Regulated secretion of neurotrophins by metabotropic glutamate group I (mGluRI) and Trk receptor activation is mediated via phospholipase C signalling pathways

Marco Canossa[1,2,3], Annette Gärtner[1], Gabriele Campana[1,2], Naoyuki Inagaki[4] and Hans Thoenen[1]

[1]Department of Neurobiochemistry, Max-Planck Institute of Neurobiology, Am Klopferspitz 18A, D-82152 Martinsried, Germany, [2]Department of Pharmacology, University of Bologna, Irnerio 48, I-40126 Bologna, Italy and [4]Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma 630-0101, Japan

[3]Corresponding author
e-mail: ashdown@neuro.mpg.de

Neurotrophins (NTs) play an essential role in modulating activity-dependent neuronal plasticity. In this context, the site and extent of NT secretion are of crucial importance. Here, we demonstrate that the activation of phospolipase C (PLC) and the subsequent mobilization of Ca^{2+} from intracellular stores are essential for NT secretion initiated by both Trk and glutamate receptor activation. Mutational analysis of tyrosine residues, highly conserved in the cytoplasmic domain of all Trk receptors, revealed that the activation of PLC-γ in cultured hippocampal neurons and nnr5 cells is necessary to mobilize Ca^{2+} from intracellular stores, the key mechanism for regulated NT secretion. A similar signalling mechanism has been identified for glutamate-mediated NT secretion—which in part depends on the activation of PLC via metabotropic receptors—leading to the mobilization of Ca^{2+} from internal stores by inositol trisphosphate. Thus, PLC-mediated signal transduction pathways are the common mechanisms for both Trk- and mGluRI-mediated NT secretion.

Keywords: adenovirus/BDNF/Ca^{2+} stores/hippocampal neurons/NGF

Introduction

Neurotrophins (NTs), a gene family of neurotrophic molecules comprising nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5), bind with virtually identical affinity to a common receptor (p75[NTR]) and with high selectivity to the tyrosine kinase receptors (Trks). NGF preferentially binds to TrkA, BDNF and NT4/5 to TrkB, and NT3 to TrkC. NTs regulate the survival and differentiation of specific populations of neurons during embryonic development and the maintenance of specific neuronal functions in adulthood (see Bothwell, 1995; Lewin and Barde, 1996). However, there is increasing evidence that NTs also play an essential role in modulating activity-dependent neuronal plasticity (Thoenen, 1995; Bonhoeffer, 1996; Cellerino and Maffei, 1996; McAllister et al., 1999). The modulatory actions of NTs on synaptic transmission are mediated by both pre- and post-synaptic mechanisms. Pre-synaptically, NTs enhance activity-mediated neurotransmitter release (Lohof et al., 1993; Knipper et al., 1994a,b; Lessmann et al., 1994; Blöchl and Sirrenberg, 1996; Gottschalk et al., 1998; Y.X.Li et al., 1998). Post-synaptically, BDNF enhances transmission via N-methyl-D-aspartate (NMDA) receptors (Levine et al., 1995, 1998; Suen et al., 1997) and attenuates transmission via γ-aminobutyric acid (GABA)$_A$ receptors (Tanaka et al., 1997). Recently, Kafitz et al. (1999) demonstrated that NTs also activate a tetrodotoxin-resistant sodium channel within a time frame of milliseconds, resulting in the initiation of repetitive action potentials. In a relatively simply organized organotypic in vitro system, namely hippocampal slices, it has been shown that BDNF is essential for the formation of long-term potentiation (LTP) (Korte et al., 1995; Patterson et al., 1996). The fact that LTP is impaired in both homozygous and heterozygous BDNF-defective mice suggests that a critical quantity of BDNF is required for LTP formation in the CA3/CA1 hippocampal system. Either exogenous administration (Patterson et al., 1996) or local re-expression of BDNF (Korte et al., 1996) could restore LTP. How these highly selective effects are elicited in an integrated physiological system in vivo is dependent on the quantity of NTs locally available to the corresponding Trk receptors. In addition to the understanding of the mechanisms of activity-dependent NT synthesis (see Lindholm et al., 1994; Shieh et al., 1998; Tao et al., 1998), the understanding of the mechanism(s) and site(s) of NT secretion is of crucial importance. In previous experiments, it has been demonstrated that the secretion of NTs from hippocampal neurons is regulated by neuronal activity and mediated via the excitatory neurotransmitters glutamate and acetylcholine (Blöchl and Thoenen, 1995, 1996; Canossa et al., 1997; Griesbeck et al., 1999). More recently, it became apparent that NTs also regulate their own secretion (Canossa et al., 1997; Krüttgen et al., 1998). Both neurotransmitters (Blöchl and Thoenen, 1995; Griesbeck et al., 1999) and NTs (Canossa et al., 1997) initiate NT secretion with a similar time course via activation of the corresponding receptors. On the basis of the use of specific receptor antagonists, glutamate is thought to induce NT secretion via the ionotropic α-amino-3-hydroxyl-5-methyl-4-isoxazolpropionic acid (AMPA) receptors and the metabotropic glutamate receptors (mGluRs), but not NMDA receptors (Blöchl and Thoenen, 1995, 1996). The NT-mediated NT secretion can be triggered by all Trk receptors: in hippocampal neurons via TrkB and TrkC receptors (Canossa et al., 1997) and in the rat phaeochromocytoma PC12 cells via TrkA receptors (Krüttgen et al., 1998). Virtually nothing is known about the signal transduction cascade leading to NT secretion, although mobilization of Ca^{2+} from endogenous stores seems to be

the common denominator of all pathways that lead to regulated NT secretion (Blöchl and Thoenen, 1995, 1996; Griesbeck *et al.*, 1999).

The goal of the present investigation was to elucidate, for both NTs and glutamate, the signal transduction pathways resulting in NT secretion. To this end, we first used TrkA receptor constructs mutated on the tyrosine residues of the intracellular domain that are highly conserved in all Trk receptors (Inagaki *et al.*, 1995). The activation of Trk receptors results in the phosphorylation of specific tyrosine residues. These tyrosine residues initiate the binding and phosphorylation of adaptor molecules such as SHC and SNT, and the activation of enzymes such as phospholipase C-γ (PLC-γ) and phosphatidylinositol 3-kinase (PI3-K) (see Kaplan and Miller, 2000). We demonstrate that Trk-mediated NT secretion depends on the phosphorylation of PLC-γ leading to Ca^{2+} release from intracellular stores. Moreover, we demonstrate that the glutamate-induced NT secretion—mediated by mGluRI—also results from the activation of PLC and the subsequent release of Ca^{2+} from intracellular stores.

