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K252a and CEP1347 Are Neuroprotective Compounds That Inhibit Mixed-lineage Kinase-3 and Induce Activation of Akt and ERK*

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K252a is best known as a Trk inhibitor, but is also a neuroprotective compound. CEP1347, a K252a derivative, retains neuroprotective properties, but does not inhibit TrkA. CEP1347 has recently been shown to directly inhibit MAPKKKs, including MLK3, but the effect of K252a on MAPKKKs remains unknown. K252a and CEP1347 not only prevent death, but also facilitate neurite outgrowth and maintenance, somal hypertrophy, and neurotransmitter synthesis. The biochemical basis for these trophic effects remains unknown. We have compared the effects of CEP1347 and K252a on MLK and JNK signaling and on neurotrophic pathways that support survival and growth. Our data show that K252a is a potent inhibitor of MLK3 activity *in vivo* and *in vitro* (IC_{50} ~ 5 nM). However, we also found that K252a and CEP1347 activate Akt and ERK and show that blockade of phosphatidylinositol 3-kinase or MEK activity ablates the effect of K252a and CEP1347 on cell survival. Activation of Akt and ERK occurs through an MLK-independent pathway that may involve c-Src. Together, these data show that the neuroprotective and neurotrophic effects of K252a and CEP1347 involve activation of several neurotrophic signaling pathways.

Cell death plays a crucial role during neuronal development, but in the adult, apoptotic cascades can contribute to neurodegenerative disease (1–5). The precise signaling mechanisms that induce neuronal apoptotic cascades are not yet certain, but key findings have emerged from analysis of nerve growth factor (NGF)[1]-dependent sympathetic neurons subjected to neurotrophin withdrawal. In this system, NGF deprivation results in activation of Rac and Cdc42 and their association with mitogen-activated protein kinase kinase kinases (MAPKKKs), including the mixed-lineage kinases (MLKs) (6–8). Activation of these MAPKKKs initiates a stress kinase cascade that leads to activation of c-Jun N-terminal kinase (JNK), which then phosphorylates and activates c-Jun. c-Jun-dependent transcription results in Bak/Bax-dependent release of cytochrome *c* from mitochondria and the activation of caspase-9 (9, 10).

In healthy neurons, apoptosis is suppressed by several signaling pathways. Phosphatidylinositol 3-kinase (PI3K)-dependent activation of Akt, a serine/threonine kinase (11), promotes cell survival by phosphorylating several substrates, including Bad, caspase-9, Forkhead family members, glycogen synthase kinase-3, and Ask1 (12–19). Activation of the ERK1/2 MAPKs via the Ras/Raf/MEK pathway supports peripheral and central neuron cell survival (20–22) and facilitates neurite outgrowth (23, 24).

CEP1347 is a synthetic compound that inhibits cell death of motoneurons (25–27), cortical neurons (28, 29), and auditory hair cells (30). CEP1347 inhibits JNK activation within motoneurons and sympathetic neurons (25, 28, 29, 31–33), but does not directly inhibit JNK *in vitro* (25). This suggests that CEP1347 targets a regulatory kinase that lies on the JNK activation cascade; and consistent with this, a recent study has demonstrated that CEP1347 directly inhibits MLK family members (34).

CEP1347 was derived from K252a, a glycosylated indolocarbazole alkaloid. K252a is a potent Trk inhibitor, but paradoxically also exerts neurotrophic effects on primary sensory neurons, neuroblastoma cells, PC12 cells, and central neurons derived from embryonic spinal cord, basal forebrain, and striatum (35–39). It is not known if K252a can function as a JNK pathway inhibitor or if it targets MLKs and therefore if K252a and CEP1347 mediate neuroprotection through similar mechanisms.

Both CEP1347 and K252a do not simply inhibit apoptosis, but also exert potent neurotrophic effects that include neurite outgrowth and maintenance, somal hypertrophy, and neurotransmitter synthesis (26, 27, 39–42). In this study, we compared the effects of CEP1347 and K252a on MLK and JNK signaling pathways and examined their effects on neurotrophic signaling pathways that support survival and growth. We show that K252a is a potent inhibitor of MLK3 activity *in vitro* and *in vivo* and is capable of blocking cellular JNK activation induced by MLK3 overexpression, serum withdrawal, or staurosporine treatment, indicating that CEP1347 and K252a inhibit JNK pathway activation through similar mechanisms. In addition, we have found that both compounds induce activation of Akt and ERK through a Src-dependent, but MLK-independent pathway. Blockade of PI3K or MEK activity ablates K252a-

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[1] The abbreviations used are: NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; MAPKKKs, mitogen-activated protein kinase kinase kinases; MLK, mixed-lineage kinase; JNK, c-Jun N-terminal kinase; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; BDNF, brain-derived neurotrophic factor; HA, hemagglutinin; MKK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FAK, focal adhesion kinase; PP, protein phosphatase; Csk, C-terminal Src kinase; Chk, Csk homologous kinase.

