (Maroney et al., 1995). Although these compounds are not pharmacologically clean, it may be possible to dissect the potentiation effect observed with K-252b on NT-3 activation of TrkA from the other activities by examining related structures. We report here the identification of one such compound, L-753,000 (NB-506), that potentiated NT-3 action on TrkA to produce a range of neurotrophic effects without the kinase inhibitory response of K-252b.

Experimental Procedures

Materials. The DNA encoding human TrkA was obtained from D. Kaplan (McGill University, Montreal, Quebec, Canada). Human recombinant NTs were purchased from Peprotech (London, UK) and ^{125}I-labeled NGF and NT-3 (custom labeled) were obtained from Amersham Life Science Ltd. (Little Chalfont, UK). Cell culture media were obtained from Gibco BRL (Paisley, UK), except for Ham's F14 medium, which was obtained from Imperial Laboratories (Andover, UK). The anti-Trk antibody sc-139 and its agarose conjugate were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antiphosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Secondary antibodies, peroxidase-conjugated avidin-biotin complex (ABC) kits, Vector SG kits used for outgrowth studies, and the rabbit antisheep secondary antibody were purchased from Vector Laboratories (Peterborough, UK). The horseradish peroxidase (HRP) substrate K-Blue was from Bionostics Ltd. (Wyboston, UK). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham. K-252b was purchased from Calbiochem (Beeston, UK). L-753,000 (NB-506; 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6*H*)-dione) was supplied through a collaboration with the Banyu Tsukuba Research Institute (Arakawa et al., 1995; Yoshinari et al., 1995). Aprotinin (#A6279), sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), antigrowth-associated protein-43 (GAP-43; clone no. GAP-7B10), and all other chemicals were purchased from Sigma-Aldrich (Poole, UK) and HRP-coupled secondary antibodies were purchased from Amersham (Western blotting) or Sigma-Aldrich. The MAP kinase pathway inhibitors PD98059 and U0126 were obtained from Calbiochem and Promega Life Science (Southampton, UK), respectively.

Culture of Dissociated Dorsal Root Ganglia (DRG) Neurons and GAP-43 Enzyme-Linked Immunosorbent Assay (ELISA). DRG were dissected and removed from either E16 rat embryos or 10-day incubation chick embryos. For rat DRG, ganglia were removed from the whole length of the spinal cord, whereas for chick, only the easily accessible lumbar ganglia from the abdominal region were removed. Ganglia were collected in Hanks' balanced salt solution and dissociated in 0.25% trypsin and cultured in poly-D-lysine/laminin-coated, 96-well plates in Ham's F14 medium supplemented with either SATO [final concentration: 4.3 mg/ml BSA, 0.77 μg/ml progesterone, 20 μg/ml putrescine, 0.49 μg/ml L-thyroxine, 0.048 μg/ml selenium, and 0.42 μg/ml triiodothyronine (Bottenstein and Sato, 1979)] for 30 min (rat DRG) or 10% fetal calf serum for 2 h (chick DRG). The cultures were then treated with compounds and/or NTs made up in F14 supplemented with SATO for 48 h at 37°C/5% CO_2. After 48 h, cultures were then fixed with 4% paraformaldehyde. Fixed cultures were treated with 5% normal horse serum in PBS/0.3% Triton X-100 for 1 h, followed by monoclonal antibody raised against GAP-43 (1:500) overnight. Cultures were then treated with an HRP-conjugated sheep antimouse secondary antibody (1:1000 in blocking serum) for 1 h. K-blue, a peroxidase substrate, was added, and color change was read at 650 nm on an ELx 800 plate reader (Biotek Instruments Inc, Winooski, VT). For each plate, a standard curve with NGF was incorporated, and data obtained for all compounds were expressed as a percentage of the NGF response. To visualize the DRG for the purpose of cell counting, a biotinylated rabbit antisheep antibody (1:200) was added to the wells for 30 min, followed by incubation with a peroxidase-conjugated avidin-biotin complex for 30 min. Surviving neurons were then visualized using an insoluble peroxidase substrate (Vector SG), and the entire well was counted by eye.

Culture and Image Analysis of Chick DRG Explants. Explants of E10 chick DRG were cultured in three-dimensional collagen gels for 2 h at 37°C/5% CO_2 to allow gels to set. Compounds and NTs were then added in Ham's F14 medium supplemented with SATO, and the cultures returned to the incubator for 48 h. The cultures were then fixed with 4% paraformaldehyde and treated with 10% horse serum in PBS/1% Triton X-100 overnight. Thereafter, explants were incubated with a monoclonal antibody raised against GAP-43 (1:500) overnight. A peroxidase-conjugated avidin-biotin complex was then added overnight to the cultures. Staining was visualized using Vector SG peroxidase substrate. Explants were then mounted

onto glass slides. Neurite outgrowth in these cultures was quantified using MicroComputer Imaging Device (MCID) image analysis software (Imaging Research, St. Catharines, Ontario, Canada) to measure the amount of pixels occupied by neurites (Bilsland et al., 1999). The area of the whole DRG was established using densitometry, from which the area occupied by the body of the DRG was subtracted. Data are expressed as mean ± S.E.M.

Cell Line Construction and Binding Assays. The DNA encoding human TrkA was cloned into the pcDNA-3 expression vector. Chinese hamster ovary (CHO) cells were transfected with the construct and a positive clonal cell line was selected under neomycin resistance with 2 mg/ml G418 using in situ hybridization to detect TrkA expression. Positive clones were confirmed by Western blotting, probing with the anti-Trk antibody sc-139 (0.5 μg/ml), and recloned to ensure a single cell origin. NGF binding assays were used to quantify TrkA receptor density. Cells were seeded overnight in 24-well tissue culture plates at 150,000 cells per well (1 ml) in normal media (Iscove's with 2× hypoxanthine/thymidine (HT) supplement, 10% fetal clone II, 1% penicillin-streptomycin, and 0.4 mg/ml G418). The cells were washed once with ice-cold buffer A (50 mM HEPES, 150 mM NaCl, pH 7.4, 0.3 mM $CaCl_2$) and incubated for 6 h at 4°C in 0.5 ml of binding buffer (100 mM HEPES, pH 7.8, 120 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 150 mM sodium acetate, 10 mM glucose, 1% BSA, and 1 mM PMSF) containing various concentrations of unlabeled NGF and approximately 100,000 dpm (50 μl of a 1 μCi/ml stock) of ^{125}I-labeled NGF. Under these conditions, samples containing only labeled NT (no unlabeled NT) typically had NT concentrations of approximately 40 pM. Nonspecific binding was determined at 500 nM NT. Cells were washed twice with buffer A, lysed with 0.5 ml of 0.05% SDS at 37°C, and the NT bound was determined by γ-counting and Scatchard analysis. The same protocol was followed using ^{125}I-labeled NT-3 and a range of unlabeled NT-3 concentrations (40 pM to 2 nM) to evaluate the effects of L-753,000 on NT-3 binding. L-753,000 (1 μM final concentration) was added in a 2% (v/v) dimethyl sulfoxide (DMSO) vehicle, which was also used in the control wells.

