Pituitary adenyl cyclase-activating polypeptide 38 (PACAP38) plays an important role in the proliferation and differentiation of neural cells. In the present study, we have investigated how PACAP38 inhibits the proliferation of cultured neocortical astroglial cells. When applied to synchronized cells during the G1 phase of the cell cycle, PACAP38 diminished the subsequent nuclear uptake of bromodeoxyuridine. When applied for 2 days, it reduced the cell number. PACAP38 did not exert its antiproliferative effect by activating protein kinase A. It also did not reduce the activity of mitogen-activated protein kinases essential for G1 phase progression. Instead, PACAP38 acted on a member of the Rho family of small GTPases. It reduced the activity of RhoA as was shown with a Rhotekin pull-down assay. The decrease in endogenous RhoA activity induced by treatment of the cells with C3 exotoxin or by expression of dominant negative RhoA also reduced the nuclear uptake of bromodeoxyuridine. In contrast, expression of constitutively active RhoA prevented the effect of PACAP38. Our data show a novel signal transduction pathway by which the neuropeptide influences cell proliferation.

Experimental Procedures

Received for publication, February 11, 2005, and in revised form, April 1, 2005
Published, JBC Papers in Press, May 3, 2005, DOI 10.1074/jbc.M501630200

Dieter K. Meyer‡, Catharina Fischer, Ulrike Becker, Isabel Göttsching, Stephanie Boutillier,
Christian Baermann, Gundula Schmidt, Norbert Klugbauer, and Jost Leemhuis

From the Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Zentrum für Neurowissenschaften,
Albert-Ludwigs-Universität, D-79104 Freiburg, Germany

Pituitary adenyl cyclase-activating polypeptide 38 (PACAP38) and vasoactive intestinal polypeptide (VIP) are neuropeptides of the secretin-glucagon family (1). Whereas both peptides stimulate the G protein-coupled receptors VPAC1 and VPAC2 at subnanomolar concentrations, only PACAP38 stimulates PAC1 receptors with a potency in the same range (2, 3). During the embryonic and postnatal period, the neuropeptides and the receptors are expressed in cells of the rodent neocortex and its growth zones. There is strong evidence for the involvement of these peptides in neural cell proliferation, phenotypic determination, differentiation, and survival (4–12).

Activation of PAC1 receptors by PACAP38 can change the proliferation of neural cells. However, inhibitory as well as stimulatory effects mediated by protein kinase A (PKA) have been observed in cultured neocortical or cerebellar neurons and spinal cord glial cells (3, 4, 12–16). These contradictory effects may be related to the diversity in receptor isoforms and their signal transduction pathways. The 7 isoforms reported differ in their third intracellular loop and can couple to adenyl cyclase as well as phospholipases C and D (12, 17).

In the cellular proliferation cycle, cyclins and cyclin-dependent kinases (CDKs) determine the progression through the G1 phase into the S phase. Thus, the association of cyclin D with CDK4/6 and of cyclin E with CDK2 is important for G1 progression and G1/S transition, respectively. The complexes induce the phosphorylation of the retinoblastoma gene product that allows the transcription of E2F-regulated genes (18). Upon activation, extracellular signal-related kinases 1 and 2 (ERK1 and ERK2) increase the activity of cyclin D1. However, the sustained activation of ERKs and cyclin D1 leads to G1 progression (19, 20). In primary cells, such a sustained activation is only observed if growth factors are applied to cells attached to the matrix (21–25). The small GTPases of the Rho family, Rac, and Cdc42 organize the actin cytoskeleton and thereby cell attachment (26–28). RhoA has been shown to be necessary for sustained activation of ERKs and cyclin D1 (29). In addition, RhoA supports G1/S transition by preventing the action of the CDK inhibitors p21Cip1 and p27Kip1, which inactivate the cyclin E-CDK2 complex (30–35).