Results

Evidence that PLC-γ mediates NT secretion in nnr5 cells

We analysed the TrkA-mediated signalling pathways of NT secretion by exploring the functional importance of individual tyrosine residues in the cytoplasmic domain. A set of TrkA receptor mutants had been produced previously by systematically replacing the tyrosine residues (Y499, Y594, Y643, Y704, Y726, Y732, Y760 or Y794) by phenylalanines (Inagaki *et al.*, 1995). In preliminary experiments, we transiently transfected nnr5 cells, which are variants of PC12 cells (Green *et al.*, 1986) that express p75[NTR] receptors but no Trk receptors (Loeb *et al.*, 1991), with different TrkA receptor mutants. We obtained evidence that the replacement of Y794 by phenylalanine abolished NGF-mediated BDNF secretion. All the other mutants in which individual tyrosines were replaced by phenylalanines did not interfere with NGF-mediated BDNF secretion. In order to substantiate further the role played by Y794 in NT-mediated NT secretion, we produced nnr5 cells stably expressing wild-type or mutated TrkA constructs (Figure 1A). We used either a construct in which Y794 of the wild-type is replaced (Y794F) or one in which only the Y794 (Re794Y) is preserved together with residues Y679, Y683 and Y684, which are putative autophosphorylation sites and are required for the receptor tyrosine kinase activity (Stephens *et al.*, 1994). We selected stable clones (nnr5-TrkA, nnr5-Y794F and nnr5-Re794Y) that expressed about the same levels of receptor protein as evaluated by western blotting with an anti-pan-Trk antibody (Figure 1B). Cells were exposed for 0, 5 or 10 min to 100 ng/ml NGF and the level of receptor phosphorylation was determined by western blot using an anti-phosphotyrosine antibody. Wild-type TrkA shows a weak tyrosine kinase activity (Figure 2A) in the absence of NGF (0 min). However, the signal is strongly increased after 5 and 10 min exposure to NGF. The Y794F mutant showed a similar pattern of tyrosine phosphorylation. The 'rescue mutant' Re794Y showed a clear tyrosine phosphorylation signal after 5 and 10 min of NGF exposure,
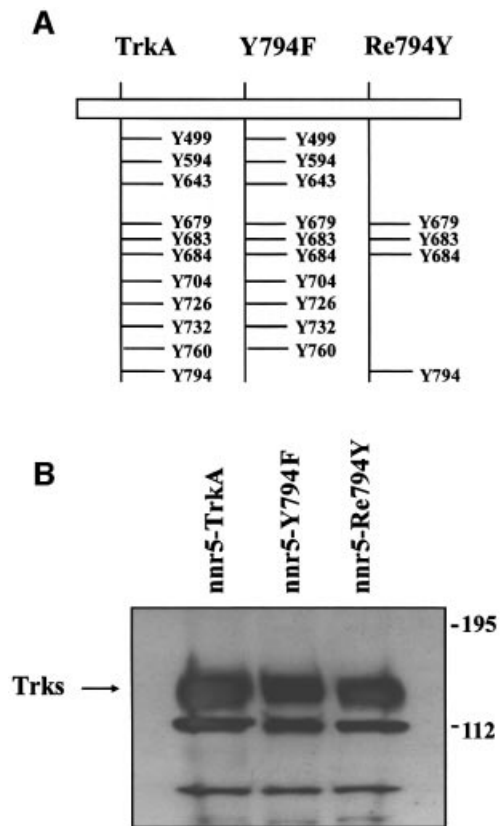

Fig. 1. Stable expression of the wild-type TrkA and receptor mutants in nnr5 cells. (**A**) Schematic representation of the cytosolic domain of the wild-type TrkA receptor and selected receptor mutants. Y794F represents a TrkA receptor in which the PLC-γ-binding site was inactivated by substituting the tyrosine residue Y794 with a phenylalanine, while in Re794Y all tyrosine residues have been replaced by phenylalanines, with the exception of Y794. Tyrosine residues Y679, Y683 and Y684 are essential for receptor autophosphorylation and were maintained in all mutants. (**B**) Western blot of wild-type (nnr5-TrkA) and mutated TrkA receptors (nnr5-Y794F and nnr5-Re794Y) stably expressed in nnr5 cells. Wild-type receptor and mutants were assayed for their levels of expression. We selected clones that, by probing with an anti-pan-Trk antibody, showed comparable levels of receptor protein.

although it was distinctly weaker than that in wild type and Y794F mutants. Tyrosine residue Y794 has been identified by Obermeier *et al.* (1993) as the binding site for PLC-γ. After exposure of the different stably transfected nnr5 clones to 100 ng/ml NGF, the cells were lysed and immunoprecipitated with an anti-phosphotyrosine antibody. The precipitates were subjected to western blotting and evaluated by a specific anti-PLC-γ antibody. In the nnr5-Re794Y clone, the PLC-γ phosphorylation after NGF exposure was as strong as in the wild-type clones. Conversely, NGF could not induce any PLC-γ phosphorylation in the nnr5-Y794F clone (Figure 2B).

In order to evaluate whether there is a causal relationship between the selective PLC-γ phosphorylation and the NT-mediated NT secretion, we investigated whether and to what extent in the different clones NGF could promote BDNF secretion in the absence or presence of PLC-γ phosphorylation. Owing to the lack of detectable BDNF expression in nnr5 cells, we expressed BDNF using an adenoviral vector (AdCMV-BDNF). After transduction, the cells were placed in a perfusion chamber and the
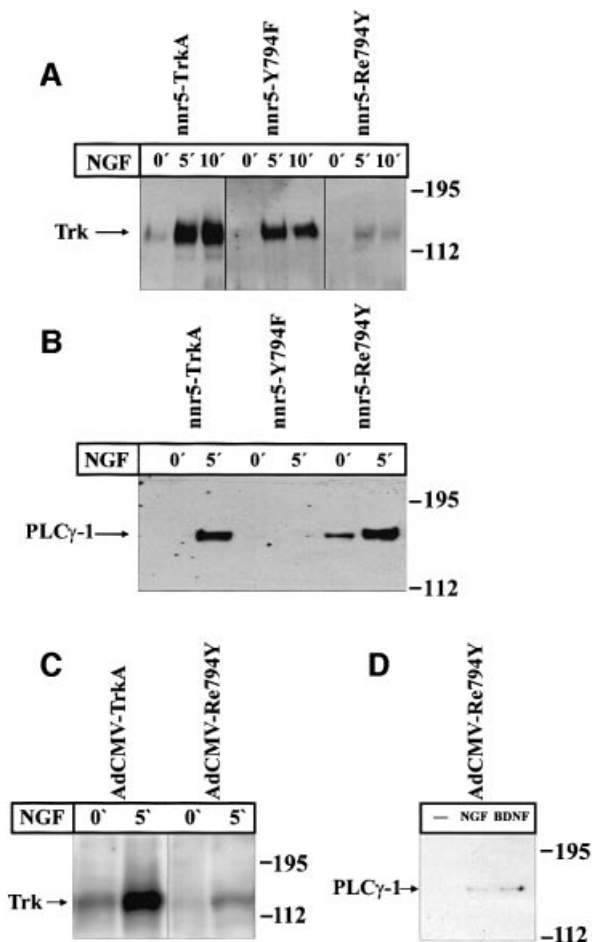
Fig. 2. Comparison between receptor tyrosine and PLC-γ phosphoryl-ation in wild-type and mutated TrkA receptors in stably transfected nnr5 cells and adenovirally transduced hippocampal neurons. (**A**) NGF-mediated tyrosine phosphorylation in nnr5-TrkA, nnr5-Y794F and nnr5-Re794Y clones. Each clone was stimulated for 0, 5 or 10 min with 100 ng/ml NGF and assayed for receptor tyrosine phosphorylation. Wild-type and mutated receptors were immunoprecipitated from cell lysates by an anti-pan-Trk antibody and the samples separated on an SDS–polyacrylamide gel. Western blots were probed with an anti-phosphotyrosine antibody. NGF administered for 5 or 10 min produced a marked tyrosine phosphorylation in wild-type and receptor mutants. (**B**) NGF-mediated PLC-γ tyrosine phosphorylation in nnr5 clones. The individual clones were stimulated with 100 ng/ml NGF for 0 or 5 min. The cell lysates were immunoprecipitated by agarose-linked anti-phosphotyrosine antibodies and subjected to western blot analysis with a specific anti-PLC-γ antibody. NGF treatment for 5 min was sufficient to induce PLC-γ phosphorylation in nnr5-TrkA and nnr5-Re794Y clones, whereas nnr5-Y794F clones did not show a PLC-γ phosphorylation signal in either the presence or absence of NGF. (**C**) NGF-mediated tyrosine phosphorylation in cultured hippocampal neurons transduced with TrkA and Re794Y receptors. Transduced neurons were stimulated for 0 and 5 min with 100 ng/ml NGF and assayed for receptor tyrosine phosphorylation. The western blot was performed as in (A). NGF produced a marked tyrosine phosphorylation in the wild-type receptor and mutant. (**D**) NGF- and BDNF-mediated PLC-γ tyrosine phosphorylation in cultured hippocampal neurons transduced with Re794Y. Cultured hippocampal neurons infected with the adenovirus coding for the Re794Y were stimulated for 0 or 5 min with 100 ng/ml either NGF or BDNF. Lysates of hippocampal neurons were immunoprecipitated by an agarose-conjugated anti-phospho-tyrosine antibody. PLC-γ phosphorylation was identified by western blotting as described in (A). Both NGF and BDNF induced PLC-γ tyrosine phosphorylation to a similar extent.

perfusate was collected at 5 min intervals. BDNF was quantified in each fraction by a two-site enzyme-linked immunosorbent assay (ELISA). The NGF-induced BDNF secretion was strictly correlated with the ability of NGF to phosphorylate PLC-γ. NGF induced BDNF secretion in nnr5-TrkA and nnr5-Re794Y, but not in nnr5-Y794F cells (Figure 3). However, all the clones evaluated secreted BDNF in response to high K+ (50 mM).

