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Pituitary Adenylate Cyclase Activating Polypeptide Prevents Apoptosis in Cultured Cerebellar Granule Neurons

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SUMMARY

The two forms of pituitary adenylate cyclase-activating polypeptide, PACAP27 and PACAP38, are two neuropeptide hormones related to the vasoactive intestinal peptide/secretin/glucagon family of peptides. PACAP receptors that are positively coupled to adenylyl cyclase and phospholipase C have been identified in cultured cerebellar granule cells. Using the reverse transcription-polymerase chain reaction methodology, we demonstrated the expression of the PACAP-R and PACAP-R-hop mRNAs in cultured granule cells. When grown in the absence of serum or in low K^+ concentrations, these neurons underwent apoptosis, a naturally occurring process characterized by cell shrinkage and internucleosomal DNA cleavage. We used these models of programmed cell death to study the relationship between PACAP receptor activation and neuronal apoptosis. Treatment with PACAP27 and PACAP38 reduced the development of apoptosis in a dose-dependent manner. The neuroprotective activity of PACAP was mimicked by high concentrations of vasoactive intestinal peptide or forskolin but not by carbamylcholine. Thus, we suggest that the activation of type I PACAP receptors may contribute to the survival of cerebellar granule neurons.

PACAP is a member of the secretin/glucagon/VIP family of peptides and exists in two α-amidated forms, PACAP27 and PACAP38, that share the same 27 amino-terminal amino acids and arise from a precursor peptide via post-translational processing (1–4).

Based on the relative affinities for PACAP and VIP, PACAP binding sites have been divided into at least two types (5–7). Type I PACAP receptors specifically bind to PACAP, whereas type II PACAP receptors bind with similar affinity to both PACAP and VIP. In the central nervous system, type I PACAP receptors are abundant, whereas the amount of type II PACAP receptors is negligibly low (8, 9).

The cDNAs of five type I PACAP receptors have been isolated and shown to originate from a common gene by an alternative splicing mechanism (10–15). The shortest cDNA form encodes a 467-amino acid protein with seven transmembrane domains characteristic of G protein-coupled receptors. The other cDNAs diverge from each other at the presence of one or two cassettes, designated "hip" and "hop" (14). The insertion of these cassettes occurs in the carboxyl-terminal end of the third intracellular loop, a domain considered crucial for interaction with G proteins (16), and introduces functional diversity into the PACAP receptors. Indeed, when transiently expressed in porcine renal epithelial LLC PK1 cells or in *Xenopus* oocytes, the PACAP receptor splice variants differently regulate cAMP and inositol phosphate levels on PACAP stimulation, thus indicating coupling to different G proteins (14).

In cultured cerebellar granule cells, activation of type I PACAP receptors leads to cAMP accumulation and polyphosphoinositide hydrolysis (17–19). Although the physiological role of PACAP receptors in cerebellar granule cells is still unknown, previous *in vivo* studies suggest that they may subserve important functions during histogenesis of the cerebellum, such as neuronal survival, proliferation, differentiation, and migration (17, 20).

In the current study, we determined the mRNA expression of type I PACAP receptor variants and examined the relationship between PACAP receptor activation and apoptosis

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ABBREVIATIONS: PACAP, pituitary adenylate cyclase activating polypeptide; VIP, vasoactive intestinal peptide, RT, reverse transcription; PCR, polymerase chain reaction; FCS, fetal calf serum; PGK, phosphoglycerate kinase 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LDH, lactate dehydrogenase; DIV, day(s) of maturation *in vitro*; bp, base pair(s).

in cultured cerebellar granule cells grown either in the absence of serum or in low K^+, according to models reported previously (21–23).

Experimental Procedures

Materials. Synthetic PACAP27, PACAP38, and VIP were purchased from Calbiochem (Milano, Italy). Hoechst 33258, forskolin, and carbamylcholine were obtained from Sigma (Milano, Italy). Basal Eagle's medium and fetal bovine serum were purchased from GIBCO-BRL (Milano, Italy). Tissue culture dishes were obtained from Nunc (Kamstrup, Denmark). Oligonucleotides used for RT-PCR were synthesized by Pharmacia Biotech (Milano, Italy). All of the following materials were purchased from Boehringer Mannheim (Milano, Italy): RNase-free DNase, Moloney murine leukemia virus RT and its buffer, dNTP mix, and *Thermus aquaticus* polymerase and its buffer. Molecular weight markers (100-bp ladder) were obtained from Pharmacia Biotech (Milano, Italy).