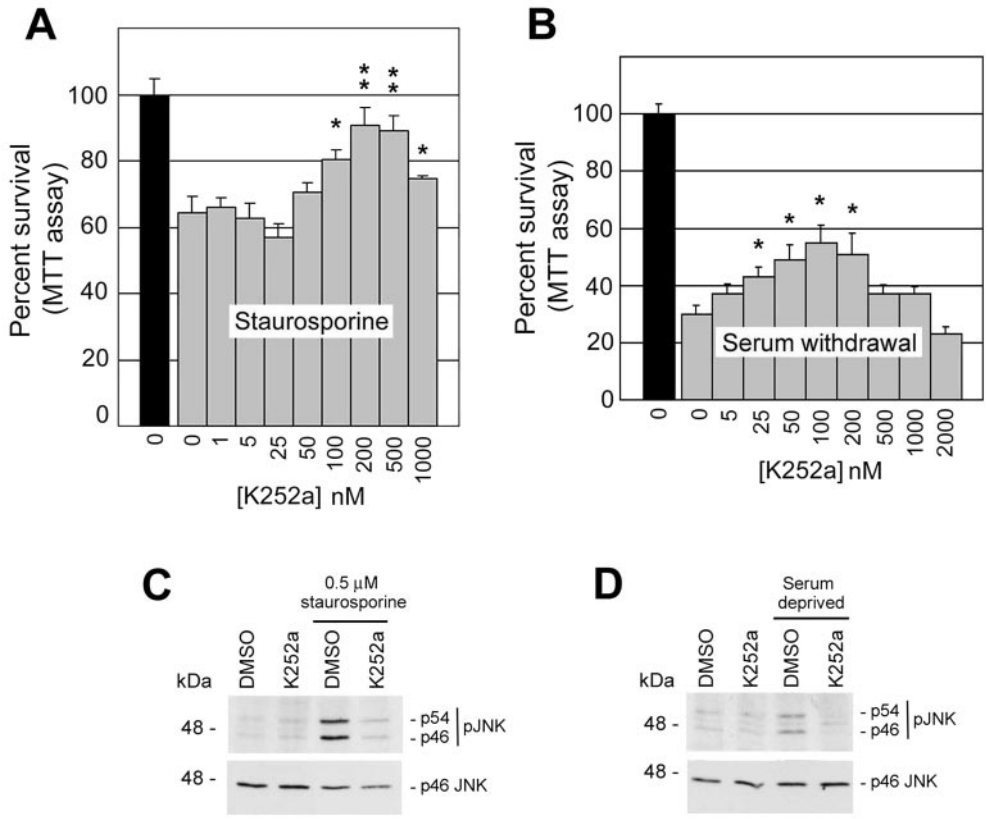

FIG. 1. **K252a promotes survival of primary cortical neurons and PC12nnr5 cells at nanomolar concentrations.** *A*, primary mouse cortical neurons maintained 4 days *in vitro* were exposed to 500 nM staurosporine for 24 h together with increasing concentrations of K252a and assayed for survival by MTT assay. Data are expressed as percent survival of untreated cells (*black bar*). *B*, PC12nnr5 cells were deprived of serum and incubated with increasing concentrations of K252a for 24 h, and cell survival was determined by MTT assay. Data are expressed as percent survival of cells grown in serum-containing medium (*black bar*). *C*, primary mouse cortical neurons exposed to 500 nM staurosporine for 24 h in the absence or presence of 200 nM K252a were lysed and analyzed for phospho-JNK and total JNK levels by immunoblotting. *D*, PC12nnr5 cells deprived of serum for 24 h in the absence or presence of 200 nM K252a for 24 h were lysed and analyzed for phospho-JNK and total JNK levels by immunoblotting. All experiments were repeated at least three times, and the final Me_2SO (*DMSO*; vehicle) concentration was identical under all conditions. For MTT assays, results represent the mean of three separate experiments. Statistically significant differences were detected by multiple analysis of variance and are indicated by *one* ($p < 0.05$) or *two* ($p < 0.001$) *asterisks*.

and CEP1347-dependent survival, indicating that activation of Akt and ERK is important for the neuroprotection mediated by these compounds. Together, these data show that K252a and CEP1347 mediate neuroprotective effects through the activation of neurotrophic signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Brain-derived neurotrophic factor (BDNF) was purchased from Collaborative Research (Bedford, MA). CEP1347 was supplied by Aegera Therapeutics Inc. K252a was purchased from Calbiochem. Cell culture reagents were from BioWhittaker, Inc. (Walkersville, MD) or Invitrogen. Primary antibodies directed against total and phospho-Akt, phospho-ERK, phospho-JNK, and phospho-Jun were from Cell Signaling (Beverly, MA). Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology, Inc. (Lake Placid, NY). The monoclonal anti-Src antibody (clone 327) was from Oncogene Research Products (Boston, MA). The anti-phospho-Src Tyr^{418} antibody was from BioSource International (Camarillo, CA). Monoclonal anti-hemagglutinin (HA) antibody 12CA5 was from Roche Diagnostics. Anti-FLAG antibody M2 was from VWR (Montreal). The polyclonal anti-TrkB antibody (directed against the extracellular domain) was a generous gift of Louis Reichardt (University of California, San Francisco, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG and protein A were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other reagents were from Sigma, ICN Biochemicals (Costa Mesa, CA), or Calbiochem. Expression plasmids encoding MKK4 and MKK7 were a kind gift from Roger Davis (University of Massachusetts, Worcester, MA).

Cell Culture—PC12 and PC12nnr5 cells were maintained in 7.5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, 5% horse serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. HEK293A cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Primary cortical cultures were prepared from embryonic day 15–16 CD1 mouse telencephalon as described (43) and maintained 3–5 days *in vitro* in Neurobasal medium supplemented with a final concentration of 0.5× B27 supplement, 0.5× N2 supplement, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Sympathetic neuron cultures were prepared from postnatal day 1 Sprague-Dawley rat sympathetic superior cervical ganglia essentially as described (44), and 10^5 cells/well were plated in six-well plates precoated with rat tail collagen. The cells were maintained in Ultraculture medium containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3% rat serum (Harlan Bioproducts, Madison, WI). Neurons were cultured for 4 days in the presence of 50 ng/ml NGF (Cedarlane, Hornby, Ontario, Canada) and 7 μM cytosine arabinoside.

Preparation of Recombinant Adenoviruses—N-terminally HA-tagged wild-type MLK3 was subcloned in pAdTrack-CMV, and viruses were generated by homologous recombination in bacteria and packaged in HEK293A cells as described (45). Crude viruses derived from viral plaques were used to infect HEK293A cells, and HA-MLK3 expression was confirmed by immunoblot analysis. Recombinant adenoviruses were amplified in HEK293A cells, purified on sucrose gradients, and titered by plaque assay in HEK293A cells. Control recombinant adenovirus expressing green fluorescent protein was generated using the same viral backbone and purification techniques.