Western Blotting. CHO cells expressing human TrkA were serum-deprived for 2 h and then treated in serum-free medium with either vehicle (0.25% DMSO) or L-753,000 in the presence or absence of NT-3 (added from a 100× stock solution in 1% BSA in PBS), each

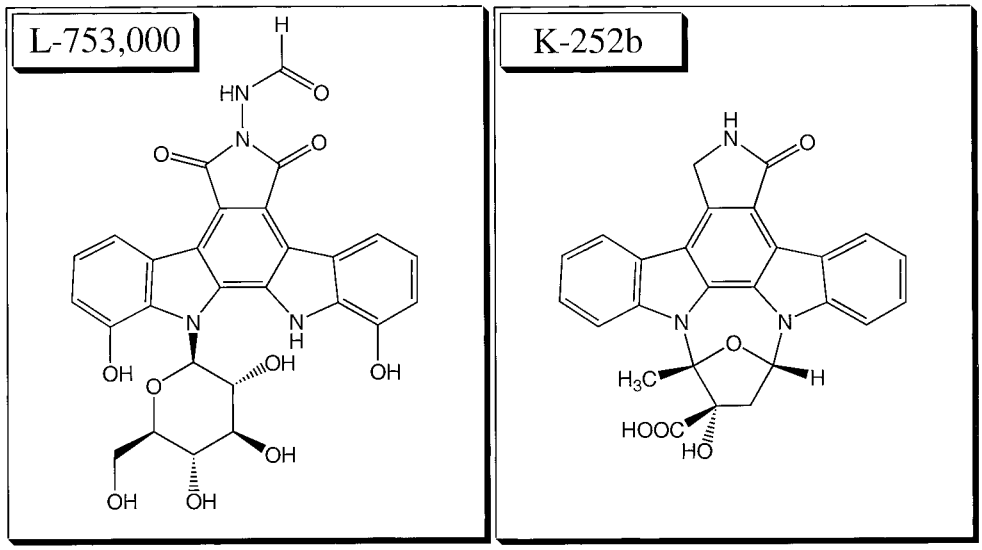

Fig. 1. The structures of L-753,000 and K-252b.

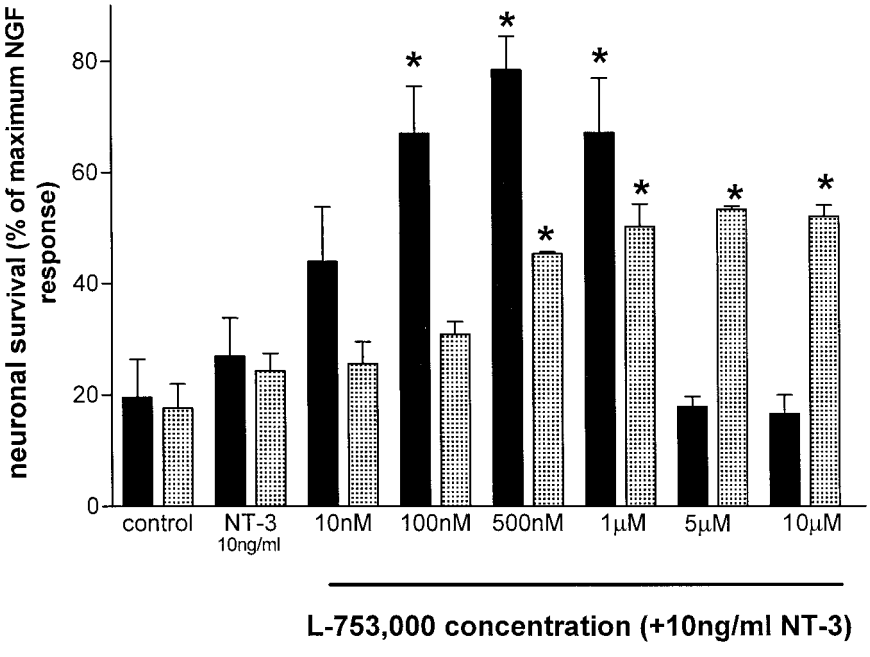

Fig. 2. Effect of K-252b or L-753,000 in combination with NT-3 on survival of rat dissociated DRG, as determined by GAP-43 ELISA. Results are expressed as percentage of maximum NGF response (100 ng/ml; $n = 4$ per experiment) and the mean of three independent experiments ± S.E. Data were analyzed using one-way ANOVA followed by Dunnett's test comparing all groups with NT-3. Asterisks denote significance ($p < .05$). Results demonstrate that there is a small increase in neuronal survival as shown by an increase in GAP-43 expression in cultures treated with NT-3 compared with control cultures and that there is a further dose-dependent increase in survival in cultures treated with increasing concentrations of either L-753,000 (dotted columns) or K-252b (solid columns) combined with NT-3.

at the indicated final concentration. After 10 min, cells were lysed in radioimmunoprecipitation assay buffer [PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.25 mM PMSF (from a 100 mM stock solution in isopropanol), 30 μl/ml aprotinin, and 1 mM sodium orthovanadate] with sonication for 15 min. The lysates were then subjected to immunoprecipitation using an agarose-conjugated anti-Trk antibody, sc-139 (20 μl). The resulting immunoprecipitated pellets were then electrophoresed on a 6% Tris-glycine gel and probed by Western blotting using the antiphosphotyrosine antibody 4G10 (0.1 μl/ml) and a secondary anti-mouse HRP conjugate (1/1000; Amersham). The phosphorylated bands were then visualized by ECL. Blots were then stripped and reprobed for Trk immunoreactivity using the anti-Trk rabbit polyclonal antibody sc-139 (0.5 μg/ml) followed by antirabbit HRP-coupled antibody and ECL detection. Densitometric measurements of the bands were carried out using the MCID image analysis software.

