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PACAP/PAC1 Autocrine System Promotes Proliferation and Astrogenesis in Neural Progenitor Cells

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KEY WORDS

pituitary adenylate cyclase-activating polypeptide (PACAP); PAC1; neural progenitor cell; autocrine proliferation factor

ABSTRACT

The Pituitary adenylate cyclase-activating peptide (PACAP) ligand/type 1 receptor (PAC1) system regulates neurogenesis and gliogenesis. It has been well established that the PACAP/PAC1 system induces differentiation of neural progenitor cells (NPCs) through the Gs-mediated cAMP-dependent signaling pathway. However, it is unknown whether this ligand/receptor system has a function in proliferation of NPCs. In this study, we identified that PACAP and PAC1 were highly expressed and co-localized in NPCs of mouse cortex at embryonic day 14.5 (E14.5) and found that the PACAP/PAC1 system potentiated growth factor-induced proliferation of mouse cortical NPCs at E14.5 via Gq-, but not Gs-, mediated PLC/IP_3-dependent signaling pathway in an autocrine manner. Moreover, PAC1 activation induced elongation of cellular processes and a stellate morphology in astrocytes that had the bromodeoxyuridine (BrdU)-incorporating ability of NPCs. Consistent with this notion, we determined that the most BrdU positive NPCs differentiated to astrocytes through PAC1 signaling. These results suggest that the PACAP/PAC1 system may play a dual role in neural/glial progenitor cells not only differentiation but also proliferation in the cortical astrocyte lineage via Ca^{2+}-dependent signaling pathways through PAC1.

INTRODUCTION

Multipotent and proliferative neural progenitor cells (NPCs) represent the epigenetic and intrinsic origin of neurons, astrocytes, and oligodendrocytes in the central nervous system (CNS) (Altman and Bayer, 1990a,b; Reynolds et al., 1992; Reynolds and Weiss, 1996). During brain development, neurogenesis and gliogenesis occur as distinct temporal events with only some overlap. In the mouse embryonic cortex, neurogenesis takes place between embryonic days (E) 12 and 17 to generate neurons from neuronal progenitors. In contrast, astrocytic differentiation begins mainly at E16 and continues in the postnatal days. In each embryonic stage, NPC proliferation or differentiation is mostly regulated by locally produced or peripherally circulating soluble paracrine factors such as growth factors (e.g. basic fibroblast growth factor (b-FGF) and epidermal growth factor (EGF)) and cytokines as well as several autocrine factors such as bone morphogenic protein-4 (BMP4), interleukin 6, glycosylated cystatin C, and insulin-like growth factors (Eccleston et al., 1991; Liu et al., 2004; Wislet-Gendebien et al., 2004). Although glial progenitors are known to arise from NPCs predominantly at a delay on neurogenesis, the underlying spatiotemporal regulatory mechanisms of proliferation and differentiation of glial progenitors are not yet defined.

The effects of pituitary adenylate cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP), which are members of the VIP/secretin/glucagon peptide family have been well characterized in the CNS. For example, these factors affect neurotransmitter release and survival of hippocampal neurons as well as controlling cerebellar maturation (Arimura, 1998; Basille et al., 1993; Bluet-Pajot et al., 1998; Otto et al., 2001; Rayan et al., 1991; Vaudry et al., 2002; Zhou et al., 2002). These PACAP and VIP functions are mediated by three PACAP receptors, PAC1, VPAC1, and VPAC2 (Christophe, 1993; Muller et al., 1995; Tatsuno et al., 1994; Zhou et al., 2002). In particular, PACAP and PAC1 are highly expressed and distributed ubiquitously in the embryonic CNS and peripheral nervous system (Tatsuno et al., 1994; Zhou et al., 2002). Accordingly, PACAP is considered to influence the regulation of NPC proliferation and/or differentiation during embryonic development (Dicicco-Bloom et al., 1998). The PAC1 gene encodes a

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G-protein-coupled receptor that has four splice variants, depending on the presence or absence of either one or two of the cassettes, "hip" and "hop," in the third intercellular loop (Bresson-Bepoldin et al., 1998; Jaworski and Proctor, 2000; Zhou et al., 2000a,b). These splice variants are involved in multiple and response-specific second messenger cascades (Pisegna et al., 1996). Evidence from studies with transfected cells indicates that each splice variant activates different cell signaling pathways involving adenylate cyclase (AC) and/or phospholipase C (PLC) and activation of these two pathways has opposite effects on the proliferation of cerebral cortical precursor cells (Basille et al., 1995; Cazillis et al., 2004; Lu et al., 1998; Mercer et al., 2004; Suh et al., 2001; Waschek et al., 2000). Recently it was shown that the PACAP/PAC1 system inhibits NPC proliferation and promotes neurogenesis and gliogenesis by activation of the Gs-mediated cAMP-dependent signal transduction pathway in the embryonic brain (Lelievre et al., 2002; Suh et al., 2001; Waschek et al., 1998). In contrast, PACAP was reported to promote adult NPC proliferation via PAC1 both in vivo and in vitro. Thus the precise effect of direct activation of PAC1 signaling on embryonic NPCs, and the mechanism thereof, remains unknown.

To elucidate the function of PACAP in embryonic cortical NPCs, we investigated regulatory mechanisms of PAC1 signaling for cell proliferation and differentiation using NPCs of mouse cortex at E14.5 when cortical NPCs in the ventricular zone (VZ)/subventricular zone (SVZ) contain not only neuronal progenitors but also glial progenitors. In this study, we identified that PACAP and PAC1 were highly expressed and co-localized in NPCs. Surprisingly, we found that the PACAP/PAC1 system potentiated growth factor-induced proliferation in mouse cortical NPCs at E14.5 via Gq-mediated—but not Gs-mediated—PLC/IP_3-dependent signaling pathways in an autocrine manner. Moreover, we showed that direct activation of PAC1 induced astrocyte-like morphological changes in embryonic cortical NPCs. Together with our present results and the previously identified role of PACAP, we suggest a dual role of the PACAP/PAC1 system for NPC proliferation during cortical astrogenesis by different signaling pathways of PAC1 variants at E14.5.

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal and polyclonal antibodies used in this study were as follows: mouse monoclonal anti-nestin (Becton Dickinson, Lexington, KY, and RaZ 401; Developmental Studies Hybridema Bank, Iowa City, IA), mouse monoclonal anti-neuronal class III β-tubulin (anti-β III tubulin (Tuj1); COVANCE, Berkeley, CA), mouse monoclonal anti-galactocerebroside (anti-Gal C; Chemicon International, Temecula, CA), rabbit polyclonal anti-glial fibrillary acidic protein (anti-GFAP: DAKO, Carpinteria, CA), goat polyclonal anti-PAC1 (gift from A. Arimura, Tulane University, New Orleans), rabbit polyclonal anti-PACAP38 (Calbiochem, San Diego, CA), rat monoclonal anti-bromodeoxyuridine (BrdU; Becton Dickinson, Lexington, KY). The secondary antibodies conjugated to Alexa Fluor fluorescein (goat anti-mouse Alexa Fluor 488, 568, or 633, goat anti-rabbit Alexa Fluor 488 or 568, rabbit anti-goat Alexa Fluor 594, and goat anti-mouse Alexa Fluor 488 or 568) were purchased from Molecular Probes (Eugene, OR). PACAP38, PACAP(6–38) (Peptide Institute, Osaka, Japan), VIP (Sigma, St. Louis, MO), maxadilan and M65 (gifts from Dr. Richard G. Titus, Colorado State University) were dissolved in distilled water. H89, 2-aminoethoxydiphenyl borate (2-APB; Calbiochem, San Diego, CA), chelerythrine (Sigma) and *O,O'*-bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (BAPTA-AM; Sigma) were dissolved in DMSO. Each solution was added to the medium, and the final concentration of organic solvent (DMSO) in the medium was adjusted to no more than 0.1% (v/v). Each control medium contained the same amount of each organic solvent.

Animals

Pregnant C57BL/6J mice were purchased from CLEA Japan. All experiments were performed in the laboratory for animal experiments according to NIH Standards for Treatment of Laboratory Animals.

Culture of Mouse Embryonic Cortical NPCs

Cortical NPCs were cultured as previously described (Li et al., 2001; Nakashima et al., 1999). Briefly, embryos were removed from pregnant C57BL/6J mice (CLEA Japan, Tokyo, Japan) and staged according to morphological criteria to confirm gestational day (Kaufman, 1998). Developing mouse cerebral cortices were dissected at embryonic day 14.5 (E14.5). Cells were mechanically dissociated by trituration and plated at a density of 3.0×10^6 cells in 10-cm dishes (BD) that were precoated with 15 μg/mL poly-×L-ornithine (Sigma) and 1 μg/mL fibronectin (Nitta Gelatin, Osaka, Japan). Cells were expanded for 4 days in serum-free Neurobasal (NB) medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), 0.5 mM L-glutamine (Invitrogen), 100 μg/mL penicillin and 100 μg/mL streptomycin (Invitrogen). This medium was supplemented with 10 ng/mL b-FGF (PeproTech, Rocky Hill, NJ), except where indicated otherwise. Cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. For secondary culture, b-FGF-expanded cortical NPCs were washed in warm Hanks' balanced salt solution, detached via mechanically pipetting, and resuspended in NB medium. Cells were then reseeded in 24-well plates (Nunc; 1×10^5 cells per well), 48-well plates (Nunc; 4.5×10^4 cells/well) or 96-well plates (Nunc; 1×10^4 cells/well) precoated with poly-L-ornithine and fibronectin.

Conditioned Medium Preparation

Subconfluent embryonic cortical NPCs in secondary cultures and control cultures maintained without

NvvPCs were incubated in serum-free NB/B27 medium for 48 h with b-FGF (5 ng/mL). After this period, conditioned medium derived from either NPCs or control cultures was collected and centrifuged at 1,000*g* for 5 min at 4°C to remove nonadherent cells.

Real-Time RT-PCR

Total RNA was isolated from cultured embryonic cortical NPCs and E14 mouse cerebral cortex. These RNAs (1 μg) were treated with DNase I and converted to cDNA using Superscript reverse transcriptase (Invitrogen) and random hexamer primers, according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed with the SYBR Green-based method (ABI PRISM 7700 Sequence Detection System, Perkin-Elmer). The quantitative RT-PCR method (User Bulletin no. 2, Applied Biosystems, Foster City, CA) was modified to establish an expression level index for mRNA, and the SYBR green signal for the hypoxanthine-guanine phosphoribosyl transferase (hprt) gene amplicon was used as a reference. Amplification efficiency was determined and confirmed in a control PCR experiment using serially diluted cDNAs as templates. Real-time RT-PCR reactions were run on an ABI PRISM 7700 Sequence Detection System device using the following program: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The real-time RT-PCR products were analyzed using the sequence detection system software 1.7 (Applied Biosystems). The analysis and calculations were performed as described above. The efficiency of reverse transcription and the quality of cDNA was assessed by the efficiency of amplification of the hprt gene (upper primer, 5′-TCTTTGCTGACCTGCTGGATT-3′, corresponding to bases 222–241; lower, 5′-TATGTCCCCCGTTGACTGATC-3′, corresponding to bases 322–342, GenBank accession no. NM-013556). PCR amplification was then performed with specific primers for PAC1, VPAC1, VPAC2, PACAP, and VIP, for which primers were designed using Primer Express software (Perkin-Elmer, Torrance, CA), as follows. PAC1: upper, 5′-CTTCGATGCTTGTGGGTTTGA-3′, corresponding to bases 543–563 and lower, 5′-AAGCGGCACAAGATGACCAT-3′, bases 667–686, GenBank accession no. D82935; VPAC1: upper, 5′-TCCCCCATTCACGGCTATAA-3′, bases 413–423, and lower, 5′-CAGTCTGTTGCTGCTCATCCAT-3′, bases 525–540, GenBank accession no. NM011703; VPAC2: upper, 5′-CTTCTCCAGATGTTGGTGGCA-3′, bases 981–1,001, and lower, 5′-CCAATAGGGAAGGCAGCAAAC-3′, bases 1,078–1,098, GenBank accession no. D28132; PACAP: upper, 5′-GGCATGTGGGACAATATCACAT-3′, bases 319–340, and lower, 5′-ACTTGGTCCGGGTTGAAGATC-3′, bases 399–419, GenBank accession no. NM009625; VIP: upper, 5′-GGAACAGACTGGTGGAGCCTT-3′, bases 55–75, and lower, 5′-TTCCATCTCGGTGCCTCCT-3′, bases 152–170, GenBank accession no. NM011702. To determine the expression of PAC1 splice variants, we used primer pairs and the condition of PCR amplification described previously (Jamen et al., 2002). A scheme for the design of primers for PAC1 splice variants is shown in Fig. 5A. For short or hip-hop variants: upper, 5′-CATCCTTGTGCAGAAGCTGC-3′, corresponding to bases 1,456–1,475, and lower, 5′-GGTGCTTGAAGTCCATAGTG-3′, bases 1,825–1,844; hip variants: upper, 5′-ACAAATTTAAGACTGAGAGT-3′, bases 1,456–1,475, and lower, 5′-GGTGCTTGAAGTCCATAGTG-3′, bases 1,825–1,844. PCR was performed with an initial step at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and then a final cycle of 72°C for 10 min. Amplification products were stored at 4°C and then electrophoresed on a 2% agarose gel; the bands were visualized by ethidium bromide staining.

