The neuroprotective effect of pituitary adenylate cyclase-activating polypeptide on cerebellar granule cells is mediated through inhibition of the CED3-related cysteine protease caspase-3/CPP32

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Communicated by Howard A. Bern, University of California, Berkeley, CA, September 12, 2000 (received for review June 8, 2000)

Caspase-3 knockout mice exhibit thickening of the internal granule cell layer of the cerebellum. Concurrently, it has been shown that intracerebral injection of pituitary adenylate cyclase-activating polypeptide (PACAP) induces a transient increase of the thickness of the cerebellar cortex. In the present study, we have investigated the possible effect of PACAP on caspase activity in cultured cerebellar granule cells from 8-day-old rat. Incubation of granule neurons with PACAP for 24 h promoted cell survival and prevented DNA fragmentation. Exposure of cerebellar granule cells to the specific caspase-3 inhibitor N-benzylxoycarbonyl-Asp-Glu-Val-Asp fluoromethylketone (Z-DEVD-FMK) for 24 h markedly enhanced cell survival and inhibited apoptotic cell death. Time-course studies revealed that PACAP causes a prolonged inhibition of caspase-3 activity without affecting caspase-1. Administration of graded concentrations of PACAP for 3 h induced a dose-dependent inhibition of caspase-3 activity. Incubation of granule cells with both dibutyryl-cAMP (dbcAMP) and phorbol 12-myristate 13-acetate (PMA) mimicked the inhibitory effect of PACAP on caspase-3. Cotreatment of cultured neurons with the protein kinase A inhibitor H89 and the protein kinase C inhibitor chelerythrine abrogated the effect of PACAP on caspase-3 activity. In contrast, the ERK kinase inhibitor U0126 did not affect the action of PACAP on caspase-3 activity. These data demonstrate that PACAP prevents cerebellar granule neurons from apoptotic cell death through a protein kinase A- and protein kinase C-dependent inhibition of caspase-3 activity.

Cerebellum | apoptosis | neurotrophic factors | neuropeptides

The development of the nervous system involves a delicate balance among the processes of proliferation, differentiation, and programmed cell death (1, 2). Neuronal death also occurs in neuropathological conditions such as Alzheimer’s disease (3) or ischemia (4). Programmed cell death is a complex regulatory process that involves a series of cysteinyl aspartate-specific proteases termed caspases (5). Caspases participate in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic structural disassembly of dying cells (6). Activation of caspases occurs after various neuronal insults, including accumulation of amyloid precursor protein (7, 8), ischemia (9), trophic factor withdrawal (10, 11), exposure to radiations (12, 13), or treatment with chemotherapeutic agents (14).

To date, fourteen mammalian homologs of CED-3 (proapoptotic gene of Caenorhabditis elegans) have been characterized (15). Among the identified caspases, two members, caspase-1/ICE and caspase-3/CPP32 appear to be activated during apoptosis of cerebellar granule neurons (14, 16–24). Mice deficient in the caspase-3 gene exhibit a decrease of the number of apoptotic neural cells during development (25). In particular, in the cerebellar cortex of these caspase-3-knockout mice, thickening of the internal granule cell layer has been observed, indicating that the caspase cascade plays a crucial role in the morphogenesis of the cerebellum. Because apoptosis can be blocked or at least delayed by caspase inhibition (26), neurotrophic factors able to decrease caspase activity generate hopes for a more efficient treatment of neuronal disorders.

The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors are actively expressed in the rat cerebellar cortex during postnatal development (27–30). In particular, high concentrations of PACAP binding sites are found in the external granule cell layer (EGL) between postnatal day 4 and postnatal day 20 (27, 28), a period of intense neurogenesis (31). These binding sites correspond to functional receptors positively coupled to adenylyl cyclase and phospholipase C (32, 33). In vivo experiments performed on immature cerebellar granule cells have shown that PACAP is able to delay cell death and to stimulate neurite outgrowth (34). Recent in vivo studies have confirmed that PACAP acts as a neurotrophic factor that prevents cerebellar granule cell death (35). In differentiated cultured neurons, PACAP has also been found to prevent apoptotic cell death, suggesting that PACAP could have a therapeutic value for treatment of neuronal diseases (36–38). The aim of the present study was to investigate the possible action of PACAP on caspase-1 and caspase-3 activity in cerebellar granule cells, and to determine the transduction pathways mediating the effect of PACAP.