MAP Kinase Assays. MAP kinase activity was determined using a kit from Upstate Biotechnology (#17–184), following the manufacturer's instructions. Briefly, cells were serum-starved for 16 h, stimulated as described above for Western blotting experiments, and lysed in 50 mM Tris, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% β-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerol phosphate, 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml microcystin. MAP kinase was immunoprecipitated using protein A agarose/anti-rat MAP kinase R2 antibody. A kinase assay was run on the washed pellets using myelin basic protein as a substrate and [γ-^{33}P]ATP in place of [γ-^{32}P]ATP. The kinase assay mixtures were spotted on phosphocellulose filter squares, which were then washed and scintillation counted to determine the amount of the phosphorylated polypeptide product of the MAP kinase reaction.

Protein Kinase C (PKC). PKC activity was determined in a cell-free assay using a kit from Upstate Biotechnology (#17–139), following the manufacturer's instructions. Briefly, the kinase assay was run in the presence of the indicated concentrations of compound or vehicle (2% DMSO) using purified PKC from rat brain (containing α, β, and γ isoforms; Upstate Biotechnology), the supplied synthetic PKC substrate and [γ-^{33}P]ATP in place of [γ-^{32}P]ATP. The kinase assay mixtures were spotted on phosphocellulose filter squares, which were then washed and scintillation counted to determine the amount of the phosphorylated peptide product of the PKC reaction.

Statistical Analyses. Statistical significance was determined by performing an ANOVA followed by Dunnett's t test. Results are considered significantly different when $p < .05$.

TABLE 1

Cell counts of surviving neurons from rat dissociated DRG cultures obtained from an ELISA plate after GAP-43 analysis

Data were analyzed by one-way ANOVA followed by Dunnett's t test comparing all groups to NT-3. Data confirm results obtained using GAP-43 ELISA, illustrating potentiation of survival effect of NT-3 with nanomolar concentrations of both K-252b and L-753,000; however, at micromolar concentrations, only L-753,000 maintains this effect.

	Control	NGF 10	NT-3 10	K-252b + NT-3 (10 ng/ml)					
				10 nM	100 nM	500 nM	1 μM	5 μM	10 μM
		ng/nl							
	3		51	245	727	866	564	7	0
	5		32	192	485	589	489	10	0
	4		53	259	539	697	403	9	1
	2		56	173	314	449	439	13	0
Mean	3.5*		48	217.25*	516.25*	650.25*	473.75*	9.75*	0.25*
S.E.M.	0.65		5.43	20.63	85.06	88.03	34.87	1.25	0.25
				L-753,000 + NT-3 (10 ng/ml)					
	3	900	34	37	58	153	315	631	365
	1		27	44	87	224	368	355	397
	2	734	37	33	73	228	382	397	411
	4	476	66	58	97	306	272	355	226
Mean	2.5*	663.75*	41	43	78.75*	227.75*	334.25*	434.5*	349.75*
S.E.M.	0.65	95.79	8.59	5.49	8.49	31.26	25.27	66.24	42.36

* $p < .05$ vs. NT-3.

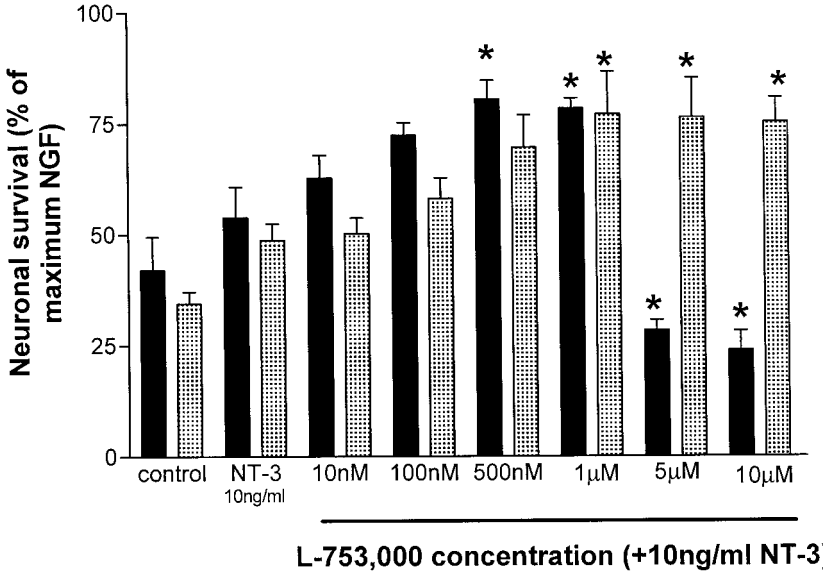

Fig. 3. Effect of K-252b or L-753,000 in combination with NT-3 on survival of chick-dissociated DRGs, as determined by GAP-43 ELISA. Results are expressed as percentage of maximum NGF response (100 ng/ml; $n = 4$ per experiment) and the mean of four independent experiments ± S.E. Data were analyzed using one-way ANOVA followed by Dunnett's test comparing all groups with NT-3. Asterisks denote significance ($p < .05$). Both K-252b (solid columns) and L-753,000 (dotted columns) showed a similar potentiation of NT-3 induced survival in chick DRG neurons; however, at higher concentrations, K-252b caused survival to significantly decrease below that induced by NT-3.