Immunohistochemistry and Immunocytochemistry

On embryonic day 14.5, C57BL/6J mouse brains were removed and fixed with 4% paraformaldehyde (PFA) for 8 h, cryoprotected in 20% sucrose in PBS, and frozen. Twenty-micrometer-thick coronal sections were cut with a cryostat, placed on APS-coated glass slides and then fixed with 4% PFA, washed three times with PBS, permeabilized with 0.1% (w/v) Triton X-100/PBS for 5 min and finally washed three times with PBS. Fixed sections were incubated for 30 min with 3% bovine serum albumin (Sigma). Sections were incubated overnight at 4°C with diluted primary polyclonal anti-PAC1 (1:1,000), see earlier for manufacturer's details, anti-PACAP38 (1:1,000) and anti-nestin (1:500) for triple staining. These sections were incubated for 1 h with diluted secondary antibodies (goat anti-mouse Alexa Fluor 633, goat anti-rabbit Alexa Fluor 488, and rabbit anti-goat Alexa Fluor 594) and washed with PBS. Confocal microscopy was performed using the Leica TCS SP2 spectral confocal scanning system (Leica Microsystems). For immunofluorescence measurements, cultured mouse embryonic NPCs were grown on poly-L-ornithine- and fibronectin-coated dishes. All incubations and washes were performed at room temperature. Cells were fixed with 4% PFA, washed three times with PBS, permeabilized with 0.1% Triton X-100/PBS for 5 min and then washed three times with PBS. Fixed cells were incubated for 30 min with 3.3% goat or rabbit serum (Nichirei, Tokyo, Japan). Cells were incubated for 0.5–1 h with diluted primary polyclonal or monoclonal antibody (both were used for double-staining). Next, these cells were incubated for 0.5–1 h with diluted secondary antibodies conjugated to fluorescein and washed with PBS. For BrdU labeling, cells were incubated with 2 M HCl at 37°C for 30 min, rinsed in 0.1 M sodium borate buffer and processed for immunocytochemistry. As a negative control in immunohistochemistry and immunocytochemistry, we performed the omission of either primary or secondary antibodies. Confocal microscopy was performed using the FLUOVIEW confocal microscope system (Olympus, Tokyo, Japan).

[^{3}H]Thymidine Incorporation Assay

Cortical embryonic NPCs were seeded in 96-well plates (1×10^4 cells/well) in 100 μL of medium and cultured for 24 h at 37°C with b-FGF (5 ng/mL). Vehicle or peptide (PACAP38, 0–100 nM; maxadilan, 0–10 nM; or M65, 10 nM) was then added; after 1 h, 1 μCi/well [^{3}H]thymidine was also added. After a 7-h incubation, the cells were washed extensively with medium, and [^{3}H]thymidine incorporation was measured using a β-counter.

For the CM proliferation assay, embryonic cortical NPCs were seeded in 96-well plates (1×10^4 cells/well) in 100 μL of medium and cultured for 24 h at 37°C. Medium was then replaced with NPC conditioned medium or control medium. [^{3}H]thymidine incorporation and measurement were performed as described above.

To assess PAC1 signaling, cortical embryonic NPCs were seeded in 96-well plates (10^4 cells/well) in 100 μL of medium and cultured for 24 h at 37°C. Cells were pre-incubated for an additional 1 h with vehicle and signal transduction inhibitors (H89, 10 μM 2-APB, 20 μM; chelerythrine, 50 μM), and then maxadilan (10 nM) was added. [^{3}H]thymidine incorporation was measured as described above.

Intracellular ATP Assays

It has been previously reported that intracellular ATP levels correlate with cell number (Crouch et al., 1993). Embryonic cortical NPCs were seeded in 48-well plates (4.5×10^4 cells/well) in 400 μL of medium and cultured for 24 h at 37°C with b-FGF (5 ng/mL). After 24 h, each peptide (PACAP38, 0–100 nM; maxadilan, 0–10 nM; PACAP(6–38), 10 nM) was added to these NPC cultures. After a 6 or 24-h incubation with the peptides, intracellular ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Radioimmunoassay for PACAP38

PACAP38 concentration was measured in conditioned medium derived from NPCs using a radioimmunoassay (RIA). Cell-free conditioned medium (100 μL) and conditioned medium derived from NPCs (100 μL) were incubated with polyclonal anti-PACAP38, which was rehydrated in RIA buffer (Phoenix Pharmaceuticals, Belmont, CA) for 24 h at 4°C. These reactions were incubated with ^{125}I-PACAP(31–38) (50 cpm/μL) for an additional 24 h at 4°C. Second antibody reaction/separation and detection of ^{125}I in the pellets were performed in a scintillation well gamma counter according to the instrument manufacturer's instructions (Phoenix Pharmaceuticals, Belmont, CA).

Intracellular cAMP Measurement

Cortical embryonic NPCs were seeded in 96-well plates and treated with PACAP38 (100 nM) or maxadilan (10 nM) for 24 h. cAMP levels were assayed by the cAMP-Screen™ System (Applied Biosystems).

Intracellular Calcium Imaging

Cortical embryonic NPCs were seeded in 96-well plates and cultured for 24 h as described above for the intracellular cAMP measurement. The intracellular concentration of free calcium ([Ca^{2+}]i) in cultured NPCs was monitored using the Calcium Kit-Fluo 3 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. For a negative control, cells were pre-incubated for an additional 1 h with BAPTA-AM (100 μM), and then PACAP38 (100 nM) or maxadilan (10 nM) was added.

RESULTS

Expression of PAC1 and PACAP in Embryonic Cortical NPCs In Vivo and In Vitro

To generate a highly purified population of embryonic cortical NPCs, we prepared a secondary culture of NPCs (Li et al., 2001; Nakashima et al., 1999). Most of these cells were positive for nestin, a specific marker for NPCs in developmental brain, but less than 0.5% were immunoreactive for β III tubulin and GFAP. Gal C positive cells were not found (data not shown).

We used RT-PCR and immunohistochemical methods to investigate PAC1 and PACAP expression in cultured mouse embryonic cortical NPCs. RT-PCR detected both PAC1 and PACAP mRNAs in the NPCs and mouse telencephalon at embryonic day 14.5 (Fig. 1A). Other PACAP receptors, VPAC1 and VPAC2, mRNAs were expressed at a much lower level [(0.01 ± 1.7)% and (0.11 ± 2.3)% (mean ± S.E.M.), respectively] than PAC1 mRNA (33.1 ± 0.23)% (Fig. 1A). In addition, the mRNA for another PAC1 ligand, VIP, was not detected in embryonic cortical NPCs (Fig. 1A). In immunohistochemistry experiments, PACAP protein was expressed in nestin-immunoreactive NPCs, and most nestin-immunoreactive NPCs expressing PAC1 colocalized with PACAP in the VZ and SVZ (Fig. 1B). We also detected PACAP and PAC1 in nestin-immunoreactive cultured embryonic cortical NPCs. Over 99.8% of the PAC1-expressing cells co-expressed PACAP (Fig. 1C).

Activation of PAC1 Signaling Induces Embryonic Cortical NPC Proliferation in the Presence of b-FGF in an Autocrine Manner

We performed a [^{3}H]thymidine incorporation assay and an ATP assay, which measures intracellular ATP

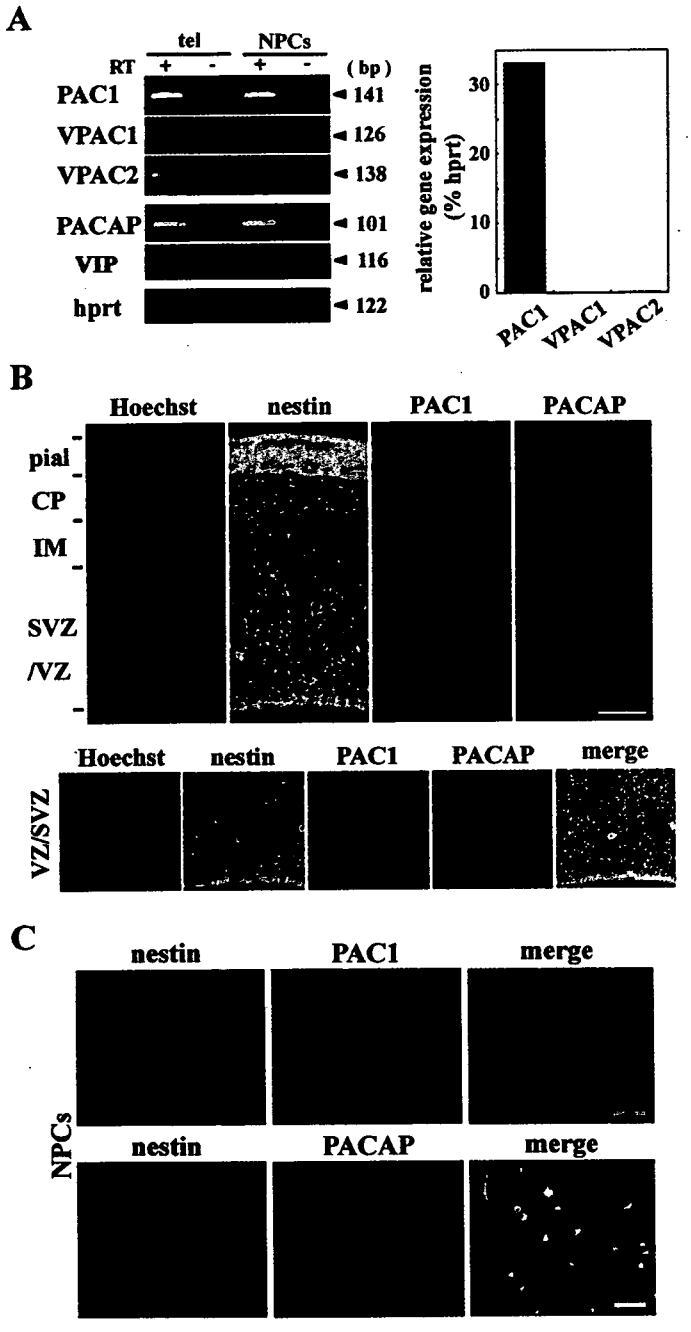

Fig. 1. NPCs express the PACAP receptors PAC1, VPAC1 and VPAC2, and their ligand, PACAP. **A:** RT-PCR was performed as described in Material and Methods. PCR products corresponding to these receptor and ligand genes were loaded onto a 3% agarose gel, as indicated at the left. The size of the PCR products is indicated on the right. Quantitative analysis of mRNA transcripts for PACAP receptors in NPCs by SYBER green-based PCR 7700 system. Data represents expression level of individual PACAP receptors relative to the expression level of hprt. **B,C:** PACAP is an autocrine factor of NPCs. PAC1 and PACAP protein expression was determined by immunofluorescence in vivo (B) and in vitro (C). Nestin-immunoreactive cells (cyan) in E14.5 mouse embryonic telencephalon also expressed PAC1 (green) and PACAP (red). The lower panels are high magnifications of the upper panels (B). Cell nuclei were counterstained with Hoechst 33258 (blue, B). Almost all nestin-immunoreactive NPCs (red, C) expressed PAC1 (green, top panel in C) and PACAP (green, lower panel in C). Cell nuclei were counterstained with Hoechst 33258 (blue, C). Scale bars: (B, C) 50 μm.

