Effect of Synthetic Cell-Penetrating Peptides on TrkA Activity in PC12 Cells

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Abstract. As TrkA, a high-affinity receptor of nerve growth factor (NGF), is a potential target for relieving uncontrolled inflammatory pain, an effective inhibitor of TrkA has been required for pain management. To identify a specific inhibitor of TrkA activity, we designed cell-penetrating peptides combined with amino-acid sequences in the activation loop of TrkA to antagonize tyrosine kinase activity. To select a peptide inhibiting TrkA activity, we examined the effect of cell-penetrating peptides on tyrosine kinase activity of recombinant TrkA in vitro and studied their effects on NGF-stimulated neurite outgrowth and protein phosphorylation in PC12 cells. Thereafter we investigated the effect of the selected peptide on NGF-stimulated TrkA activity and the expression of transient receptor potential channel 1 in PC12 cells. The selected peptide inhibited TrkA activity, but did not inhibit tyrosine kinase activities of other receptor-type tyrosine kinases in vitro. It also suppressed NGF-stimulated responses in PC12 cells. The selected synthetic cell-penetrating peptide antagonizing TrkA function would be a candidate for inflammatory pain therapy.

Keywords: cell-penetrating peptide, nerve growth factor (NGF), Tat, TrkA, tyrosine phosphorylation

Introduction

Nerve growth factor (NGF) plays pivotal roles in inflammatory pain (1). Single injection of NGF either systemically or locally induces persistent thermal hyperalgesia in rodents (2). The early phase of thermal hyperalgesia (1 – 6 h post-NGF) involves peripheral mechanisms, followed by the late phase (7 h – 4 days post-NGF) involving central mechanisms (2). NGF binding to the extracellular portion of TrkA, a high-affinity receptor of NGF, leads to autophosphorylation of five different tyrosine residues in the intracellular portion, located in the juxtamembrane domain (Y490), the activation loop of the tyrosine kinase domain (Y670, Y674, Y675), and the C-terminal (Y785) (3). NGF-stimulated autophosphorylation of three tyrosines in the activation loop of TrkA regulates both overall and specific downstream signaling and promotes biological responses, including neurite outgrowth (3). After NGF binds to TrkA, NGF signaling containing NGF, TrkA, and activated signaling proteins is transmitted retrogradely through axonal transport of TrkA from the peripheral terminals of nociceptive neurons to the cell bodies of the dorsal root ganglion (DRG) (4). In several persistent pain states, NGF sensitizes transient receptor potential channel 1 (TRPV1) in nerve terminals, and retrograde transport of TrkA to DRG neurons activates mitogen-activated protein kinases and enhances expres-
sion of several proteins (e.g., substance P, tetrodotoxin-resistant voltage-gated sodium channel Nav1.8, TRPV1 channel, brain-derived neurotrophic factor) in DRG neurons with modulation of spinal N-methyl-D-aspartate receptors (1, 2, 5).

Under the severe inflammatory pain state in which neither non-steroidal anti-inflammatory drugs nor opiate drugs have any therapeutic effect, novel agents to antagonize NGF are required because the dominant role of NGF as a pain mediator is recognized (1). Although anti-NGF antibody (6) and TrkAd5, which binds NGF (7), are effective for several models of the pain state, repetitive administration is required to suppress the long-lasting pain state, as interaction with NGF occurs primarily at extracellular sites.

Our previous studies indicated that there is a close resemblance of amino acid sequences between the activation loop of the insulin receptor (1154 TRDIYETDYYR 1164) and that of TrkA (666 SRDIYSTDYYR 676) (8, 9). As the synthetic peptide DIYST, an analogue of the amino-acid sequence in the activation loop of insulin receptor, inhibited tyrosine kinase activity of the insulin receptor (8), we hypothesized that a synthetic peptide analogous to the amino-acid sequence in the activation loop of TrkA would be a new agent to antagonize TrkA function intracellularly and would suppress pain state more effectively. In the present study, we developed synthetic cell-penetrating peptides to inhibit tyrosine kinase activity of TrkA and investigated the effects of the peptide on NGF-stimulated responses in PC12 cells.

