Gabapentin-Lactam Induces Dendritic Filopodia and Motility in Cultured Hippocampal Neurons

Frank Henle, Jost Leemhuis, Catharina Fischer, Hans H. Bock, Kerstin Lindemeyer, Thomas J. Feuerstein, and Dieter K. Meyer

Zentrum für Neurowissenschaften, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie (F.H., J.L., C.F., K.L., D.K.M.), Zentrum für Neurowissenschaften, Abteilung Innere Medizin II (H.H.B.), and Sektion Klinische Neuropharmakologie der Neurochirurgischen Universitätsklinik (T.J.F.), Albert-Ludwigs-Universität, Freiburg, Germany

Received April 13, 2006; accepted July 13, 2006

ABSTRACT

Gabapentin is currently used as a therapeutic agent against epilepsy as well as neuropathic pain. In contrast to gabapentin, its derivative gabapentin-lactam has a pronounced neuroprotective activity. We have studied in cultured hippocampal neurons whether gabapentin-lactam has also neurotrophic effects. Gabapentin-lactam enhanced the formation of dendritic filopodia, which are necessary for synapse formation. It also induced a network of F-actin-containing neurites. In studies with time lapse microscopy, gabapentin-lactam increased the addition but also the elimination of new branches. Affinity precipitation assays showed that gabapentin-lactam increased the GTP binding of the small GTPases Rac and Cdc42, which facilitate branch addition. Gabapentin-lactam also activated RhoA and phosphatidylinositol 3-kinases. In neurons transfected with dominant-negative RhoA or treated with the RhoA-inactivating C3 toxin, gabapentin-lactam increased the number of dendrites and branches. In the presence of Y-27632, which inhibits Rho kinase, newly added branches induced by gabapentin-lactam were no longer eliminated so that gabapentin-lactam increased the number of branches. Y-27632 ([(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cycohexanecarboxamide] also prevented the gabapentin-lactam induced activation of phosphatidylinositol 3-kinases. The phosphatidylinositol 3-kinase inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride] reduced the elimination of newly added branches caused by gabapentin-lactam and thus facilitated branch formation. In contrast to gabapentin-lactam, gabapentin had no effect on dendritic filopodia or motility. The effects exerted by gabapentin-lactam on dendritic arborization may be of potential therapeutic interest.
traction or elimination of the new extensions (Van Aelst and Cline, 2004). The Rho family of small GTPases is of special importance in the regulation of dendrite addition ( Luo, 2002; Van Aelst and Cline, 2004). These molecular switches are active in the GTP-bound form and are inactivated by GTP hydrolysis ( Hall, 1998). Rac and Cdc42 induce the formation of lamellipodia and filopodia, respectively ( Hall, 1998). They also support the addition of new dendrites and branches by acting on the actin cytoskeleton, whereas RhoA attenuates this process via its effector Rho kinase (ROCK) ( Li et al., 2000; W ong et al., 2000; Leemhuis et al., 2004). The retraction of newly formed extensions is under the control of class I phosphatidylinositol 3 (PI3) kinases, as recently shown in hippocampal neurons ( Leemhuis et al., 2004).

We have studied in cultured embryonic hippocampal neurons how GBP-L affected filopodia formation as well as the formation of new dendrites and branches. Since ROCK can inhibit the formation of new extensions, we also studied the effects of GBP-L in the presence of ROCK inhibitors. Time lapse analysis was used to study the dynamics of dendrite and branch formation. The activities of Rho GTPases and PI3-kinases were measured with affinity precipitation assays and Western blot analysis, respectively.

Materials and Methods

Materials. Y-27632 and HI-1152 were from Calbiochem (Bad Soden, Germany). Poly-L-lysine, Dubelco's modified Eagle’s medium, fetal calf serum (fetal calf serum), and St aarV medium were prepared from brains of embryonic rats at day 17. The dissociated cells were seeded on glass coverslips coated with poly-L-lysine at a density of approximately 500 cells/mm². The incubation medium consisted in Dubelco's modified Eagle’s medium plus 10% fetal calf serum with an endothelin level < 0.5 EU/ml. Cultures were incubated at 37°C in a humidified atmosphere with low oxygen conditions (6.5% CO₂ and 9% O₂) to simulate in vivo conditions. After 1 day in vitro (DIV), the incubation medium was replaced with serum-free differentiation medium, which consisted of neurobasal A, pH 7.3 (HEPES) supplemented with 2% B27. If not mentioned otherwise, the neurons were used at DIV1 for morphological studies. For biochemical studies, the cultures were incubated until DIV3 and then treated with the respective drugs. The neuronal cultures contained less than 5% astrocytes as determined by glial fibrillary acidic protein immunostaining.