levels and hence indicates the viable cell number, to determine whether PAC1 signaling promotes NPC proliferation. A 6-h incubation neither with PACAP38 (0–100 nM), the natural ligand for PAC1, nor with maxadilan (0–1 nM), a specific agonist for PAC1, induced DNA synthesis in the absence of b-FGF (Fig. 2A). In contrast, both reagents increased [^{3}H]thymidine incorporation in a dose-dependent manner in the presence of b-FGF without altering the viable cell number as assessed by the ATP assay (Figs. 2A,B). After incubation for 24 h, maxadilan increased intracellular ATP levels in the presence of b-FGF, and this increase was completely inhibited by the PAC1-specific antagonist, PACAP(6–38) (Fig. 2C). The PAC1 ligand, VIP, also increased [^{3}H]thymidine incorporation in NPCs (Fig. 2D) after incubation for 6 h. However, the DNA synthesis-promoting activity of VIP was lower than that of PACAP38 or maxadilan, and was inhibited by PACAP(6–38) (data not shown). These results indicate that activation of PAC1 promotes DNA synthesis in 6 h followed by NPC proliferation in 24 h in cultures supplemented with b-FGF.

To investigate whether PACAP secreted from embryonic cortical NPCs induces DNA synthesis in the presence of b-FGF, we analyzed [^{3}H]thymidine incorporation in NPCs cultured with conditioned medium derived from embryonic cortical NPCs supplemented with b-FGF. After 7 h, [^{3}H]thymidine incorporation increased by 140% relative to NPCs cultured with NPC-free media, and this activity was inhibited by another PAC1-specific antagonist, M65 (Fig. 3). Similar inhibition was observed when PACAP(6–38) was used (data not shown). We also performed a radioimmunoassay to detect PACAP38 in the conditioned medium derived from NPCs. The PACAP concentration was substantially higher in conditioned medium derived from NPCs (284 ± 2.3 pg/mL, Mean ± SEM) than in NPC-free medium (0.072 ± 0.25 pg/mL, Mean ± SEM).

PLC/IP$_3$ Signaling Pathway are Activated by Maxadilan Via PAC1 Splice Variants in Embryonic Cortical NPCs

To determine which splice variant (or variants) was expressed in our embryonic cortical NPCs, we performed RT-PCR using splice variant-specific primer pairs. We detected four PAC1 variants (Fig. 4).

To determine whether PAC1 couples to the cAMP pathway, we measured intracellular cAMP concentration in NPCs after treatment with PACAP38 or maxadilan. Both reagents elicited a 3.5–4-fold increase in intracellular cAMP concentration (Fig. 5A). We used the Fluo-3 ratio method to determine the effects of PAC1 activation on calcium signaling. The intracellular calcium level ([Ca^{2+}]i) increased rapidly in NPCs following treatment with PACAP38 or maxadilan (Fig. 5B). This activity was inhibited by BAPTA-AM, which was an intracellular Ca^{2+}-chelator (Fig. 5B). We used pathway-specific in-

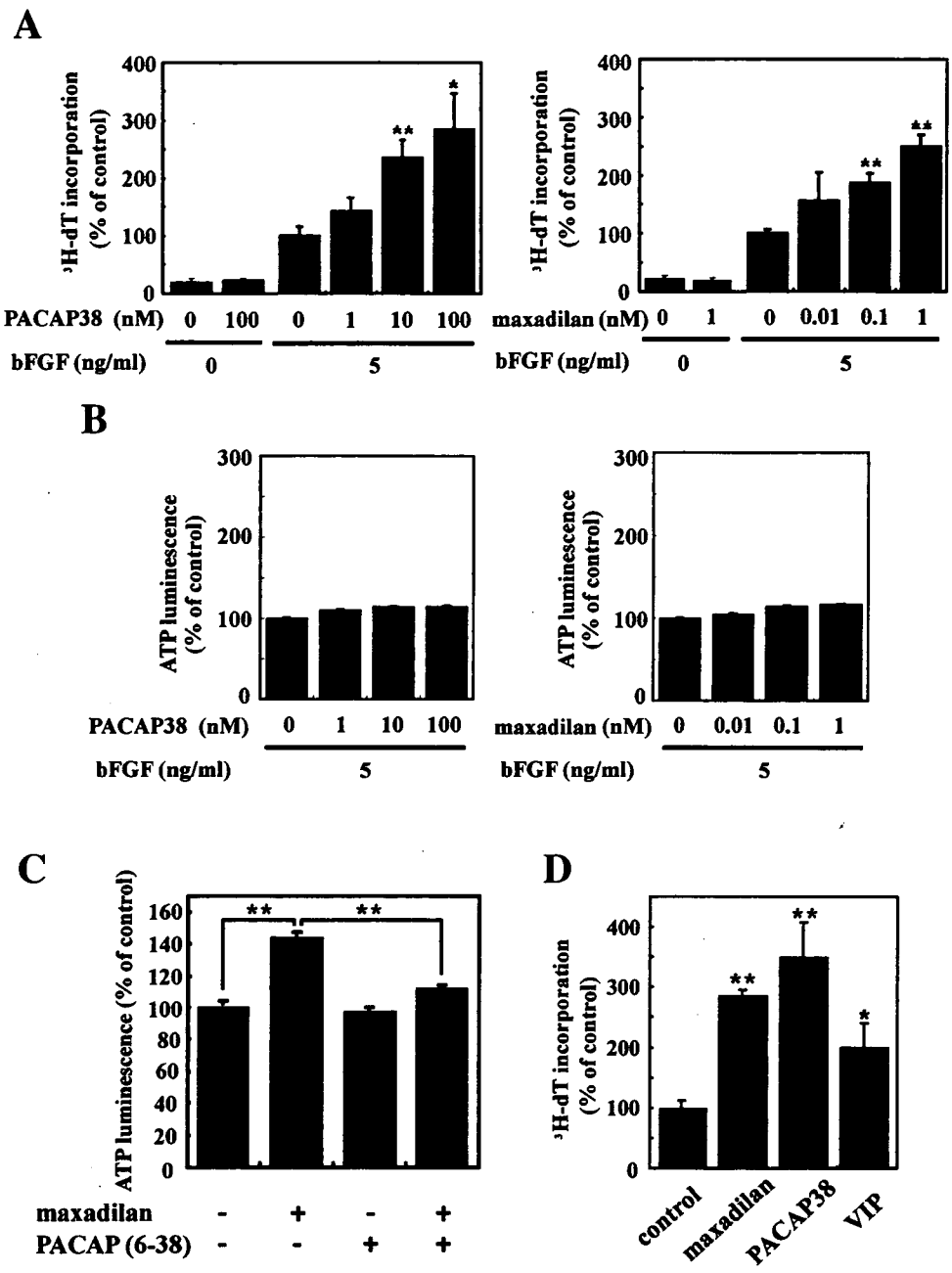

Fig. 2. PAC1 signaling promotes NPC growth in the presence of b-FGF. **A,B:** PACAP38 and maxadilan promote DNA synthesis and cell growth of NPCs in a dose-dependent manner in the presence of b-FGF. NPCs were cultured with PACAP38 (0–100 nM) (left) or maxadilan (0–1 nM) (right) in the presence or absence of b-FGF (5 ng/mL) for 7 h, and then [^{3}H]thymidine was added during the last 6 h of culture (A). NPCs were cultured with PACAP38 (left) or maxadilan (right) in the presence of b-FGF (5 ng/mL) for 6 h, and then the growth rate was examined by an ATP luminescence assay (B). **C:** The PAC1-specific agonist, maxadilan, promotes NPC growth; blockade of PAC1 with the PAC1-specific antagonist, PACAP (6–38), completely cancels this growth-promoting activity. NPCs were incubated with or without maxadilan (1 nM) for 24 h. Maxadilan-induced cell growth was inhibited by co-incubation with PACAP (6–38) (10 nM). **D:** NPCs were cultured with or without PACAP38 (100 nM), maxadilan (10 nM) or VIP (1 μM) in the presence of b-FGF for 7 h, and then [^{3}H]thymidine was added during the last 6 h of culture. Bars represent mean ± SD (n = 4). Significant differences from control (without agonist or antagonist) are indicated by asterisks (*P < 0.05; **P < 0.01, ANOVA).

hibitors for protein kinase A (PKA) or inositol 1,4,5-trisphosphate (IP3)/C kinase (PKC) to determine which signaling pathway mediates PAC1 activity during embryonic cortical NPC proliferation. H89, which inhibits cAMP-dependent PKA, did not inhibit the maxadilan-induced increase in [^{3}H]thymidine incorporation; however, 2-APB, which inhibits the IP3 receptor, had a strong inhibitory effect (Fig. 5C). In addition, chelerythrin, which inhibits PKC, did not inhibit the effect of maxadilan. Taken together, these results indicate that NPC proliferation involved the PLC/IP_3-dependent signaling pathway and a downstream Ca^{2+}-dependent pathway.

PAC1 Activation Induces NPC Proliferation and Morphological Changes in Embryonic Cortical NPCs

Upon maxadilan stimulation for 48 h, we observed a morphological change, which was an elongation of cell processes with stellate and astrocyte-like morphology, in nestin-immunoreactive NPCs (Fig. 6A). To determine the relationship between NPC proliferation and the morphological changes seen by PAC1 signaling, we identified nestin-immunoreactive NPCs in the mitotic phase with a BrdU incorporation assay. Immediately after 10 min of

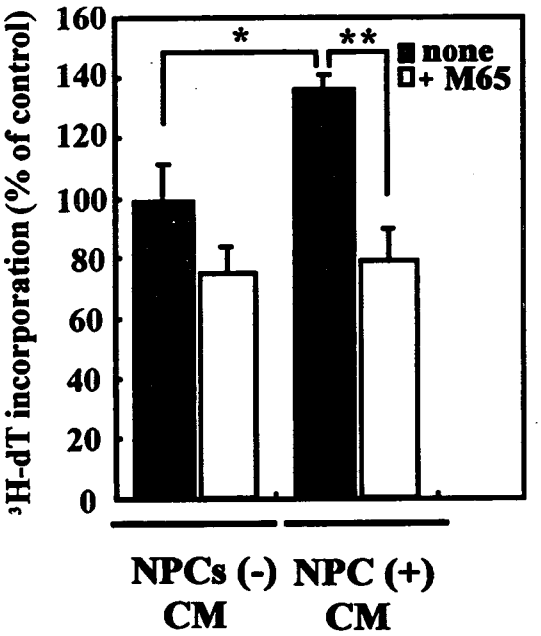

Fig. 3. The activation of PAC1-induced NPC proliferation in autocrine system. NPCs were cultured for 24 h, and the conditioned medium (CM) was collected. The CM or control cell-free media was added to freshly prepared NPC cultures and incubated for 7 h. [^{3}H]thymidine was added during the last 6 h, and incorporation was later measured. DNA synthesis-promoting activity in CM was observed and this activity was inhibited by the PAC1-selective antagonist, M65 (10 nM). Bars represent mean ± SD (n = 4). Significant differences from the control are indicated by asterisks (*P < 0.05, **P < 0.01; ANOVA).