Immunoblotting—To produce lysates for immunoblots, cell cultures were washed twice with cold phosphate-buffered saline, lysed in Nonidet P-40 lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate), and centri-

fuged to remove insoluble material. The protein content of supernatants was determined using the BCA assay (Pierce), and 25 μg of lysate was combined with sample buffer (46), separated on SDS-polyacrylamide gels, and electroblotted onto nitrocellulose. Blocking, primary antibody, and secondary antibody incubations were performed in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20 with 5% (w/v) dry skim milk powder using recommended dilutions of commercially available antibodies or a 1:2000 dilution of rabbit polyclonal anti-TrkB antibody. For the phospho-specific antibodies, 5% (w/v) bovine serum albumin was used instead of milk during the primary antibody incubation. For anti-phosphotyrosine immunoblotting using antibody 4G10, 2% (w/v) bovine serum albumin was used for the blocking step instead of milk powder, and blocking agents were omitted during the primary antibody incubation. Secondary antibodies were used at a dilution of 1:10,000, and immunoreactive bands were detected using enhanced chemiluminescence (DuPont) according to the manufacturer's instructions.

Immunoprecipitation—After treatment, cells were washed with cold phosphate-buffered saline and lysed in Nonidet P-40 lysis buffer. Immunoprecipitations were performed at 4 °C using the monoclonal anti-HA or polyclonal anti-TrkB antibody. Complexes were precipitated using 45 μl of protein G- or protein A-Sepharose (Amersham Biosciences), incubated for 90 min at 4 °C, and then subjected to multiple washes with Nonidet P-40 lysis buffer. Samples were lysed in Laemmli sample buffer and analyzed by immunoblotting as described above.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Survival Assays—Analysis of cell survival was performed using MTT, which was added at a final concentration of 1 mg/ml for 4 h following a 24-h incubation. The reaction was ended by addition of 1 volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% acetic acid). After overnight solubilization, specific and nonspecific absorbances were read at 570 and 690 nm, respectively. Each condition was tested six times, and results were analyzed for statistical significance by multiple analysis of variance.

Immune Complex Kinase Assay—Upon activation, MLK3 becomes phosphorylated at several serine and threonine residues (47), some of which are phosphorylated by MLK3 itself. MLK3 autophosphorylation has been shown to tightly correlate its activity (48) and therefore was used to determine MLK3 kinase activity. Recombinant adenovirus encoding human HA-tagged MLK3 was overexpressed in HEK293A cells, and immunoprecipitations were performed as described above using the monoclonal anti-HA antibody at 1 μg/ml. Beads were washed three times with Nonidet P-40 lysis buffer, followed by two washes with kinase buffer (50 mM HEPES (pH 7.4) and 10 mM $MgCl_2$). Prior to the last wash, the lysate was divided into several equal aliquots, and 30 μl of kinase buffer was added to the beads together with K252a, CEP1347, or Me_2SO such that the Me_2SO concentration was constant across all conditions tested. Complexes were preincubated with the compounds for 10 min at 30 °C, and the reaction was initiated by addition of 10 μl of kinase buffer containing 40 μM ATP and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences). After 20 min at 30 °C, kinase reactions were terminated by addition of Laemmli sample buffer, boiled for 5 min, separated by SDS-PAGE, dried, and autoradiographed. Levels of total MLK3 were assessed by immunoblotting for the HA epitope using antibody 12CA5.

RESULTS

The Structurally Related Compounds K252a and CEP1347 Promote Survival and Inhibit the JNK Pathway—K252a has potent protein kinase C and Trk inhibitory activities, but paradoxically also has neurotrophic effects on a variety of neuronal cells. The mechanisms that mediate K252a-induced survival remain unknown, and to address this, we established two systems amenable to biochemical analysis. Primary mouse cortical neurons treated with 0.5 μM staurosporine for 24 h normally showed a 40% reduction in survival, which was accompanied by an increase in JNK activity (Fig. 1, *A* and *C*). In the presence of K252a, staurosporine-treated neurons showed no increase in JNK activation, and survival levels were significantly improved (Fig. 1, *A* and *C*). As an alternative system, we used PC12nnr5 cells, a variant PC12 line that lacks TrkA receptors and that rapidly undergoes cell death when deprived of serum. Fig. 1*B* shows that PC12nnr5 cells deprived of serum for 24 h showed 70% reduction in viability, which was accompanied by increased JNK phosphorylation. K252a significantly protected these cells from death induced by serum deprivation while simultaneously suppressing JNK activation (Fig. 1, *B* and *D*).

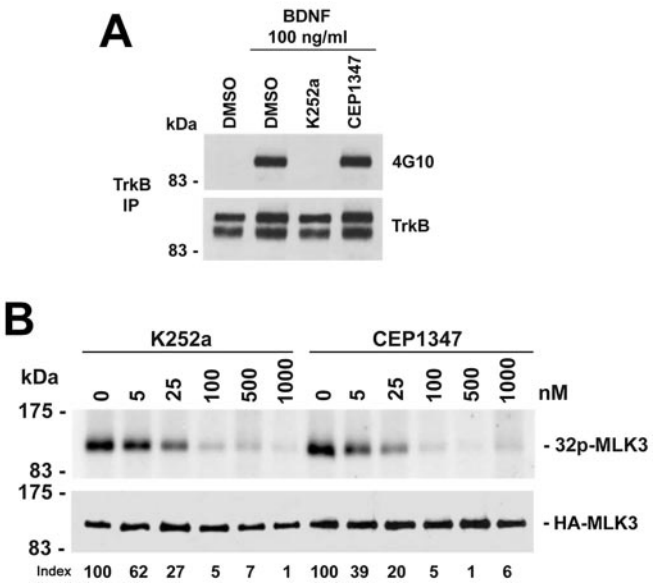

FIG. 2. **K252a and CEP1347 are potent inhibitors of MLK3.** *A*, primary mouse cortical neurons maintained 4 days *in vitro* were incubated with either 100 nM K252a or CEP1347 for 45 min, exposed to BDNF (100 ng/ml) for an additional 15 min, and lysed for immunoprecipitation. TrkB immunoprecipitates (*IP*) were assayed by immunoblotting for phosphotyrosine levels (antibody 4G10) and TrkB protein levels as indicated. *B*, HA-tagged MLK3 was overexpressed in HEK293A cells, immunoprecipitated, and incubated with increasing concentrations of K252a and CEP1347. Immune complex kinase assays were initiated by addition of [γ-^{32}P]ATP and proceeded for 20 min at 30 °C prior to SDS-PAGE and autoradiography. The same samples were subjected to anti-HA immunoblotting to confirm equivalent MLK3 protein levels under each condition. All experiments were repeated at least three times. Autoradiographs were scanned and quantified using NIH Image. *DMSO*, Me_2SO.