Materials and Methods

Chemicals. The 38-amino acid form of PACAP was synthesized by solid phase methodology as previously described (39).
PACAP (6–38) was obtained from the American Peptide (Sunnyvale, CA). N-benzoylcarbonyl-Asp-Glu-Val-Asp fluoromethylketone (Z-DEVD-FMK) was supplied by R & D Systems. H89 was provided by ICN, U0126 was from Promega. Fluorescein diacetate (FDA), propidium iodide, chelerythrine chloride, and cycloheximide were purchased from Sigma Aldrich.

**Cell Culture.** Granule cell suspensions were prepared from cerebellums of 8-day-old Wistar rats, as previously described (40). For cell survival experiments, dispersed cells were seeded in multiwell plates (Costar) at a density of 3.5 × 10⁵ cells per well. For measurements of caspase activity, cells were plated in Falcon 3003 dishes (Becton Dickinson) at a density of 1 × 10⁶ cells per dish. For Western blot analysis, cells were plated in Falcon 3001 dishes at a density of 4 × 10⁶ cells per dish.

**Cell Survival.** Cells were preincubated for 3 h and then cultured for 48 h in the absence or presence of PACAP (10⁻¹¹ to 10⁻⁶ M) or the cell-permeable caspase-3 inhibitor Z-DEVD-FMK (10⁻⁸ to 10⁻⁴ M). Cells were incubated for 5 min with 15 μg/ml FDA (producing green fluorescence in living neurons) and 15 μg/ml propidium iodide (producing red fluorescence in dead cells) and examined on a confocal laser scanning microscope (Noran Instruments, Middleton, WI). For quantification of surviving neurons, cells were incubated for 8 min with 15 μg/ml FDA, rinsed once with PBS, and lysed with a Tris-HCl solution. Fluorescence intensity was measured with an FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT). Pilot experiments have shown that the fluorescence intensity is proportional to the cell number (in the range 5 × 10⁴ to 1 × 10⁶ cells/ml).

**Assessment of Apoptosis.** Detection and quantification of apoptotic cells were performed by the terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) technique with an in situ cell death detection kit (Roche, Nutley, NJ). Cultured cells were examined under a fluorescence microscope (Leitz). Quantification of apoptotic cells was performed by using a flow cytometer (FACSCalibur; Becton Dickinson). Internucleosomal DNA cleavage was assessed by conventional gel electrophoresis after extraction of nuclear DNA by using the Wizard Plus Miniprep DNA purification system (Promega). Intact genomic DNA, which remained trapped in the nuclei, was removed by centrifugation.

**Caspase Activity.** Cultured cells were washed with PBS at 37°C and resuspended in PBS at 4°C. Cells were harvested by centrifugation at 350 × g for 10 min at 4°C and treated with the fluorometric caspase assay system (Promega). Briefly, the cell pellet was resuspended in 10 μl hypotonic cell lysis buffer and centrifuged (16,000 × g, 4°C, 20 min). The supernatant was preincubated for 30 min at 30°C with 32 μl of the caspase assay buffer, 2 μl DMSO, 10 μl DTT (100 mM), and deionized water to a final volume of 100 μl. The samples were then incubated with 2 μl of the caspase-3 substrate Ac-YVAD-AMC (2.5 mM; AMC = 7-amino-4-methylcoumarin) or the caspase-3 substrate Ac-DEVD-AMC (2.5 mM) for 1 h at 30°C. Fluorescence intensity was measured with the microplate reader. The specificity of the assay was verified by adding 2.5 mM of the caspase-3 inhibitor Ac-YVAD-CHO (D-CHO = aspart-1-al) or the caspase-3 inhibitor Ac-DEVD-CHO to the incubation mixture.

**Western Blot Analysis.** Total cellular protein was extracted by incubating neurons in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, and 10 mM EDTA. The homogenate was centrifuged (20,000 × g, 4°C, 15 min), and the supernatant was precipitated by addition of ice-cold 10% trichloroacetic acid. The extract was centrifuged (15,000 × g, 4°C, 15 min) and washed with alcohol/ether. The pellet was denatured in 50 mM Tris-HCl (pH 7.5) containing 20% glycerol, 0.7 M 2-mercaptoethanol, 0.004% (wt/vol) bromophenol blue, and 3% (wt/vol) SDS at 100°C for 5 min, and electrophoresed on a 10% SDS/PAGE. After separation, proteins were electroblotted onto a nitrocellulose membrane (Amersham). The membrane was incubated with blocking solution (1% BSA in Tris-buffered saline containing 0.05% Tween 20) at room temperature for 1 h and revealed with antibodies against the active forms of ERK (Promega) by using a chemiluminescence detection kit (ECL system, Amersham).