The PLC-γ signal transduction pathway is also responsible for NT-mediated NT secretion in hippocampal neurons

In hippocampal neurons, NGF does not initiate NT secre-tion (Canossa *et al.*, 1997), owing to the absence of functioning TrkA receptors (Aibel *et al.*, 1998). This experimental situation provided the opportunity to analyse the TrkA-mediated signalling pathways. We constructed adenoviral vectors that carry the cDNA for either wild-type TrkA (AdCMV-TrkA) or the mutant receptors Y794F (AdCMV-Y794F) and Re794Y (AdCMV-Re794Y). The use of these viruses, together with AdCMV-BDNF, necessitated double infection procedures. Since we had no reliable anti-TrkA-specific antibody for immunohisto-chemical analysis, we estimated the degree of double transduction in nnr5 cells that do not express any Trk receptors, and hence an anti-pan-Trk antibody could be used specifically to identify wild-type TrkA and the different mutants. Nnr5 cells transduced with the Re794Y construct showed a predominant signal at the plasma membrane (Figure 4B, middle panel). In contrast, BDNF shows the characteristic discontinuous scattered pattern (Figure 4B, left panel), reflecting its localization in the endoplasmic reticulum (ER) and Golgi (Gärtner *et al.*, 2000). Similar patterns of intracellular distribution were obtained for doubly transduced AdCMV-BDNF/AdCMV-TrkA or AdCMV-BDNF/AdCMV-Y794F nnr5 cells (data not shown). The intensity of expression of both BDNF and wild-type TrkA, or Y794F and Re794Y mutants varies from one cell to another, but the quantitative evaluation of clearly double-stained cells showed a level of double infection of ~90%. Importantly, the double-infected nnr5 cells showed the same BDNF secretion characteristics (Figure 4A) as stably transfected nnr5 cells (Figure 3). In adenovirally transduced hippocampal neurons, NGF applied for 5 min mediated tyrosine phosphorylation of TrkA wild-type receptors and its mutant Re794Y with a ratio that was similar to that obtained in nnr5 cells (Figure 2C). Furthermore, Re794Y proved to be sufficient to elicit NGF-mediated PLC-γ phosphorylation to a level comparable to that mediated by BDNF via endogenous TrkB receptors (Figure 2D). PLC-γ phosphorylation was in accordance with the observation that NGF resulted in BDNF secretion to a similar extent to that demonstrated by NT4/5 via the activation of endogenous TrkB receptors (Figure 5B). Cultured hippocampal neurons expressing wild-type TrkA or the Re794Y mutant have shown enhanced BDNF secretion in response to NGF, whereas neurons that were transduced with AdCMV-Y794F have not. These experiments were conducted under 'static' conditions (see Materials and methods) with the intention of excluding the possibility of a difference between 'static' and 'perfusion' conditions, an aspect that has been
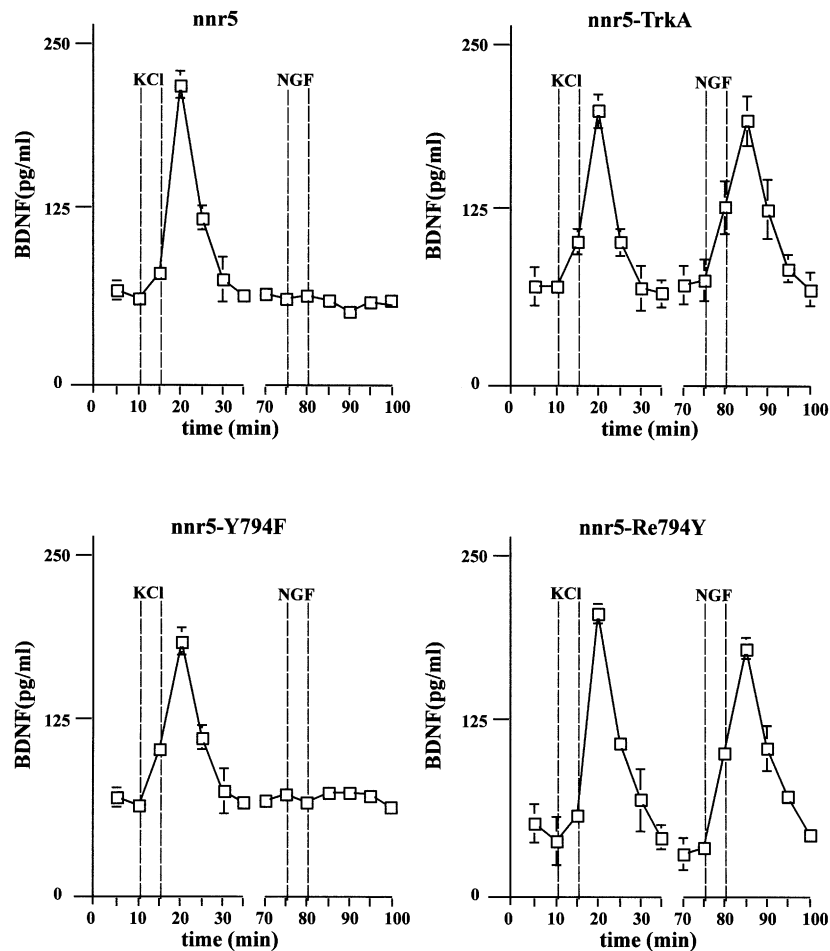
Fig. 3. High potassium- and NGF-mediated BDNF secretion from nnr5-TrkA, nnr5-Y794F and nnr5-Re794Y. Cells were perfused at a flow rate of 0.1 ml/min. Fractions were collected every 5 min. After collecting two fractions, a first stimulation by 50 mM KCl was initiated and four additional fractions collected. After a recovery of 30 min, cells were stimulated by 100 ng/ml NGF. BDNF concentrations were measured by two-site ELISA. Increased BDNF concentrations (expressed in pg/ml) occurred immediately after stimulation. The peak concentration occurred in the following fraction. The 5 min delay between the onset of stimulation and the peak of BDNF secretion is due to the dead volume of the perfusion system. NGF induced BDNF secretion in nnr5-TrkA and nnr5-Re794Y cells, but not in nnr5 or nnr5-Y794F cells. The values given represent the mean ± SE ($n = 6$).

analysed and discussed extensively by Griesbeck *et al.* (1999).