Preparation of cell cultures. Primary cultures of cerebellar granule cells were prepared from 8-day-old rats as described previously (24, 25). In brief, cerebella were sliced (450 × 450 μm), and the tissue was dissociated through trypsinization in 0.025% trypsin solution (15 min at 37°) and trituration in the presence of DNase (0.01%) and trypsin inhibitor (0.05%). Dissociated cells were collected through centrifugation and resuspended in standard medium: basal Eagle's medium containing 10% FCS, 2 mM glutamine, gentamycin (0.05 mg/ml), and 25 mM KCl. Cells were plated at a density of 1.8×10^6 onto 35-mm dishes coated with 10 μg/ml poly-L-lysine. Cytosine arabinofuranoside (10 μM) was added after 18 hr of culture to inhibit the growth of non-neuronal cells (21) and did not affect the survival of cultured neurons as assessed through morphological and biochemical analyses (data not shown). Cultures set up this way contain 90–95% granule cells, 2–5% GABAergic interneurons, and a small amount (2–3%) of glial and endothelial cells as contaminants (21, 22).

Experimental procedure. To induce programmed cell death in cerebellar granule cells, we used three different experimental paradigms. Neurons were cultured after their plating in (i) serum-free medium containing 25 mM KCl, (ii) in medium containing 10% FCS and 10 mM KCl (in both of these paradigms, control cells were grown in medium containing 10% FCS and 25 mM KCl), and (iii) in standard medium for 6 days and then washed twice and maintained in serum-free medium containing 5 mM KCl (control cells were washed identically but maintained in serum-free medium supplemented with 25 mM KCl).

Analysis of PACAP receptor mRNAs with RT-PCR. The analysis of PACAP receptor mRNAs with RT-PCR was performed as described previously (26, 27). Primers used to identify type I PACAP receptor mRNAs through RT-PCR were based on the reported sequence for rat PACAP-R-hip-hop1 isoform (14). Oligonucleotide sequences and location of the primers with respect to the initiation of translation were as follows: p1, 5′-CATCCTTGTACAGAAGCTGC-3′ (forward primer, matching the beginning of the third intracellular loop and corresponding to bases 984-1003); p2, 5′-CCTCAGACCAGCATTCACC-3′ (forward primer, matching the end of the "hip" cassette and corresponding to bases 1100–1118); p3, 5′-TCCACCATTACTCTACGGCT-3′ (forward primer, matching the end of the "hop" cassette and corresponding to bases 1198–1217); and p4, 5′-GGTGCTTGAAGTCCATAGTG-3′ (reverse primer, matching a region of the cytoplasmic tail and corresponding to bases 1437–1456). The following is the size of the expected PCR products using primer pair p1/p4: 305 bp for the PACAP-R, 386 bp for the PACAP-R-hop2, 389 bp for the PACAP-R-hip and the PACAP-R-hop1, and 473 bp for the PACAP-R-hip-hop1. The predicted PCR products using primer pair p2/p4 are 273 bp for the PACAP-R-hip and 357 for the PACAP-R-hip-hop1, whereas those obtained with primer pair p3/p4 are 256 bp for the PACAP-R-hop2 and 259 bp for either PACAP-R-hop1 or PACAP-R-hip-hop1.

Primers for rat PGK (28) were included as internal control (for integrity of RNA) in each PCR. The primers used to detect rat PGK were 5′-AGGTGCTCAACAACAACATGGAG-3′ (forward primer, corresponding to bases 759–778) and 5′-TACCAGAGGCCACAGTAGCT-3′ (reverse primer, corresponding to bases 922–941), which generated a 183-bp cDNA fragment.