Materials and Methods

Synthetic peptides containing Tat protein transduction domain

We designed 5- to 11-mer synthetic peptides originating from the amino-acid sequence of the activation loop including three tyrosine residues (664-GMSRDIYSTYYRVGGR-680) in TrkA to inhibit TrkA activity (DIYST, RDIYSTD, SRDIYSTDYYR). Moreover, to develop synthetic peptides capable of interacting with the intracellular portion of TrkA, these peptides need to include the amino acid sequence of a cell-penetrating peptide to facilitate transfer of peptide molecules into living cells. The cell-penetrating peptide sequence used was based on human immunodeficiency virus type I Tat (transactivator of transcription)-derived peptide (47-YGRKKRRQRRR-57) (10). Epsilon-aminocaproic acid (acp) was inserted as a highly flexible spacer between inhibitory peptide of TrkA activity and Tat peptide (11). Cell-penetrating peptides (YGRKKRRQRRR-acp, YGRKKRRQRRR-acp-DIYST-NH$_2$, YGRKKRRQRRR-acp-RDIYSTD-NH$_2$, YGRKKRRQRRR-acp-SRDIYSTDYYR-NH$_2$) were synthesized and purified by HPLC (Peptide Institute, Osaka).

Receptor-type tyrosine kinase activities in vitro

Using the recombinant catalytic domain of TrkA (474–796), which is the intracellular portion of the receptor, direct effects of cell-penetrating peptides on TrkA activity were evaluated in vitro (TrkA Kinase Assay/Inhibitor Screening Kit; Cyclex, Nagano). After adding synthetic peptide, followed by recombinant catalytic domain of TrkA with ATP, to a 96-well plate coated with tyrosine-kinase substrate, tyrosine phosphorylation of the substrate was evaluated by enzyme-linked immunoabsorbant assay (ELISA) using anti-phosphotyrosine antibody. The effect of K252a (Calbiochem, Darmstadt, Germany), which reportedly inhibits NGF-stimulated tyrosine phosphorylation of TrkA almost completely at over 100 nM (12), on TrkA kinase activity was also evaluated in the same manner.

Amino acid sequence of the activation loop in TrkA reveals homology with that of the insulin receptor (8, 9). On the other hand, there is no homology in amino acid sequences among the autophosphorylation sites of TrkA and epidermal growth factor (EGF) receptor (EGFR) (841 EEKEYHAE 848, 988 DADEYLI 994, 1101 RDPHYQD 1107, 1144 DNPDYQQDFF 1153, 1169 ENAEYLR 1175) (13). To evaluate the effect of cell-penetrating peptides on these receptor-type tyrosine kinase activities, the effect of peptides on both the insulin-stimulated autophosphorylation of the insulin receptor and EGF-stimulated autophosphorylation of EGFR were examined in vitro as described before (14, 15). Briefly Insulin (100 nM) or EGF (100 ng/ml) with synthetic peptide was added to 1 µg of the purified insulin receptor or the purified EGFR (Sigma, St. Louis, MO, USA), respectively. The purified receptors were phosphorylated with 0.2 mM of ATP for 5 min at 37°C. After the incubation, the samples were subjected to Western blot analysis with anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA).

NGF-stimulated protein phosphorylation and neurite outgrowth

The PC12 cell line derived from rat pheochromocytoma is a useful model for studying NGF signaling (16). NGF induces tyrosine phosphorylation in proteins in PC12 cells (17). Inhibitory effects of cell-penetrating peptides on NGF stimulated-tyrosine phosphorylation of the whole cell were evaluated in PC12 cells. After cells were seeded on 96-well plates, synthetic peptide and/or NGF (100 ng/ml) was added. Tyrosine phosphorylation
of the whole cell was evaluated by ELISA using anti-phosphotyrosine antibody (Cellular Phosphotyrosine ELISA Kit, Upstate Biotechnology).

NGF stimulation of PC12 cells induces autophosphorylation of TrkA and causes neurite outgrowth. PC12 cells were maintained in RPMI1640 medium supplemented with 5% fetal bovine serum, 10% horse serum. After incubating under reduced concentrations of serum (0.5% fetal bovine serum, 1% horse serum) for 6 h, cell-penetrating peptide was added, followed by 100 ng/ml of NGF (NGF 2.5S, Upstate Biotechnology). Outgrowth of neurites was monitored under phase-contrast microscopy after 24 h. Processes with lengths equivalent to one or more diameters of a cell body were counted as neurites. A minimum of 100 cells was examined for each datum point.