Cytoskeletal Staining. Cultured hippocampal neurons were fixed for 15 min with 4% paraformaldehyde in cytoskeletal stabilization buffer (CSB) ( Schaefer et al., 2002) containing 80 mM Pipes, 5 mM EGTA, 1 mM MgCl₂, and 4% polyethylene glycol (mol. wt. 35,000). After washing with CSB, neurons were permeabilized with 0.1% (v/v) Triton X-100 in CSB. Normal goat serum in PBS was used to block nonspecific binding. For staining of β-tubulin III, neurons were incubated with a monoclonal mouse anti-β-tubulin III antibody ( Sigma, Deisenhofen, Germany). The resulting immune complex was visualized with an Alexa 488-conjugated F(ab)₂ fragment goat anti-mouse ( Molecular Probes, Heidelberg, Germany). For actin staining, cells were incubated with Alexa 594-conjugated phalloidin ( Molecular Probes) and washed again with CSB.

Pull-Down Experiments with the GST-PAK-CRIB Domain or GST-C21 Domain. The fusion protein GST-C21 contains the N-terminal 90 amino acids of the Rho-binding region of the Rho effector Rhotekin. The fusion protein GST-PAK-CRIB contains the Rac/Cdc42 binding domain of the common effector PAK. The GST-fusion proteins used for the pull-down assays were expressed in Escherichia coli. BL21 cells grown at 37°C. Expression in the BL21 cells was induced by adding 0.1 mM isopropyl β-d-thiogalactoside (final concentration) at OD₆₀₀ 1.0. Two hours after induction, the cells were collected and sonicated in lysis buffer (50 mM Trys-HCl, pH 8.0, 2 mM MgCl₂, 2.0 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 10,000g. Then, the GST-PAK-CRB and domain and the GST-C21 domain, respectively, were affinity purified from the supernatant with glutathione-Sepharose beads (Pharmacia, Piscataway, NJ). Beads loaded with the GST-fusion proteins were washed twice with GST-fishing buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1% (v/v) Nonidet P-40, and 25 μg/ml aprotinin) at 4°C.

Approximately 1 × 10⁶ neurons were used for the pull-down experiments on Rac or Cdc42 activity; approximately 4 × 10⁶ neurons were used to determine RhoA activity. After the actual experiments, neurons were washed twice with PBS and harvested after addition of 250 μl of ice-cold GST-fishing buffer. The detergent-soluble supernatant was recovered after centrifugation (14,000 × g) for 15 min. GTP-Rac or GTP-Cdc42 proteins were precipitated for 1 h with GST-PACK fusion protein at 4°C. GTP-RhoA was precipitated for 1 h with GST-C21 fusion protein. The complexes were washed three times with ice-cold PBS, resuspended, and boiled in Laemmli buffer. GTP-bound Rac, Cdc42, or RhoA proteins were detected by Western blotting using anti-Rac1 ( BD Bioscience, Heidelberg, Germany), anti-Cdc42 ( Upstate, Milton Keynes, UK), or anti-RhoA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Preparation of Transfection Vectors and Cell Transfection. The coding regions of the RhoAN19 and Rac1N17 genes were excised from the plasmid pGEX with BamHI and EcoRI and inserted in-frame into the BglIII/EcoRI sites of pEGFP-C1 (Clontech, Heidelberg, Germany). The coding region of the Cdc42N17 gene was excised from the plasmid pCDNA3 with BamHI and EcoRI and inserted in-frame into the BglII/EcoRI sites of pEGFP-C1. All plasmids were confirmed by restriction digest analysis and sequencing. Cells were transfected for 1.5 h at 2.5% CO₂ using the calcium phosphate/DNA coprecipitation procedure. After transfection, neurons were grown for 16 h prior to use.

Measurement of Akt Phosphorylation. After the actual experiment, neurons were washed twice with PBS and harvested after ice-cold lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1% (v/v) Nonidet P-40, and 25 μg/ml aprotinin) had been added. The detergent-soluble supernatant was recovered after centrifugation for 15 min at 14,000g and 4°C. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis. To determine the phosphorylation of Akt, anti-PhosphoSer473 Akt and anti-Akt antibodies were used (Cell Signaling, Beverly, MA).