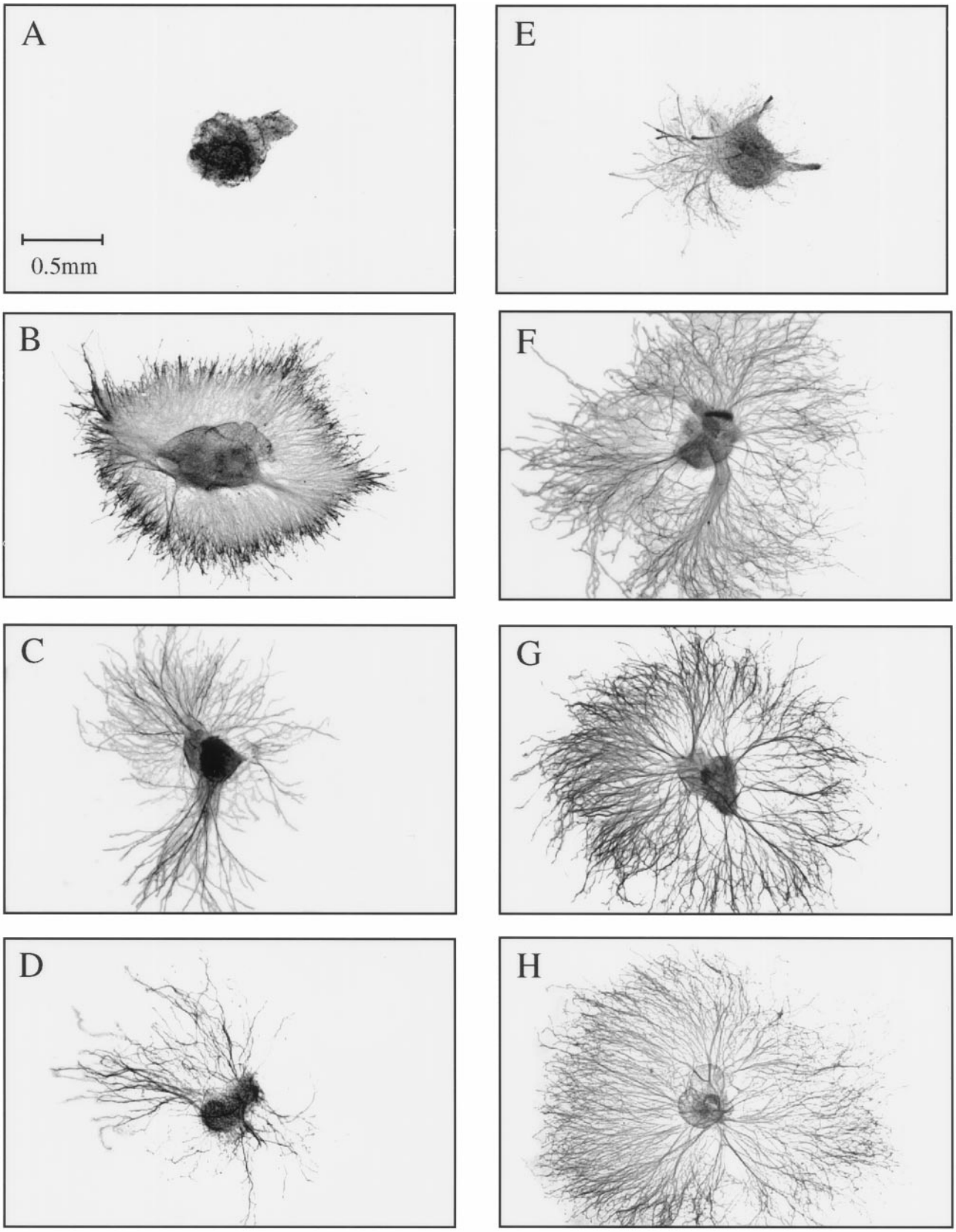

Fig. 4. Photomicrographs of E10 chick DRG explants cultured in DMEM supplemented with SATO. Ganglia were cultured for 48 h, fixed and stained with an antibody raised against GAP-43. A, control; B, NGF (10 ng/ml); C, 10 ng/ml NT-3; D–H, a combination of 10 ng/ml NT-3 and increasing concentrations of L-753,000. D, 10 nM; E, 100 nM; F, 500 nM; G, 1 μM; H, 10 μM. The calibration bar shown in A indicates the magnification for all panels.

Results

L-753,000 and K-252b Potentiate the Effect of NT-3 on Neuronal Survival. L-753,000 (NB-506) (Fig. 1), originally developed as an anticancer agent (Arakawa et al., 1995; Yoshinari et al., 1995), was identified from a search of the Merck and Banyu chemical collections using staurosporine as the starting structure. Because E16 DRG cultured for 48 h and assayed for neuronal survival using the GAP43 ELISA method produces a result that correlates with numbers of surviving neurons (L.Y., J.G.B. and S.J.H., unpublished observations), we have used this method to assess neuronal survival in response to combinations of NT-3 and K-252b or L-753,000. Both K-252b and L-753,000 were tested for neuronal survival effects on rat DRG in the absence of any additional neurotrophic factors; neither compound promoted neuronal survival at concentrations ranging from 1 nM to 10 μM (data not shown). However, when L-753,000 or K-252b was added at nanomolar concentrations in combination with NT-3 (10 ng/ml), there was an increase in survival compared with survival with NT-3 alone as measured in the GAP-43 ELISA assay (Fig. 2). This potentiation effect on survival reached a maximum at 500 nM for K-252b and declined thereafter. This decline is probably caused by inhibition of TrkA activation at higher concentrations of K-252b. L-753,000 caused a potentiation of NT-3-induced survival, which reached a plateau at 1 μM; in contrast to K-252b, no decline in activity was seen. L-753,000 was, on the average, not as efficacious as K-252b in potentiating the NT-3-induced survival in rat DRG neurons. Cell counts of surviving neurons from a representative experiment confirm the potentiation effects of submicromolar concentrations of L-753,000 and K-252b observed in the GAP-43 ELISA assay, indicating that either method gives an equivalent measure of cell survival (Table 1). Comparison of surviving neurons in K-252b and L-753,000 treated cultures, analyzed using this method, also indicates that L-753,000 is not as efficacious as K-252b.

Chick DRGs have different neurotrophic factor responsiveness to rat DRGs. For example, more neurons respond to BDNF with survival and neurite outgrowth and fewer neurons respond to NGF (Barde et al., 1982). Chick DRGs also show a survival response to K-252a, unlike rat DRG (Borasio, 1990). Despite these differences, both L-753,000 and K-252b potentiated the NT-3-induced survival of chick DRG neurons. In this case, L-753,000 was as efficacious as K-252b in enhancing neuronal survival in the presence of NT-3 (Fig. 3); with each compound, the survival response approached that observed with NGF (70% of maximum NGF response). L-753,000 achieved a maximum response at 1 μM and stayed constant up to 10 μM. However, the effect with K-252b declined to control values at 5 and 10 μM. As with rat DRG neurons, neither compound had any effect on chick DRG survival in the absence of NT-3 (data not shown).

L-753,000 and K-252b Potentiate Effect of NT-3 on Neurite Outgrowth. Neurite outgrowth from embryonic chick DRG is another measure of 'neurotrophic' effects. Therefore we tested L-753,000 in combination with NT-3 for potentiating effects on neurite outgrowth and compared effects with those seen with K-252b. As in previous experiments, there was no effect of either K-252b or L-753,000 on neurite outgrowth from chick DRGs in the absence of NT-3 (data not shown). In the absence of neurotrophic factors, there was virtually no outgrowth from chick DRG (Fig. 4A). NGF (10 ng/ml) caused substantial neurite outgrowth (Fig. 4B) and NT-3 (10 ng/ml) also significantly increased the neurite outgrowth response compared with control (Fig. 4C). Both L-753,000 and K-252b potentiated the neurite outgrowth response of NT-3, with the magnitude almost as great as in explants treated with NGF (Fig. 4, C–H, and Fig. 5).