Total RNA from cultured cerebellar granule cells was isolated through use of the acid-guanidinium isothiocyanate method (29) and treated with RNase-free DNase to remove residual genomic DNA. Single-stranded cDNAs were synthesized with the use of 1 μg of total RNA, Moloney murine leukemia virus RT, and 200 μM of the reverse primer for PACAP receptors (p4) and PGK. To the cDNA reaction we added a PCR master mix to yield 1 μM concentration of specific primers, 200 μM concentration of dNTPs, and 2.5 units of *T. aquaticus* DNA polymerase and *T. aquaticus* buffer containing 1.5 mM $MgCl_2$. PCR was performed with a Perkin-Elmer/Cetus thermal cycler (40 cycles of 94° for 30 sec, 61° for 30 sec, and 72° for 45 sec). Amplification products arising from RT-PCR were separated through electrophoresis (1.8% agarose gel in 0.045 M Tris-borate/1 mM EDTA buffer) and visualized with the use of ethidium bromide staining. At the end of PCR, samples were maintained at 72° for 10 min for final extension and stored at 4°.

The use of RT-PCR was validated on the bases of size and sequence of the amplification products (data not shown).

Microscopic analysis of apoptotic neuronal death. The typical morphological features of apoptotic degeneration were analyzed through the use of fluorescence microscopy with the nuclear dye Hoechst 33258 (30). Cells were fixed in a methanol/acetic acid (3:1 v/v) mixture for 30 min, washed three times in phosphate-buffered saline, and then incubated for 15 min at 37° with 0.4 μg/ml Hoechst 33258. After being washed in water, cells were viewed for determination of nuclear chromatin morphology with the use of a Leitz fluorescence microscope. Apoptotic neurons were recognized on the basis of nuclear condensation and/or fragmented chromatin. Each condition was represented in three dishes per experiment. Both normal and apoptotic neurons were counted from three fields per dish in a fixed pattern.

Analysis of DNA fragmentation. Cells were scraped in phosphate-buffered saline containing 5 mM EDTA and collected through centrifugation. DNA isolation was performed as followed: cell pellets were incubated at 37° for 20 hr in lysis buffer (200 mM Tris·HCl, pH 8.5, 100 mM EDTA, 50 μg/ml proteinase K, 1% sodium dodecyl sulfate). Proteins were removed through phenol/chloroform extraction, and the aqueous phase was precipitated in ethanol and incubated overnight at −20°. RNase A (2 μg/ml) was added to the DNA solution and incubated at 37° for 1 hr. Then, the DNA solution was extracted with phenol/chloroform and precipitated with ethanol. The DNA was resuspended in Tris/EDTA buffer (consisting of 10 mM Tris·HCl, pH 8.0, 1 mM EDTA), and the DNA concentration was determined through absorbance at 260 nm. DNA samples (10 μg/lane) were electrophoresed through a 2% agarose gel and visualized by UV illumination after staining with ethidium bromide.

Immunodetection of oligonucleosomes. Mononucleosomes and oligonucleosomes released from the nucleus into the cytoplasm of apoptotic neurons were detected with the use of a sandwich enzyme-linked immunosorbent assay (cell death detection ELISA, Boehringer Mannheim). The assay is based on quantitative sandwich enzyme-linked immunosorbent assay principle, with mouse monoclonal antibodies directed against DNA and histones, respectively. For sample preparation, cerebellar granule cells were washed in phosphate-buffered saline and then scraped into 400 μl of cold buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol, pH 7.9. Cells were placed on ice for 10 min, added to Nonidet P-40 (0.1% final concentration), and vortex-mixed for 10 sec. Individual homogenates were centrifuged for 10 min with an Eppendorf microfuge at 13,000 × *g*. The supernatant was diluted to yield 13×10^3 cell equivalents/ml and used for immunodetection. The assay was performed as follows: (i) an antibody

that reacts with the histones H1, H2A, H2B, H3, and H4 was fixed on the wall of a microtiter plate module provided with the kit; (ii) samples prepared as described above were added to the plate containing the immobilized anti-histone antibody; (iii) anti-DNA monoclonal antibodies conjugated to peroxidase were added, to allow their binding to the DNA part of nucleosomes; and (iv) after removal of unbound peroxidase conjugate, the amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2′-azino-di(3-ethylbenzthiazoline sulfonate) as a substrate.

LDH release. When indicated, LDH released into the culture medium was used as a measure of cell death. LDH activity was determined spectrophotometrically, as described previously (31).