Time Lapse Imaging. Hippocampal neurons were plated and incubated on poly-L-lysine-coated glass-bottom microwell dishes (MatTek, Ashland, MA). For the imaging procedure, the dishes were placed in a chamber that provided a humidified atmosphere (6.5% CO₂, 9% O₂) at 37°C. It was staged on a Zeiss Axiovert 200M microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a digital camera (CoolSnap HQ; Roper Scientific, Tucson, AZ). DIC images were acquired every 60 s with a Zeiss ×40; A: 1.4 oil immersion lens for 8 h. The Metamorph 6.25 software (Universal Imaging, Downingtown, PA) was used to acquire and process the resulting stacks of images. To determine the number of added and retracted branches, processes > 5 μm were counted that persisted longer than 30 min. In this way, dendritic filopodia were not included in the analysis. Finally, all newly added and retracted branches were summed up. Shown are the average values of at least 10 randomly.
selected cells. After acquisition of the time lapse movies, in some experiments, neurons were fixed and stained for β-tubulin III and F-actin to secure that dendrites had been analyzed. For the analysis of dendrite elongation, DIC photographs were used that had been taken at 20-min intervals during a period of 8 h. The position of the growth cone center was traced with the help of Metamorph Software (VisiGer Systems, Puchheim, Germany). For each time point, the distance was calculated, which had been covered by the growth cone during the respective period.

Morphometry of Fixed Cells. After staining for β-tubulin III and F-actin, cells were imaged with a Zeiss Axiocvert 200 microscope equipped with a digital camera (Cool snap HQ; Roper Scientific). For data processing, Metamorph 6.5.2 software (Universal Imaging) was used. All images were thresholded. After subtraction of the cell body, the remaining dendrites were analyzed. The Integrated Morphometry Analysis tool was used to measure the length of dendrites or branches. Only structures positive for both β-tubulin III and F-actin were considered as dendrites or branches. Extensions were taken for filopodia if they contained only F-actin and were <10 μm. Dendrites were defined as structures that arose from the soma and were longer than 10 μm. Structures originating from dendrites were considered as branches. Only primary branches were counted. To quantify dendritic filopodia formation, we did not only count the filopodia per 10 μm but also measured the F-actin content in filopodia. For this purpose, Metamorph was used, and the F-actin content of all filopodia was related to that of the total dendrite. The relative tubulin content of dendrites was measured by relating the area that showed a β-tubulin III signal to the area positive for F-actin.

Statistics. Whenever applicable, analysis of variance was used followed by Scheffe’s test. Student’s t test was used when two groups had to be compared. The Kruskal-Wallis and Mann-Whitney U or Wilcoxon test were applied for processed values.

Results

After 1 day in culture, most hippocampal neurons had developed three to four neurites. After phalloidin staining for F-actin and β-tubulin III immunostaining for microtubules, neurites containing β-tubulin III immunoreactivity up to their tips were considered as axon-like. Neurites that contained β-tubulin III immunoreactivity predominantly in their proximal part and F-actin in their tips were counted as dendrites. A dendritic branch was defined as a β-tubulin III-positive process originating from a dendrite. In contrast, dendritic filopodia contained F-actin but not β-tubulin III immunoreactivity and were shorter than 10 μm.

GBP-L Enhances Filopodia Formation. When added to the neurons at DIV1 for 8 h, GBP-L (10 μM) increased the average number of dendritic filopodia from 0.55 ± 0.07/10 μm in controls to 0.92 ± 0.08/10 μm (n = 50; p < 0.0018; see also Fig. 1A). To further quantify filopodia formation, we calculated the dendritic F-actin contained in filopodia. In controls, 43% of the dendritic F-actin content was found in filopodia. GBP-L increased this value to 63% (Fig. 1B). Next, we determined whether the generated filopodia were able to make contact with neighboring neurons. To assist interactions between neurites of different neurons, we plated the neurons on coverslips that had been coated with 50% poly-lysine. In addition, GBP-L caused the formation of a neurite network (Fig. 1C). Contact points were formed by extensions that contained only F-actin and extensions that also contained β-tubulin III (Fig. 1C). GBP-L increased the number of contacts formed by extensions which contained only F-actin by a factor of 6.25 but did not change the number of contacts formed by β-tubulin III-containing extensions. Taken together, these data showed that GBP-L not only mediated the formation of filopodia but also of filopodial networks.

GBP-L Activates Rho Family GTPases in Hippocampal Neurons. The increase in filopodial activity caused by GBP-L suggested that it activated the GTPase Cdc42 (see Introduction). Surprisingly, GBP-L had no effect on the number of dendrites and branches per neuron, when applied for 8 h (Fig. 1, A and D). To find out whether GBP-L indeed activated the Rho GTPases, we used affinity precipitation assays to quantify the GTP-bound, active Rho proteins. For these experiments, neurons seeded at an approximate density of 500 cells/mm² were cultured for 3 days to increase the amount of neuritic tissue (see also Materials and Methods). When applied for 30 min, GBP-L raised the level of GTP-bound Cdc42 by 40% compared with controls (Fig. 2A). In addition, GBP-L increased the GTP-binding of Rac1 and RhoA by 31 and 54%, respectively (Fig. 2, B and C). Thus, GBP-L activated all GTPases of the Rho family. Although Cdc42 and Rac1 are known to support dendrite and branch formation, RhoA can attenuate this process by acting via its effector ROCK (see Introduction).