**Statistical Analysis.** Data are presented as the mean ± SEM from three independent experiments performed in triplicate or quadruplicate. Statistical analysis of the data were performed using Student’s t test.

**Results**

**Effects of PACAP on Apoptosis of Immature Cerebellar Granule Cells.** The viability of cultured cerebellar granule cells was visualized by double-staining with FDA/propidium iodide (Fig. 1 A and B). After 48 h of culture in control conditions, relatively few cells were still alive, as revealed by green fluorescence staining with FDA (Fig. 1A). Addition of PACAP (10⁻⁷ M) markedly increased the number of surviving cells and enhanced neurite outgrowth (Fig. 1B). Incubation of granule cells with graded concentrations of PACAP (10⁻¹¹ to 10⁻⁶ M) induced a dose-dependent increase in the number of living cells (Fig. 1C).

Fragmentation of DNA in immature granule neurons was visualized on 24-h cultured cells by the TUNEL histochemical technique (Fig. 1D). Exposure of granule cells to 10⁻⁷ M PACAP during the 24-h culture period elicited a marked reduction of the number of apoptotic cells (Fig. 1E). Quantitative analysis by flow cytometry showed that PACAP reduced by 43% the number of labeled cells (data not shown). The occurrence of intense DNA laddering after 24 h of culture confirmed that granule cells undergo apoptotic cell death (Fig. 1F). Incubation of granule cells with 10⁻⁷ M PACAP induced a 60% decrease of the intensity of DNA fragmentation (Fig. 1F). Exposure of cultured granule neurons to the cell-permeable caspase-3 inhibitor Z-DEVD-FMK for 24 h induced a concentration-dependent increase in the number of living cells (Fig. 2A) and a concomitant decrease of DNA fragmentation (Fig. 2B).

**Effects of PACAP on Caspase Activity.** Incubation of cultured granule cells with 10⁻⁷ M PACAP for durations ranging from 1 to 9 h had no effect on caspase-1 activity (Fig. 3A). In contrast, the same dose of PACAP induced a significant inhibition in caspase-3 activity within the first 3 h of incubation (P < 0.001), and the inhibitory effect of PACAP was still observed after 9 h of treatment (Fig. 3B).

A 3-h incubation of granule cells with graded concentrations of PACAP (10⁻¹¹ to 10⁻⁸ M) did not affect caspase-1 activity (Fig. 4A). Conversely, in the same conditions, PACAP induced a concentration-dependent decrease of caspase-3 activity (Fig. 4B). Half-maximum inhibition was observed at a concentration of 0.02 μM, and the maximum effect (P < 0.001) occurred at a dose of 1 μM PACAP. For concentrations ranging from 10⁻¹¹ to 10⁻⁸ M, a 3-h treatment with vasoactive intestinal polypeptide (VIP) did not affect caspase-3 activity (Fig. 4C). A significant decrease of caspase-3 activity was observed only when cultured cells were exposed to 10⁻⁶ M VIP (P < 0.01). Incubation of cultured cells with the PACAP antagonist PACAP (6–38) (10⁻⁶ M) significantly attenuated (P < 0.01) the inhibitory effect of PACAP (10⁻⁸ M) on caspase-3 activity (Fig. 5A). In the presence of the selective caspase-3 inhibitor Ac-
DEVD-CHO, the fluorescence signal was totally abolished (Fig. 5B), thus demonstrating the specificity of the caspase-3 assay.