Results

The mRNA expression of type I PACAP receptors in cultured cerebellar granule cells. To identify the type I PACAP receptor isoform(s) expressed in cultured cerebellar granule cells, we analyzed the expression of their transcripts with the use of RT-PCR (26, 27). To discriminate among the five known receptor splice variants, we used three pairs of primers. The first primer pair (p1/p4) is external to the site of insertion of the hip/hop exons and produces a PCR product for each of the PACAP receptor isoforms. The other two primer pairs, p2/p4 and p3/p4, were used to discriminate the PACAP-R-hip and the PACAP-R-hop variants, respectively. To control for the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, the levels of mRNA transcripts for rat PGK were simultaneously determined in each RT-PCR.

Fig. 1 shows a representative agarose gel stained with ethidium bromide. With primers p1/p4 (*lane 1*), two amplification products of ~300 and ~390 bp were observed in cerebellar granule cells at 1 DIV. The size of the first fragment is indicative of the PACAP-R variant, whereas the latter, as confirmed by the amplification with primer pairs p2/p4 and p3/p4 (*lanes 2* and *3*, respectively), represents the PACAP-R-hop isoform.

PACAP stimulation reduces apoptosis induced by serum deprivation in cultured cerebellar granule cells. Cultured granule cells grown in the presence of 10% FCS and 25 mM KCl were healthy and viable up to 9 DIV (Fig. 2A; see also Fig. 6A). Almost all of the cells grown in the absence of FCS underwent apoptosis within 48–72 hr (Figs. 2B and 3). Microscopic analysis of cultures grown in the absence of FCS with the fluorescent nuclear dye Hoechst 33258 revealed the typical features of apoptotic degeneration (32, 33), including chromatin condensation and fragmentation (Fig. 2B).

The presence of an apoptotic process in cerebellar granule cells grown in the absence of FCS was further supported by

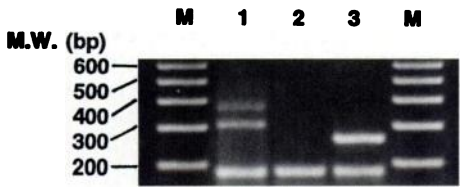

Fig. 1. RT-PCR analysis of PACAP receptor mRNAs. Total RNA from cultured cerebellar granule cells at 1 DIV was reverse transcribed and PCR amplified with three primer pairs: p1/p4 (*lane 1*), p2/p4 (*lane 2*), and p3/p4 (*lane 3*). Primers for PGK were used as internal control in each PCR and generated a 183-bp DNA fragment. *M*, DNA standard lane. For experimental details, see Experimental Procedures.