PLC-γ tyrosine phosphorylation correlates with IP₃ accumulation and Ca²⁺ release from intracellular stores

In previous experiments, it has been shown that NT secretion is dependent on intact intracellular Ca^{2+} stores and the release of Ca^{2+} from them (Blöchl and Thoenen, 1995, 1996; Canossa *et al.*, 1997; Griesbeck *et al.*, 1999). Hence, the most likely relationship between PLC-γ activation and NT secretion is the formation of D-myo-inositol 1,4,5-trisphosphate (IP_3) and subsequent mobilization of Ca^{2+} from the ER via activation of IP_3 receptors (Obermeier *et al.*, 1996; Tinhofer *et al.*, 1996). In nnr5-Re794Y cells, NGF (100 ng/ml) elicited a 3-fold IP_3 increase (18.8 ± 0.1 pg/10⁶ cells versus 5.5 ± 0.05 pg/10⁶ cells). In contrast, in control nnr5 cells, NGF did not induce any IP_3 formation above the basal level. NGF-mediated Ca^{2+} mobilization from the ER was assessed by Ca^{2+} imaging procedures, using the Ca^{2+} fluorophor Fura-2. In hippocampal neurons infected with an adenovirus carrying the Re794Y mutant, application of either

BDNF or NGF elicited intracellular Ca^{2+} signals in Ca^{2+}-free medium supplemented with 10 µM of the high-affinity Ca^{2+} chelator BAPTA (Figure 6A). In accordance with previous experiments (Canossa *et al.*, 1997), no Ca^{2+} signal was obtained after administration of NGF to native, non-transduced cultivated hippocampal neurons (Figure 6A). That the observed increase of cytosolic Ca^{2+} resulted from the release of Ca^{2+} from intracellular stores was supported further by the observation that depletion of these stores by pre-treatment with a combination of caffeine and thapsigargine blocked both NGF- and BDNF-mediated Ca^{2+} signalling in AdCMV-Re794Y-infected hippocampal neurons (Figure 6B).

PLC-γ-mediated BDNF secretion is dependent on Ca²⁺ release from intact intracellular Ca²⁺ stores

After showing that NGF induces an increase in cytosolic Ca^{2+} in hippocampal neurons expressing the Re794Y construct, we next evaluated the role of PLC-γ-mediated Ca^{2+} release in initiating NT secretion. In previous experiments, we have demonstrated that BDNF, acting via endogenous TrkB receptors, could mediate NGF secretion from hippocampal neurons in the absence of
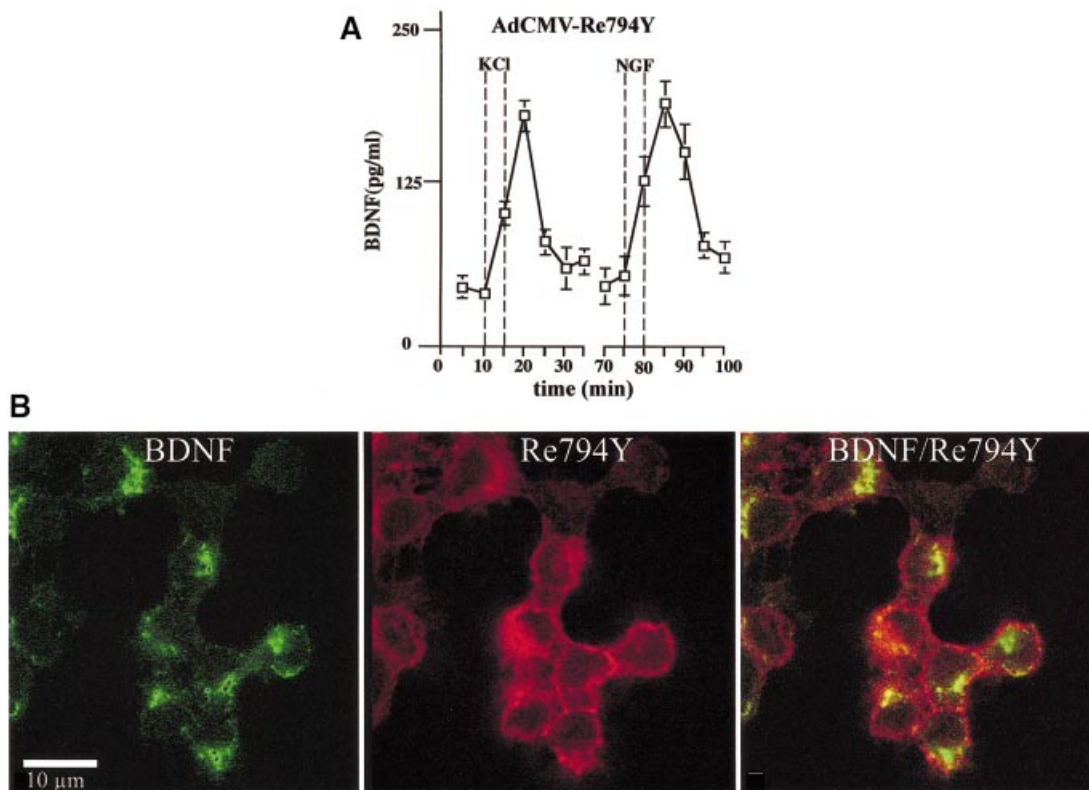
Fig. 4. Adenovirally transduced nnr5 cells. (**A**) Time course of BDNF secretion from nnr5 cells double transduced with AdCMV-Re794Y and AdCMV-BDNF. Perfusion was performed as described in Figure 3. Two subsequent stimulations by 50 mM KCl and 100 ng/ml NGF were separated by a recovery period of 30 min. Fractions were collected every 5 min and the BDNF concentrations were determined by ELISA. The values presented are the mean ± SE (*n* = 4). (**B**) Expression of BDNF and Re794Y mutant in nnr5 cells co-transduced with AdCMV-BDNF and AdCMV-Re794Y. The expression of both BDNF and Re794Y was detected by probing with the hybridoma supernatant (9E10), which recognizes the myc epitope added to the C-terminal domain of BDNF, and with the anti-pan-Trk antibody, which recognizes the C-terminus of Re794Y. Cells were analysed by confocal microscopy. The pictures represent a single confocal scan along a given *x–y* plane. The BDNFmyc-infected nnr5 cells showed a discontinuous staining pattern characteristic for the localization of BDNF in ER and Golgi compartments (green). The staining obtained with an anti-pan-Trk antibody was localized predominantly at the plasma membrane (red).

extracellular Ca^{2+} in a manner similar to that of neurotransmitter-mediated NT secretion (Canossa *et al.*, 1997). A membrane-permeable form of the high-affinity Ca^{2+} chelator BAPTA-AM abolished the secretion of NGF by sequestering cytosolic Ca^{2+}, indicating that Ca^{2+} release from intracellular stores is critical in NT-mediated NT secretion. This interpretation is now supported further by the observation that the selective activation of PLC-γ initiates BDNF secretion under Ca^{2+}-free conditions. We compared the response of cells infected with AdCMV-Re794Y to NT4/5 or NGF (Figure 7A). Removal of extracellular Ca^{2+} from the perfusion medium supplied with 10 μM of the high-affinity Ca^{2+} chelator BAPTA did not prevent NGF-mediated BDNF secretion (Figure 7A). However, pre-treatment with the membrane-permeable Ca^{2+} chelator BAPTA-AM abolished the secretion of BDNF in a manner similar to that of emptying the stores by pre-treatment with caffeine and thapsigargine (Figure 7A). Similar results were obtained with nnr5-TrkA and nnr5-Re794Y clones (Figure 7B).

Evidence that mGluRI mediates NT secretion

We now approached the question of whether the activation of the PLC signal transduction pathway might also be responsible for glutamate-mediated NT secretion. It is known that mGluRI is coupled specifically to PLC-