CEP1347 is an ethylthiomethyl derivative of K252a that has neuroprotective properties, but does not block Trk receptor action. Several studies have shown that CEP1347 blocks activation of the JNK pathway (25, 28, 29, 31–33). Recent work has established that CEP1347 is a potent inhibitor of MLK3, a pro-apoptotic member of the MLK family that lies upstream of MKK4 and MKK7, MAPK kinases that activate JNK (34). The structural homology between K252a and CEP1347, together with the finding that K252a inhibited staurosporine- and serum deprivation-induced JNK activation (Fig. 1, *C* and *D*), prompted us to hypothesize that K252a and CEP1347 may mediate their neuroprotective effects through similar mechanisms. To address this, we compared the effects of these compounds on TrkB activation and MLK3 activity. Fig. 2*A* shows the phosphotyrosine content of TrkB immunoprecipitated from cortical neurons treated with BDNF in the presence of 200 nM K252a or CEP1347. CEP1347 had no discernible effect on the activation of TrkB, whereas K252a strongly inhibited ligand-mediated TrkB activation. However, when the compounds were compared for their ability to directly inhibit MLK3 using immune complex assays, K252a and CEP1347 showed essentially identical inhibitory dose profiles, with both showing an *in vitro* IC_{50} for MLK3 inhibition of ~5 nM (Fig. 2*B*). These data indicate that the protective properties of K252a correlate with its ability to inhibit JNK activation and reveal that K252a, like CEP1347, is an effective inhibitor of MLK3 activity.

K252a and CEP1347 Induce Akt and ERK Phosphorylation—K252a and CEP1347 do not simply inhibit apoptosis, but also induce somal hypertrophy, neurite extension, and neurotransmitter synthesis and maintain metabolic rates (26, 27, 39–41). The Akt and ERK signaling cascades are critical for

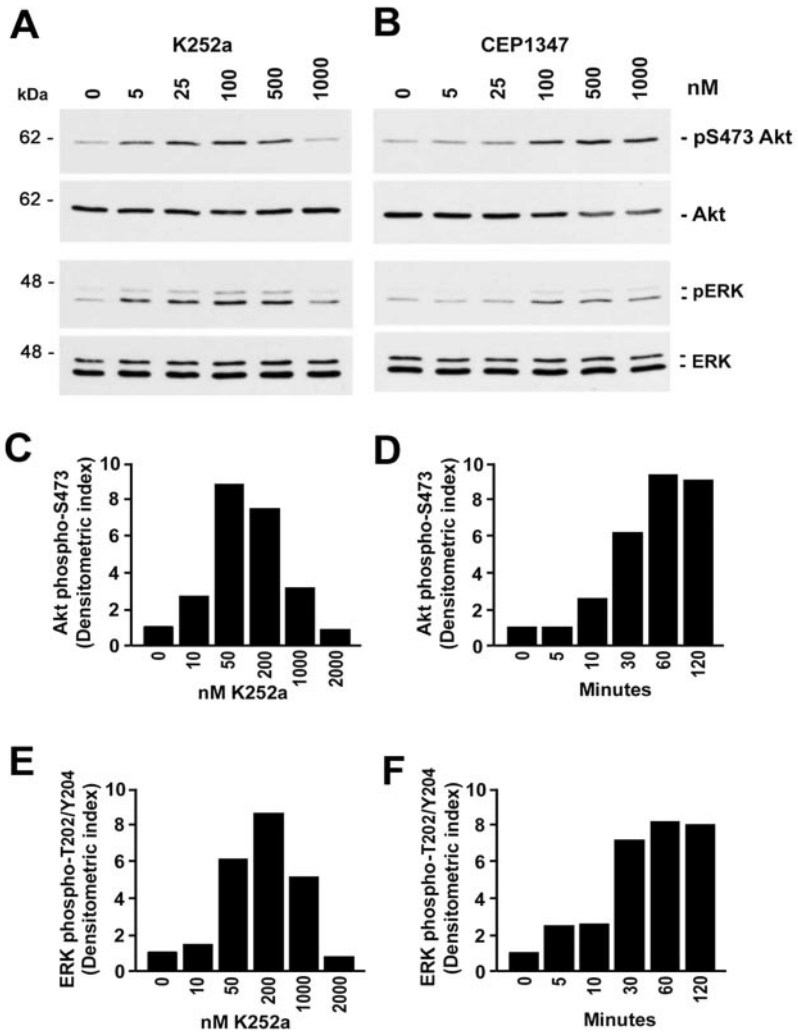

FIG. 3. **K252a and CEP1347 induce Akt and ERK phosphorylation in primary cortical neurons and PC12nnr5 cells.** Mouse primary cortical neurons were exposed to increasing concentrations of K252a (*A*) and CEP1347 (*B*) for 1 h and then assayed by immunoblotting for Akt and ERK phosphorylation at Ser^{473} and Thr^{202}/Tyr^{204}, respectively. The dose curve (*C* and *E*) and time course (*D* and *F*) of K252a-induced Akt and ERK phosphorylation in PC12nnr5 cells were determined as described for *A* and *B*. For the dose curve, cells were exposed to K252a for 1 h; 200 nM K252a was used in the time course experiment. Films were scanned, and images were quantified using NIH Image (*C–F*). All experiments were repeated at least four times with similar results. All experiments in Fig. 2 were done using Me_2SO as vehicle at maximal concentrations of 0.2%. Control experiments revealed that Me_2SO at concentrations up to 1% had no effect on Akt or ERK phosphorylation (data not shown).