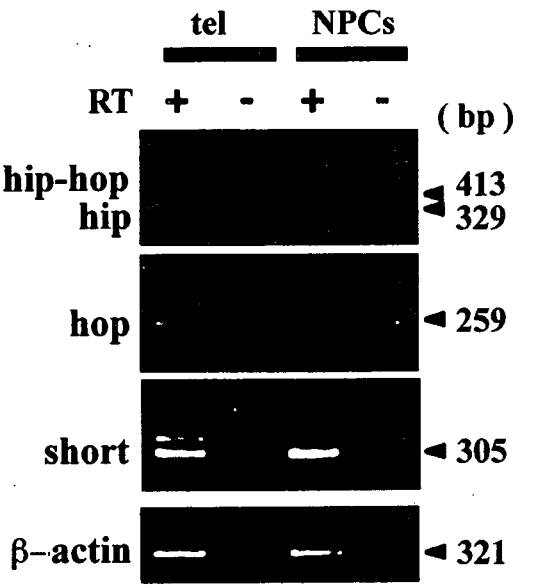

Fig. 4. NPCs express all PAC1 isoforms. RT-PCR was performed as described in Material and Methods. RT-PCR of E14.5 mouse embryonic telencephalon (tel) was used for comparison. The PCR products corresponding to the PAC1 isoforms were loaded onto a 3% agarose gel, as indicated at the left. The size of the PCR products is indicated on the right.

exposure to BrdU, the unincorporated BrdU was washed off the NPCs and maxadilan (10 nM) was added in BrdU-free and b-FGF contained medium. After 24 h, maxadilan increased the number of BrdU/nestin immunoreactive NPCs with long cell processes compared with non-treated NPCs (Fig. 6B). Next, we addressed the differentiation of BrdU immunoreactive NPCs to neurons, astrocytes, or oligodendrocytes. We observed a gradual increase of BrdU immunoreactive NPCs by the 24-h treatment of maxadilan during 7 days, compared with non-treated NPCs. The treatment of maxadilan did not increase the number of β III tubulin immunoreactive neurons, whereas maxadilan induced marked increase of GFAP immunoreactive astrocytes. Moreover, we observed the significant increase in the percentage of BrdU/GFAP double immunoreactive astrocytes, compared with that of non-treated NPCs at day 7 (Figs. 6B–E). These data suggested that PAC1 signaling regulates the proliferation of glial progenitor cells to generate astrocytes in E14.5 NPCs.

DISCUSSION

The PACAP/PAC1 system plays an important role in regulating differentiation of embryonic NPCs at E12–17 (Lee et al., 2001; Lelievre et al., 2002; Lu and DiCicco-Bloom, 1997; Lu et al., 1998; Suh et al., 2001). In our results, the PACAP/PAC1 autocrine system potentiated growth factor-promoted proliferation of E14.5 mouse cortical NPCs. Moreover, we found that the activation of PAC1 initiated morphological changes, which were cell process elongation of typical astrocytes in embryonic cortical NPCs. In the BrdU incorporation assay, most of BrdU positive NPCs differentiated to astrocytes. We suggest dual aspects of PAC1 signaling in the regulation of not only differentiation but also proliferation of NPCs at E14.5 via PLC/IP_3 signaling pathways through PAC1 variants in NPCs committed to an astrocytic lineage.

We found that the PACAP/PAC1 system potentiated growth factor-induced proliferation of E14.5 mouse cortical NPCs in an autocrine manner. Many trophic factor-generated microenvironments control NPC proliferation or differentiation. In particular, growth factors such as b-FGF, EGF, and TGFα promote NPC proliferation in the embryonic and postnatal brain (Gritti et al., 1996; Kilpatrick and Bartlett, 1993; Richards et al., 1992; Vescovi et al., 1999). Although these growth factors are soluble and thus diffuse widely in the CNS, their regulation during development has not been fully understood. In our results, immunoreactivity for PACAP and PAC1 in VZ/SVZ as well as the detection of PACAP38 in conditioned medium derived from NPCs supported the evidence of a PACAP/PAC1 autocrine loop in embryonic NPCs. Several molecules function in an autocrine manner in embryonic NPCs. BMPs and noggin are autocrine factors that regulate NPC proliferation and differentiation (Mabie et al., 1999; Nadarajah et al., 2002; Panchision et al., 2001; Sauvageot and Stiles, 2002). In adult brain, Cystatin C, IGF-I and stem cell-derived stem/progenitor cell-supporting factor have been charac-

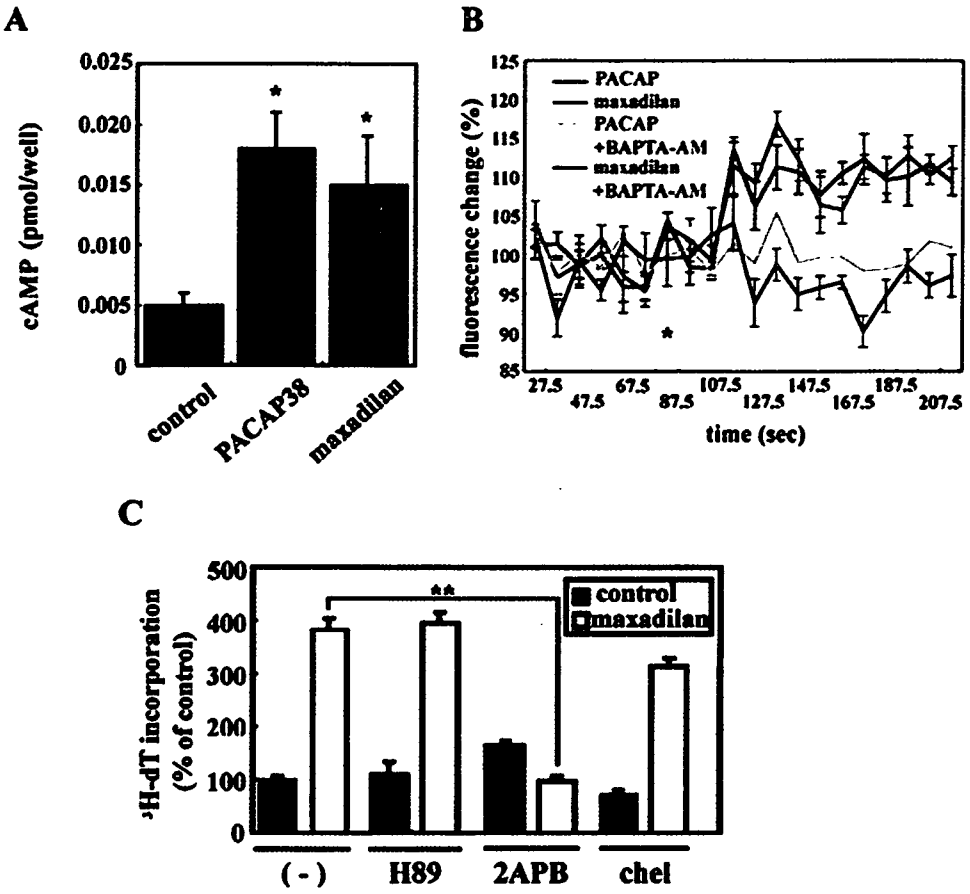

Fig. 5. PACAP promotes DNA synthesis in NPCs via PKC and IP3, but the PAC1-selective agonist, maxadilan, promotes the activity via IP3 only. **A:** The activation of PAC1 increases intracellular cAMP level in NPCs. The intracellular cAMP content in NPCs was measured after treatment with PACAP38 (100 nM) or maxadilan (10 nM) for 15 min by ELISA system. **B:** The activation of PAC1 increases intracellular calcium level ($[Ca^{2+}]i$) in NPCs. The ratio of ($[Ca^{2+}]i$ was analyzed by Fluo-3 AM imaging at the steady state and after the treatment* of PACAP38 (100 nM) or maxadilan (10 nM). **C:** NPCs were incubated with 10 μM H89, 2.5 μM 2-APB or 100 nM chelerythrine in the presence of 5 ng/mL b-FGF for 1 h before the addition of 100 nM PACAP38 or 10 nM maxadilan. [^{3}H]thymidine was added during the last 6 h of culture. Data represent mean values ± SD (n = 4). Significant differences from the control are indicated by asterisks (*P < 0.05, **P < 0.01; ANOVA).

terized as autocrine/paracrine growth-supporting factors for adult NPC proliferation, which is promoted by b-FGF/EGF (Toda et al., 2003). These factors are required to maintain multipotent adult NPC proliferation (Toda et al., 2003). These autocrine systems, including the PACAP/PAC1 system, are considered to play an important role in restricting or amplifying growth factor-mediated signals and in controlling NPC proliferation and differentiation.

At an early developmental stage (E12–14) during which the mouse cortex undergoes neurogenesis, the PACAP/PAC1 system promotes neuronal differentiation of cortical NPCs (Dicicco-Bloom et al., 1998; Lee et al., 2001; Lu and DiCicco-Bloom, 1997; Suh et al., 2001). In contrast, cortical NPCs derived from a late stage (E17) promote astrocyte differentiation via PAC1 (Vallejo and Vallejo, 2002). Furthermore, at the postnatal stage, when oligogenesis occurs, PAC1 signaling regulates both the growth and differentiation of oligodendrocyte progenitor cells (Lee et al., 2001). Thus the PACAP/PAC1 system plays multiple roles in the different cell lineages during development, and this fact suggests that it may function via several signaling pathways. Recent studies have shown that Gs- or Gq-mediated intracellular signal via PAC1 splice variants induced a specific biological activity, differentiation or proliferation, in cortical progenitor cells (Bresson-Bepoldin et al., 1998; Jaworski and Proctor, 2000; Lu et al., 1998; Zhou et al., 2000a,b). Our RT-PCR experiment showed that PAC1 splice variants were expressed in mouse embryonic cortical NPCs at E14.5. Moreover, we detected intracellular cAMP accumulation and $[Ca^{2+}]i$ increase via PAC1 activation in NPCs (Figs. 5A,B). These results suggested that the activation of PAC1 stimulated both Gs-mediated AC/PKA and Gq-mediated PLC/IP3 signaling pathways in embryonic cortical NPCs at E14.5 via PAC1 variants. Although many studies have reported that AC/PKA signal induced NPC differentiation into neuron or glial cells via PAC1 (Zhou et al., 2001), the effects of PLC/IP3 signal in NPC lineage is unknown. We found that IP3 inhibitor curtailed PAC1-mediated NPC proliferation (Fig. 5C), suggesting that Gq-mediated PLC/IP3, not Gs-mediated AC/PKA, signaling pathway modulated NPC proliferation via the PAC1 variant which might give rise to different signaling pathways for NPC differentiation and proliferation.