In preliminary experiments, we have observed that PACAP38 strongly inhibited the S phase-dependent nuclear uptake of bromodeoxyuridine (BrdUrd) in cultured astroglial cells from the rat neocortex. This apparent antiproliferative effect was in contrast to our previous observation of a PACAP38-induced activation of ERKs (36). In the present study, we have examined the antiproliferative effect of PACAP38 in cultured astroglial cells. The antiproliferative effect of PACAP38 was shown to be because of inactivation of the small GTPase RhoA that was essential for the proliferation of cultured astroglial cells.
Biosciences) and incubated with the FCS-containing Dulbecco’s modified essential medium. After 4 days, the culture medium was renewed. Experiments were performed 6 to 7 days after seeding, when the cells were pre-confluent. At this time, more than 96% were astroglial cells of type I, as indicated by the presence of glial fibrillary acidic protein and the absence of A2B5 antigen (37).

**Nuclear Uptake of BrdUrd**—The nucleotide BrdUrd is taken up during the S phase of the cell cycle. Unless indicated otherwise, experiments were performed in astroglial cells synchronized by FCS withdrawal from the incubation medium for 24 h. To start the G1 phase progression, FCS (final concentration 10%) was added for 16 h. PACAP38 and other agents to be tested were present during this period. BrdUrd (10 μM; Roche Diagnostics GmbH, Mannheim, Germany) was added to the cultures for the last 60 min of the incubation. Cells were fixed with methanol (−20 °C), washed with phosphate-buffered saline for 5 min, and then incubated with 2 N HCl (10 min, 37 °C). After the medium had been neutralized with borate buffer (0.1x, pH 8.5), the cells were washed and incubated for 3 h with a mouse anti-BrdUrd antibody (Roche Diagnostics). The immune complex was detected with a Cy3-labeled secondary goat anti-mouse antibody (Dianova, Hamburg, Germany).

To estimate the total cell number, fixed cells were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics) for 5 min. Apoptotic cells were stained with propidium iodide (Sigma, Taufkirchen, Germany) added to the incubation medium at a concentration of 5 μg/ml for 20 min prior to fixation.

**MAP Kinase Assays**—ERKs were determined in their activity with a non-radioactive procedure (New England BioLabs, Schwalbach, Germany). After incubation with FCS for 15 h, the astroglial cells were lysed in buffer. After sonication and centrifugation protein concentrations were determined. Aliquots containing 300 μg of protein were used to immunoprecipitate active ERKs with an immobilized monoclonal anti-phospho-ERK antibody (overnight at 4 °C). To estimate the activity of the precipitated ERKs, the washed precipitate was incubated for 30 min with an Elk-1 fusion protein at 30 °C in the presence of ATP. Reactions were stopped by addition of 3x Laemmli buffer. After electrophoresis in 15% SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. Unspecific binding was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T).

**Western Blotting**—Protein samples (15 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% Skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS), the membranes were incubated overnight with a specific primary antibody. Detection was performed by Western blotting using anti-RhoA antibodies (C-19, Santa Cruz, CA).

**Expression of GST-C21 Protein**—The fusion protein GST-C21 contains the N-terminal 90 amino acids of Rhotekin (42). It was a gift of Prof. Collard (Amsterdam, The Netherlands). The expression plasmid was expressed in insect cells in the presence of nystatin (Sigma, Griesheim, Germany) at 37 °C. Expression was induced by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) at A600 1.0. One hour after induction, the cells were collected and lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 2.0 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 10,000 x g, and the supernatant was used for purification of the GST-C21 protein by affinity purification using glutathione-Sepharose beads (Amersham Biosciences). Loaded beads were washed twice with GST-fishing buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 1% Nonidet P-40, 10% glycerol) at 4 °C.

**Growth Curves**—To estimate the total cell number, fixed cells were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics) for 5 min. Apoptotic cells were stained with propidium iodide (Sigma, Taufkirchen, Germany) added to the incubation medium at a concentration of 5 μg/ml for 20 min prior to fixation.

**Western Blotting**—Protein samples (15 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% Skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), the membranes were incubated overnight with a specific primary antibody. Detection was performed by Western blotting using anti-RhoA antibodies (C-19, Santa Cruz, CA).