Cytotoxicity

Several cell-penetrating peptides are known to have cellular cytotoxicity (18). To investigate the cytotoxicity of each synthetic peptide, we measured the activity of lactate dehydrogenase (LDH), released from dead cells, to quantify cell death. PC12 cells were exposed to NGF (100 ng/ml), synthetic peptide (6, 30, 60, or 300 µM), or both under a reduced concentration of serum (0.5% fetal bovine serum and 1% horse serum). LDH released from dead cells was measured by the LDH-Cytotoxic Test (Wako, Osaka) after incubation for 1 day with NGF and peptides.

Tat-acp-SRDIYSTDYYR

After Tat-acp-SRDIYSTDYYR was found to show inhibitory effects on both TrkA activity and NGF-stimulated neurite outgrowth in the above experiments, cell permeability of this peptide and the effects of Tat-acp-SRDIYSTDYYR on both NGF-stimulated autophosphorylation of TrkA and TRPV1 expression were examined in PC12 cells.

To confirm cell permeability of Tat-acp-SRDIYSTDYYR, the peptide labeled with fluorescein isothiocyanate (FITC) was synthesized (Peptide Institute, Osaka). PC12 cells without NGF stimulation were incubated with both FITC-acp-Tat-acp-SRDIYSTDYYR (6 µM) and Hoechst 33342 (1 µg/ml) for 10 min and then examined under fluorescence microscopy to evaluate cell permeability of the peptide.

To evaluate NGF-stimulated autophosphorylation of TrkA (9), the cells were cultured on 60-mm diameter dishes to be 80% confluent. After the synthetic peptide were added, followed by incubation with NGF (100 ng/ml) for 10 min, each medium was replaced with 5 ml ice-cold phosphate-buffered saline, and each sample was scraped and harvested into the tubes. After the supernatant was removed by centrifugation, each sample was suspended in a lysis buffer [50 mM HEPES, (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM phenylmethylsulphonyl fluoride, 10 mM sodium pyrophosphate, 5 µg/ml aprotinin, and 0.5 µg/ml pepstatin]. Insoluble material was removed by centrifugation at 15,000 rpm for 15 min. Aliquots of the supernatants containing equal amounts of protein, as determined by the Bradford protein assay, were subjected to immunoprecipitation with anti-Trk antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following the addition of protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ, USA), the immunoprecipitate was washed three times in a wash buffer and then subjected to Western blot analysis with anti-Trk antibody or anti-phosphotyrosine antibody. Although anti-trk antibody detects all three trks (TrkA, TrkB, and TrkC), tyrosine-phosphorylated protein stimulated by NGF with a molecular weight of around 140-kDa level can be identified as autophosphorylated TrkA because NGF binds to only TrkA.

As NGF increases TRPV1 expression in PC12 cells (5), the effect of Tat-acp-SRDIYSTDYYR on NGF-induced TRPV1 expression was also examined in PC12 cells. After the peptide was added to PC12 cells incubated in 60-mm diameter dishes, NGF stimulation was performed. The cells were incubated for 2 days, and then they were suspended in the lysis buffer. Cell lysates containing equal amounts of protein were subjected to Western blot analysis with rabbit anti-TRPV1 antibody (Alamone Labs, Jerusalem, Israel).

Statistical analysis

Data were analyzed using one-way analysis of variance with Bonferroni post hoc analysis. Statistical significance was established at the P<0.05 level. All values are reported as the mean ± S.D.

Results

Effect of cell-penetrating peptides on receptor-type tyrosine kinase activities in vitro

Tyrosine phosphorylation of the tyrosine-kinase substrate, induced by the recombinant catalytic domain of TrkA, was not significantly affected by any cell-penetrating peptides with the exception of Tat-acp-SRDIYSTDYYR at above 30 µM (Fig. 1A). Tat-acp-SRDIYSTDYYR at 300 µM inhibited TrkA kinase activity to the same level as 300 nM K252a, a concentration that induces almost complete inhibition of TrkA activity (12). No synthetic peptides (300 µM) had any
significant effects on either insulin-stimulated auto-
phosphorylation of insulin receptor or EGF-stimulated
autophosphorylation of EGFR (Fig. 1B). Tat-acp-
SRDIYSTDDYYR thus showed inhibitory effects on
TrkA activity without effects on either insulin receptor
kinase or EGFR kinase activities.