mediated IP_3 production (Frenguelli *et al.*, 1993). Hence, this signal transduction pathway lends itself to a more detailed analysis. We first analysed the secretion of endogenous BDNF from hippocampal slices of adult rats in a perfusion chamber. The perfusate was collected in 5 min fractions and the BDNF concentrations measured by a two-site ELISA. Administration of 50 μM 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD), an agonist of mGluRI and II receptors, resulted in an increase of endogenous BDNF secretion after 5 min stimulation (Figure 8). We obtained a similar pattern of secretion in dissociated hippocampal cultures infected with AdCMV-BDNF (Figure 9A). In order to demonstrate that the specific activation of mGluRI is responsible for BDNF secretion, we pre-treated hippocampal neurons with the specific mGluRI inhibitor 1-aminoindan-1,5-dicarboxyl-acid (AIDA). It has been shown that this inhibitor lowers the production of IP_3 elicited by t-ACPD (100 μM) in hippocampal slices without affecting the activation of group II receptors (Moroni *et al.*, 1997). In agreement with the reduction of IP_3 accumulation, 20 min pre-treatment with 500 μM AIDA resulted in a strong reduction of BDNF secretion mediated by t-ACPD (Figure 9B). Accordingly, the effects of t-ACPD proved to be strictly dependent on intact Ca^{2+} stores. Indeed, the addition of thapsigargine and caffeine (Figure 9C), which themselves
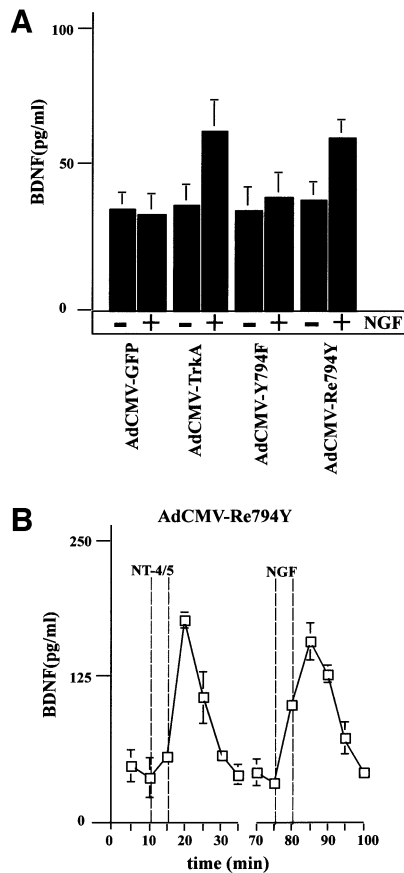
Fig. 5. BDNF secretion from cultured hippocampal neurons expressing TrkA, Y794F and Re794Y receptors. (**A**) Hippocampal neurons transduced with AdCMV-TrkA, AdCMV-Y794F and AdCMV-Re794Y were stimulated with 100 ng/ml NGF for 10 min under 'static' conditions. NGF-induced BDNF secretion occurred only in neurons expressing wild-type TrkA and the Re794Y receptors, but not Y794F or the green fluorescence protein (GFP) taken as a control of virus infection. The values given are the mean ± SE ($n = 10$). (**B**) Time course of BDNF secretion induced by NT4/5 via endogenous TrkB or by NGF via transuced Re794Y. Cells were perfused and fractions were collected before, during and after stimulation with 100 ng/ml NT4/5. A second stimulation was initiated by the administration of 100 ng/ml NGF. NT4/5 activated endogenous TrkB receptors, and NGF the TrkA receptor mutant Re794Y. The values given are the mean ± SE ($n = 6$).

initiate BDNF secretion (Griesbeck *et al.*, 1999), abolished the BDNF secretion induced by t-ACPD in the presence of extracellular Ca^{2+}. Thus, mGluRI-mediated BDNF secretion in hippocampal slices and transduced hippocampal neurons is initiated by activation of mGluRI receptors, resulting in IP_3-mediated Ca^{2+} mobilization from intracellular stores.

BDNF secretion is mediated by AMPA but not NMDA glutamate receptors

In previous experiments (Blöchl and Thoenen, 1996), it has been demonstrated that the glutamate-mediated NT secretion could be impaired by antagonists of AMPA receptors, but not those of NMDA receptors. We now used specific agonists for AMPA and NMDA receptors and demonstrated that the selective activation of AMPA, but not NMDA receptors also mediated a 3- to 4-fold increase of BDNF secretion from hippocampal slices (Figure 8) and

cultured neurons (Figure 9A). The stimulatory effect of AMPA resulted from the selective activation of AMPA receptors and not from an indirect *trans*-activation of mGluRI. Indeed, the selective AMPA receptor antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) could only prevent BDNF secretion induced by AMPA, but not that by t-ACPD (Figure 9B). Conversely, AIDA could only prevent BDNF secretion induced by t-ACPD without affecting the stimulatory effect of AMPA (Figure 9B). Similarly to mGluRI, AMPA receptor-mediated BDNF secretion was prevented by depletion of intracellular Ca^{2+} stores (Figure 9C).

Discussion

Use of nnr5 cells and cultured hippocampal neurons as analytical systems for studying the signal transduction pathway of TrkA-mediated NT secretion

TrkA receptors that were mutated in their intracellular tyrosine residues (Inagaki *et al.*, 1995) were used to identify the signal transduction pathways leading to BDNF secretion from both nnr5 cells and cultured hippocampal neurons. Wild-type TrkA and selected mutants were stably expressed in nnr5 cells (defective PC12 cells expressing p75[NTR] but no Trk receptors) (Loeb *et al.*, 1991). These cells were tested for their ability to mediate regulated BDNF secretion after administration of NGF. The presence of p75[NTR] alone was not sufficient to induce BDNF secretion by NGF (Figure 3). These data are in agreement with our previous observations (Canossa *et al.*, 1997), but are partially in disagreement with those of Krüttgen *et al.* (1998) in PC12 cells, in which NT secretion was obtained by not only TrkA receptor stimulation, but also activation of p75[NTR], i.e. after blockade of TrkA receptors. These discordant results can most probably be explained by the differing properties of the different PC12 cells used.

In order to validate the results obtained in nnr5 cells, we took advantage of the fact that hippocampal neurons do not express detectable levels of functional TrkA receptors (Aibel *et al.*, 1998) and, accordingly, they do not show any response to NGF. However, after transfection with TrkA, NGF promoted hippocampal neuron differentiation, as reflected by stimulation of fibre outgrowth (Aibel *et al.*, 1998). Hence, cultivated hippocampal neurons represent a valid cellular system with appropriate contextual properties for investigating signal transduction via (transduced) TrkA receptors and their mutants. Adenoviral gene transfer (Figure 5) accomplished expression of wild-type TrkA and corresponding mutants in hippocampal neurons. The quantity of BDNF secreted by activation of TrkA receptors with NGF was comparable to that mediated by NT4/5 through the activation of endogenous TrkB receptors (Figure 5). In accordance with the results obtained in nnr5 cells, the signal transduction via the PLC-γ-activating pathway of the TrkA receptor and the corresponding mutants also proved to be crucial for the NGF-mediated BDNF secretion in hippocampal cultures.