**RESULTS**

**PACAP38 Reduces the Nuclear Uptake of BrdUrd in Neocortical Astroglial Cells**—In the presence of FCS (10%) in the incubation medium, astroglial cells passed through the G1 phase and entered the S phase, as indicated by nuclear BrdUrd immunostaining. When either PACAP38 or VIP was present in the incubation medium during the 16 h incubation, the peptides decreased the nuclear uptake of BrdUrd in a concentration-dependent manner, with EC₅₀ values of ~0.2 and 50 nM, respectively (Fig. 1A). The lower potency of VIP indicated...
that it did not act via VPAC1 and VPAC2 receptors. To test whether PAC1 receptors were involved, we used the PAC1 receptor-specific antagonist PACAP-(6–38), which blocks adenylyl cyclase activation by PACAP38 at a concentration of 1.5 nM (44). When used at a concentration of 100 nM, PACAP-(6–38) had no effect of its own on nuclear BrdUrd uptake. Despite the high concentration, it did not block the inhibitory effect of 0.5 nM PACAP38, indicating that PAC1 receptors coupled to adenylyl cyclase were not involved (Table I).

To test the long-term effects of PACAP38, we added the neuropeptide to free-cycling cells for 12, 24, or 48 h. Compared with controls, PACAP38 (10 nM) reduced the subsequent nuclear immunostaining of BrdUrd by 72% independent of the duration of its presence (Fig. 1B). Because PACAP38 was active over 2 days, we tested whether it reduced the total cell number. When applied for 48 h, PACAP38 reduced the total cell number by 18.5% (Fig. 1C). To exclude that this decrease was because of PACAP38-induced cell death, we used propidium iodide (5 μM; added for 20 min) to stain dead cells. Treatment with 10 nM PACAP38 for 16 h did not significantly increase the number of propidium iodide positive cells (controls 1.7 ± 0.33%; PACAP38 2.8 ± 0.52% (p > 0.05; n = 15). We concluded that PACAP38 indeed exerted an antiproliferative effect.

PACAP38 Inhibits Growth Factor-induced BrdUrd Uptake—The EGF and bFGF enhance proliferation in the absence of fetal serum constituents. So does tetradecanoylphorbol acetate (TPA), which activates protein kinase C. We examined whether PACAP38 changed the effects of EGF (5 nM), bFGF (100 nM), or TPA (100 nM) on the nuclear uptake of BrdUrd. Glial cells were

FIG. 1. PACAP38 and VIP reduce the nuclear uptake of BrdUrd and exert an antiproliferative effect. Panel A, PACAP38 (●) and VIP (○) act in a concentration-dependent manner. In free-cycling astroglial cells, PACAP38 and VIP were added to the incubation medium for 16 h. BrdUrd was present during the last hour of incubation. Total cell number was determined by DAPI staining. In the control group, 12.4% of the cells showed nuclear uptake of BrdUrd. In cells treated with 10 nM PACAP38, 5.2% of the cells still showed nuclear BrdUrd uptake. The range between both values was taken as 100% to calculate the concentration-response curves (n ≥ 24). Panel B, PACAP38 (10 nM) inhibits the nuclear uptake of BrdUrd when added to the incubation medium for 12, 24, or 48 h. Free-cycling cells were used. In all experiments, BrdUrd was added to the incubation medium during the last hour of the treatment with PACAP38. BrdUrd positive cells were counted in an area of 0.1 mm². The mean value of controls was set at 100%. Panel C, treatment of neocortical astroglial cells with PACAP38 (10 nM) for 48 h reduced the total cell number as determined by DAPI staining. Positive cells were counted in an area of 0.1 mm². The mean value of controls was set at 100%. In B and C, mean ± S.E. are shown; n ≥ 30 in the respective experiments. a, indicates significant difference (p < 0.05) compared with controls. Panel D, PACAP38 (10 nM) reduces the increase in BrdUrd uptake induced by TPA (100 nM), EGF (5 nM), or bFGF (100 nM). Cells were incubated in serum-free medium for 24 h while drugs were present. BrdUrd was present during the last 60 min of the incubation period. The mean value of BrdUrd positive cells in the control group was taken for 100%. Mean ± S.E. are shown. a, indicates significant difference (p < 0.05) compared with controls; b, indicates significant difference to respective mutagen; n ≥ 24.
PACAP38, RhoA, and Astroglial Cell Cycle