Fig. 1. In vitro analyses of the effects of cell-penetrating peptide on receptor-type tyrosine kinase activities. A: Effect of cell-penetrating peptides on TrkA kinase activity. None of the cell-penetrating peptides, with the exception of Tat-acp-SRDIYSTDDYYR above 30 μM, suppresses tyrosine phosphorylation of the tyrosine kinase substrate stimulated by the recombinant catalytic domain of TrkA (474-796). K252a over 6 nM significantly suppressed TrkA kinase activity. *P<0.05, **P<0.01, compared with recombinant catalytic domain of TrkA-induced autophosphorylation without cell-penetrating peptides and K252a (100%). B: Tyrosine phosphorylation of purified insulin receptor (upper panel) and that of purified epidermal growth factor (EGFR) receptor (EGFR) (lower panel) in the presence or absence of cell-penetrating peptides in vitro. Tyrosine phosphorylation of purified receptors was affected by none of the peptides at 300 μM. *P<0.05, compared with percent phosphorylation of each insulin-stimulated or EGF-stimulated phosphorylation without cell-penetrating peptide (100%). Results represent means ± S.D. of four separate experiments.

Fig. 2. Effects of cell-penetrating peptide on nerve growth factor (NGF)-stimulated tyrosine phosphorylation of protein and neurite outgrowth of PC12 cells stimulated by NGF. A: Tat-acp-SRDIYSTDDYYR above 60 μM suppresses NGF-stimulated tyrosine phosphorylation in proteins in PC12 cells. *P<0.05, compared with NGF-stimulated tyrosine phosphorylation without cell-penetrating peptide. B: PC12 cells, incubated with NGF (100 ng/ml) for 24 h, induced marked increases in neurite outgrowth. Tat-acp-SRDIYSTDDYYR above 30 μM significantly suppressed NGF-stimulated increases in neurite outgrowth. *P<0.05, **P<0.01, compared with NGF-stimulated neurite outgrowth without cell-penetrating peptide. Results represent means ± S.D. of four separate experiments.

Effect of cell-penetrating peptides on NGF-stimulated protein phosphorylation and neurite outgrowth in PC12 cells

In the examination to evaluate the effect of synthetic peptides on NGF-stimulated tyrosine phosphorylation of proteins in PC12 cells, only 60 and 300 μM of Tat-acp-SRDIYSTDDYYR inhibited phosphorylation (Fig. 2A).

All cell-penetrating peptides (300 μM) with the exception of Tat protein transduction domain alone (YGRKKRRQRRR-acp) suppressed NGF-stimulated neurite outgrowth in PC12 cells. Tat-acp-SRDIYSTDDYYR, however, showed significant suppression at the lower concentrations of 30 and 60 μM (Fig. 2B).
Effect of cell-penetrating peptides on cell death in PC12 cells

Deprivation of NGF under the reduced concentration of serum increased cell death in PC12 cells because of lack of the cell protective effect of NGF (black column in Fig. 3A). Tat-acp-SRDIYSTDYYR at 30 and 60 µM increased cell death, to levels that were not significantly different from that due to NGF deprivation. LDH releases under 300 µM of this peptide surpassed the release induced by deprivation of NGF significantly (Fig. 3A). Although high concentration of Tat-acp-SRDIYSTDYYR (300 µM) shows cytotoxicity in PC12 cells, cell death under incubation with lower concentrations of this peptide (30 or 60 µM) is likely caused by the suppression of the cell protective effect of NGF.

Cell permeability of Tat-acp-SRDIYSTDYYR

Figure 3B shows that this peptide labeled with FITC penetrates into PC12 cells effectively.

Analysis of Tat-acp-SRDIYSTDYYR on NGF-stimulated autophosphorylation of TrkA and TRPV1 expression in PC12 cells

After Tat-acp-SRDIYSTDYYR was found to show inhibitory effects on both TrkA activity and NGF-stimulated neurite outgrowth in the above experiments, we tested the effects of Tat-acp-SRDIYSTDYYR both on NGF-stimulated tyrosine phosphorylation of TrkA and on a downstream signal of TrkA, TRPV1 expression in PC12 cells, at a concentration under 60 µM, because 300 µM of this peptide showed cytotoxicity in this study and also Tat peptide conjugates at concentrations above 100 µM reportedly exhibit some cytotoxicity (18). The peptide significantly inhibited NGF-stimulated TrkA phosphorylation (Fig. 4A), and it also suppressed NGF-induced TRPV1 expression in PC12 cells (Fig. 4B).