Cortical NPCs at E14.5 may have heterogeneous subpopulations. Some of these are multipotent and others are committed to neuronal or glial progenitors which generate neurons or astrocytes (Sauvageot and Stiles, 2002). As indicated by our morphological study and BrdU labeling, PAC1 signaling potentiated the pro-

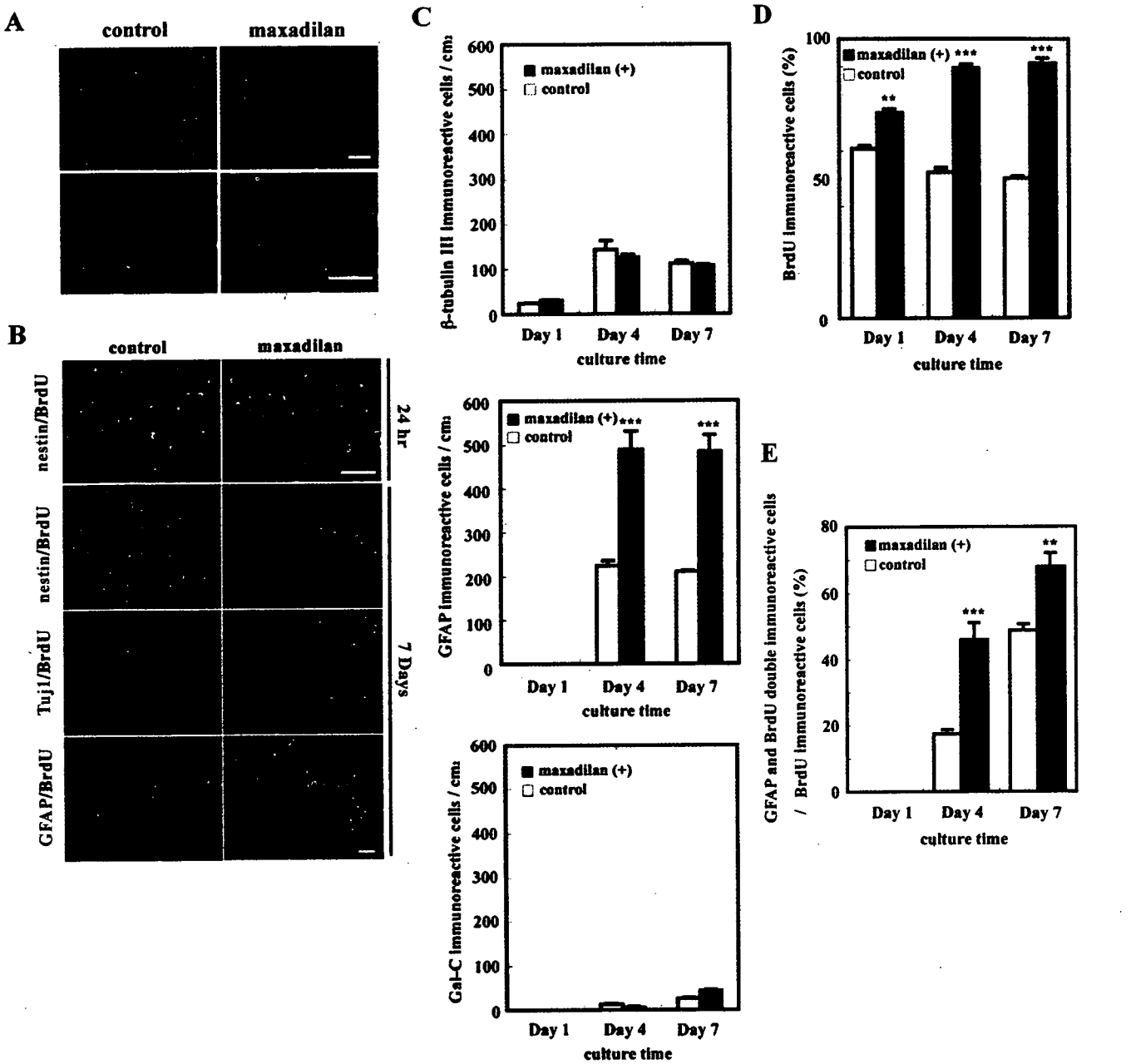

Fig. 6. PAC1 signaling promotes the proliferation of embryonic cortical NPCs committed to astrocytes. **A:** After maxadilan treatment for 24 h, cells were fixed and stained for nestin (green), Hoechst (blue) and BrdU (red). **B:** After maxadilan treatment for 7 days, cells were fixed and stained for nestin (green), β III tubulin (b III tub) (green), GFAP (green), Hoechst (blue) and BrdU (red). **C:** Quantitative analysis of differentiation of embryonic cortical NPCs treated or non-treated with maxadilan for 7 days. **D:** Proliferation of embryonic cortical NPCs treated or non-treated with maxadilan assessed by BrdU incorporation assay. **E:** Time course of astrogenesis from BrdU immunoreactive embryonic cortical NPCs treated or non-treated with maxadilan. Total cellular counts were obtained as described in Materials and Methods. Data represent mean values ± SD (n = 4). Significant differences from the control are indicated by asterisks (*P < 0.05, **P < 0.01, ***P < 0.001; ANOVA). Scale bars: (A, B) 50 μm.

liferation of NPCs with longer cell processes manifesting astrocyte-like shapes. Moreover, most BrdU immunoreactive NPCs were differentiated into GFAP immunoreactive astrocytes via PAC1. One of the autocrine factor families, BMPs, are reported to promote astrocytic differentiation and to change the proliferative activities of cortical NPCs in the different developmental stages (Gross et al., 1996; Mabie et al., 1999). Our data may reflect a dual role for the PACAP/PAC1 system; potentiating glial progenitor cell proliferation and subsequent astrogenesis as well as astrocytic differentiation in NPCs similar to BMPs. The PACAP/PAC1 autocrine system is critical for the regulation of NPC and glial lineage, depending on the stage of brain development.

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Alpha 1-adrenoceptor agonists protect against stress-induced death of neural progenitor cells

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Abstract

Here, we show that α_1-adrenoceptor agonists suppress stress-induced death of mouse embryonic brain-derived neural progenitor cells (NPCs). NPCs highly expressed both α_{1A}- and α_{1B}-adrenoceptor genes, whereas the gene encoding α_{1D}-adrenoceptor was expressed at low levels. Application of the α_1-adrenoceptor agonists phenylephrine and cirazoline significantly promoted cell survival of embryonic NPCs that had been exposed to stress, as measured by a lactate dehydrogenase release assay, but had no remarkable effect on differentiation of the NPCs. Both phenylephrine and cirazoline protected NPCs from death induced by growth factor deprivation, N2 nutrient deprivation, tunicamycin treatment or staurosporine treatment. Phenylephrine and cirazoline treatments both maximally reduced stress-induced cell death by ~60% but did not change the percentage of undifferentiated cells as measured by nestin staining. Moreover, phenylephrine and cirazoline treatments did not affect the cellular activities of caspase-3 and caspase-7 but markedly reduced propidium iodide penetration into the cytoplasm, suggesting that α_1-adrenoceptor agonists inhibit caspase-3/7-independent death of the embryonic NPCs.

Keywords: α_1-adrenoceptor; GPCR; Neural progenitor cell; Cell death; Cell stress; Phenylephrine; Cirazoline

1. Introduction

The noradrenergic system is proposed to play multiple roles in the adult central nervous system (CNS). Apart from its classical transmitter signaling action, noradrenaline has important roles in attention, arousal, and memory reviewed in Murchison et al., (2004); Southwick et al., (1999). Furthermore, it was proposed that noradrenaline influences the survival, maintenance and plasticity of CNS neurons, including the regulation of endogenous neurotrophin systems, glial function, CNS energy utilization and extracellular homeostasis, and has anti-inflammatory and anti-oxidant effects reviewed in Marien et al., (2004). All cell surface adrenoceptors are members of the G protein-coupled receptor family and mediate responses to extracellular noradrenaline. To date, three subfamilies of adrenoceptors (α_1, α_2 and β) have been identified (Bylund et al., 1995). The adrenoceptors are expressed in many tissues, particularly in the cardiovascular, genitourinary and nervous systems. High levels of adrenoceptors are also present in the neocortex during embryogenesis (Lidow and Rakic, 1992), and there are regional concentrations of α_1, α_2, and β adrenoceptors in the fetal forebrain (Lidow and Rakic, 1994). There are three subtypes of α_1-adrenoceptors, the α_{1A}, α_{1B}, α_{1D}-adrenoceptor with varying degrees of efficiency of G protein (Gq/11) coupling ($\alpha_{1A}>\alpha_{1B}>\alpha_{1D}$ adrenoceptor) reviewed in Hieble et al., (1995). This leads to activation of downstream signa

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transduction pathways, including Ca^{2+}, arachidonic acid, phospholipase C and phospholipase D signals reviewed in Zhong and Minneman (1999). α_1-adrenoceptors are specifically localized to NPCs located in the ventricular zone and subventricular zone in the embryonic rat forebrain, and noradrenaline-containing fibers are also present in both the ventricular zone and subventricular zone (Pabbathi et al., 1997). In addition, noradrenaline has been suggested to regulate development of the murine forebrain. The β-adrenoceptor agonist isoproterenol alters proliferation and differentiation of neural precursors in the cerebral cortex (Slotkin et al., 1988). α_1-adrenoceptors were also implicated in controlling cell proliferation and survival in a rat cortical mixed cell culture and in a heterochronic coculture system of the rat neocortex and rostral pons (Pabbathi et al., 1997; Popovik and Haynes, 2000). Although the functions of α_1-adrenoceptors in the mixed-culture NPCs and in the organ culture were investigated, the function of α_1-adrenoceptors and the direct effects of α_1-adrenoceptor-selective agonists such as phenylephrine and cirazoline in isolated pure embryonic NPCs are not known.

The precise role of the α_1-adrenoceptor-mediated signal in embryonic cortical NPCs remains obscure, because the effects observed in the mixed-cell and organ culture experiments possibly reflect the secondary and tertiary effects mediated by multiple cellular interactions (glial cell-progenitor, neuronal cell-progenitor and neuronal cell-glial cell-progenitor interactions). As such, the aim of this study is to know the primary biological effect of α_1-adrenoceptor activation in the NPCs. We addressed the biological effects of α_1-adrenoceptor-selective agonists on highly purified embryonic NPCs. Our data indicate that these agonists inhibit death of NPCs cultured under various stress conditions but do not affect proliferation, differentiation or caspase-3/7-activity.

2. Materials and methods

2.1. Animals

Pregnant C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Animal care and handling were in accordance with institutional regulations for animal care and public law, and were approved by the Animal Investigation Committee of the National Institute of Neuroscience, Japan.

2.2. Antibodies and reagents

Monoclonal and polyclonal antibodies used in this study were as follows: monoclonal anti-nestin (Becton Dickinson, Lexington, KY), monoclonal anti-tuj1 (Covance, Berkeley, CA), polyclonal anti-glial fibrillary acidic protein (Dako, Carpinteria, CA), monoclonal anti-galactocerebroside (Chemicon International, Temecula, CA). The secondary antibodies conjugated to Alexa Fluor dye were purchased from Molecular Probes (Eugene, OR). α_1-adrenoceptor agonists used in this study were (*R*)-3-[1-hydroxy-2-(methylamino) ethyl] phenol (phenylephrine; Sigma, St. Louis, MO) and 2-[(2-cyclopropyl-phenoxy) methyl]-4, 5-dihydro-1H-imidazole (cirazoline; Tocris, Ellisville, MO). Each agonist was dissolved in Neurobasal™ medium (Invitrogen, Carlsbad, CA). Staurosporine and tunicamycin (Sigma) were dissolved in dimethyl sulfoxide (DMSO). Each solution was added to the medium, and the final concentration of DMSO in the medium was adjusted to at most 0.1% (v/v). Medium containing the same amount of organic solvent was used as a negative control.