PACAP-(6–38) does not block the effect of PACAP38 on nuclear uptake of BrdUrd

After FCS removal for 24 h, astroglial cells were incubated in FCS-containing medium for 16 h. During this time, PACAP38 and/or PACAP-(6–38) were present at the concentrations shown. BrdUrd was added for the last hour of the incubation period. Total cell number was determined by nuclear staining with DAPI. In controls, BrdUrd was found in 8.7 ± 0.7% of the cells. This value was taken as 100%. All other values are expressed as a percentage of this mean value. Mean ± S.E. are shown (n ≥ 24).

| Treatment          | BrdUrd positive cells % of controls |
|--------------------|-------------------------------------|
| Controls           | 100 ± 8                             |
| PACAP38, 0.5 nM    | 55 ± 9*a                            |
| PACAP-(6–38), 100 nM | 88 ± 7                             |
| PACAP38, 0.5 nM, + PACAP-(6–38), 100 nM | 63 ± 12a                           |

*a indicates significant difference (p < 0.05) compared with controls.

incubated in FCS-free incubation medium for 24 h in the absence or presence of the growth factors. Compared with controls, TPA, EGF, and bFGF enhanced the number of BrdUrd positive cells by 106, 62, and 65%, respectively. PACAP38 (10 nM) reduced the effects of the 3 different stimuli by 64% (Fig. 1D), suggesting that it did not act on the receptors of the growth factors but on a signal transduction pathway shared by the 3 stimuli.

**PACAP38 Can Reduce Proliferation Independent of PKA**—In neuroblasts, PACAP38 reduces proliferation by activating adenylyl cyclase and thus PKA (4). To study whether PACAP38 decreased astroglial proliferation via this pathway, we used the PKA inhibitor protein PKI (45), which was expressed in glial cells using an adenoviral mediated expression system. After infection with either Ad.EGFP or Ad.PKI/EGFP, 91.5 ± 1.3 and 93.6 ± 2%, respectively, of the glial cells showed green fluorescence. Compared with untreated cells, expression of EGFP alone did not change nuclear BrdUrd uptake in synchronized FCS-stimulated astroglial cells (Fig. 2A). Also the inhibitory effect of PACAP38 (10 nM) was not changed by EGFP expression (Fig. 2A). Compared with cells expressing only EGFP, those expressing PKI/EGFP showed an unchanged nuclear uptake of BrdUrd, indicating that active PKA was not necessary for the FCS-induced proliferation (Fig. 2A). Expression of PKI/EGFP did not prevent but only slightly reduced (by 13.3%; p = 0.086) the inhibitory effect of PACAP38 on nuclear BrdUrd uptake (Fig. 2A). To confirm that PKI expression blocked PKA activity, we studied the effect of the adenyl cyclase activator forskolin on proliferation. In EGFP expressing cells, forskolin (10 μM) reduced the nuclear uptake of BrdUrd by 76.8%. This effect was indeed no longer present in cells expressing PKI (Fig. 2B). These findings suggested that PACAP38 reduced gial proliferation mainly by an action independent of PKA.

To test this hypothesis, we simultaneously applied FSK and PACAP38. The strong reduction in FCS-induced BrdUrd uptake caused by forskolin was indeed further enhanced by additional application of PACAP38 (Fig. 2C). Finally, we used the pharmacological PKA inhibitor H89. It neither changed FCS-stimulated nuclear BrdUrd uptake nor its reduction caused by PACAP38 (Fig. 2D). Taken together, these data indicated that PACAP38 exerted an antiproliferative effect, which was independent of PKA, although active PKA was able to reduce gial proliferation.

