Regulated secretion of neurotrophins by metabotropic glutamate group I (mGluR1) and Trk receptor activation is mediated via phospholipase C signalling pathways

Marco Canossa1,2,3, Annette Gärtner1, Gabriele Campana1,2, Naoyuki Inagaki4, and Hans Thoenen1

1Department of Neurobiochemistry, Max-Planck Institute of Neurobiology, Am Klopferspitz 18A, D-82152 Martinsried, Germany, 2Department of Pharmacology, University of Bologna, Inerino 48, I-40126 Bologna, Italy and 3Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma 630-0101, Japan

Corresponding author
E-mail: ashdow@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 phospholipase C (PLC) and the subsequent mobilization of Ca2+ 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 mnr5 cells is necessary to mobilize Ca2+ 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 Ca2+ from internal stores by inositol trisphosphate. Thus, PLC-mediated signal transduction pathways are the common mechanisms for both Trk- and mGluR1-mediated NT secretion.

Keywords: adenovirus/BDNF/Ca2+ 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 (p75NTR) 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; Blöchel 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) 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/C1 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 (Blöchel 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 (Blöchel 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-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) receptors and the metabotropic glutamate receptors (mGluRs), but not NMDA receptors (Blöchel 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 Ca2+ from endogenous stores seems to be

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the common denominator of all pathways that lead to regulated NT secretion (Böchel 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 Ca²⁺ 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 Ca²⁺ from intracellular stores.

Results

Evidence that PLC-γ mediates NT secretion in nmr5 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 nmr5 cells, which are variants of PC12 cells (Green et al., 1986) that express p75NTR 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 nmr5 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, 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 nmr5 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-<s>Re794Y</s> 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 nmr5 cells, we expressed BDNF using an adenoviral vector (AdCMV-BDNF). After transduction, the cells were placed in a perfusion chamber and the
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 secretion (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 immunohistochemical analysis, we estimated the degree of double transduction in nmr5 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. Nmr5 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 nmr5 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 nmr5 cells showed the same BDNF secretion characteristics (Figure 4A) as stably transected nmr5 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 nmr5 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...
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 Ca²⁺ stores and the release of Ca²⁺ from them ( Bölch 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-myoinositol 1,4,5-trisphosphate (IP₃) and subsequent mobilization of Ca²⁺ from the ER via activation of IP₃ receptors (Obermeier et al., 1996; Tinhofer et al., 1996). In nrr5-794Y cells, NGF (100 ng/ml) elicited a 3-fold IP₃ increase (18.8 ± 0.1 pg/10⁶ cells versus 5.5 ± 0.05 pg/10⁶ cells). In contrast, in control nrr5 cells, NGF did not induce any IP₃ formation above the basal level. NGF-mediated Ca²⁺ mobilization from the ER was assessed by Ca²⁺ imaging procedures, using the Ca²⁺ fluorophor Fura-2. In hippocampal neurons infected with an adeno-virus carrying the Re794Y mutant, application of either

BDNF or NGF elicited intracellular Ca²⁺ signals in Ca²⁺-free medium supplemented with 10 μM of the high-affinity Ca²⁺ chelator BAPTA (Figure 6A). In accordance with previous experiments (Canossa et al., 1997), no Ca²⁺ signal was obtained after administration of NGF to native, non-transduced cultivated hippocampal neurons (Figure 6A). That the observed increase of cytosolic Ca²⁺ resulted from the release of Ca²⁺ from intracellular stores was supported further by the observation that depletion of these stores by pre-treatment with a combination of caffeine and thapsigargin blocked both NGF- and BDNF-mediated Ca²⁺ 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 Ca²⁺ in hippocampal neurons expressing the Re794Y construct, we next evaluated the role of PLC-γ-mediated Ca²⁺ 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
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-\(\gamma\) 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 \(\mu\)M 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 thapsigargin (Figure 7A). Similar results were obtained with mr5-TrkA and mr5-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 \(\mu\)M 1S,3R-1-amino-cyclopentane-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-dicarboxylic acid (AIDA). It has been shown that this inhibitor lowers the production of IP\(_3\) elicited by t-ACPD (100 \(\mu\)M) 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 \(\mu\)M 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 thapsigargin and caffeine (Figure 9C), which themselves
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\textsuperscript{2+} stores (Figure 9C).