Combined Application of GBP-L and Y-27632 Generates Dendrites and Branches. Next, we examined whether GBP-L enhanced dendrite and branch formation in the presence of a pharmacological inhibitor of ROCK. In all experiments, Y-27632 was used at a concentration of 10 μM, which effectively blocks the kinase (Leemhuis et al., 2002). When applied for 8 h, Y-27632 did not increase filopodia activity (Fig. 1, A and B) or the numbers of dendrites and branches (Fig. 1, A and D). Compared with controls, the combination of Y-27632 and GBP-L increased the average number of dendrites by 38% and that of branches even 12-fold (Fig. 1, A and D). In contrast, Y-27632 did not change the effect of GBP-L on filopodia formation (Fig. 1, A and B). When we measured the GTP binding of the Rho GTPases, Y-27632 applied alone had no effect on the level of GTP-bound Cdc42 but significantly raised the level of GTP-bound Rac1 (Fig. 2, A and B). In combination with GBP-L, Y-27632 did not further enhance the levels of GTP-bound Cdc42 or GTP-bound Rac1 (Fig. 2, A and B). In the presence of Y-27632, we tested several concentrations of GBP-L on dendrite and branch formation. Although 1 μM GBP-L was ineffective, 100 μM GBP-L was more effective by 46% than 10 μM (data not shown). However, 100 μM GBP-L caused cytotoxicity as indicated by pearl string-like neurites. Therefore, 10 μM GBP-L was used for the subsequent experiments.
tion, at a concentration of 2 μM, Ro 318220 was without effect (data not shown).

As shown in Fig. 1, GBP-L increased filopodia formation in neurons cultured for 1 or 3 days. Next, we studied whether the effects of GBP-L plus Y-27632 on dendrite and branch formation were influenced by the cultivation time or the seeding density (Table 2A). In cultures seeded at low or high density, GBP-L plus Y-27632 increased the number of dendrites per neuron by approximately 30%, when used at DIV1 (Table 2A). At DIV3, however, the neurons had already spontaneously formed a larger number of dendrites so that GBP-L plus Y-27632 had no additional effect (Table 2A). In addition, the number of spontaneously formed branches increased from DIV1 to 3. Again, GBP-L plus Y-27632 had a smaller effect at DIV3 than at DIV1 (Table 2A).

Next, we studied whether the dendrites and branches produced by the combination of GBP-L and Y-27632 persisted after removal of both drugs. In one group of cultured neurons, we added the drug combination for 1 day and then replaced it with vehicle during the following day. In the second group,
both agents were present during both days. Compared with controls treated with vehicle for 2 days, both types of treatment with GBP-L plus Y-27632 enhanced the average number of dendrites and branches (Table 2B).

Since the neurotrophic effects of GBP-L can be prevented by 100 μM 5-hydroxydecanoate (5-HD) (Piel et al., 2004), we studied whether this agent also blocked the effects of GBP-L on dendrite formation (Table 1A). Used alone for 8 h, 5-HD (100 μM) had no effect on the average number of dendrites or branches. However, it prevented the dendrite and branch formation induced by GBP-L plus Y-27632 (Table 1A). Lower 5-HD concentrations of 1 and 10 nM proved to be ineffective (data not shown).

Role of Rho Proteins in the Induction of Dendrites and Branches by GBP-L. To find out whether Rac and Cdc42 were necessary for the GBP-L-induced dendrite formation, we transfected hippocampal neurons with EGFP fu-
sion proteins of dominant-negative (dn) Rho GTPases (Fig. 3A). These recombinant isoforms sequester the respective GEFs so that the endogenous GTPases become inactive (Diekmann et al., 1991; Ridley and Hall, 1992). To increase the transfection rates, neurons were used at DIV4 for the calcium phosphate/DNA coprecipitation procedure. In our hippocampal neurons, the transfection rate was 3 to 8%. Positive cells were mostly found in groups of several neurons. The neurites formed a dense network so that the analysis of single dendrites by β-tubulin III immunostaining was impossible. Therefore, we counted the EGFP-positive extensions, not all of which may have been dendrites.

EGFP control neurons showed an average number of 5.8 extensions (Fig. 3, A and B). The number increased to 7.5, when the neurons were incubated for 8 h with GBP-L plus Y-27632. In neurons transfected with dnRac1 or dnCdc42, however, GBP-L plus Y-27632 did not change the number of extensions (Fig. 3, A and B). Neurons transfected with dnRhoA had a significantly higher average number of extensions than EGFP controls, i.e., 8.1 (Fig. 3B). In such neurons, treatment with GBP-L alone for 8 h further increased the average number of extensions by 17%. According to these results, Rac1 and Cdc42 seemed to be necessary for the GBP-L-induced formation of extensions, whereas RhoA had an inhibitory effect.