the trophic actions of NGF and related factors. To test whether K252a and CEP1347 mediate their trophic effects through activation of these pathways, primary cortical neurons were exposed to dose ranges of K252a and CEP1347, lysed, and assessed for phosphorylation of Akt at its hydrophobic motif (Ser^{473}) and dual phosphorylation of ERK at Thr^{202} and Tyr^{204}. Fig. 3 (*A* and *B*) shows that treatment with K252a or CEP1347 induced phosphorylation of Akt and ERK, with maximal effects at concentrations of 50–200 nM. The effect of CEP1347 was sustained at concentrations up to 1 μM, but K252a had a more narrow dose response. Densitometric analysis revealed that Akt and ERK phosphorylation was maximally increased ~8–9-fold by 200 nM K252a or CEP1347 (Fig. 2, *C* and *E*) (data not shown). The induction of Akt and ERK phosphorylation was observed within 10 min, was maximal by 30–60 min, and was maintained for at least 2 h (Fig. 2, *D* and *F*).

To compare the magnitude of Akt phosphorylation induced by K252a with that induced by neurotrophins, primary cortical neurons were exposed to 100 nM K252a, 100 ng/ml BDNF, or the two together, lysed, and examined for Akt phosphorylation (in lysates) and for TrkB tyrosine phosphorylation content (after immunoprecipitation). Fig. 4*A* shows that phosphorylation of Akt induced by K252a in cortical neurons was ~50% of that induced by 100 ng/ml BDNF. When K252a and BDNF were added together, BDNF-mediated TrkB activation was strongly attenuated, but Akt phosphorylation was maintained, suggesting that K252a acts downstream of TrkB to induce Akt.

Primary peripheral neurons are sensitive to the neuroprotective effects of K252a and CEP1347 (42, 49, 50). To determine whether Akt is activated in peripheral neurons, primary rat sympathetic neurons prepared from postnatal day 0 pups were maintained *in vitro* for 4 days, exposed to CEP1347 or K252a for 1 h, and then lysed and analyzed by immunoblotting. Fig. 4*B* shows that both compounds induced a modest (~2-fold) phosphorylation of Akt in sympathetic neurons. PC12 cells and PC12nnr5 cells (see below) and cerebellar granule neurons and primary fibroblasts[2] were examined in similar assays; and in all cases, exposure to K252a and CEP1347 induced phosphorylation of Akt and ERK.

PI3K and MEK Inhibitors Block K252a- and CEP1347-mediated Survival—These data show that nanomolar concentrations of K252a and CEP1347 induce Akt and ERK phosphorylation and raise the possibility that the trophic activity of K252a and CEP1347 involves activation of the Akt and ERK pathways. To address this, specific inhibitors of the PI3K/Akt and MEK/ERK pathways were assessed for their impact on Akt and ERK phosphorylation and on survival induced by the compounds. Fig. 5*A* shows that phosphorylation of ERK induced by K252a in PC12nnr5 cells was blocked in the presence of the MEK inhibitor PD98059. In complementary experiments, the PI3K inhibitors LY294002 and wortmannin were found to effectively inhibit K252a-dependent Akt phosphorylation (Fig. 5*A*). To test whether PI3K or MEK activity is necessary for the

[2] P. P. Roux and P. A. Barker, unpublished data.

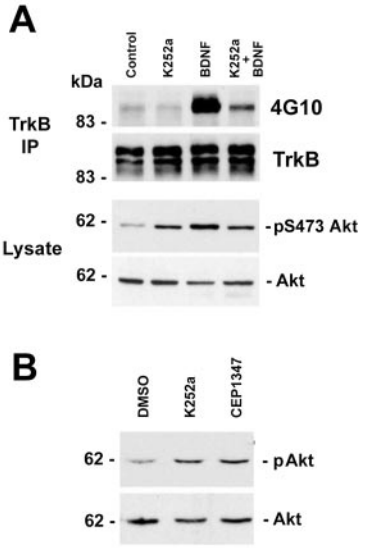

FIG. 4. **K252a treatment activates Akt in central and peripheral neurons.** *A*, primary mouse cortical neurons maintained 4 days *in vitro* were incubated with 100 nM K252a for 45 min, exposed to BDNF (100 ng/ml) for an additional 15 min, and lysed for immunoprecipitation. TrkB immunoprecipitates (*IP*) were immunoblotted for phosphotyrosine levels (antibody 4G10) and TrkB protein levels as indicated. Akt phosphorylation and protein levels were monitored in the total cell lysate. *B*, primary rat sympathetic neurons maintained 4 days *in vitro* in 50 ng/ml NGF were deprived of NGF for 12 h, incubated with either 100 nM K252a or CEP1347 for 60 min and then lysed. Akt phosphorylation and protein levels in total cell lysates were determined by immunoblotting. Experiments shown in *A* were repeated three times, and those in *B* were repeated twice with similar results. *DMSO*, Me_2SO.