2.3. Cortical NPCs culture

Cortical NPCs were cultured as previously described (Fukazawa et al., 2006). Briefly, embryos were removed from pregnant C57BL/6J mice and were staged according to morphological criteria to confirm gestational age. Developing mouse brain and cerebral cortex containing the ventricular and subventricular zones were dissected from embryonic day 14 (E14) embryos. Cells were mechanically dissociated by trituration and plated at 3.0×10^6 cells per 10-cm dish (BD) precoated with 15 μg/ml poly-L-ornithine (Sigma) and 1 μg/ml fibronectin (Nitta Gelatin, Osaka, Japan). Cells were expanded for 4 days in serum-free Neurobasal medium supplemented with 0.5 mM L-glutamine (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and B27 (biotin, L-carnitine, corticosterone, ethanolamine, D(+)-galactose, glutathione (reduced), linoleic acid, linolenic acid, progesterone, putrescine, retinyl acetate, selenium, T3 (triodo-l-thyronine), DL-α-tocopherol (vitamin E), DL-α-tocopherol acetate, bovine serum albumin, catalase, insulin, superoxide dismutase, transferrin, vitamin A (Brewer et al., 1993); Invitrogen). N2 supplement (100 mg/l apo-transferrin, 5 mg/l insulin, 16 mg/l putrescine, 6.3 μg/l progesterone, 5 μg/l selenite; Sigma) were used for stress experiments instead of the B27 supplement. This medium was supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ) except when mentioned otherwise. Cultures were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. For secondary cultures, bFGF-expanded cortical NPCs were washed in warm Hanks' balanced salt solution, detached with mechanically pipetting, and resuspended in Neurobasal medium. Cells were then re-seeded on 24-well plates (Nunc; 1.8×10^5 cells/well), or 48-well plates (Nunc; 1.5×10^5 cells/well) precoated with poly-L-ornithine and fibronectin.

2.4. Real-time quantitative reverse-transcription (RT)-PCR

Real-time quantitative RT-PCR with the SYBR Green-based detection method was performed as previously described (Aoki et al., 2002). Total RNA was isolated from cultured cortical NPCs and E14 mouse cerebral cortex. These RNAs (1 μg) were treated with DNase I and converted to cDNA with Superscript II reverse transcriptase (Invitrogen) and random hexamer primers according to the manufacturer's instructions. The efficiency of reverse transcription and the quality of cDNA was compared with the efficiency of PCR amplification of the hypoxanthine guanine phosphoribosyl transferase (hprt) gene (GenBank accession ID. NM_013556; forward primer, 5′-TCTTTGCTGACCTGCTGGATT-3′; reverse primer, 5′-TATGTCCCCCGTTGACTGATC-

3′). Primers were designed for the α_{1A} (NM_013461), α_{1B} (NM_007416) and α_{1D} (NM_013460) adrenoceptor genes using Primer Express software (Perkin-Elmer, Torrance, CA). The forward and reverse primer sequences were as follows: 5′-TTTCAAGCCACCGGAAACA-3′ and 5′-ACTGGATTCGCAGCACATTCT-3′ (α_{1A}); 5′-AACCCTTCTACGCCCTCTTTTC-3′ and 5′-CCAGATTCTTGGTGGTCCTCTT-3′ (α_{1B}); and 5′-TCGCTCAAGTATCCAGCCATT-3′ and 5′-AACCTAGTAGCGGTCCCACAGA-3′ (α_{1D}). SYBR Green-based real-time RT-PCR was performed in 12.5-μl reactions (ABI PRISM 7700 Sequence Detection System, Perkin-Elmer). PCR products were analyzed with agarose gel electrophoresis. We checked each primer individually to ensure that the primer was selective for the target (data not shown). We also ensured that no band was observed in gel electrophoresis of PCRs that included distilled water or total RNA preparation without reverse transcriptase as template. The quantitative RT-PCR method (User Bulletin #2, Applied Biosystems, Foster City, CA) was modified to establish an expression level index for mRNA (Aoki et al., 2002), and the SYBR Green signal for the hprt amplicon was used as a reference. Amplification efficiency was determined and confirmed in a control PCR experiment using serial cDNA dilutions as templates. The real-time RT-PCR products were analyzed using the Applied Biosystems sequence detection system software 1.7.

2.5. LDH and ATP assay

The number of non-viable cortical NPCs was quantitatively assayed by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH) released into the culture medium after membrane rupture. LDH activity was measured using the cytotoxicity assay CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI). To quantify the number of viable cells in cultured cortical NPCs, the amount of cellular ATP was measured using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega). These assays were performed in accordance with the manufacturer's protocol and on secondary cultured cortical NPCs as described above. Four hundred μl (1.5×10^5 cells/well) of cell suspension was added to each well of a 48-well plate (Nunc) precoated with poly-L-ornithine and fibronectin. After 24 h, cells were treated without or with 10 μM phenylephrine or cirazoline with different concentrations of bFGF or under different stress conditions as described in the figure legends. Cultures were then returned to the 37 °C incubator for 24 h, and assessment of LDH release in the media and amount of ATP was conducted with a Wallac 1420 multilabel counter (Perkin-Elmer, Finland).

2.6. Immunocytochemistry

Cells were stained as we have previously described with minor modifications (Sakurai et al., 2006). Briefly, all incubations and washes were performed at room temperature. Cells were fixed with 3.8% formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.02% (w/v) Triton X-100 in PBS for 5 min. Fixed cells were blocked with 3.3% goat serum in PBS for 30 min. Cells were incubated for 30 min with anti-nestin (neural progenitor marker; 1:500), anti-tuj1 (early neuronal cell marker; 1:500)(Sakurai et al., 2006), anti-glial fibrillary acidic protein (astrocyte marker; 1:1000) or anti-galactocerebroside (immature oligodendrocyte marker; 1:200)(Fukazawa et al., 2006). These cells were incubated with diluted secondary antibody (1:200) conjugated to Alexa Fluor for 30 min. All primary and secondary antibodies were diluted in 1% goat serum in PBS before use. The fluorescence microscopy images were obtained with an IX70 microscope (Olympus).

2.7. Quantification of enzymatic activities of caspases

Caspase-3 and caspase-7 protease activities were determined using the Caspase-Glo™ 3/7 Assay kit (Promega). All assays were performed on secondary cultured cortical NPCs as described above. Four hundred μl (1.5×10^5 cells/well) of cell suspension was added to each well of a 48-well plate (Nunc) precoated with poly-L-ornithine and fibronectin. After 24 h, cells were treated with or without 10 μM phenylephrine in medium lacking the N2 supplement as described in the figure legends. Cultures were then returned to the 37 °C incubator for 24 h, and caspase-3 and -7 activities were assessed with a Wallac 1420 multilabel counter.

2.8. Measurement of cell death using propidium iodide

All assays were performed on secondary cultured cortical NPCs as described above. Four hundred μl (1.5×10^5 cells/well) of cell suspension was added to each well of a 48-well plate (Nunc) precoated with poly-L-ornithine and fibronectin. After 24 h, cells were treated with or without 10 μM phenylephrine in medium lacking the N2 supplement. Cultures were then returned to the 37 °C incubator and maintained for 24 h, then stained with 1 μg/ml propidium iodide. Only dead cells with permeable plasma membranes were stained with propidium iodide. Positive controls were stained with propidium iodide after fixing with 3.8% formaldehyde in PBS for 10 min and permeabilized with 0.02% Triton X-100 in PBS. Dead cells and positive controls stained with propidium iodide were counted by fluorometry (Wallac 1420 multilabel counter).

2.9. Statistical analysis

Results are expressed as the mean±standard error of the mean (S.E.M.). Either the Student's *t*-test or Dunnett's multiple range test was used to evaluate the data using Prism software version 4.03 (GraphPad, San Diego, CA). Values of $P<0.01$ and $P<0.05$ were considered statistically significant depending on the specific experiment.

3. Results

3.1. Embryonic cortical NPCs express α_1-adrenoceptor genes

We analyzed gene expression levels of the three α_1-adrenoceptors, and all were expressed both in the E14 embryonic

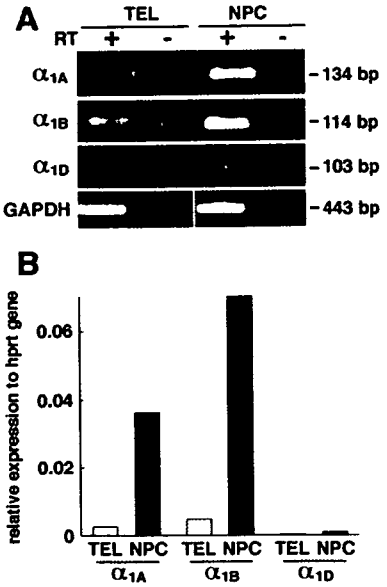

Fig. 1. NPCs derived from E14 telencephalon express α_{1A}-adrenoceptor (α_{1A}), α_{1B}-adrenoceptor (α_{1B}) and α_{1D}-adrenoceptor (α_{1D}). Total RNA isolated from cultured NPCs and E14 mouse telencephalon (TEL) was reverse-transcribed, and the resultant cDNA was used in RT-PCR analysis. (A) As indicated on the left, the PCR products corresponding to the α_{1A}, α_{1B}, and α_{1D} adrenoceptor genes and *GAPDH* were separated on a 3% agarose gel. The size of PCR products are indicated on the right. (B) Gene expression levels of the α_{1A}, α_{1B} and α_{1D} adrenoceptor were analyzed by quantitative RT-PCR, and the expression levels relative to *hprt* are presented. The results are representative of three separate experiments that yielded similar results.

telencephalon and E14 telencephalon-derived cultured NPCs (Fig. 1A). Semi-quantitative analysis of expression levels of the α_1-adrenoceptor genes (normalized to the internal control, *hprt*) showed that the isolated NPCs highly expressed α_{1A}, α_{1B} and α_{1D} adrenoceptor genes as compared with the E14 telencephalon (Fig. 1B). Among the α_1-adrenoceptor genes, the α_{1A} and α_{1B} genes were highly expressed in the cultured NPCs, whereas a low level of expression was detected for the α_{1D} gene in both the E14 telencephalon and cultured NPCs (Fig. 1B).

3.2. Effect of the α_1-adrenoceptor agonist phenylephrine on NPC differentiation

E14 telencephalon-derived NPCs have potencies to differentiate into multiple neural cell types, including neurons, astrocytes and oligodendrocytes, in the absence of bFGF (Fig. 2). The effect of phenylephrine, an α_1-adrenoceptor-selective agonist, on NPC differentiation was examined using the neural cell differentiation marker tuj1 for neuronal cells, glial fibrillary acidic protein for astroglial cells and galactocerebroside for oligodendrocytes. At 72 h after bFGF deprivation, 41.8±1.0% of the NPCs had differentiated into tuj1-positive (tuj1$^+$) cells, 40.6±6.0% of the NPCs had differentiated into glial fibrillary acidic protein-positive (GFAP$^+$) cells and 9.4±1.1% were galactocerebroside-positive (GC$^+$) (Fig. 2A and B). Phenylephrine treatment did not significantly change the percentages of neuronal and glial cells (tuj1$^+$, 38.1±3.2%; GFAP$^+$, 28.7±3.1%; and GC$^+$, 6.2±1.0%) in cultures lacking bFGF (Fig. 2A and B). These results indicate that phenylephrine did not affect NPC differentiation induced by bFGF deprivation. We also examined the effect of phenylephrine on neural differentiation of NPCs in cultures containing bFGF and again found no effect on neuronal or glial differentiation (data not shown).