To confirm the involvement of Rho proteins in dendrite formation, we inactivated the GTPases with clostridial toxins (Fig. 3, C and D). Toxin B of \textit{C. botulinum} inactivates RhoA, Rac1, and Cdc42 (Aktories and Barbieri, 2005). When added to the neuronal cultures at DIV1 for 8 h at a concentration of 10 ng/ml, it did not affect the number of dendrites and branches but prevented their increase induced by GBP-L plus Y-27632 (Fig. 3D). In contrast, C3 toxin of \textit{C. botulinum} selectively inactivates RhoA (Aktories and Barbieri, 2005). Its application for 8 h at a concentration of 100 ng/ml slightly enhanced the formation of dendrites and branches (Fig. 3, C and D). In the presence of C3 toxin, GBP-L as strongly increased the number of dendrites and branches as in the presence of Y-27632 (Fig. 3, C and D). When the two components of C3 toxin (see Materials and Methods) were used alone, they had no effects of their own and did not facilitate the effects of GBP-L (data not shown).

**Fig. 3.** Inactive Rac1 and Cdc42 abolish the effects of GBP-L (10 μM) plus Y-27632 (10 μM) on dendrite formation in hippocampal neurons. A, at DIV4, neurons were transfected with plasmids coding for EGFP alone or EGFP/dominant-negative Rac1, EGFP/dominant-negative Cdc42, and EGFP/dominant-negative RhoA. Changes in the number of EGFP-positive extensions were quantified 8 h after the addition of Y-27632 (Y) and/or GBP-L by using EGFP fluorescence microscopy; vertical projections of z-stacks of neurons. Scale bar, ~10 μm. B, quantification of the experiment: transfected neurons treated with vehicle (white columns) as well as after additional treatment with Y-27632 plus GBP-L (gray columns); neurons transfected with dominant-negative RhoA and treated with GBP-L (black column). EGFF-containing extensions > 10 μm were counted; mean ± S.E.M.; n = 25, †, significant difference (p < 0.05) from EGFP control. †, significant difference (p < 0.05) from dnrhoA. C, at DIV1, neurons were treated with C3 fusion toxin (C3FT; 100 ng/ml) from \textit{C. botulinum} or C3 toxin plus GBP-L. Cells were seeded at a density of approximately 500/mm². In one panel, the same neuron is shown after phalloidin staining for F-actin and β-tubulin III immunostaining as well as after merging; scale bar, 10 μm. D, at DIV1, neurons were treated with C3FT (100 ng/ml) or toxin B (ToxB; 10 ng/ml) from \textit{C. botulinum}. GBP-L was applied simultaneously. Cells were fixed 8 h later. Dendrites and branches are shown as white and black columns, respectively. † and ††, significant differences from controls (p < 0.05 and p < 0.001, respectively; n = 25). †††, significant differences from neurons treated with GBP-L alone (p < 0.001).
Gabapentin. In contrast to GBP-L, the anticonvulsant gabapentin (10 μM) did not change the levels of GTP-bound Cdc42, Rac1, or RhoA (Fig. 2, A–C). Gabapentin did not increase the filopodial content of dendritic F-actin [controls, 42.9 ± 2.3% (n = 46) versus 49.1 ± 2.1 (n = 37); p = 0.085], when applied for 8 h. Neither alone nor together with Y-27632 (10 μM), gabapentin (10 μM) changed the average number of dendrites (controls, 3.3 ± 0.5; gabapentin, 3.3 ± 0.3; gabapentin plus Y-27632, 3.0 ± 0.2; n = 60) or branches (controls, 0.65 ± 0.15; gabapentin, 0.85 ± 0.13; gabapentin plus Y-27632, 0.3 ± 0.1). Also at concentrations of 100 or 1000 μM, gabapentin did not increase dendrite and branch formation (data not shown).

GBP-L Enhances Branch Motility. To study the effects of GBP-L on the dynamics of branch formation, we used time lapse microscopy and determined the average number of branches added and eliminated during a period of 8 h. Extensions were counted as branches if they were longer than 5 μm and persisted for more than 30 min. In control neurons, approximately four branches were added, and three were eliminated, corresponding to a ratio of 1.23 (Fig. 4A). Compared with controls, Y-27632 did not affect branch addition and elimination. In contrast, GBP-L increased the number of added branches by 54% compared with controls. However, a similar number was eliminated, so that the ratio of both values was 1.2 (Fig. 4A). Thus, GBP-L pronouncedly enhanced dendrite motility but did not increase the number of persistent branches. When GBP-L and Y-27632 were combined, the number of added branches was increased by 67% compared with controls, but the number of eliminated branches remained similar to that of controls. This divergence increased the ratio of added to eliminated branches and raised the number of persistent branches.