**Discussion**

**Use of nrr5 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 nrr5 cells and cultured hippocampal neurons. Wild-type TrkA and selected mutants were stably expressed in nrr5 cells (defective PC12 cells expressing p75\textsuperscript{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\textsuperscript{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\textsuperscript{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 nrr5 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 cell culture 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 nrr5 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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} concentrations were determined by the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm. In the absence of extracellular Ca\textsuperscript{2+} (BAPTA), hippocampal neurons expressing the TrkA receptor mutant showed an NGF-mediated increase in [Ca\textsuperscript{2+}], 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\textsuperscript{2+} stores by thapsigargin and caffeine on the subsequent Ca\textsuperscript{2+} signalling by BDNF or NGF. In the absence of extracellular Ca\textsuperscript{2+} (BAPTA), caffeine/thapsigargin initiated a strong, prolonged Ca\textsuperscript{2+} signal resulting from the depletion of the Ca\textsuperscript{2+} stores. Subsequent administration of BDNF and NGF did not elicit a detectable Ca\textsuperscript{2+} signal, in distinct contrast to the hippocampal neurons not treated with caffeine and thapsigargin. 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\textsuperscript{2+} from intracellular stores rather than Ca\textsuperscript{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\textsuperscript{2+} and subsequent NT secretion. In both primary cultures of hippocampal neurons and nmr5 cells, the signal transduction mediated by Y794, the binding site for PLC-\gamma, proved to be crucial for TrkA receptor-mediated NT secretion (Figures 3 and 4A). The binding of PLC-\gamma to Y794 results in PLC-\gamma activation by phosphorylation, which then cleaves phosphatidylinositol-4,5-bisphosphate to generate IP\textsubscript{3}. IP\textsubscript{3} mobilizes Ca\textsuperscript{2+} from intracellular ER stores by activating IP\textsubscript{3} receptors, as demonstrated by Obermeier et al. (1996) in NIH 3T3 cells. Here we demonstrate that in nmr5 cells stably transfected with the ‘rescue mutant’ Re794Y (nmr5-Re794Y), NGF produced an increase in IP\textsubscript{3} comparable to that of wild-type TrkA receptors. NGF-mediated IP\textsubscript{3} production via Re794Y was correlated with the Ca\textsuperscript{2+} release from intracellular ER stores and the Ca\textsuperscript{2+} mobilization was correlated with the NGF-mediated BDNF secretion, supporting the concept of a causal relationship between IP\textsubscript{3} production, Ca\textsuperscript{2+} mobilization from intracellular stores and NT secretion. In order to provide the most direct evidence for the involvement of IP\textsubscript{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\textsubscript{3} (W.H.Li et al., 1998). However, the intensity of the UV flash necessary to uncage intraneuronal IP\textsubscript{3}, and to produce a detectable Ca\textsuperscript{2+} signal, itself resulted in a massive BDNF secretion from hippocampal neurons that did not contain caged IP\textsubscript{3} (O.Griesbeck and M.Canossa, unpublished results). Hence, the relationship between Ca\textsuperscript{2+} mobilization and NT secretion had to rely on pharmacological evidence, namely that regulated NT secretion was blocked by pharmacologically depleting Ca\textsuperscript{2+} stores with caffeine/thapsigargin or by loading the cells with the intracellular high-affinity Ca\textsuperscript{2+} chelator BAPTA-AM (Figure 7).
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)
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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 Ca²⁺ stores on AMPA- and t-ACPD-mediated BDNF secretion. In hippocampal neurons, treatment with thapsigargin (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 IP₃ formation (Fengueli et al., 1993) and subsequent Ca²⁺ release from intracellular stores by the activation of IP₃ receptors. Although the glutamate-mediated NT secretion is mediated by mGluRI, previous experiments conducted by Böehl 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 (Böehl 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 Ca²⁺ from endogenous stores seems to be the only common denominator of all pathways that lead to regulated NT secretion. As previously demonstrated for glutamate (Böehl and Thoenen, 1995), AMPA-mediated NT secretion depends on intact Ca²⁺ stores, as demonstrated by the observation that pre-treatment with thapsigargin and caffeine prevented BDNF secretion (Figure 9C). This suggests that, as with mGluRI, AMPA triggers intracellular signalling pathway(s) that lead to Ca²⁺ 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 nrr5 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% CO₂. Nnr5 cells, nrr5-TrkA, nrr5-Y794F or nrr5-RE794Y were kept under G418 (100 μg/ml) 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 Böehl 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 nrr5 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 nrr5 cells using a conventional calcium phosphate method. For the generation of stably expressing clones, nrr5 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 μg/ml), the resistant colonies were expanded in a medium with 100 μg/ml G418.
Recombinant adenosin virus construction
The cDNA coding for wild-type TrkA, or the mutants Y794F and Re794Y, were subcloned into the XbaI site of the pXCI1-MV-BCG vector (provided by C.Gravel, Quebec, Canada). Recombinant replication-deficient virus was obtained by homologous recombination in 293 cells (McGregory et al., 1988, Graham and Prevec, 1992). AdCMV-TrkA, AdCMV-Y794F and AdCMV-RC794Y 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 HindIII–NotI fragment of the N1-EFGP vector (Clontech). Virus titres estimated by a plaque assay were in the range of 10^6–10^7 plaque-forming units/ml.

Characteristics of the perfusion set up and release experiments
Acute slices, cultured hippocampal neurons or nr5 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-ACP-DP (Biotrend, 100 µM), NGF or NT/4/5 (100 ng/ml). The treatment was initiated during the recovery phase with several inhibitors: CNQX (Biotrend, 50 µM), AIDA (Biotrend, 500 µM), caffeine (Sigma, 3 mM), BAIPA-AM (Molecular Probes, 10 µM), BAIPA (Sigma, 10 µm) and thapsigargin (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 × 10^5) infected with AdCMV-TrkA, AdCMV-Re794Y or AdCMV-Y794F were stimulated with 100 ng/ml NFG for 0, 5 or 10 min and lysed. Overnight immunoprecipitation was performed by using either anti-phosphotyrosine antibody (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 unspecified sites, the membranes were incubated overnight at 4°C with the primary antibody: anti-PLC-γ (4 µg/ml, Upstate Biotechnology) or pan-Trk antisera (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).

IP₃ determination
IP₃ was measured using the Biotrak [H]Pi assay system (Amersham).

Ca²⁺ imaging
Free intracellular Ca²⁺ concentrations were determined by Ca²⁺ imaging procedures according to Canossa et al. (1997).

Immunohistochemistry and confocal microscopy
For intracellular detection of Re794Y and BDNF/Fyn, 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 BDNF/Fyn. For Re794Y, rabbit polyclonal antisera 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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