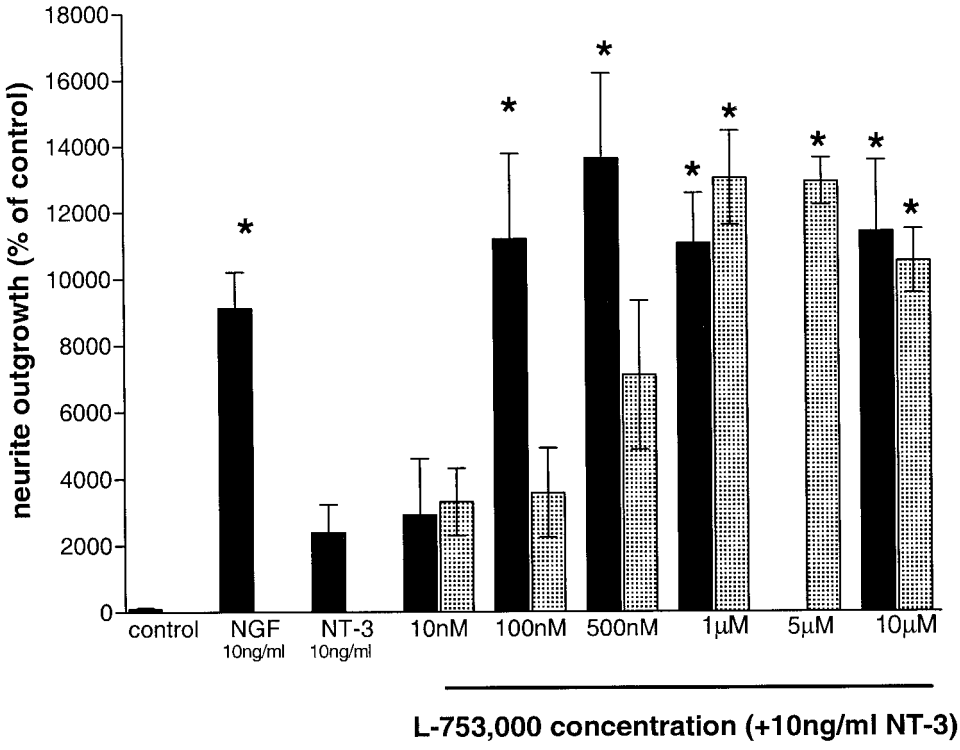

Fig. 5. Quantification of neurite outgrowth from chick DRG explants by MCID image analysis. Cultures were treated with NGF (10 ng/ml), NT-3 (10 ng/ml), or a combination of 10 ng/ml NT-3 and L-753,000 (1 nM to 10 μM; dotted columns) or K-252b (1 nM to 10 μM; solid columns). Data presented are from a single representative experiment comprising three or four ganglia per treatment and are expressed as percentage of control (100%). There is a significant increase in neurite outgrowth in all treatment groups compared with control. Asterisks denote significance compared with NT-3 treatment groups. K-252b caused a potentiation of the NT-3-induced neurite outgrowth at concentrations of 100 nM and above, whereas L-753,000 caused significant potentiation at 1, 5, and 10 μM. Both compounds increased neurite outgrowth to a similar degree as NGF.

Quantification of the extent of outgrowth from DRGs was performed (Fig. 5) and confirmed 1) the dose-dependent increase in neurite outgrowth in ganglia treated with NT-3 in the presence or absence of L-753,000 or K-252b, and 2) that the magnitude of the outgrowth is nearly as great as that seen with NGF.

L-753,000 Potentiates Activation of TrkA by NT-3. A CHO cell line was constructed that stably expresses human TrkA at a density of approximately 10,000 receptors per cell. Untransfected CHO cells lack both Trk receptors and the low-affinity NT receptor p75 (M. Chao, personal communication), so the transfected cell line has a homogeneous population of NT-binding TrkA receptors. The dissociation constant (K_d) for NGF binding TrkA in this cell line was determined to be 0.41 ± 0.23 nM (data not shown), consistent with reported values for NGF-binding to TrkA expressed in other cell lines in the absence of p75 (Kaplan et al., 1991; Chao and Hempstead, 1995).

NGF caused a dose-dependent TrkA activation, as measured by phosphorylation of the Trk receptor (Western blotting) in the CHO-TrkA cell line (Fig. 6). NT-3 also showed a suboptimal TrkA response. Similar results were obtained in rat PC12 cells that express both TrkA and p75 (Fig. 7). The ability of NT-3 to activate TrkA was potentiated by L-753,000 and, to a lesser extent, by K-252b (each at 250 nM). The compounds had a negligible effect on TrkA activation in the absence of NT-3 in both cell lines (Fig. 8). The potentiation effect of L-753,000 on TrkA activation in CHO-TrkA cells was shown to occur at concentrations up to 10 μM, unlike K-252b, which was inhibitory at this concentration (Fig. 8). These differences between the compounds are consistent with the results of the DRG survival studies. L-753,000 did not have any effect on its own on NT-3-induced TrkB or TrkC autophosphorylation in similar CHO cell lines expressing these receptors (data not shown). Likewise, L-753,000 did not affect NT-induced responses of NGF or BDNF in TrkA, TrkB, or TrkC-expressing CHO cell lines (data not shown).

L-753,000 Selectively Potentiates NT-3 Binding to TrkA. To determine whether effects on NT-3 binding to TrkA are the basis for the effects of L-753,000 on TrkA activation, binding assays were carried out on the CHO-TrkA cell line using ^{125}I-labeled NT-3 in the presence or absence of L-753,000. Although specific binding was negligible in the absence of L-753,000 in the concentration range of NT-3 employed in this study, it increased dramatically in the presence of L-753,000 (1 μM) (Fig. 9). Similar findings were observed with K-252b but not staurosporine (data not shown). In contrast to the effects of L-753,000 on NT-3 binding, the binding of ^{125}I-labeled NGF was not affected by L-753,000 ($K_d \sim 400$ pM; $B_{max} \sim 11{,}000$ receptors/cell in the presence or absence of L-753,000). Moreover, NT-3 binding to TrkB or TrkC (expressed in CHO cell lines) was not affected by L-753,000 (data not shown).