PI3-Kinases Are Involved in the GBP-L-Induced Dendrite and Branch Formation. Since PI3-kinases can mediate the elimination of dendrites and branches (Leemhuis et al., 2004), we next studied the effect of GBP-L on PI3-kinase activity. We measured the phosphorylation of Akt at Ser473, which depends on PI3-kinase activity. Control neurons showed a low amount of phosphorylated Akt protein (P-Akt). Treatment of the cultures for 30 min with the PI3-kinase inhibitor LY294002 (20 μM) reduced the level of P-Akt (Fig. 4B), confirming that it was due to active PI3-kinase. GBP-L increased the amount of P-Akt, whereas the ROCK inhibitor Y-27632 had no effect. However, simultaneous ap-

![Fig. 4. GBP-L (10 μM) and Y-27632 (Y, 10 μM) change the dynamics of branch motility by acting via PI3-kinases. A, cultured neurons were treated with Y-27632 (Y), GBP-L, and LY294002 (LY, 20 μM) alone or in combination. Every minute, DIC photographs were taken with time lapse video microscopy. Newly formed and eliminated branches were counted every 30 min. Branches added (white box) or eliminated (gray box) during a period of 8 h were summed up. The difference between added and eliminated branches indicates number of persistent branches. The quotient of added/eliminated branches was also calculated, n = 10. B, Akt phosphorylation assay to determine PI3-kinase activity was performed with cultured hippocampal neurons at DIV3. Left, Western blot of AktSer473. Right, quantification of four Western blots. In all panels, mean ± S.E.M. are shown. * and †, significant differences from controls at the levels of p < 0.05, p < 0.01, and p < 0.001. † and ††, significant differences from GBP-L at the levels of p < 0.05 and p < 0.01. C, effect of LY294002 (LY, 20 μM) on GBP-L increase in GTP binding of Rac1 and Cdc42 as determined by PAK-CRIB pull-down assay; inputs are shown in lower lane. Right, effect of LY294002 (LY) on GBP-L increase in AktSer473 phosphorylation.
plication of Y-27632 prevented the increase in P-Akt induced by GBP-L (Fig. 4B).

To study whether the increase in PI3-kinase activity was responsible for the elimination of the GBP-L-induced branches, we used a PI3-kinase inhibitor (Fig. 4A). Since the experiments lasted for 8 h, we did not use the light-sensitive wortmannin but LY294002. Compared with controls, treatment of the neurons with 20 μM LY294002 reduced the average number of added and eliminated branches by 50 and 66%, respectively. The rise in the ratio of added to eliminated branches from 1.23 in controls to 1.8 after treatment with LY294002 explained the resulting average net formation of 0.7 branches (Fig. 4A). Y-27632 prevented the LY294002-induced decrease in branch addition but not that in elimination. This divergence was reflected in a ratio of 3.1 and resulted in the average net formation of 2.7 branches. When LY294002 was combined with GBP-L, approximately five branches were added. This value was slightly lower than that observed with GBP-L alone. In this combination, LY294002 reduced the average number of eliminated branches to 2.1. The resulting ratio of 2.83 was again higher than that of controls or GBP-L alone so that more persistent branches were formed (Fig. 4A). The additional application of Y-27632 only slightly enhanced branch addition but did not change branch elimination. In independent experiments, we confirmed that LY294002 applied together with GBP-L for 8 h increased the number of dendrites and branches (data not shown). To find out whether the PI3-kinase inhibitor affected dendrite and branch formation by changing the activities of the Rho GTPases, we studied its effect on GTP binding of Rac1 and Cdc42. LY294002 prevented the increase in GTP-bound Rac1 caused by GBP-L but did not change the respective effect on Cdc42 (Fig. 4C). Our additional finding that LY294002 inhibited the increase in AKT phosphorylation caused by GBP-L was in agreement with the observed morphological effects (Fig. 4C). Taken together, these data suggested that PI3-kinases played a role in both branch addition and elimination.

**GBP-L Does Not Affect Dendrite Elongation.**

The dendritic tree does not only grow by adding new dendrites and branches but also by elongating existing extensions. Next, we studied whether GBP-L changed the dynamics of dendrite elongation. For this purpose, we measured with time lapse microscopy the total outgrowth and the total retraction distance over an 8-h period. Subtraction of both values corresponded to the actual elongation (Fig. 5, A and B). Total average dendrite outgrowth amounted to 15 μm in control neurons. Since 13 μm was again retracted, only 2 μm persisted (Fig. 5, A and B). During treatment with Y-27632, average outgrowth was 22 μm, of which only 6 μm was again retracted, so that net elongation amounted to 16 μm. In contrast, GBP-L neither affected outgrowth nor retraction compared with controls. When Y-27632 and GBP-L were used together, the average outgrowth and retraction distances did not differ from those induced by Y-27632 alone (Fig. 5, A and B). According to these results, GBP-L had no effect on dendrite elongation.