PACAP38 Does Not Reduce Proliferation by an Action on ERKs—Kinases of the MAP kinase family, i.e. ERK1 and ERK2, are essential for G1 progression and entry of the S phase. First, we confirmed the role of the ERKs in astroglial proliferation. We used U0126 to inhibit the dual-specificity kinases MEK1/2, which prevents the activation of the ERKs (46). U0126 (10 μM) indeed reduced the FCS-stimulated nuclear BrdUrd uptake of synchronized cells by 83.5% (Fig. 3A). When used alone, PACAP38 (10 nM) reduced the BrdUrd uptake by 66.2%. Together with U0126, however, PACAP38 reduced the nuclear BrdUrd uptake by 93.6%. This significant additional effect suggested that U0126 and PACAP38 acted via different mechanisms. Next, we examined whether PACAP38 (10 nM) reduced the activity of the ERKs using an immunoprecipitation assay. We added PACAP38 (10 nM) for up to 4 h to the FCS-containing incubation medium of astroglial cells. It slightly reduced ERK activity only after 1 h, but had no effect at later times (Fig. 3B). In a third approach, we investigated the activity of ETS2 repressor factor (ERF), which is phosphorylated by ERK2 (41, 47–49). When localized in the nucleus, ERF can block access of the transcription factor ETS2 to its binding site. Upon phosphorylation at multiple sites by ERK2 within the nucleus, ERF translocates to the cytosol and thus allows the transcription necessary for the initiation of the proliferative cycle (41, 47, 49). Astroglial cells were transiently transfected with a plasmid coding for an EGFP/ERF fusion protein. Two days later, EGFP immunofluorescence showed the cellular localization of the transcription factor. In cells incubated with FCS-containing medium, ERF was mainly located in the cytosol. Only a few cells showed a nuclear localization of ERF (Fig. 3C). One hour after serum deprivation, we found ERF mainly in the cell nucleus (Fig. 3C), confirming previous findings (41). When astroglial cells in FCS-containing medium were treated for 1 h with PACAP38 (10 nM), most cells still showed a cytosolic localization of ERF (Fig. 3C). Apparently, PACAP38 did not decrease the activity of ERK2 so that ERF re-entered the nucleus. Taken together, our results provided no evidence that PACAP38 reduced ERK activity.

The Small GTPase RhoA Is Involved in the Anti proliferative Effect of PACAP38—The small GTPase RhoA supports the G1 phase progression via 2 mechanisms. It can contribute to the sustained increase in ERKs activity necessary for cyclin D/CDK4/6 activation as well as prevent the inhibitory effects of CKIs p21Cip1 and p27Kip1 on the cyclin E-CDK2 complex (29, 31, 33, 35). Because morphological observations indicated that PACAP38 may affect RhoA activity, we examined the role of RhoA.

In FCS-containing medium, proliferating astroglial cells are widely spread and show numerous stress fibers, which depend on RhoA activity. PACAP38 had a pronounced effect on the morphology of most glial cells. Within 1 h, the polygonal cells started to lose their stress fibers. After 4 h the stress fibers were completely destroyed (Fig. 4), suggesting that RhoA was inactive. C3 toxin of Clostridium limosum, which ADP-ribosylates and inactivates RhoA but not Rac1 or Cdc42 (for review, see Aktories et al. (50)) also destroyed the stress fibers in most cells (Fig. 4), when used together with a carrier system that facilitates permeation of the cell membrane (51). In addition, C3 toxin (200 ng/ml) diminished nuclear BrdUrd uptake by 63% (Table II). In contrast to treatments with forskolin or U0126, PACAP38 had no additional effect, when applied together with C3 toxin (Table II). This finding suggested that PACAP38 induced proliferation by an action on RhoA.

To study whether RhoA supported nuclear BrdUrd uptake by organizing the actin cytoskeleton, we destroyed the latter with cytochalasin D, i.e. without disturbing Rho activity (1 μM; Fig. 4). Cytochalasin D decreased by 84.1% the nuclear uptake of BrdUrd (Table II). Simultaneous application of PACAP38, however, further decreased BrdUrd uptake. This finding suggested that PACAP38 did not reduce proliferation by destroying the actin cytoskeleton.
To further study whether active RhoA was necessary for astroglial BrdUrd uptake, we expressed dominant negative RhoA in glial cells using an adenovirus vector. dnRhoA scavenges the guanine-nucleotide exchange factors (GEFs) necessary for the activation of endogenous RhoA (26). In cells infected with Ad.EGFP or Ad.dnRhoA, 91.7 ± 0.4% and 94.7 ± 0.5%, respectively, of the astroglial cells were infected as visualized by the co-expressed EGFP protein. In such cells, stress fibers were no longer observed, indicating that endogenous Rho was indeed inactive (Fig. 5 A). dnRhoA reduced the nuclear uptake of BrdUrd by 51.5%, i.e. to an extent similar to the effect of PACAP38 alone. When the neuropeptide was added to such cells, it had no additional effect (Fig. 5 D). Taken together, these results indicated that inactivation of RhoA and PACAP38 had similar and non-additive effects on BrdUrd uptake.