Ca^{2+} signalling and regulated NT secretion

In previous studies, it has been demonstrated that regulated NT secretion initiated by glutamate (Blöchl
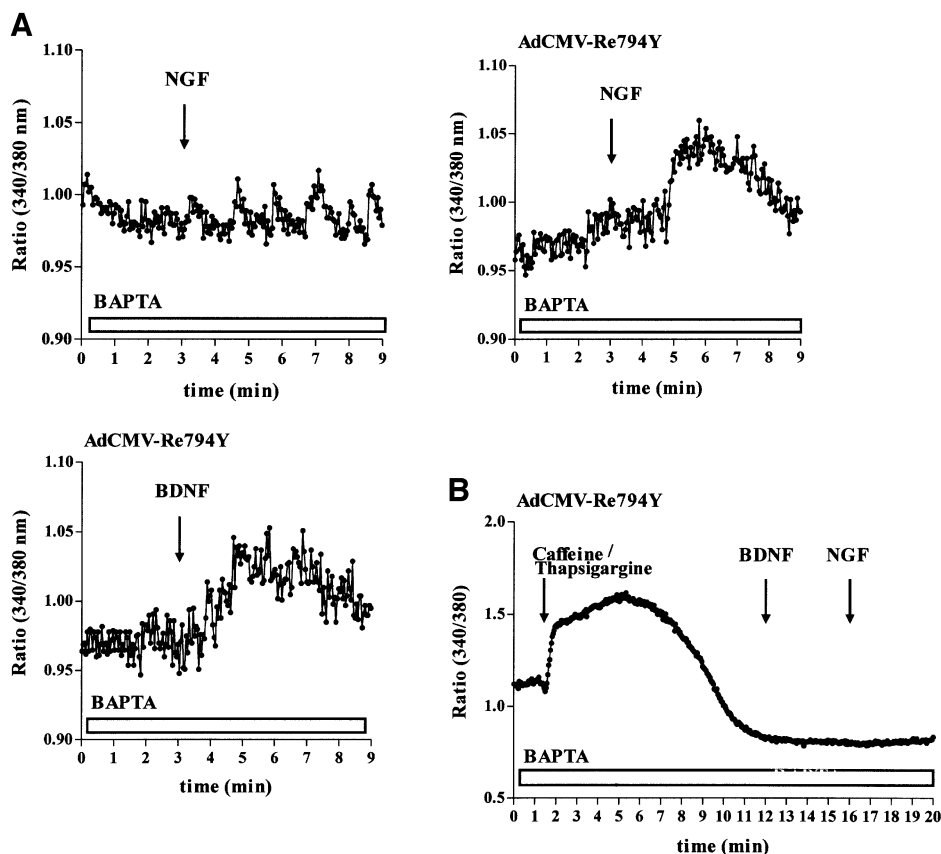
Fig. 6. Changes of intracellular Ca^{2+} concentrations in hippocampal neurons expressing the Re794Y receptors. (**A**) Non-transduced hippocampal neurons (controls) and those transduced by Re794Y were loaded with Fura-2/AM. Changes in intracellular Ca^{2+} concentrations were determined by the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm. In the absence of extracellular Ca^{2+} (BAPTA), hippocampal neurons expressing the TrkA receptor mutant showed an NGF-mediated increase in $[Ca^{2+}]_i$ that could not be distinguished from that obtained by BDNF acting via endogenous TrkB receptors. This is a representative example of eight independent experiments. (**B**) Effect of the depletion of the intracellular Ca^{2+} stores by thapsigargine and caffeine on the subsequent Ca^{2+} signalling by BDNF or NGF. In the absence of extracellular Ca^{2+} (BAPTA), caffeine/thapsigargine initiated a strong, prolonged Ca^{2+} signal resulting from the depletion of the Ca^{2+} stores. Subsequent administration of BDNF and NGF did not elicit a detectable Ca^{2+} signal, in distinct contrast to the hippocampal neurons not treated with caffeine and thapsigargine. This is a representative example of three independent experiments.

and Thoenen, 1995, 1996; Griesbeck *et al.*, 1999) and the activation of Trk receptors by NTs (Canossa *et al.*, 1997) are mediated by the release of Ca^{2+} from intracellular stores rather than Ca^{2+} influx. This represents a mechanism that is distinctly different from the activity-mediated secretion of conventional neurotransmitters and the majority of neuropeptides (Hökfelt *et al.*, 1980; Thureson-Klein and Klein, 1990; Matteoli and DeCamilli, 1991; Südhof, 1995; Berridge, 1998), raising pertinent questions concerning the storage/release compartments of NTs and the mechanism(s) of their secretion. In the present investigation, we have focused on the signal transduction pathway leading from the activation of Trk and glutamate receptors to the intracellular release of Ca^{2+} and subsequent NT secretion. In both primary cultures of hippocampal neurons and nnr5 cells, the signal transduction mediated by Y794, the binding site for PLC-γ, proved to be crucial for TrkA receptor-mediated NT secretion (Figures 3 and 4A). The binding of PLC-γ to Y794 results in PLC-γ activation by phosphorylation, which then cleaves phosphatidylinositol-4,5-bisphosphate to generate IP_3. IP_3 mobilizes Ca^{2+} from intracellular ER stores by activating IP_3 receptors, as demonstrated by Obermeier *et al.* (1996) in NIH 3T3 cells. Here we demonstrate that in

nnr5 cells stably transfected with the 'rescue mutant' Re794Y (nnr5-Re794Y), NGF produced an increase in IP_3 comparable to that of wild-type TrkA receptors. NGF-mediated IP_3 production via Re794Y was correlated with the Ca^{2+} release from intracellular ER stores and the Ca^{2+} mobilization was correlated with the NGF-mediated BDNF secretion, supporting the concept of a causal relationship between IP_3 production, Ca^{2+} mobilization from intracellular stores and NT secretion. In order to provide the most direct evidence for the involvement of IP_3 formation in NT secretion, we attempted to demonstrate NT secretion from hippocampal neurons by UV activation of a cell membrane-permeable form of caged IP_3 (W.H.Li *et al.*, 1998). However, the intensity of the UV flash necessary to uncage intraneuronal IP_3, and to produce a detectable Ca^{2+} signal, itself resulted in a massive BDNF secretion from hippocampal neurons that did not contain caged IP_3 (O.Griesbeck and M.Canossa, unpublished results). Hence, the relationship between Ca^{2+} mobilization and NT secretion had to rely on pharmacological evidence, namely that regulated NT secretion was blocked by pharmacologically depleting Ca^{2+} stores with caffeine/thapsigargine or by loading the cells with the intracellular high-affinity Ca^{2+} chelator BAPTA-AM (Figure 7).
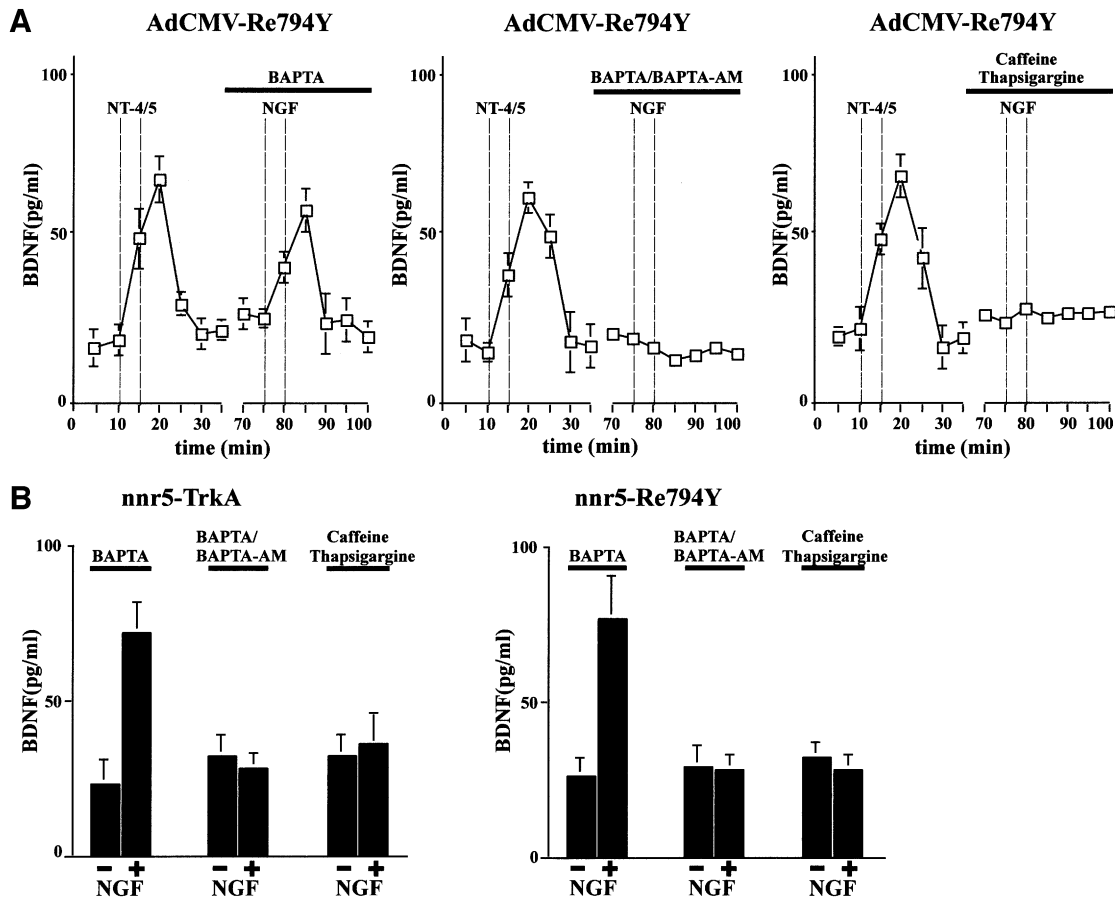
Fig. 7. Dependence on intact Ca^{2+} stores of NT-mediated NT secretion in hippocampal neurons. (**A**) BDNF secretion from hippocampal neurons in the absence of extracellular Ca^{2+} (BAPTA), chelation of intracellular calcium by BAPTA-AM and after depletion of the intracellular Ca^{2+} stores by caffeine and thapsigargine. Cultured hippocampal neurons co-infected with AdCMV-BDNF and AdCMV-Re794Y were perfused as described in Figure 3. After stimulation with 100 ng/ml NT4/5 for 5 min in normal Ca^{2+}, a second stimulation with 100 ng/ml NGF was performed in Ca^{2+}-free medium or medium containing the Ca^{2+} chelator BAPTA-AM (10 μM). Removal of extracellular Ca^{2+} (BAPTA) did not affect the ability of NGF to induce BDNF secretion. However, the effect of NGF on BDNF secretion was abolished by pre-treating hippocampal neurons for 30 min with the cell-permeable BAPTA-AM. Similarly, the depletion of intracellular Ca^{2+} stores with a combination of caffeine (3 mM) and thapsigargine (10 μM) abolished the NGF-mediated BDNF secretion. The values given represent the mean ± SE ($n = 6$). (**B**) Role of Ca^{2+} in BDNF secretion from nnr5-TrkA or nnr5-Re794Y clones. Similarly to the effects shown in hippocampal neurons, NGF induced BDNF secretion from nnr5 clones in the absence of extracellular Ca^{2+} (BAPTA). In contrast, chelating the intracellular Ca^{2+} with BAPTA-AM or depleting intracellular Ca^{2+} stores with caffeine/thapsigargine prevented NGF-mediated secretion of BDNF. The values given represent the mean ± SE ($n = 7$).
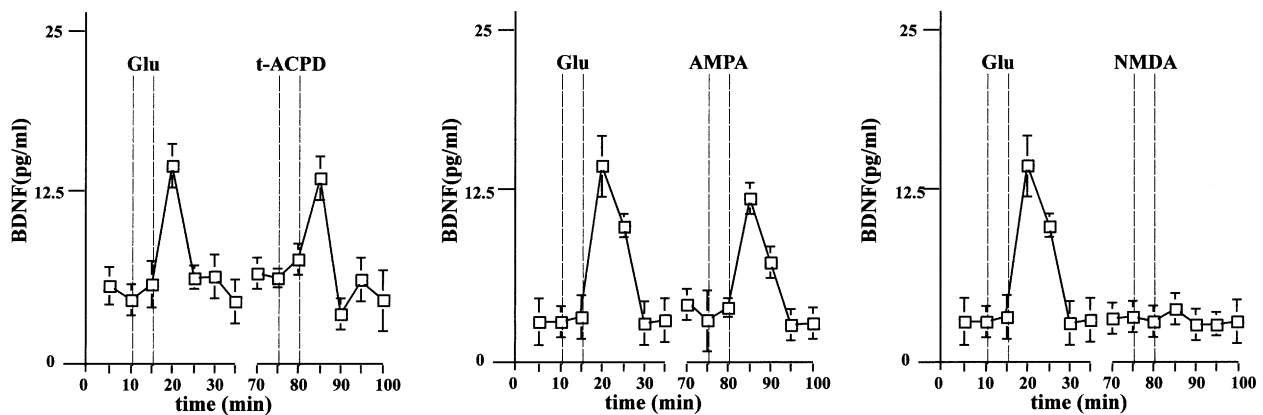