The proximal part of dendrites contains microtubules and is more stable than the distal part that contains only F-actin. In view of the effect of Y-27632 on elongation, we next examined whether Y-27632 and GBP-L changed the relative content of microtubules in the dendrites. For this purpose, we related the microtubules containing part of the dendrite to its total length. Although GBP-L caused a minor decrease in this ratio, Y-27632 significantly enhanced it (Fig. 5C). This effect was not changed by GBP-L after combined treatment. These data confirmed that GBP-L mainly affected the actin cytoskeleton.

**Discussion**

In cultured hippocampal neurons, GBP-L enhanced the formation of dendritic filopodia and dendritic networks. It also increased the addition and elimination of new branches, i.e., dendritic motility. GBP-L activated the Rho GTPases Rac, Cdc42, and RhoA as well as PI3-kinases. In neurons
as well as PRK2. In contrast to Y-27632 or HI-1152, dnRhoA induced dendrite and branch formation by inhibiting ROCK, excluding that Y-27632 and HI-1152 facilitated the GBP-L-induced stress fibers (Vincent and Settleman, 1997). Thus, we cannot exclude that GBP-L also affected the process of neurite attachment.

Although GBP-L activated Rac and Cdc42 and induced the formation of filopodia, it did not generate more dendrites and branches. Our data indicate that the simultaneous activation of RhoA blocked the GBP-L-induced dendritic changes. Thus, GBP-L was able to generate extensions in neurons transfected with dnRhoA. In neurons treated with C3 toxin from *C. botulinum*, which selectively inactivates RhoA (Aktories and Barbieri, 2005), GBP-L induced dendrites and branches. To study the involvement of the RhoA effector ROCK, we used Y-27632 and HI-1152. Both agents are widely used as ROCK inhibitors but also inactivate the serine/threonine kinases PRK2, MSK1, and MAPKAP-K1b (Davies et al., 2000). The MSK1 and MAPKAP-K1b inhibitor Ro 318220 did not facilitate the GBP-L-induced formation of dendrites and branches, suggesting that the kinases were not involved. Like ROCK, PRK2 is a RhoA effector that may organize stress fibers (Vincent and Settleman, 1997). Thus, we cannot exclude that Y-27632 and HI-1152 facilitated the GBP-L-induced dendrite and branch formation by inhibiting ROCK as well as PRK2. In contrast to Y-27632 or HI-1152, dnRhoA and C3 toxin slightly enhanced the number of neuronal extensions, suggesting that additional RhoA effectors may be involved. The essential roles of Rac and Cdc42 in neurite formation were confirmed in experiments with neurons transfected with dnRac or dnCdc42 as well as after treatment with toxin B, which inactivates all GTPases of the Rho family (Aktories and Barbieri, 2005). Under all these conditions, the combination of GBP-L plus Y-27632 no longer increased dendrite formation.

Time lapse microscopy shows the dynamics of branch formation. In retinal neurons, Rac and RhoA enhance and reduce, respectively, addition and elimination of new extensions (Wong et al., 2000). In rat retinal neurons as well as tectal neurons from *Xenopus laevis*, RhoA inhibits the outgrowth of new dendrites and branches induced by active Rac and/or Cdc42 but has no effect on retraction or elimination of newly added branches (Li et al., 2000; Wong et al., 2000; Leemhuis et al., 2004). In our cultured hippocampal neurons, RhoA was involved in branch addition. However, this action became only apparent when branch addition was diminished by PI3-kinase inhibitors. Under these conditions, Y-27632 prevented the decrease (see also Leemhuis et al., 2004). GBP-L modulated this complex regulation. It activated the three Rho GTPases as well as PI3-kinases, thereby enhancing branch addition as well as elimination (Fig. 6). We will first discuss the data concerning branch addition.

GBP-L enhanced the addition of branches, although it increased the activity of RhoA. Moreover, Y-27632 did not further increase the branch addition caused by GBP-L. GBP-L even increased branch addition in the presence of the PI3-kinase inhibitor LY-294002, although to a slightly lower extent, which was prevented by Y-27632. Taken together, these results showed that RhoA had only a negligible inhibitory effect on the branch addition caused by GBP-L.

In contrast, Rac and Cdc42 were essential for branch formation as shown by the effects of dnRac, dnCdc42, and toxin B (Fig. 6). It is difficult to evaluate which of the two GTPases was more important for branch addition. Y-27632 increased the level of GTP-bound Rac over that of controls but had no effect on GTP-bound Cdc42, confirming previous data (Yamaguchi et al., 2001; Tsuji et al., 2002; Leemhuis et al., 2004). However, Y-27632 did not enhance branch addition, suggesting that Rac alone was not sufficient for this action. In contrast, the selective activation of Cdc42 by forskolin can increase branch addition (Leemhuis et al., 2004).