3.3. α_1-adrenoceptor agonists protect NPCs from cell death

It has been reported that activation of α_1-adrenoceptors stimulates DNA synthesis of embryonic NPCs in mixed culture conditions (Pabbathi et al., 1997). Primary NPC cultures prepared from the telencephalon contain a considerable number of neuronal cells (>10%) that also express α_1-adrenoceptors (Papay et al., 2006). To determine whether the previously reported activation of DNA synthesis was indicative of NPC proliferation or proliferation by secondary effect via other cell types contaminating the culture, we re-seeded cultured NPCs from a primary culture to prepare highly purified secondary

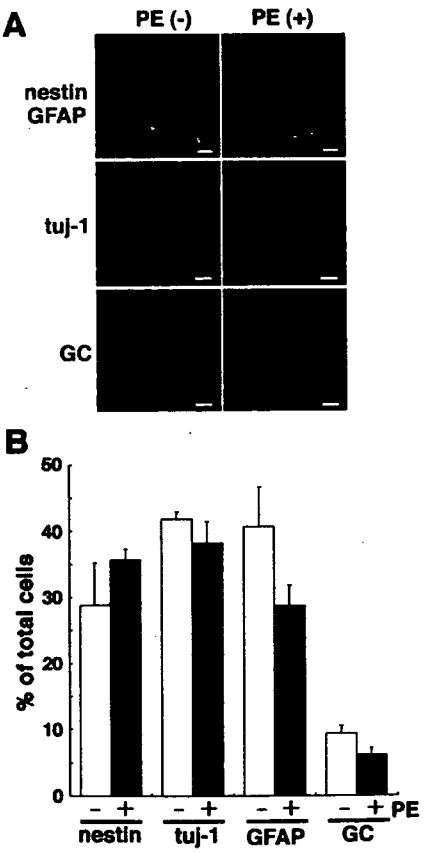

Fig. 2. Effect of the α_1-adrenoceptor agonist phenylephrine on NPC differentiation. (A) Immunofluorescence staining was carried out after 72 h on NPC cultures with or without phenylephrine. Fluorescence microscopic images of cells labeled with anti-nestin (green), anti-GFAP (red), anti-tuj1 (green) and anti-galactocerebroside (green) are shown; nuclei are stained with Hoechst (blue). Scale bar=20 μm. Similar results were obtained in two independent experiments. (B) Secondary cultured NPCs from the E14 mouse telencephalon were maintained *in vitro* for 72 h without or with 10 μM phenylephrine (PE). After 72 h, cells were fixed and immunostained for tuj1, glial fibrillary acidic protein (GFAP), nestin and galactocerebroside (GC). The number of nestin$^+$, tuj1$^+$, GFAP$^+$ and GC$^+$ cells were counted, and the percentages are presented. Nestin was used as a marker for undifferentiated NPCs. No significant differences were observed.

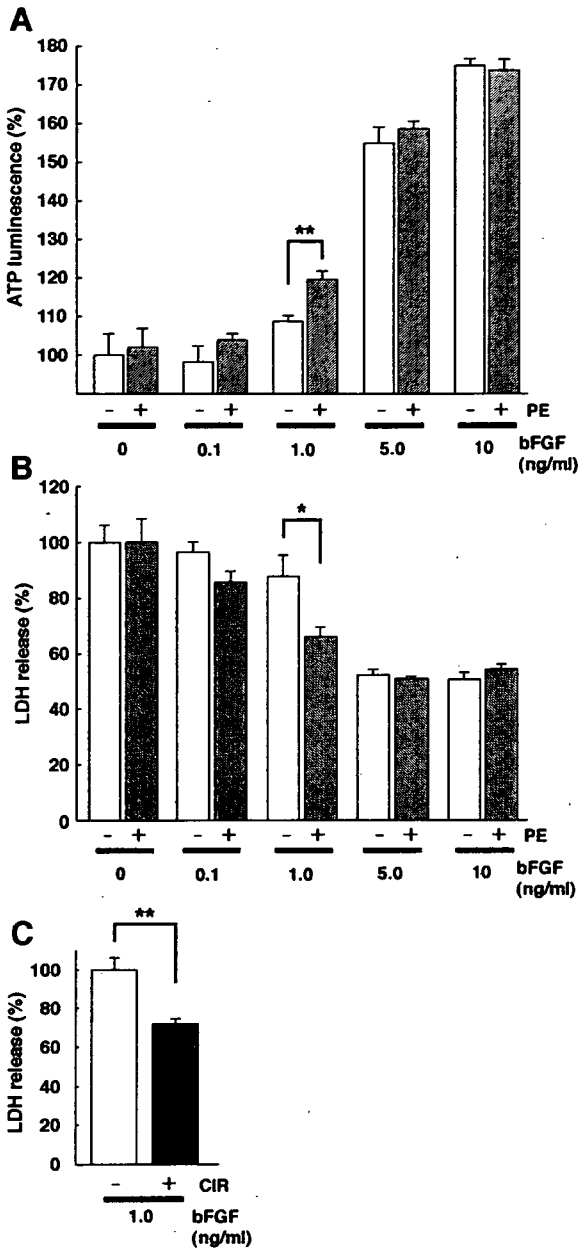

Fig. 3. Effects of α_1-adrenoceptor agonists on the number of cells and cell death of NPCs at various doses of bFGF. Secondary cultured NPCs from the E14 mouse telencephalon were treated without or with 10 μM phenylephrine at different concentrations of bFGF for 24 h. (A) The number of NPCs was examined with an ATP luminescence assay. (B) The viability of NPCs was examined with the LDH release assay. (C) Secondary cultured NPCs from the E14 mouse telencephalon were treated without or with 10 μM cirazoline in 1 ng/ml bFGF for 24 h. Cell survival was assessed by the ATP luminescence assay. Bars represent mean±S.E.M. (n=4). Significant differences are indicated by single or double asterisks (*P<0.05, **P<0.01, Student's t-test).

NPC cultures that contained over 99±0.4% nestin$^+$ undifferentiated NPCs and no more than 0.5% tuj1$^+$ neuronal cells. Using the secondary NPC culture, we examined whether phenylephrine increased the number of NPCs at various doses of bFGF (0~10 ng/ml) using an intracellular ATP luminescence assay (Crouch et al., 1993; Petty et al., 1995). We found that phenylephrine significantly increased the number of NPCs only at a moderate dose (1.0 ng/ml) of bFGF (P<0.01; Fig. 3A) and had no significant effect at high doses (5–10 ng/ml), a low dose (0.1 ng/ml) of bFGF, or no bFGF. To determine whether the effect of phenylephrine on NPCs at 1 ng/ml bFGF was due to promotion of cell growth or cell survival, we examine the effect of phenylephrine on cell death using the LDH release assay (Decker and Lohmann-Matthes, 1988), which measures destruction of the plasma membrane. Phenylephrine also significantly decreased LDH release at 1 ng/ml bFGF (P<0.05; Fig. 3B), indicating that the difference between ATP luminescence (cell numbers) of phenylephrine-treated and

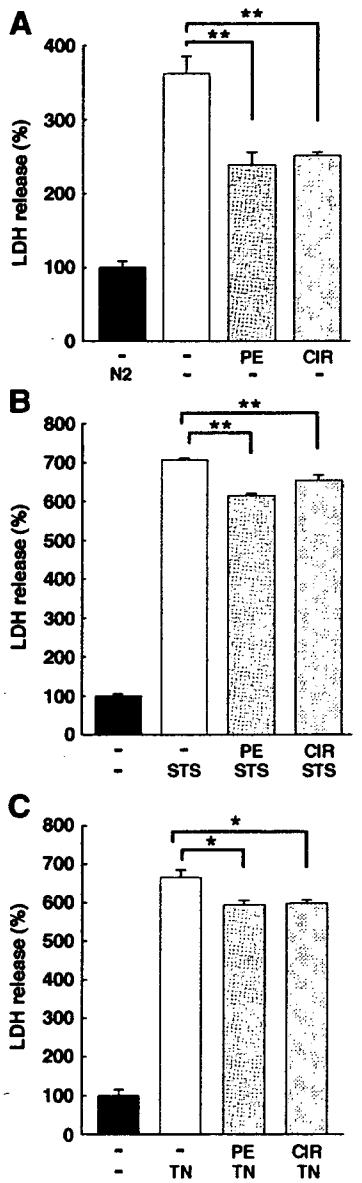

Fig. 4. Phenylephrine and cirazoline promote survival of NPCs under various stress conditions. Secondary cultured NPCs from the E14 mouse telencephalon were incubated in medium lacking N2 (A), 50 nM staurosporine (STS) (B) or 30 ng/ml tunicamycin (TN) (C) in the presence or absence of 10 μM phenylephrine (PE) or 10 μM cirazoline (CIR) for 24 h. Quantification of cell death was performed with the LDH release assay. Bars represent mean±S.E.M. (n=3~5). Significant differences are indicated by single or double asterisks (*P<0.05, **P<0.01, Dunnett's test).

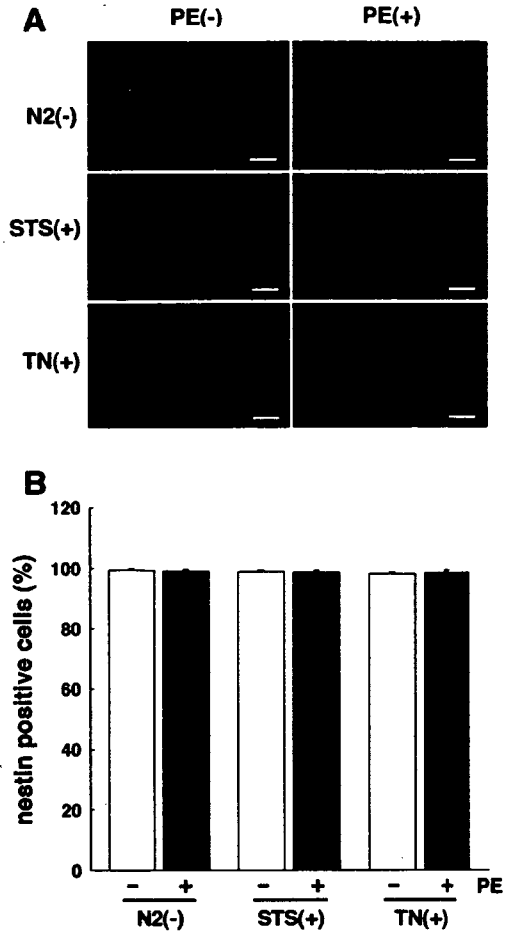

Fig. 5. Effect of phenylephrine on the proportion of nestin-positive cells under various stress conditions. Secondary cultured NPCs were exposed for 24 h to stress conditions without or with 10 μM phenylephrine: N2 deprivation medium, or exposure to 50 nM staurosporine (STS) or 30 ng/ml tunicamycin (TN). After 24 h, the NPCs were fixed and stained with anti-nestin and Hoechst. (A) Fluorescence microscopic images of cells labeled with anti-nestin (green) and Hoechst (blue). Scale bar = 50 μm. (B) The percentages of nestin-positive cells in the cultures were quantified. No significant differences were seen among the groups.

untreated NPCs correlated with the difference in the amount of cell death. These results also indicated that phenylephrine protected NPCs from death during bFGF deprivation-induced stress. Moreover, we confirmed that α_1-adrenoceptor agonists specifically protected NPCs from death using another α_1-adrenoceptor agonist, cirazoline. Cirazoline treatment of NPCs under the same culture conditions resulted in a significant decrease in LDH release ($P<0.01$; Fig. 3C), indicating that α_1-adrenoceptor agonists promote survival of NPCs cultured in 1 ng/ml bFGF. A [^{3}H]thymidine incorporation assay showed that phenylephrine and cirazoline did not induce DNA synthesis of NPCs (data not shown).