PACAP38 Reduces BrdUrd Uptake by Acting on RhoA—To examine whether PACAP38 reduced the activity of RhoA, we used an affinity precipitation assay. The C21 domain of the RhoA effector Rhotekin was used to precipitate the active GTPase, because it interacts only with the GTP bound, active form of RhoA. Compared with controls, treatment with PACAP38 (10 nM) strongly reduced the amount of RhoA bound to Rhotekin beads (Fig. 5 B), whereas expression of dnRhoA abolished the binding of RhoA to Rhotekin (Fig. 5 C). To find out whether enhanced activity of RhoA prevented the reduction in nuclear BrdUrd uptake caused by PACAP38, we...
infected glial cells with caRhoA. Like V12Ras V14RhoA no longer has endogenous GTPase activity (52, 53). Once it binds GTP after its synthesis, it remains active. Of the cells infected with Ad.EGFP or Ad.caRhoA, 91.5 ± 2.1 and 94.2 ± 0.2%, respectively, expressed EGFP. Determination of GTP binding by caRhoA with the Rhotekin affinity precipitation assay indeed showed that PACAP38 did not reduce GTP binding (Fig. 6A). Moreover, in cells expressing caRhoA PACAP38 was no longer able to reduce nuclear uptake of BrdUrd (Fig. 6D). Also stress fibers produced by caRhoA were not destroyed by PACAP38 (data not shown).

PACAP38 Reduces RhoA Activity in Astroglial Cells Treated with CNFy— CNFy is a cytotoxic necrotizing factor from Y. pseudotuberculosis. It deamidates glutamine 63 of RhoA and thus inhibits the GTP hydrolyzing activity of the latter (54). When used alone, CNFy strongly enhanced GTP binding of RhoA as measured by the Rhotekin affinity precipitation assay (Fig. 6C). However, PACAP38 applied simultaneously was still able to diminish GTP binding, suggesting that it decreased the activation of RhoA by a GEF. The PKA inhibitor H89 did not prevent this inhibitory effect indicating that PKA was not involved in this action (Fig. 6C). The persistent inhibitory effect of PACAP38 on RhoA activity was also observed in a morphological study. PACAP38 destroyed stress fibers in cells treated with CNFy (Fig. 6D). Because CNFy alone reduced nuclear BrdUrd in FCS-stimulated synchronized cells, the effect of PACAP38 could not be evaluated under these conditions (data not shown).

**DISCUSSION**

There is increasing evidence that the neuropeptide PACAP38 acts as a growth factor that regulates the proliferation and differentiation of neuronal and glial cells in the neocortex and other brain areas (see Introduction). In the present study, PACAP38 reduced the number of cultured neocortical astroglial cells that incorporated BrdUrd during the S phase of the proliferative cycle. This effect was independent of the cAMP/PKA signaling pathway. Also an action on the ERKs of the MAP kinase family could not be shown. Instead, we obtained evidence that PACAP38 inhibited the small GTPase RhoA that supports progression through the G1 phase into the S phase.

Whereas PACAP38 reduced nuclear BrdUrd uptake at sub-nanomolar concentrations, VIP was 250 times less potent, suggesting that PAC1 but not VPAC1 or VPAC2 receptors were involved, because the latter are stimulated by similar, low nanomolar concentrations of PACAP38 and VIP (2, 3). PAC1 receptors are indeed expressed at low density by neocortical astroglial cells (11, 55–57). PAC1 receptor mRNA was also found in our cells with use of reverse transcriptase-PCR (data not shown). However, our further results did not confirm an exclusive role of PAC1 receptors. Thus, PAC1 receptors coupled to adenylyl cyclase or phospholipase A are stimulated only by micromolar concentrations of VIP (2, 3), whereas in our cultures VIP was active at a concentration of 10 nM. Moreover, the competitive antagonist PACAP (6–38), which blocks PAC1 receptor-mediated CAMP production at low nanomolar concentrations (44), did not prevent the inhibitory effect of PACAP38 on nuclear uptake of BrdUrd. By stimulating PAC1 or VPAC1/2 receptors, nanomolar concentrations of PACAP38 or VIP can also activate phospholipase D via an ARF-dependent mechanism, which is blocked by brefeldin A (58). In preliminary experiments, however, brefeldin A abolished astroglial proliferation (data not shown). Because this result confirmed previous findings (59), the involvement of ARF was not further tested. Thus, the second messenger involved in the antiproliferative effect of the neuropeptides remained unclear.