Discussion

The present study shows that Tat-acp-SRDIYSTDYYR, derived from studies to select a cell-penetrating peptide inhibiting TrkA activity, suppresses NGF-stimulated responses in PC12 cells. Tat-acp-SRDIYSTDYYR at 60 and 300 µM suppressed NGF-stimulated neurite outgrowth almost completely. Although the cytotoxic effect of this peptide contributes to this suppression at 300 µM, 60 µM of Tat-acp-SRDIYSTDYYR likely suppresses neurite outgrowth by direct inhibition of TrkA activity, as this peptide at 60 µM inhibited NGF-stimulated protein phosphorylation and NGF-induced TRPV1 expression to the same levels without NGF. Incomplete suppression of either in
vitro TrkA kinase activity or NGF-stimulated autophosphorylation of TrkA, however, was observed after administration of 60 µM Tat-acp-SRDIYSTDDYYR. These discrepancies might be caused by methodological differences.

We previously developed the synthetic peptide DIYET, an analogue of the amino-acid sequence in the activation loop of insulin receptor, and then showed that DIYET inhibits insulin-stimulated autophosphorylation of the purified insulin receptor (8). Cell-penetrating peptides including DIYST, containing the sequence in the activation loop of TrkA, was thus initially thought to suppress TrkA activity in the present study. Tat-acp-DIYET, however, had no effect on TrkA activity. As a longer synthetic peptide (RDIYETDYYYRK) compared to DIYET has been shown to inhibit insulin-stimulated autophosphorylation of purified insulin receptor (19), we also synthesized two more cell-penetrating peptides containing RDIYST or SRDIYSTDYYR, both which are longer than DIYET, and eventually found that 60 µM Tat-acp-SRDIYSTDYYR directly suppressed TrkA activity with inhibition of NGF-stimulated responses in PC12 cells. This synthetic peptide showed no inhibitory effect on tyrosine kinase activities of both insulin receptor and EGFR, and Tat-acp-SRDIYSTDYYR might be recognized as a specific inhibitor of TrkA kinase.

In the kinase domain of the insulin receptor, the unphosphorylated activation loop works as a pseudosubstrate to the catalytic site (20). Although the crystal structure of the intracellular portion of TrkA has not yet been revealed, homologous residues in the insulin receptor superfamily (e.g., insulin receptor, TrkA) lead to the speculation that the unphosphorylated activation loop of TrkA also interacts with the catalytic site and contributes to the stability of catalytic activity in TrkA. Tat-acp-SRDIYSTDYYR, which displays the same amino-acid sequence as the unphosphorylated activation loop of TrkA, is thus likely to bind to the catalytic site as a pseudosubstrate to inhibit TrkA activity. Further in vitro studies using SRDIYSTDYYR or smaller peptide is needed to clarify the precise binding site of this peptide in TrkA.

Mitogen-activated protein kinases, activated by TrkA kinase activity, are retrogradely transported in signaling endosomes containing phosphorylated TrkA and NGF is transported to DRG neuronal cell bodies after NGF stimulation (4) and enhance the expression of several proteins associated with central sensitization of inflammatory pain, such as TRPV1 (1). Increased TrkA activity stimulated by NGF augments expression of TRPV1 in DRG neurons and PC12 cells (5). As the present study showed that Tat-acp-SRDIYSTDYYR suppressed NGF-induced TRPV1 expression in PC12 cells, Tat-acp-SRDIYSTDYYR likely suppresses the downstream signals of TrkA, which play a crucial role for prolonged inflammatory pain (1, 2, 5). Cell-penetrating peptides including the Tat-derived amino acid sequence administered intravenously are reportedly detected intracellularly in all brain regions (21, 22). Therefore in vivo administration of Tat-acp-SRDIYSTDYYR could distribute into peripheral and central neurons and could affect TrkA activity and the downstream signaling. To investigate the usefulness of this peptide for potential pain therapy, we are planning...
to examine the effect of Tat-acp-SRDIYSTDYYR administered systemically or locally on inflammatory pain in vivo.

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