Fig. 8. Time course of BDNF secretion in native, non-transduced hippocampal slices. Acute hippocampal slices were perfused and fractions were collected over consecutive 5 min periods. BDNF concentrations were determined by ELISA. After a recovery period of 30 min, an initial stimulation by 50 μM glutamate was followed by a second stimulation with 100 μM t-ACPD, AMPA or NMDA. The values given are the mean ± SE ($n = 4$).

Stimulated BDNF secretion was reduced under all these experimental conditions. In contrast, the absence of extracellular Ca^{2+} did not interfere with the regulated secretion of NTs. Since PLC-γ activation leads to the formation of not only IP_3, but also diacylglycerol, leading in turn to the activation of protein kinase C (PKC)
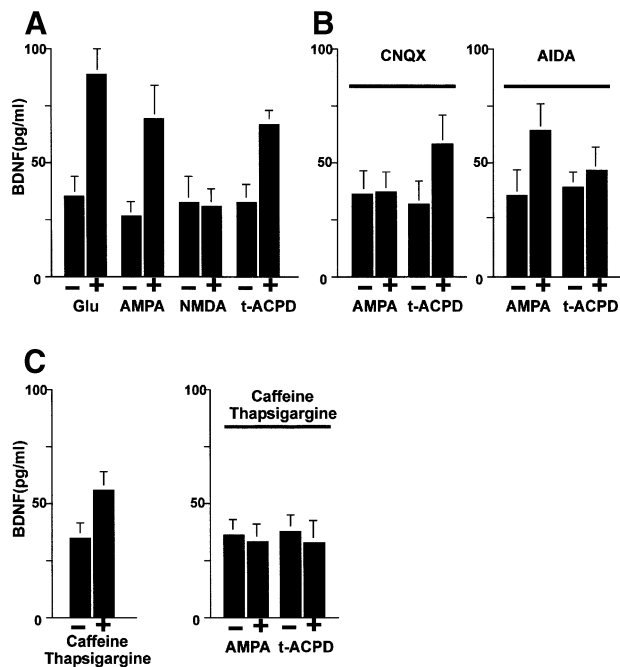
Fig. 9. Analysis of the signal transduction pathway(s) leading to BDNF secretion by glutamate. (**A**) BDNF secretion initiated by glutamate receptor agonists in cultured hippocampal neurons. Primary cultures of hippocampal neurons were infected with AdCMV-BDNF for 36–48 h. After an equilibration time of 60 min, basal levels of secreted BDNF were determined in the medium collected over a 10 min period under 'static' conditions. Stimulation of neurons for 10 min with glutamate (50 µM), AMPA (100 µM) or t-ACPD (100 µM) resulted in increased concentrations of BDNF in the incubation medium. NMDA had no effect. (**B**) Hippocampal neurons, pre-treated with the specific antagonists of AMPA and mGluRI receptors, CNQX (50 µM) and AIDA (500 µM), respectively, were tested for the effects of AMPA and t-ACPD. (**C**) Influence of Ca^{2+} stores on AMPA- and t-ACPD-mediated BDNF secretion. In hippocampal neurons, treatment with thapsigargine (10 µM) and caffeine (3 mM) initiated BDNF secretion but abolished the subsequent secretion of BDNF induced by AMPA and t-ACPD. The values given represent the mean ± SE ($n = 6$).

(Kikkawa *et al.*, 1989; Nishizuka, 1992), we evaluated the possible involvement of this signal transduction pathway by pre-treating hippocampal neurons with specific PKC inhibitors. This, however, did not interfere with the NGF-mediated BDNF secretion (data not shown).