L-753,000 Potentiates Activation of MAP Kinase by NT-3. To examine the downstream signaling elements that lead to the observed neurotrophic effects of L-753,000 and

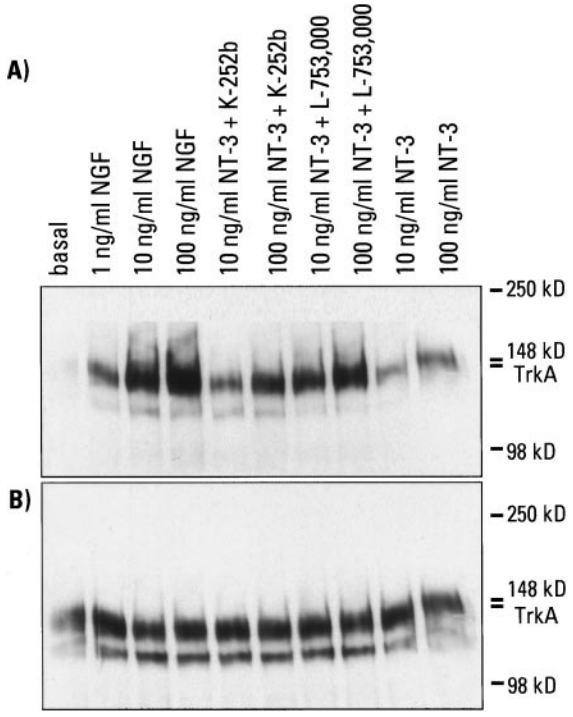

Fig. 6. Western blotting illustrating TrkA autophosphorylation in a CHO cell line expressing human TrkA. A, TrkA basal phosphorylation (band at 140 kDa) increased in a dose-dependent fashion in response to NGF. Phosphorylation also increased moderately in the presence of NT-3 (5 and 12% of the optimal NGF response at 10 and 100 ng/ml NT-3, respectively). K-252b (250 nM) showed a small potentiation of the NT-3 response (14 and 48% of the optimal NGF response), whereas a much stronger potentiation (50 and 77% of the optimal NGF response) was seen with L-753,000 (250 nM). B, the blot was reprobed for Trk immunoreactivity, demonstrating approximately equivalent Trk receptor levels among the different treatment conditions.

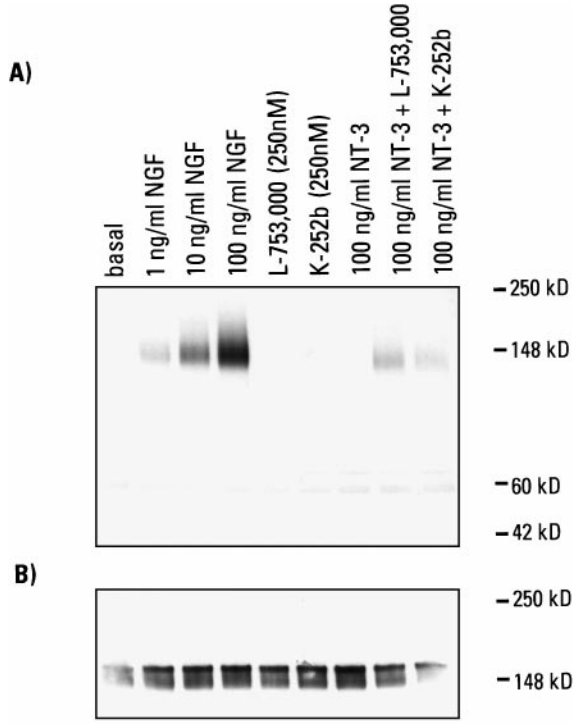

Fig. 7. Western blotting illustrating TrkA autophosphorylation in rat PC12 cells. A, TrkA basal phosphorylation (band at 140 kDa) increased in a dose-dependent fashion in response to NGF. Phosphorylation was not detectable in the presence of NT-3 alone (100 ng/ml) or in the presence of K-252b or L-753,000 (each at 250 nM) alone (<0.3% of the optimal NGF response in each case). K-252b showed a small potentiation of the NT-3 response (3% of the optimal NGF response), whereas a stronger potentiation was seen with L-753,000 (7% of the optimal NGF response). B, the blot was reprobed for Trk immunoreactivity.

K-252b, effects of the compounds on MAP-kinase activation in CHO-Trk A cells were examined in the presence and absence of NT-3. MAP kinase is a key downstream signaling component of TrkA-mediated signaling and plays an essential role in the cell survival and neurite outgrowth responses at least in some neuronal cell types (Cowley et al., 1994; Greene and Kaplan, 1995; Kaplan, 1998). L-753,000 and K-252b were both found to potentiate the action of NT-3 on MAP kinase activation in the same concentration range as the TrkA autophosphorylation and DRG effects were observed (Fig. 10). This effect rendered the NT-3 response on MAP kinase activation equivalent to an optimal NGF response. To determine whether sustained MAP kinase activation is involved in the effect of L-753,000 on DRG survival, the effects of two MAP kinase pathway inhibitors (PD98059 and U0126) were examined. Treatment of DRG cultures with NT-3 (10 ng/ml) and L-753,000 (250 nM) in the presence of either inhibitor (10 to 100 μM) led to complete inhibition of the survival effects of the combined treatment on the DRG cultures and of the neurite outgrowth effects in dissociated DRG explants compared with similar treatment in the absence of PD98059 or U0126 (data not shown). Treatment of DRG cultures with NGF (10 or 100 ng/ml) in the presence of either inhibitor (10 to 100 μM) led to a similar complete loss of the survival and outgrowth responses of NGF alone.

K-252b But Not L-753,000 Inhibits PKC. Because it is known that K-252a and K-252b are relatively nonselective kinase inhibitors, to examine the specificity of K-252-like compounds toward Trk, a cell-free PKC assay was used with purified rat PKC (α, β, and γ isoforms present). Whereas both K-252a and K-252b were found to inhibit PKC with IC_{50} values in the mid-nanomolar range, L-753,000 had no effect on PKC activity at concentrations up to 10 μM (Fig. 11).