3.4. α_1-adrenoceptor agonists prevent NPC death upon exposure to various stresses

To determine the extent to which α_1-adrenoceptor agonists could prevent NPC death, we employed other stress conditions: N2 deprivation, or exposure to 50 nM staurosporine or 30 ng/ml tunicamycin. The results of the LDH release assay for these stress conditions showed that N2 deprivation, staurosporine treatment, and tunicamycin treatment induced LDH release from NPCs (361±24%, 706±5% and 664±21%, respectively, relative to the controls; Fig. 4). However, application of the agonists to the NPC cultures under these stress conditions significantly reduced LDH release (N2 deprivation + phenylephrine, 238±18% $P<0.01$; N2

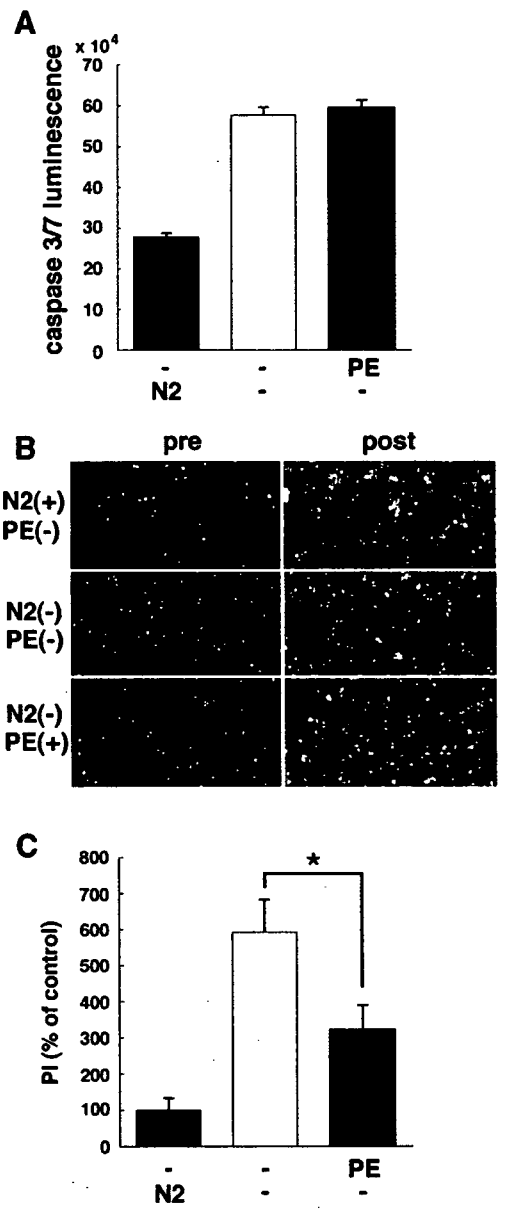

Fig. 6. Phenylephrine does not alter cellular caspase activity but changes propidium iodide penetration into NPCs. Secondary cultured NPCs were incubated without or with 10 μM phenylephrine (PE) under N2 deprivation conditions for 24 h. (A) Caspase-3 and caspase-7 activities in cell lysates were measured by luminometry. (B) Fluorescence microscopy images of dead cells stained with propidium iodide are presented (left). For positive controls, cells were fixed and stained with PI after permeabilization with 0.02% Triton X-100 (right). Scale bar = 100 μm. (C) The intensity of propidium iodide staining was measured by fluorometry. Bars represent mean±S.E.M. ($n=4$). Significant differences are indicated by an asterisk (*$P<0.05$, Dunnett's test).

deprivation+cirazoline, 251±5% $P<0.01$; staurosporine+phenylephrine, 614±9% $P<0.01$; staurosporine+cirazoline, 654±16% $P<0.01$; and tunicamycin+phenylephrine, 594±13% $P<0.05$; tunicamycin+cirazoline, 597±10% $P<0.05$) (Fig. 4). Under the three stress conditions, the differences in the efficacies of phenylephrine and cirazoline on LDH release did not differ greatly. However, both agonists were most effective against N2 deprivation stress, where phenylephrine and cirazoline reduced LDH release 34% and 31%, respectively, as compared with the unstressed control (Fig. 4A). These data suggest that α_1-adrenoceptor agonists protected NPC death under several different stress conditions but exhibited different efficacies depending on the particular stress.

3.5. α_1-adrenoceptor agonists do not modulate the proportion of nestin-positive NPCs under stress conditions

Phenylephrine did not affect differentiation of NPCs in cultures with or without bFGF (Fig. 1), and the high proportion (over 98%) of nestin-positive cells remained for ~24 h even in 1 ng/ml bFGF (data not shown). The ability of the agonists to prevent cell death in 1 ng/ml bFGF was thus a direct effect on the nestin-positive NPCs and not an artifact due to contaminating cells. We also examined the effects of stress induced by N2 deprivation, 50 nM staurosporine or 30 ng/ml tunicamycin on NPC differentiation to exclude the possibility that the increased cell numbers observed following α_1-adrenoceptor agonist exposure, as measured by ATP production, were derived from the differentiated cells induced by the stresses. The proportion of nestin-positive NPCs was not changed by N2 deprivation, staurosporine-or tunicamycin-induced stress, and treatment of NPCs with phenylephrine or cirazoline under these stress conditions did not decrease the proportions of nestin-positive cells (Fig. 5).

3.6. Phenylephrine inhibits caspase-3/7-independent cell death

Of the three stress conditions tested, α_1-adrenoceptor agonists most effectively suppressed stress caused by N2 deprivation, as measured by LDH release (Figs. 3 and 4). Whereas cell death induced by bFGF deprivation, staurosporine treatment or tunicamycin treatment probably was caused by activation of various death signaling pathways, the stress caused by N2 deprivation mainly induced caspase-3/7-dependent cell death in NPC cultures (Fig. 6). To ascertain whether α_1-adrenoceptor agonists protect against caspase-3/7-dependent cell death, we examined the effects of α_1-adrenoceptor agonists on cellular caspase-3/7 activities. Stress caused by N2 deprivation induced elevated cellular caspase-3 and caspase-7 activities as compared with non-stress conditions (>2 fold; Fig. 6A). However, the elevated activities of caspases were not changed by treatment of NPCs with phenylephrine (Fig. 6A). Despite a lack of modulation of the cellular caspase activity, phenylephrine suppressed propidium iodide penetration into NPC cytoplasm (45.4%; $P<0.05$, as compared with untreated cells deprived of N2) (Fig. 6B and C). These data indicate that α_1-adrenoceptor agonists selectively protect against caspase-3/7-independent death of NPCs exposed to stress.

4. Discussion

In this study, we prepared highly purified embryonic NPCs (>99% nestin$^+$ cells) from the E14 mouse cortex and found that the cortical embryonic NPCs highly express α_{1A}- and α_{1B}-adrenoceptor genes but express the α_{1D}-adrenoceptor gene at low levels. Our pharmacological experiments also revealed that α_1-adrenoceptor agonists are protective against NPC death induced by various stresses without any modification of the cell differentiation state of the NPCs. Moreover we demonstrated that α_1-adrenoceptor agonists reduced NPC death caused by the N2 deprivation stress without modulation of intracellular caspase-3/7 activities.

The α_1-adrenoceptor is expressed in the ventricular zone and subventricular zone of the embryonic rat forebrain (Pabbathi et al., 1997). The ventricular zone of the embryonic cerebral cortex contains both undifferentiated NPCs and differentiated nascent neuronal cells. Our quantitative RT-PCR analysis showed that purified cortical NPCs express α_{1A}- and α_{1B}-adrenoceptor genes at high levels compared with the E14 embryonic telencephalon, suggesting that the undifferentiated NPCs highly expressed the α_{1A}- and α_{1B}-adrenoceptor genes. We could not confirm the expression of α_1-adrenoceptor proteins in NPCs because of low specificity of commercially available antibodies against α_1-adrenoceptor in immunocytochemical experiments with NPCs (data not shown). However, we demonstrated that α_1-adrenoceptor agonists have protective effects against cell death in NPCs, indicating the presence of the α_1-adrenoceptors in NPCs.

The chemical structure of phenylephrine ((*R*)-3-[1-hydroxy-2-(methylamino) ethyl] phenol) differs from that of cirazoline (2-[(2-cyclopropylphenoxy) methyl]-4, 5-dihydro-1H-imidazole). However, both α_1-adrenoceptor agonists had the same effect on NPC death induced by a moderate concentration (1 ng/ml) of bFGF, nutritional deprivation (no N2 supplementation), staurosporine treatment or endoplasmic reticulum stress (tunicamycin treatment), indicating that the effects were specifically mediated by α_1-adrenoceptors. It is well known that cell death can be induced via multiple apoptosis signaling pathways that are specifically activated by different stresses. Although the α_1-adrenoceptor agonists were able to protect NPCs from death induced by the stresses we tested, the molecular mechanism that underlies this broad protection is unknown. However, treatment of NPCs with α_1-adrenoceptor agonists failed to decrease the activities of caspase-3 and caspase-7, which are activated in the apoptosis pathway. Instead, α_1-adrenoceptor agonists reduced propidium iodide incorporation induced by stress caused by N2 deprivation. These data suggest that α_1-adrenoceptor agonists protect against necrotic NPC death but not apoptotic NPC death.

Phenylephrine or cirazoline protects against cell death induced by bFGF deprivation as well as by STS and nutritional deprivation, or treatment with staurosporine or tunicamycin. Niidome et al. (Niidome et al., 2006) showed that NPC death resulting from growth factor deprivation is caused by both caspase-dependent and -independent pathways in concert with oxidative stress, suggesting that α_1-adrenoreceptor agonists may also protect against NPC death induced by oxidative stresses. On the other hand, application of phenylephrine is effective only against cell death induced by 1.0 ng/ml bFGF. Complete

deprivation of growth factors activates multiple cell death pathways, including apoptosis, necrosis and the oxidative cell death pathway, in cultured NPCs (Niidome et al., 2006). Therefore, the application of phenylephrine is unlikely to be effective against NPC death induced by the overlapping activation of multiple death pathways after complete loss of growth factor support.

In a recent study, Hiramoto et al. (2006) reported that the stimulation of α_1-adrenoreceptors by phenylephrine or by L-epinephrine induces the proliferation of NPCs derived from cultured neurospheres. Our data indicate that α_1-adrenoreceptor agonists do not induce proliferation of purified NPCs. It is known that the neurospheres have the 3D organization in which nestin-positive (progenitor) cells surround a large core of differentiated GFAP-positive (glial) and β-tubulin III-positive (neuronal) cells (Campos, 2004). As such, the NPC cultures prepared from neurospheres could also contain glial and neuronal cells. Thus, the difference between these data may have resulted from differences in the NPC preparation methods and from the purity of the nestin-positive NPCs.

Transplantation of neural stem and progenitor cells into patients with intractable neurological diseases is considered an effective strategy for neural regeneration therapy reviewed in Lindvall et al., (2004). Studies on cell transplantation/implantation for CNS disorders have indicated that neural stem cells and progenitor cells have the ability to replace lost neurons and to repair the damaged nervous system (Chu et al., 2004). However, a large proportion of grafted cells is lost due to early necrotic death. Thus, the low rate of graft survival reduces the effectiveness of such therapies (Emgard et al., 2003). Here we demonstrated that α_1-adrenoceptor agonists protect against NPC death (probably necrotic death) induced by various stresses. We also demonstrated that α_1-adrenoceptor agonists with this protective effect did not modify the cell differentiation state of the NPCs. Thus, α_1-adrenoceptor agonists may be useful for the preparation and maintenance of neural stem and progenitor cells for transplantation therapy, as they are likely to increase cell viability without induction of unexpected cell differentiation. Moreover, phenylephrine is a popular and safe drug that is used as a non-prescription decongestant (Chua and Benrimoj, 1988) Therefore, phenylephrine may be easily applied as an additional reagent in the cultivation medium of neural stem and progenitor cells for transplantation therapy.

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