**Transduction Pathways Involved in the Effect of PACAP on Caspase-3 Activity.** Exposure of cultured granule cells to dibutyryl-cAMP (dbcAMP) (10⁻³ M) induced a robust inhibition of caspase-3 activity (Fig. 6A). Incubation of cells with PMA (10⁻⁷ M) also decreased caspase-3 activity (Fig. 6A). Concomitant administration of dbcAMP and PMA provoked an inhibition of caspase-3 activity that was virtually identical to that induced by 10⁻⁷ M PACAP (Fig. 6A). Incubation of granule cells with the selective protein kinase A (PKA) inhibitor H89 (20 μM) caused a slight attenuation of the PACAP-evoked inhibition of caspase-3 activity whereas the selective protein kinase C (PKC) inhibitor chelerythrine (5 μM) markedly reduced the effect of

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**Fig. 1.** Effect of PACAP on apoptosis of cerebellar granule cells. (A and B) Typical microphotographs illustrating the effect of PACAP on survival and neurite outgrowth. Cells were cultured for 48 h in the absence (A) or presence (B) of 10⁻⁷ M PACAP. Living cells were labeled with FDA (green fluorescence), and dead cells were labeled with propidium iodide (red fluorescence). Scale bar = 10 μm. (C) Effect of graded concentrations of PACAP (10⁻¹¹ to 10⁻⁶ M; 48 h) on survival of cultured cells. (D and E) Microphotographs illustrating the effect of PACAP on DNA fragmentation on cultured cells. Cells were cultured for 24 h in the absence (D) or presence (E) of 10⁻⁷ M PACAP, and DNA fragmentation was labeled by the TUNEL technique. Scale bar = 20 μm. (F) Representative gel electrophoresis illustrating the effect of PACAP on DNA laddering. MW, molecular weight markers in base pairs. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. control (C).
after plating, treatment of granule cells with 10 Kangaroo kinase inhibitor. 

induced inhibition of caspase-3 activity (Fig. 7 A). ERK phosphorylation was totally abolished (Fig. 7 B).

Fig. 4. Effect of graded concentrations of PACAP and VIP on caspase-1 (A) and caspase-3 (B and C) activities. Cultured cells were exposed for 3 h to PACAP (A and B) or VIP (C). **, P < 0.01; ***, P < 0.001 vs. control (Ct).

Coincubation of granule cells with both H89 and chelerythrine totally suppressed the effect of PACAP on caspase-3 activity. Preincubation of granule neurons with the selective ERK kinase inhibitor U0126 (50 Kangaroo) did not affect the PACAP-induced inhibition of caspase-3 activity (Fig. 7 A). Three hours after plating, treatment of granule cells with 10 Kangaroo M PACAP had no effect on ERK phosphorylation (Fig. 7 B). In contrast, 24 h after plating, PACAP (10 Kangaroo M) induced a rapid stimulation of ERK phosphorylation (Fig. 7 B). Incubation of granule cells with H89, chelerythrine, or a combination of H89 and chelerythrine did not abolish the PACAP-induced phosphorylation of ERK (Fig. 7 B). In the presence of U0126, the effect of PACAP on ERK phosphorylation was totally abolished (Fig. 7 C), demonstrating the efficacy of the ERK kinase inhibitor.

Incubation of cultured granule cells with cycloheximide (1 Kangaroo M) alone induced a marked decrease of the basal level of caspase-3 activity (P < 0.001) (Fig. 8). Coincubation of cells with cycloheximide and PACAP did not abolish the ability of PACAP to inhibit caspase-3 activity (P < 0.001).

Discussion
It has been previously reported that PACAP promotes survival and stimulates neurite outgrowth in cultured cerebellar granule cells (34, 36–38). It has been subsequently shown that in vivo administration of PACAP at the surface of the cerebellar cortex increases the number of surviving granule cells in the EGL and internal granule cell layer (35). The present study now demonstrates that the neurotrophic effect of PACAP is mediated through inhibition of the activity of the apoptotic enzyme caspase-3.

Exposure of nondifferentiated granule cells to PACAP for 24 h resulted in a dose-dependent increase in the number of surviving neuroblasts with a maximum effect observed at a dose of 10 Kangaroo M. At this latter concentration, PACAP decreased the intensity of DNA laddering, suggesting that the action of the peptide on granule cell survival can be accounted for by inhibition of programmed cell death. Caspases are critical effectors of apoptotic cell death in the developing brain (6, 41). In particular, it has been previously shown that caspase-1 and caspase-3 are activated during apoptosis of cerebellar neuroblasts (14, 16–24). These observations prompted us to investigate the effect of PACAP on caspase-1 and caspase-3 activity in granule neurons.