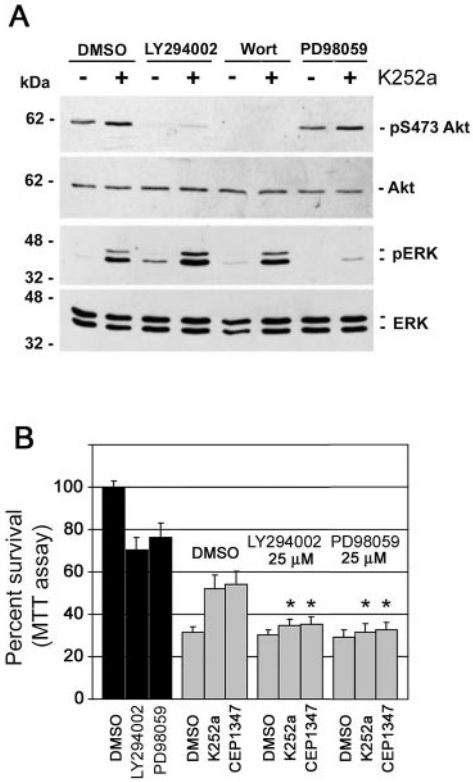

FIG. 5. **PI3K and MEK inhibitors suppress K252a-induced Akt and ERK phosphorylation, respectively.** *A*, serum-deprived PC12nnr5 cells were exposed to 100 nM K252a for 1 h in the presence of the PI3K inhibitors LY294002 (20 μM) and wortmannin (*Wort*; 100 nM) or the MEK inhibitor PD98059 (30 μM). Cells were harvested and assayed by immunoblotting for Akt and ERK phosphorylation at Ser^{473} and Thr^{202}/Try^{204}, respectively. Experiments were repeated twice with identical results. *B*, PC12nnr5 cells were deprived of serum and incubated for 24 h with 100 nM K252a, CEP1347, or vehicle in the presence of 25 μM LY294002, 25 μM PD98059, or Me_2SO (*DMSO*), and mitochondrial activity (MTT) was assayed as a marker for cell survival. Data are expressed as percent survival of cells grown in serum-containing medium (*black bars*). Conditions that are statistically different from cells deprived of serum with K252a or CEP1347 are indicated with an *asterisk* ($p < 0.001$). All experiments were repeated at least four times with similar results.

neuroprotection conferred by K252a or CEP1347, PC12nnr5 cells were deprived of serum and then exposed to K252a or CEP1347 in the absence or presence of 25 μM LY294002 or PD98059. Survival assays revealed that K252a- and CEP1347-mediated survival was strongly attenuated in the presence of inhibitors of PI3K and MEK (Fig. 5*B*). These results indicate that PI3K and MEK are required for K252a- and CEP1347-induced activation of Akt and ERK, respectively, and demonstrate that activation of both the PI3K/Akt and MEK/ERK pathways is necessary for K252a- and CEP1347-induced survival.

MLK3 Activation Does Not Modulate Akt or ERK Activation by K252a and CEP1347—It is possible that K252a and CEP1347 induce Akt and ERK activation by acting on targets distinct from MLK3 and related MAPKKKs. Alternatively, Akt and ERK activity induced by K252a and CEP1347 might be secondary to their inhibition of MLK activity. This latter possibility presumes that MLK is capable of negatively regulating an upstream activator of Akt and ERK and that the compounds may attenuate this regulation. To examine this, PC12 cells were infected with adenovirus coexpressing MLK3 and green fluorescent protein or with virus expressing green fluorescent protein alone; treated with 1 or 5 ng/ml NGF; and then examined for Akt, ERK, and JNK phosphorylation by immunoblotting. Fig. 6*A* shows that MLK3 overexpression induced the expected phosphorylation of JNK (compare the *first three lanes* and the *last three lanes*), but did not inhibit (or activate) Akt or ERK phosphorylation in quiescent cells (compare the *first* and *fourth lanes*) or in cells treated with NGF (compare the *second* and *fifth lanes* and the *third* and *sixth lanes*).

We then determined whether MLK3 influences K252a- and CEP1347-dependent Akt and ERK activation. PC12nnr5 cells were infected with MLK3 or control adenovirus for 24 h, treated with 200 nM K252a and CEP1347 for 1 h, lysed, and examined for Akt, ERK, and JNK phosphorylation by immunoblotting. Fig. 6*B* shows that JNK phosphorylation induced by MLK3 overexpression was completely blocked by CEP1347 or K252a, yet Akt and ERK phosphorylation induced by K252a or CEP1347 was not affected by overexpression of MLK3. Therefore, under conditions in which CEP1347 or K252a blocked MLK3-induced JNK activation, CEP1347 or K252a induced Akt and ERK phosphorylation.

Together, these data are consistent with the hypothesis that CEP1347 or K252a phosphorylates Akt and ERK through an MLK3-independent pathway. However, these experiments do not rule out the possibility that inhibition of the JNK signaling pathway may be a prerequisite for the activation of survival pathways induced by CEP1347 or K252a. To address this, PC12 cells were transfected with MKK4 and MKK7, MAPK kinases that lie distal to MLK3, and then exposed to 200 nM K252a for 1 h and analyzed for ERK activation. Fig. 6*C* shows that overexpression of MKK4 and MKK7 induced phosphorylation of c-Jun, but had no effect on K252a-induced activation of ERK. Interestingly, K252a at 200 nM induced a modest reduction of the MKK4- and MKK7-dependent c-Jun phosphorylation, suggesting that K252a might inhibit JNK activation through different mechanisms.

K252a and CEP1347 Promote Akt and ERK Phosphorylation through a Src-dependent Mechanism—The data presented