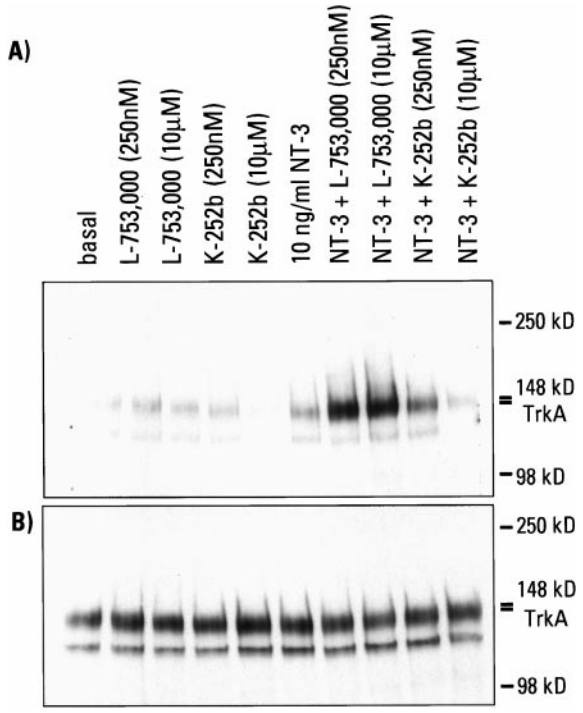

Fig. 8. Effect of different concentrations of L-753,000 or K-252b on TrkA autophosphorylation in the presence or absence of NT-3 in human TrkA-expressing CHO cells. A, negligible phosphorylation (<3% of maximal response) was observed with either compound in the absence of NT-3 (band at 140 kDa). The TrkA autophosphorylation observed in the presence of NT-3 (9% of maximal response; 10 ng/ml) increased in the presence of L-753,000 at 250 nM or 10 μM (85 and 100% of maximal response, respectively) but with K-252b, although potentiation occurred at 250 nM (24% of maximal response), an inhibitory effect was observed at 10 μM (2% of maximal response). B, the blot was reprobed for Trk immunoreactivity, demonstrating approximately equivalent Trk receptor levels among the different treatment conditions.

Discussion

Compounds similar in structure to staurosporine and K-252b were examined with the goal of identifying a compound with a greater specificity and efficacy than K-252b for activation of TrkA-mediated neurotrophic effects, including survival and neurite outgrowth. Among several compounds examined, L-753,000 was found to potentiate the action of NT-3 on cell survival in both rat and chick DRG as measured by GAP-43 expression and direct cell counting. The compound also potentiated the effects of NT-3 on neurite outgrowth in chick DRG. DRG encompass a key population of sensory neurons that respond to NGF and, to a lesser extent, NT-3, because of their expression of TrkA (Barbacid, 1994). Although NT-3, the preferred TrkC ligand, is less potent at TrkA activation than NGF, in the presence of submicromolar concentrations of L-753,000 or K-252b, the survival and neurite outgrowth responses with NT-3 are comparable with those of NGF on its own.

We have shown that L-753,000 activates TrkA in the presence of NT-3 to a greater extent than does K-252b, both in a CHO cell line expressing human TrkA and in rat PC12 cells. The compounds had a negligible effect in the absence of NT-3 consistent with the lack of effect observed on DRG survival and neurite outgrowth. The potentiation effect of L-753,000 and K-252b translated into effects on downstream signaling,

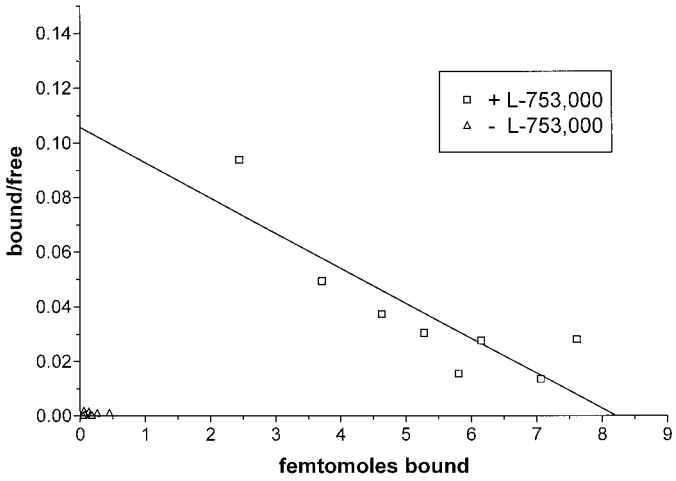

Fig. 9. Scatchard analysis of the effect of L-753,000 (1 μM) on NT-3 binding to TrkA in the CHO-TrkA cell line. Binding assays were carried out as described under *Experimental Procedures*, using eight different concentrations of NT-3. The data shown are from a representative experiment of seven experiments, each carried out with quadruplicate data points (wells). From a Scatchard analysis of the data from this experiment, K_d = 157 pM and B_{max} 33,300 receptors/cell in the presence of L-753,000. In the absence of L-753,000, binding was much lower (indistinguishable from background binding in some experiments) and so the binding parameters (affinity and maximal receptor occupancy) were difficult to determine accurately. The binding parameters among the seven experiments were: K_d = 320 ± 126 pM and B_{max} 29,444 ± 7,154 receptors/cell in the presence of L-753,000, with immeasurably low binding in the absence of L-753,000 in each case.

as manifested by the ability of the compounds to potentiate the activation of MAP kinase by NT-3. The MAP kinase cascade is instrumental in the survival and neurite outgrowth responses of PC12 cells and other TrkA-expressing cells to NGF (Cowley et al., 1994; Greene and Kaplan, 1995; Kaplan, 1998). Consistent with such a role for this pathway, we have found that selective inhibitors of the MAP kinase pathway (PD98059 or U0126) inhibit the survival and neurite outgrowth effects of L-753,000 in combination with NT-3.

Importantly, unlike K-252b, which at micromolar concentrations inhibits the TrkA activation and, therefore, DRG survival responses of NT-3, giving overall bell-shaped dose-response curves, L-753,000 has no such inhibitory action on TrkA at concentrations up to 10 μM. These differences make L-753,000 a more selective potentiator of TrkA activity without acting additionally as a broad kinase inhibitor. Indeed, L-753,000 did not inhibit PKC at concentrations up to 10 μM, whereas K-252b inhibited PKC with a submicromolar (approximately 200 nM) IC_{50} value. Agents that activate TrkA may be useful therapeutically for the treatment of peripheral sensory neuropathy, because sensory neuronal populations respond to NGF (McMahon and Priestly, 1995). Compounds that potentiate the action of NT-3 on TrkA may have potential for use by themselves or in adjunct therapy with NT-3 for the treatment of peripheral sensory neuropathy because they broaden the potential effects of endogenous or administered NT-3 on TrkA-expressing neurons. L-753,000 provides an advantage over the K-252 compounds that have Trk inhibitory and non-Trk-related effects that make them undesirable as neurotrophic agents.