Of particular interest is the glutamate-mediated NT secretion, since glutamate is the most prominent excitatory neurotransmitter in the central nervous system. We have demonstrated that activation of mGluRI resulted in an NT secretion from hippocampal slices and cultured hippocampal neurons comparable to that mediated by 50 mM potassium (Figures 8 and 9A). Conversely, specific inhibitors of mGluRI markedly reduced, although did not completely abolish, the glutamate-mediated NT secretion. mGluRI is a G-protein-coupled receptor that activates PLC, resulting in IP_3 formation (Frenguelli *et al.*, 1993) and subsequent Ca^{2+} release from intracellular stores by the activation of IP_3 receptors. Although the glutamate-mediated NT secretion is mediated by mGluRI, previous experiments conducted by Blöchl and Thoenen (1996) demonstrated that AMPA receptor inhibitors also reduced the glutamate-mediated NGF secretion. In the present experiments, we have demonstrated that selective activators of AMPA receptors also evoked NT secretion,

which did not result from cross-activation of mGluRI. This AMPA receptor-mediated NT secretion must be considered in the context of previous investigations from which it was thought to be mediated by sodium influx, given that sodium replacement by *N*-methyl-glucamine abolished the glutamate-mediated NGF secretion (Blöchl and Thoenen, 1995). This interpretation was wrong, since a more thorough analysis demonstrated that *N*-methyl-glucamine exhibited a blocking effect independent of sodium replacement (Höner, 2000). This excludes the fact that AMPA receptors initiate NT secretion via Na^+ influx, leading to a mechanism of secretion that has yet to be characterized. The mobilization of Ca^{2+} from endogenous stores seems to be the only common denominator of all pathways that lead to regulated NT secretion. As previously demonstrated for glutamate (Blöchl and Thoenen, 1995), AMPA-mediated NT secretion depends on intact Ca^{2+} stores, as demonstrated by the observation that pre-treatment with thapsigargine and caffeine prevented BDNF secretion (Figure 9C). This suggests that, as with mGluRI, AMPA triggers intracellular signalling pathway(s) that lead to Ca^{2+} mobilization from intracellular stores. However, the elucidation of the signal transduction mechanism(s) that lead to NT secretion by AMPA receptor activation requires more detailed future analysis.

Materials and methods

Cell lines
Rat nnr5 cells (Green *et al.*, 1986) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS; Gibco) and 10% horse serum (Boehringer Mannheim), and were incubated at 37°C in 10% CO_2. Nnr5 clones, nnr5-TrkA, nnr5-Y794F or nnr5-Re794Y were kept under G418 (100 mg/l) selection. Nnr5 cells and clones were plated at a density of 100 000 per collagen-coated glass coverslip (10 mm). At a confluence of 80%, cells were infected with adenoviral vectors in a reduced volume of 300 µl for 8–12 h before the release experiments.

Acute hippocampal slices
Slices of 350 µm were prepared from hippocampi of adult Wistar rats and placed in a perfusion chamber as reported by Blöchl and Thoenen (1995). Release experiments were initiated after a recovery phase of 10 min with a flow rate of 0.1 ml/min.

Primary culture of hippocampal neurons
Hippocampal neurons were prepared from E17 Wistar rats according to Zafra *et al.* (1990). Dissociated hippocampal neurons obtained from embryonic E17 rats were grown for 5–7 days in complete medium. Under our culture conditions, only 2% of the total cell population are of astroglial origin, as demonstrated by immunocytochemistry probing with glial fibrillary acidic protein (GFAP), an astroglial-specific marker (data not shown). The remaining cells were shown to express the microtubule-associated protein 2 (MAP2), an established neuronal marker. Neurons were plated at a density of 200 000 per 10 mm glass coverslip, coated with poly-DL-ornithine (0.5 mg/ml) and infected with adenoviral vectors in a reduced volume of 300 µl for 36–48 h before the release experiments.

Transfection and selection of nnr5 clones
The cDNA coding for wild-type TrkA and the mutants cloned into a mammalian expression vector (pEF-BOS), described by Inagaki *et al.* (1995), were transiently transfected in nnr5 cells using a conventional calcium phosphate method. For the generation of stably expressing clones, nnr5 cells were co-transfected with constructs expressing TrkA, Y794F or Re794Y, and a vector expressing the resistance gene for gentamycin. After selection in the presence of G418 (500 mg/l), the resistant colonies were expanded in a medium with 100 mg/l G418.

Recombinant adenovirus construction

The cDNA coding for wild-type TrkA, or the mutants Y794F and Re794Y, were subcloned into the *Xba*I site of the pXCJL1-CMV-BGH vector (provided by C.Gravel, Quebec, Canada). Recombinant replication-deficient virus was obtained by homologous recombination in 293 cells (McGrory *et al.*, 1988; Graham and Prevec, 1992). AdCMV-TrkA, AdCMV-Y794F and AdCMV-Re794Y contained the cDNA sequence coding for TrkA, Y794F and Re794Y receptors, respectively. AdCMV-BDNF, previously described in Canossa *et al.* (1997), contained the cDNA sequence coding for mouse pre-proBDNF tagged with the myc epitope at the C-terminus. AdVGFP contains the *Hin*dIII–*Not*I fragment of the N1-EGFP vector (Clontech). Virus titres estimated by a plaque assay were in the range of 10^{10}–10^{11} plaque-forming units/ml.

Characteristics of the perfusion set up and release experiments

Acute slices, cultured hippocampal neurons or nnr5 cells on glass coverslips were placed in a perfusion chamber and perfused as described by Canossa *et al.* (1997). Both slides and culture cells were stimulated by either replacing 50 mM NaCl in the perfusion medium with 50 mM KCl or adding glutamate (50 µM), AMPA (Biotrend, 100 µM), NMDA (Biotrend, 50 µM), t-ACPD (Biotrend, 100 µM), NGF or NT4/5 (100 ng/ml). Pre-treatment was initiated during the recovery phase with several inhibitors: CNQX (Biotrend, 50 µM), AIDA (Biotrend, 500 µM), caffeine (Sigma, 3 mM), BAPTA-AM (Molecular Probes, 10 µM), BAPTA (Sigma, 10 µM) and thapsigargine (Alexis, 10 µM). BDNF secretion was also quantified under 'static' conditions. In these experiments, infected hippocampal neurons were equilibrated for 60 min in the tissue culture plate in Hanks buffer. After four basal collection values of 10 min, neurons were stimulated for 10 min with several stimuli. In each sample, the amount of BDNF was determined by ELISA.

Enzyme immunoassays (ELISAs)

BDNF concentrations were determined by a two-site ELISA according to Canossa *et al.* (1997) and Kolbeck *et al.* (1999). The ELISAs showed a sensitivity of 0.5–1.0 pg/ml of BDNF.

Western blot

Confluent 10 cm dishes of nnr5-TrkA, nnr5-Re794Y and nnr5-Y794F cells and cultured hippocampal neurons (5×10^6) infected with AdCMV-TrkA, AdCMV-Re794Y or AdCMV-Y794F were stimulated with 100 ng/ml NGF for 0, 5 or 10 min and lysed. Overnight immunoprecipitation was performed by using either anti-phosphotyrosine antibody-conjugated agarose (Upstate Biotechnology) or wheat germ agglutinin (WGA)-conjugated Sepharose. Samples were then separated by 8% SDS–PAGE and transferred to Immobilon-P membranes (0.45 µm; Millipore) using standard procedures. After blocking unspecific sites, the membranes were incubated overnight at 4°C with the primary antibody: anti-PLC-γ (4 µg/ml; Upstate Biotechnology) or pan-Trk antiserum (1:1000 provided by Mariano Barbacid). Detection was performed after 1 h incubation with the appropriate secondary antibody conjugated to horseradish peroxidase (Dianova) with subsequent conversion of a chemiluminescent substrate (Pierce).

IP$_3$ determination

IP$_3$ was measured using the Biotrak [^{3}H]IP$_3$ assay system (Amersham).

Ca^{2+} imaging

Free intracellular Ca^{2+} concentrations were determined by Ca^{2+} imaging procedures according to Canossa *et al.* (1997).

Immunohistochemistry and confocal microscopy

For intracellular detection of Re794Y and BDNFmyc, nnr5 cells were fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline, permeabilized, and unspecific sites blocked. The first antibody was added overnight at 4°C; the other steps were performed at room temperature. A hybridoma (9E10) supernatant (1:10) recognizing the myc epitope was used for detection of BDNFmyc. For Re794Y, rabbit pan-Trk antiserum was used (1:500). Secondary antibodies were fluorescein isothiocyanate-conjugated anti-mouse in combination with an anti-rabbit lissamine–rhodamine antibody (both Dianova; 1:150). Immunofluorescence was analysed by confocal microscopy (Leica).

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