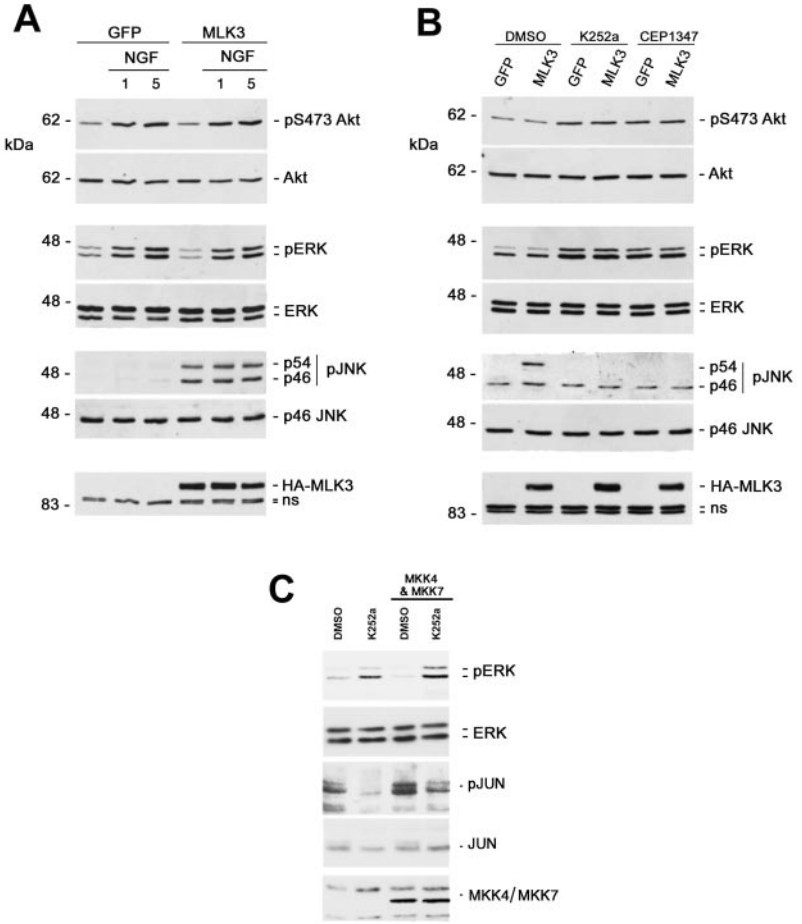

FIG. 6. **Activation of the MLK pathway does not modulate Akt or ERK phosphorylation induced by K252a or CEP1347.** *A*, PC12 cells were infected with recombinant adenovirus expressing either green fluorescent protein (*GFP*) or HA-tagged MLK3 at a multiplicity of infection of 100 for 24 h. Cells were treated with 1 or 5 ng/ml NGF for 10 min; harvested; and analyzed by immunoblotting for Akt, ERK, and JNK phosphorylation at Ser^{473}, Thr^{202}/Tyr^{204}, and Thr^{183}/Tyr^{185}, respectively. The presence of MLK3 was revealed using the anti-HA antibody (nonspecific bands (*ns*)). *B*, PC12nnr5 cells were infected as described for *A* for 24 h and then exposed to 200 nM K252a and CEP1347 for 1 h prior to harvesting. The presence of MLK3 was shown using the anti-HA antibody (nonspecific bands). Akt, ERK, and JNK activation levels were assayed using phospho-specific antibodies. These experiments were repeated three times with similar results. *C*, PC12 cells were transfected with expression plasmids encoding FLAG-tagged MKK4 and MKK7 for 24 h and then exposed to 200 nM K252a for 1 h prior to harvesting. MKK4/MKK7 expression was demonstrated using the anti-FLAG antibody. Akt, ERK, JNK, and c-Jun (Ser^{63}) activation levels were assayed using phospho-specific antibodies. These experiments were repeated three times with similar results. *DMSO*, Me_2SO.

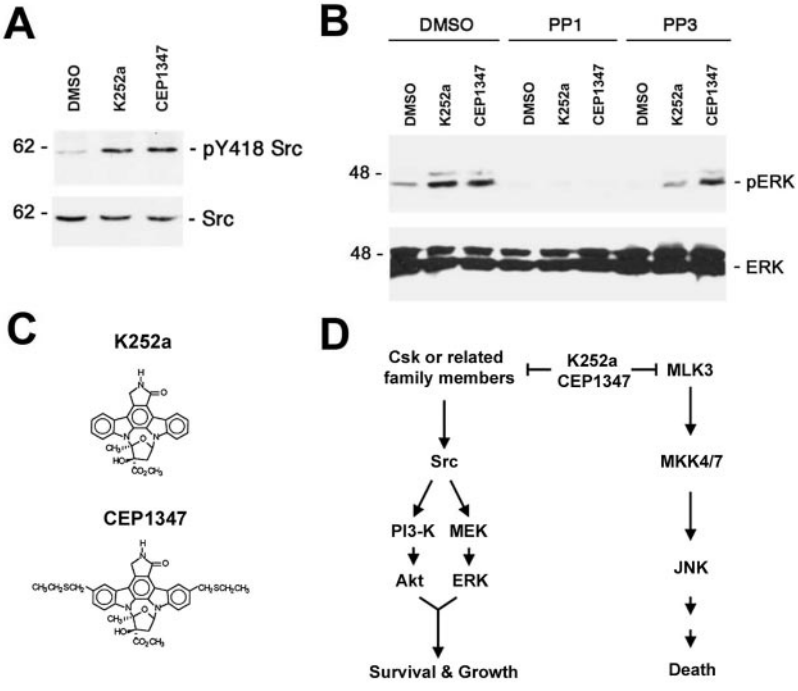

FIG. 7. **K252a and CEP1347 promote Akt and ERK phosphorylation through a Src-dependent mechanism.** *A*, mouse primary cortical neurons were exposed to K252a or CEP1347 for 1 h, lysed, and assayed for Src phosphorylation and total Src levels by immunoblotting. *B*, serum-deprived PC12nnr5 cells were exposed to 100 nM K252a for 1 h in the presence of the Src inhibitor PP1 or PP3, its inactive analog (both used at 5 μM), lysed, and assayed for ERK phosphorylation by immunoblotting. Experiments in *A* and *B* were repeated three times with similar results. *C*, shown are the chemical structures of K252a and CEP1347. *D*, shown is a schematic diagram showing the possible mechanism of K252a and CEP1347 action. K252a and CEP1347 are MLK3 inhibitors, but may also target kinases such as Csk and Chk, which negatively regulate Src and related kinases. Inhibition of Csk and Chk results in Src-dependent activation of Akt and ERK. *DMSO*, Me_2SO.

above indicate that K252a and CEP1347 affect MLK3-independent targets that activate PI3K- and MEK-dependent signaling cascades. One possible intermediary in this cascade is the Src tyrosine kinase. Previous studies have shown that tyrosine phosphorylation of focal adhesion kinase (FAK), a Src substrate, is induced by K252a (40) and that Src activation can activate ERK and Akt through FAK-dependent and FAK-independent pathways (reviewed in Ref. 51). To determine whether Src is activated by K252a, cortical neurons were exposed to K252a or CEP1347 and then examined for Src phosphorylation using phospho-specific antibodies that detect the phosphorylation status of Src at Tyr^{418}, a regulatory residue necessary for