A 3-h treatment with PACAP strongly inhibited caspase-3 but not caspase-1 activity in cultured granule cells. Dose-response experiments revealed that PACAP exhibited a high potency in promoting cell survival (ED50 = 10 Kangaroo M) and inhibiting caspase-3 activity (ED50 = 10 Kangaroo M). The inhibitory effect of PACAP on caspase-3 activity preceded the action of the peptide on cell survival (34) and [3H]thymidine incorporation (35), suggesting that the neurotrophic effect of PACAP can be ascribed to caspase-3 inhibition. In support of this hypothesis, the cell-permeable caspase-3 inhibitor Z-DEVD-FMK was found to promote granule cell survival and to inhibit DNA fragmentation in very much the same way as PACAP. The fact that caspase-3 causes activation of deoxyribonucleases that are responsible for DNA fragmentation (42, 43) is consistent with the notion that the inhibitory effect of PACAP on DNA laddering is mediated through inhibition of caspase-3 activity. Moreover, in agreement with recent data obtained in caspase-3-deficient neurons (44), we observed that exposure of granule cells to Z-DEVD-FMK only delayed apoptosis and that, after 72 h of treatment, cell death occurred even in the presence of the caspase-3 inhibitor (data not shown). These observations, together with previous in vivo and in vitro studies showing that PACAP promotes survival of granule neurons during the first 3 days of treatment (34, 35),
provide additional evidence for the involvement of caspase-3 in the neuroprotective activity of PACAP.

Recent studies have shown that immature granule cells from P8 rat cerebellum express all three types of PACAP receptors, that is, the PACAP-selective PAC1 receptor (45, 46) and the VIP/PACAP mutual receptors VPAC1-R and VPAC2-R (46). The present study revealed that VIP was at least 1000 times less potent than PACAP in inhibiting caspase-3 activity, indicating that the effect of PACAP is mediated through activation of PAC1-R. In agreement with this notion, we found that the effect of PACAP on caspase-3 activity was significantly attenuated in the presence of the PACAP antagonist PACAP(6–38). The fact that activation of PAC1-R is also responsible for the cell survival-promoting effect of PACAP (34) is consistent with the implication of caspase-3 in the neurotrophic activity of the peptide.

It has been previously demonstrated that, in cerebellar granule cells, PACAP stimulates the adenylyl-cyclase/PKA (32, 33), the phospholipase C/PKC (32, 33), and the mitogen-activated protein (MAP) kinase (38) pathways. The present data show that treatment of cultured granule cells with the PKA activator dbcAMP or the PKC activator PMA significantly reduced caspase-3 activity. Concomitant administration of dbcAMP and PMA mimicked the inhibitory effect of PACAP on caspase-3 activity. Reciprocally, coincubation of granule cells with the PKA inhibitor H89 and the PKC inhibitor chelerythrine abrogated the effect of PACAP on caspase-3. In contrast, blockage of ERK phosphorylation with the specific MAP kinase kinase inhibitor U0126 did not impair the effect of PACAP on caspase-3 activity. These data demonstrate that the PACAP-induced inhibition of caspase-3 activity in granule neurons is mediated through activation of both the PKA and the PKC but not the ERK-type MAP kinase transduction pathways.

Caspases are synthesized as inactive precursors that are subsequently processed by proteolytic cleavage to generate active heterodimeric enzymes (6, 47). Therefore, a decrease in caspase-3 activity could be ascribed either to a reduction of the biosynthesis of pro-caspase-3 or to a reduction of the processing of the proenzyme. The observation that the PACAP-induced inhibition of caspase-3 activity was not affected by cycloheximide indicates that...
Recent reports indicate that caspase-3 is involved in various pathological disorders, including Alzheimer’s and Parkinson’s diseases (7, 8, 50). Caspase-3 is also activated by ischemic injury (9, 51), and administration of caspase-3 inhibitors has been shown to decrease the infarction volume (52, 53). Similarly, it has been shown that i.v. injection of PACAP prevents the death of hippocampal neurons induced by ischemia (54). These observations, together with the present data showing that PACAP induces a long-lasting inhibition of caspase-3 activity, suggest that PAC1-R agonists that could cross the bloodbrain barrier might be of therapeutic value for preventing apoptotic cell death in neurodegenerative diseases and stroke.

This research was supported by the Institut National de la Sante et de la Recherche Medicale (U-413), an exchange program from the Institut National de la Sante et de la Recherche Medicale-Fonds de la Recherche Scientifique—Quercy (to H. Vaudry), and the Centre Regional de Haute-Normandie. H.V. is Affiliated Professor at the Institut National de la Recherche Scientifique-Institut Armand Frappier.