Class I PI3-kinases contribute to branch addition and elimination (Leemhuis et al., 2004). Indeed, GBP-L increased PI3-kinase activity and induced the elimination of newly added branches. Y-27632 did not reduce the basal activity of PI3-kinases but inhibited the stimulatory effect of GBP-L. Apparently, RhoA acted synergistically with another mechanism induced by GBP-L (Fig. 6). We suggest that Y-27632 reduced branch elimination by preventing the GBP-L-induced increase in PI3-kinase activity. The direct inhibition of PI3-kinase activity with LY294002 always strongly reduced branch elimination. LY294002 also allowed GBP-L to form numerous new branches (Fig. 6). LY294002 did not only increase the formation of branches (Fig. 4) but also of dendrites (data not shown). We assume that similar effects were involved.

The GBP-L-induced increase in PI3-kinase activity is not only of interest in the context of dendrite arborization. Since

---

**Fig. 6.** Model summarizing the effects of GBP-L on the dynamics of branch formation in cultured embryonic hippocampal neurons. Arrows, stimulation or facilitation. The balance between addition and elimination determines the formation of dendrites and branches. GBP-L induces addition by activating Rac and Cdc42 but simultaneously enhances elimination by activating PI3-kinases.
class I PI3-kinases facilitate the survival of various cells (Rameh and Cantley, 1999; Vanhaesebroeck et al., 2001), the GBP-L-induced increase in kinase activity may explain its neuroprotective effects (Jehle et al., 2001; Pielen et al., 2004).

Although Y-27632 had no effect on dendrite and branch formation, it caused the elongation of dendrites. This finding confirmed previous observations (Wong et al., 2000; Sin et al., 2002) and pointed out that different mechanisms regulate the formation of new dendrites and their elongation. For elongation, active Rac and ROCK inhibition seem to be essential, whereas Cdc42 is mainly involved in the increased formation of dendrites and branches. The finding that GBP-L not only activated Rac and but also ROCK explains why the agent did not cause elongation. Y-27632 facilitated the extension of microtubules into the dendrites, suggesting that these structures were involved in elongation. Thus, ROCK and possibly PRK2 seem to restrain the outgrowth of microtubules during dendrite formation. Since RhoA can affect microtubule organization in polarized cells via its effector mDia (Ishizaki et al., 2001; Palazzo et al., 2001), this finding indicates that RhoA may regulate microtubules via different effectors.

5-HD blocks the neuroprotective effects of GBP-L (Pielen et al., 2004). In our neurons, it prevented the effects of GBP-L on dendrite and branch formation. 5-HD affects the formation of reactive oxygen species as well as blocks mitochondrial KATP channels (Szewczyk et al., 1993; Dzeja et al., 2001; Scholz et al., 2001). This result indicates that RhoA may regulate microtubules via different effectors.

References


Scholz et al., 1996; Sarner et al., 2000). Cdc42 and RhoA share GEFs such as Dbl and Dbs (Schmidt and Hall, 2002). The dominant-negative isoforms of the Rho proteins used in our study scavenge GEFs. However, our finding that dNrhoA produced numerous neurites indicates that Cdc42 was still active. We consider this as indirect evidence that GBP-L-regulated RhoA and Cdc42 act independently. The GEFs involved have to be elucidated in future studies.

GBP-L has pronounced neuroprotective effects (Jehle et al., 2001; Pielen et al., 2004). Now, we report that it also has neurotrophic effects. In contrast, the antiangiogenic agent gabapentin did not generate filopodia as well as dendrites or branches. Gabapentin also did not change the activities of Rho GTPases or PI3-kinases. Only GBP-L induced dendritic filopodia. Since filopodia are an important early step in synapse formation (Ziv and Smith, 1996), GBP-L may facilitate synaptic interactions. GBP-L also caused the formation of neurite networks. In addition, GBP-L enhanced the outgrowth of new dendrites and branches but also their retraction. This increased motility was turned into net formation of dendrites and branches when GBP-L was combined with inhibitors of RhoA or PI3-kinases. The observed effects of GBP-L on dendritic arborization are of potential therapeutic interest, which will be further investigated. In addition, the combined effects of GBP-L on the GTPases of the Rho family and PI3-kinases make it an interesting tool for further investigations on the interactions of these proteins.


**Address correspondence to:** Dr. Dieter K. Meyer, Zentrum für Neurowissenschaften, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität, Albert-Strasse 25, D-79104 Freiburg, Germany. E-mail: dieter.meyer@pharmakol.uni-freiburg.de