Src activation. Fig. 7*A* shows that phosphorylation of ERK induced by K252a correlated with increased phosphorylation of Src Tyr^{418}. To directly test whether activated Src plays a role in the K252a- and CEP1347-induced activation of ERK, cells were treated with K252a and CEP1347 in the presence of PP1, a specific Src family inhibitor, or in the presence of PP3, a structurally related analog with reduced activity. Fig. 7*B* shows that ERK activation mediated by K252a and CEP1347 was blocked by PP1. PP3 had a modest effect on the ERK activation induced by CEP1347 and K252a, which may reflect latent Src kinase inhibitory activity. Together, these data indicate that activation of Src or related kinases plays a crucial role in mediating the survival-promoting effects of K252a and CEP1347.

DISCUSSION

K252a was originally identified as a protein kinase C inhibitor, but is now most widely used as an inhibitor of Trk tyrosine kinase receptor activity (52–54). However, numerous studies have shown that K252a is a potent neurotrophic molecule that can facilitate survival of primary neurons and neural cell lines (35–37). CEP1347 is a semisynthetic derivative of K252a and is a potent neuroprotectant *in vitro* and *in vivo* (25, 26, 28, 30). Previous studies have shown that CEP1347 does not possess protein kinase C or Trk inhibitory activity, but effectively blocks activation of JNK *in vivo* (25, 28, 31–33), most likely through inhibition of MLKs, which are MAPKKKs capable of inducing JNK within neurons (8, 34). Our data indicate that, like CEP1347, K252a efficiently blocks JNK activation *in vivo* and is a potent and direct inhibitor of MLK3 activity, with an IC_{50} of ~5 nM *in vitro*. Therefore, K252a and its derivative, CEP1347, appear to block JNK pathway activation through similar mechanisms.

Neurotrophin-deprived peripheral neurons that are maintained in caspase inhibitors or that are derived from animals rendered null for *bax* do not undergo apoptosis, but exhibit somal atrophy and neurite degeneration (55–57). Therefore, in the absence of trophic support, inhibition of apoptosis is not sufficient to maintain normal cellular function. Intriguingly, in neurons treated with K252a or CEP1347, neurites are stable; metabolic rate is maintained; and somal atrophy is prevented (26, 27, 39–42). Therefore, the effects of K252a and CEP1347 cannot be explained solely by their anti-apoptotic properties. To address this, we examined the effects of CEP1347 and K252a on neuronal signaling pathways involved in growth and survival. Our results show that nanomolar concentrations of K252a and CEP1347 rapidly activate Akt and ERK signaling in primary neurons and cell lines. The activation of Akt and ERK mediated by these compounds is blocked by inhibitors of PI3K and MEK, respectively, indicating that targets of K252a and CEP1347 lie upstream of these kinases. Furthermore, these pathways appear to play important roles in the neuroprotection induced by these compounds because inhibitors of PI3K and MEK ablate survival induced by K252a and CEP1347.

Src can activate PI3K/Akt and the MEK/ERK cascades through both FAK-dependent and FAK-independent pathways (51, 58), and previous studies have shown that K252a induces tyrosine phosphorylation of FAK, a physiological Src substrate (40). Activation of Src is associated with phosphorylation of Tyr^{418}, located in the activation loop of the Src kinase domain; and our data show that K252a induces phosphorylation of Tyr^{418} and that PP1 and PP2, inhibitors of Src activity, block K252a- and CEP1347-induced ERK activation. Together, these findings are consistent with the hypothesis that Src activation is necessary for Akt and ERK phosphorylation induced by K252a and CEP1347.

K252a and CEP1347 are kinase inhibitors that block ATP binding to target enzymes (25, 59), and it is therefore likely that the induction of Src and downstream activities is secondary to inhibition of an upstream kinase. All Src family members are negatively regulated by phosphorylation at a C-terminal tyrosine (Tyr^{527} in c-Src), which results in a "closed" enzyme conformation due to an intramolecular association between Tyr^{527} and the Src homology-2 domain (60). Enzymes responsible for phosphorylating this C-terminal residue in Src family members include the C-terminal Src kinase (Csk) and the Csk homologous kinase (Chk). Specific inhibitors of these enzymes have not been identified; but staurosporine, which is structurally related to K252a and CEP1347, directly binds Csk and has been co-crystallized with the Csk ATP-binding pocket (61). A mechanism consistent with the available evidence would have K252a and CEP1347 directly inhibiting one or more of these related kinases and thereby relieving inhibition of Src activity. A schematic diagram outlining this proposed action of K252a and CEP1347 is shown in Fig. 7*D*.

Our results show that K252a and CEP1347 are activators of Src/FAK, PI3K/Akt, and MEK/ERK signaling in primary cortical and sympathetic neurons and in two sublines of PC12 cells, and our unpublished findings has identified similar responses in cerebellar granule neurons and primary mouse fibroblasts. The results described here differ from those reported by Harris *et al.* (42), who recently reported that CEP1347 did not alter Akt and ERK phosphorylation levels within sympathetic neurons. This difference could reflect specific tissue culture conditions because, as noted above, maintaining cells in low-serum or serum-free conditions enhances Akt and ERK responses to K252a or CEP1347.

In recent years, K252a has become widely used for examining Trk signaling in complex settings that run the gamut from intact cells, to hippocampal and cortical slices, and even to intact animals. Our findings show that the effects of K252a are wide-range and include Trk inhibition, MLK3 inhibition, and activation of PI3K and MEK signaling pathways through interactions with distinct targets. Therefore, although K252a will continue to prove very useful for analyzing specific signaling events proximal to Trk and MLK3, its use for examining distal signaling events or cellular processes should proceed with caution.

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