In neuronal precursors, the short isoform of PAC1 receptors has been suggested to mediate the antimitogenic effects of PACAP via production of cAMP and activation of PKA (12, 16). In astroglial cells, however, the contribution of PKA was rather
small to the antiproliferative effect of PACAP38. When expressed in the astroglial cells using an adenovirus system, PKI, a peptide inhibitor of PKA (45), did not have a significant effect on the inhibition of the nuclear uptake of BrdUrd caused by PACAP38. Also the pharmacological PKA inhibitor H89 was without effect. In contrast, PKI prevented the strong inhibition of the nuclear BrdUrd uptake caused by the adenyllyl cyclase stimulator forskolin. This finding confirmed that PKI was expressed by the adenoviral system and efficiently blocked the activity of PKA. It also showed that active PKA had an antiproliferative effect in astroglial cells, as it has in neuroblasts (4, 12). However, PACAP38 receptors do not seem to be efficiently coupled to this signal transduction pathway.

ERKs mediate the G0/G1 phase transition and the G1 phase progression upon activation by growth factors such as EGF and bFGF (60, 61). When we prevented the activation of the ERKs with the specific MEK1/2 inhibitor U0126, the nuclear uptake of BrdUrd in the astroglial cultures was reduced by 83.5%, indicating that ERKs were indeed essential for astroglial proliferation. However, our additional findings disagreed with the hypothesis that PACAP38 reduced astroglial proliferation by inhibiting p27Kip1 (18). Three lines of evidence were in agreement with this hypothesis that PACAP38 reduced astroglial proliferation by inhibiting p27Kip1 (18). Three lines of evidence were in agreement with the inhibitory effect of dnRhoA on platelet-derived growth factor-induced proliferation in IIC9 cells (34, 63). In dnRhoA expressing cells, PACAP38 did not further inhibit BrdUrd uptake, indicating a common mechanism of action. It is noteworthy that the expression of dnRhoA caused the disappearance of stress fibers without changing
PACAP38, RhoA, and Astroglial Cell Cycle

by cytochalasin D also reduced by 84.1% the nuclear uptake of BrdUrd, but PACAP38 applied together with cytochalasin D even further reduced BrdUrd uptake.

RhoA has been shown to decrease the content in p27Kip1 (31). It does so by activating the cyclin E-CDK2 complex, which then induces the degradation of p27Kip1 (34). In this context, it is noteworthy that p27Kip1, but not p21Cip1, is strongly expressed in rat neocortical tissue during the late pre- and early postnatal period (64), when astroglial cells proliferate. However, a reduction in cyclin D1 protein content has been also observed that was induced by RhoA inactivation with C3 toxin (65).

By using the toxin CNFy, we obtained some information how PACAP38 inactivated RhoA. The bacterial toxin deamidates glutamine 63 of RhoA and thus inhibits the latter’s GTP hydrolyzing activity. Consequently, the exchange of GDP to GTP mediated by a cellular GEF leads to an irreversible activation of RhoA (43). Simultaneous treatment of the cells with PACAP38 and CNFy diminished the RhoA activity enhanced by CNFy, suggesting that PACAP38 interfered with the activation of RhoA.