We have begun to explore the mechanism for the potentiation effects of L-753,000 or K-252b. The effects of the compounds on

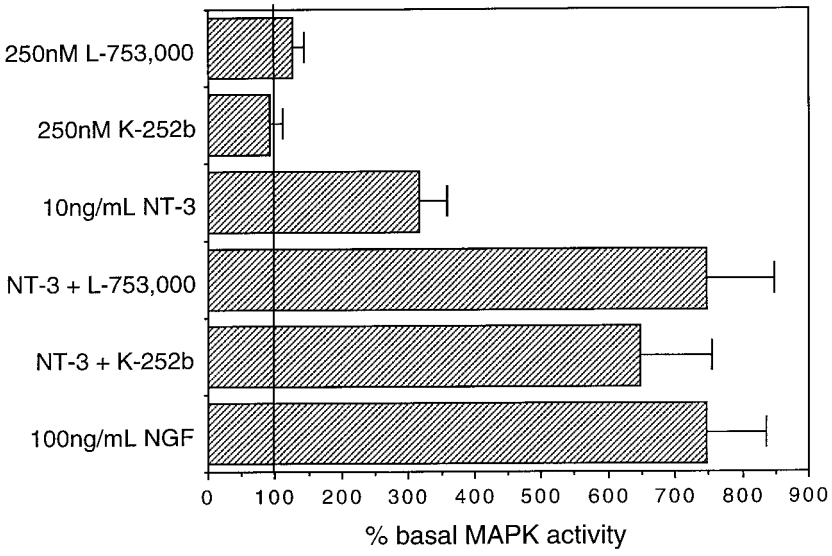

Fig. 10. Effect of L-753,000 or K-252b (each at 250 nM) in the presence or absence of NT-3 (10 ng/ml) on MAP kinase activation in the CHO-TrkA cell line. Mean data are expressed as percentage of control MAP kinase activity ± S.E.M. for three experiments, each performed with duplicate samples per treatment.

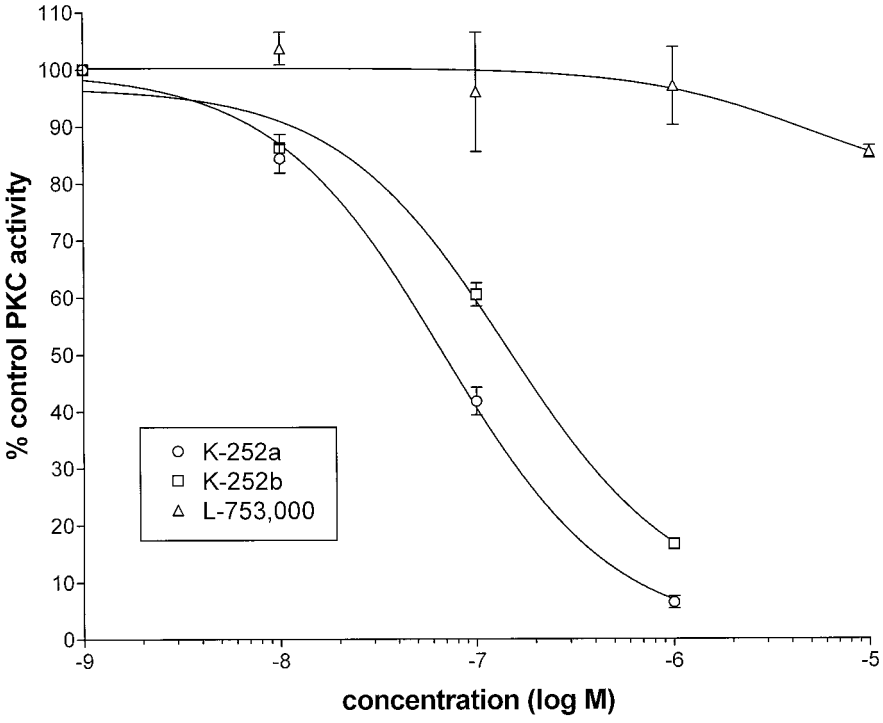

Fig. 11. Effect of K-252a, K-252b, and L-753,000 on PKC activity in a cell-free assay. Mean data are expressed as percentage of control PKC activity ± S.E.M. for three experiments, each performed with duplicate samples per treatment.

NT-3 induced TrkA activation may result from an ability of the compounds to increase the affinity of NT-3 on TrkA by modulating either the receptor itself or a TrkA-associated protein. Another possibility is that the compounds inhibit a phosphatase that dephosphorylates the activated Trk receptor. It has recently been established that the low-affinity NT receptor, p75 [which interacts with Trk to modulate NT binding (Greene and Kaplan, 1995)], is not involved in the action of K-252b, because the potentiation was observed in NIH3T3 cells expressing only TrkA in the absence of p75 (Maroney et al., 1997). Our results confirm that finding both for K-252b and L-753,000 in CHO cells that express TrkA but not p75. In fact, p75 expression has the opposite effect, limiting the NT binding specificity of TrkA to NGF (Benedetti et al., 1993; Lee et al., 1994). The potentiating effect of K-252b and L-753,000 on TrkA activation by NT-3 is unlikely to relate to the inhibitory site of action of K-252a and K-252b on the Trk kinase domain, both because L-753,000 did not produce such inhibition at high concentrations and because inhibition by the K-252 compounds required long incubations with high compound concentrations (Knüsel and Hefti, 1992; Maroney et al., 1997). Moreover, because of the very highly conserved kinase domains, one would expect a potentiation effect to occur with each of the three Trk receptors, but the effects are only observed on TrkA.

Given the specificity of the NT-3 potentiation effects of L-753,000 and K-252b for TrkA, it remains likely that L-753,000 interacts directly with Trk. To determine whether NT-3 binding to TrkA was affected by L-753,000, a cell-based NT binding assay was employed, using radio-iodinated NT-3. It was found that NT-3 binding was profoundly increased in the presence L-753,000. This effect seemed to be selective for NT-3 binding to TrkA, with no substantial effect on NGF binding to TrkA or on NT-3 binding to TrkB and TrkC.

One possibility is that L-753,000 interacts with the extracellular domain of TrkA to make it more 'TrkC-like,' allowing NT-3 to bind with greater affinity. Although the sequences of the three Trk receptors are highly similar in their intracellular domains, substantial differences in their extracellular domains (including leucine-rich regions and Ig-like regions) have been shown to contribute to NT binding specificity (Urfer et al., 1995; Windisch et al., 1995a,b; Holden et al., 1997; Ninkina et al., 1997). One recent study identified regions in and around the second Ig-like domain that were particularly important to TrkA and TrkC specificity (Urfer et al., 1998). NGF is proposed to have interactions with TrkA that are unfavorable with NT-3, based on differences in binding affinity observed with TrkA and TrkC mutants. It may be that L-753,000 affects the mode of NT-3 interacting with TrkA by forming a favorable ternary complex, possibly involving a conformational change in one or both polypeptides. Studies are currently in progress to determine the site of action of K-252b and L-753,000 in their potentiation effect.

Acknowledgments

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