More than 60 mammalian GEF proteins are already known, several of which specifically activate Rho. The subcellular localization of a GEF protein seems to be important for its activity and may regulate the effect of the activated Rho GTPase (66). The GEFs expressed in astroglial cells are not known so that we can only speculate on the mechanisms of their regulation. Of the signal transduction pathways initiated by PACAP38, only the AMP-PKA cascade has been linked to Rho activation. ARAP-Lbc is a PKA anchoring protein with an additional Rho GEF domain (67). Upon binding of active PKA, the GEF activity is reduced (68, 69). However, activation of PKA was not responsible for the antimitogenic effect of PACAP38 in our astroglial cells. Stimulation of PACAP receptors may also activate phospholipase C and thus increase intracellular free Ca2+ levels as well as protein kinase C activity (3). However, PACAP38 decreased the nuclear BrdUrd uptake induced by activation of protein kinase C with TPA, indicating that this kinase was not involved in the inhibitory effect of PACAP38 on Rho. Whether PACAP38-induced changes in intracellular Ca2+ concentrations affected the activation of RhoA remained open.

High concentrations of RhoA encoding mRNAs are present in neocortical growth zones during the embryonic and early postnatal period (70). The present data suggest that the GTGase plays a role in the regulation of proliferation of astroglial cells. Which extra- or intracellular factors maintain the activation of RhoA and thus cell proliferation is an important question to be studied in future experiments. In addition, we have found a link between the neuropeptide growth factor PACAP38 and RhoA, which seems to be specific for astroglial cells. In cultured neocortical neuroblasts, PACAP38 inhibits proliferation by activating PKA and the CKI p57Kip2. It decreases CDK2 kinase activity (64). In cerebellar granule cell precursors, it inhibits the proliferation induced by the Sonic-Hedgehog pathway (71).

Astroglial morphology. This finding indicates that inactivation of endogenous RhoA reduced proliferation independent of changes in cell morphology. Second, PACAP38 strongly reduced the cellular content of active RhoA extracted with the Rhotekin pull-down assay. The destruction of stress fibers caused by PACAP38 in the astroglial cells provides additional evidence for the inactivation of RhoA. Finally, calRhoA expressed in the astroglial cells prevented the inhibitory effect of PACAP38 on BrdUrd uptake.

In primary cultures, the sustained activation of ERKs and cyclin D1 necessary for cell cycle progression is only possible if the cells attach to the matrix (21–25). Cell attachment is mediated by the actin cytoskeleton that is organized by the Rho GTPases RhoA, Rac, and Cdc42. Although PACAP38 caused pronounced changes in the astroglial morphology, our results did not show that the peptide reduced nuclear BrdUrd uptake via this mechanism. Thus, destruction of the actin cytoskeleton

FIG. 6. Effects of calRhoA and CNFy.

A. calRhoA is active in astroglial cells as shown by a Rhotekin precipitation assay for GTP bound RhoA. Cells were cultivated in medium containing 1% FCS.

B. Because of the infection-mediated increase in RhoA input, cell density was evaluated by determination of Cdc42. Cells were incubated with PACAP38 (10 nM) for 1 h. For further explanation see Fig. 3C. Panel B. calRhoA prevents the reduction in nuclear uptake of BrdUrd caused by PACAP38 in cells synchronized by transient FCS withdrawal. Mean ± S.E. (n = 45); a, indicates significant difference (p < 0.05) to EGFP controls.

C. Panel C, CNFy increases GTP bound RhoA as shown by a Rhotekin precipitation assay. Cells were incubated with CNFy (200 ng/ml), PACAP38 (10 nM), and H89 (10 μM) for 4 h. Panel D, F-actin staining with phalloidin in astroglial cells treated with CNFy (200 ng/ml) for 4 h in the absence or presence of PACAP38 (10 nM).

REFERENCES
1. Arimura, A. (1992) Regul. Pept. 37, 287–303
2. Harmar, A. J., Arimura, A., Gozes, I., Journot, L., Laburthe, M., Pisegna, J. R., Rawlings, S. R., Robberecht, P., Said, S. I., Stredharm, S. P., Wank, S. A., and Waschek, J. A. (1998) Pharmacol. Rev. 50, 265–270
3. Vaudry, D., Gonzalez, B. J., Basille, M., Yon, L., Fournier, A., and Vaudry, H. (2000) Pharmacol. Rev. 52, 269–324
4. Lu, N., and Cicco-Bloom, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 25265

Acknowledgment—The gift of the ERF plasmid by Dr. Mavrothalas-sitis (IMBB-Forth, University of Crete, Crete, Greece) is gratefully acknowledged.