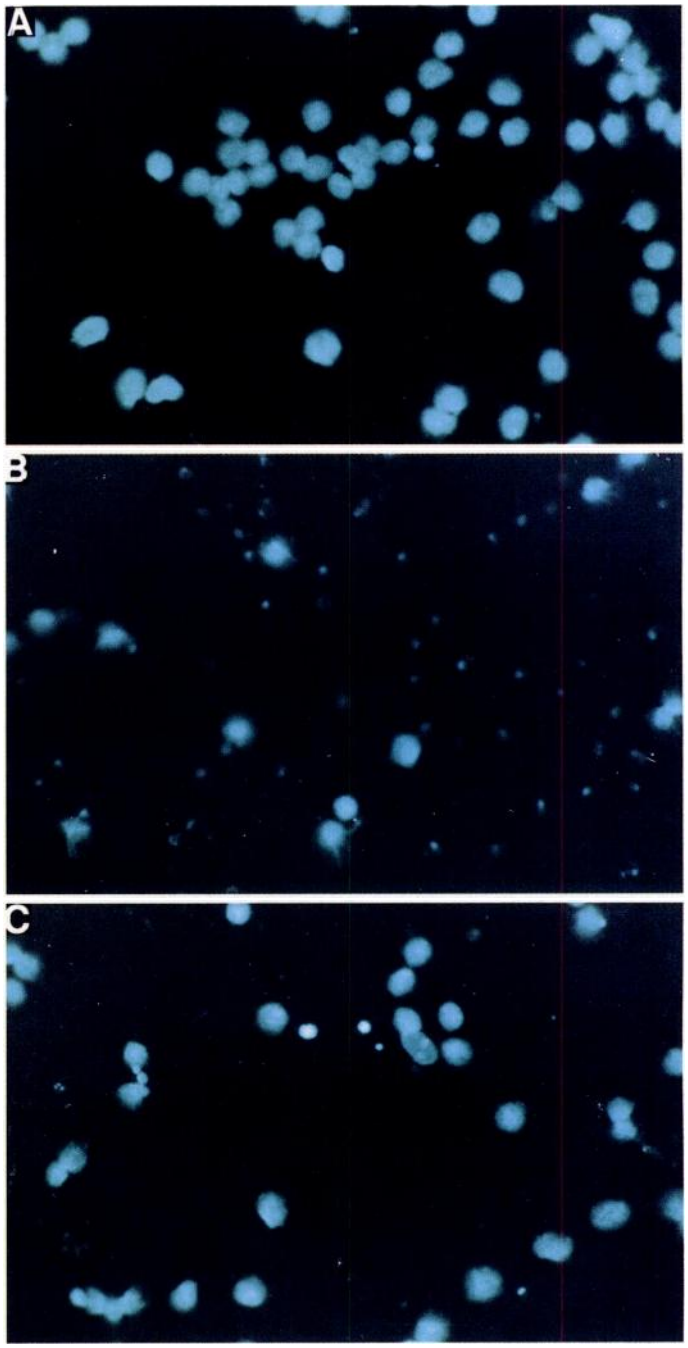

Fig. 2. Photomicrographs of cultured cerebellar granule cells stained with the fluorescent nuclear dye Hoechst 33258. Cells were grown in the presence of 10% FCS and 25 mM KCl (A), in serum-free medium containing 25 mM KCl (B), and in serum-free medium containing 25 mM KCl and 100 nM PACAP38 (C). Staining and microscopic analysis was performed in 2-DIV cultures. Cells were viewed at 40× magnification.

the characteristic fragmentation of DNA into nucleosomal-sized fragments (Fig. 4) and the release of oligonucleosomes from the nucleus to the cytoplasm (Fig. 5).

To study the effect of PACAP receptor activation on the development of apoptotic neuronal death, we observed the effect of PACAP27 and PACAP38 at a concentration (100 nM) that is able to maximally stimulate cAMP and inositol phosphate accumulation in cultured cerebellar granule cells (17–19). Exposure of cerebellar granule cells to PACAP27 and

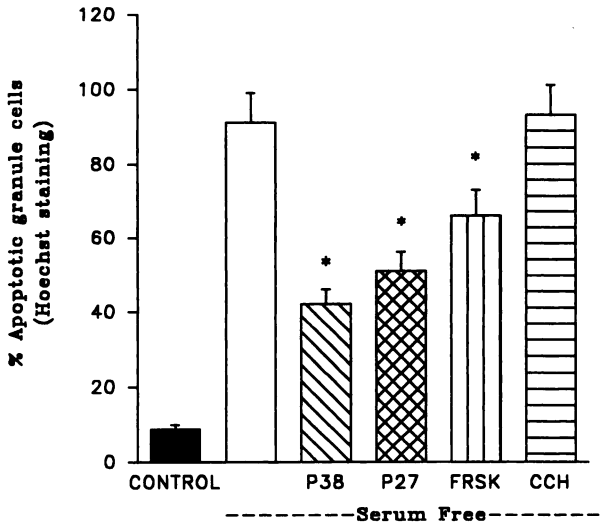

Fig. 3. Effects of PACAP, forskolin, and carbamylcholine on apoptotic cerebellar granule cells grown in the absence of serum. Cerebellar granule cells were grown in the presence (*CONTROL*) or absence of serum for 48 hr and treated with vehicle (*open bar*), PACAP38 (100 nM) (*P38*), PACAP27 (100 nM) (*P27*), forskolin (10 μM) (*FRSK*), and carbamylcholine (1 mM) (*CCH*). Apoptotic neurons were revealed and quantified through the use of Hoechst staining. Data are expressed as percentage of apoptotic neurons relative to the total number of neurons, and values are mean ± standard error of three to five determinations. *, $p < 0.05$ versus serum free cultures (Student's *t* test).

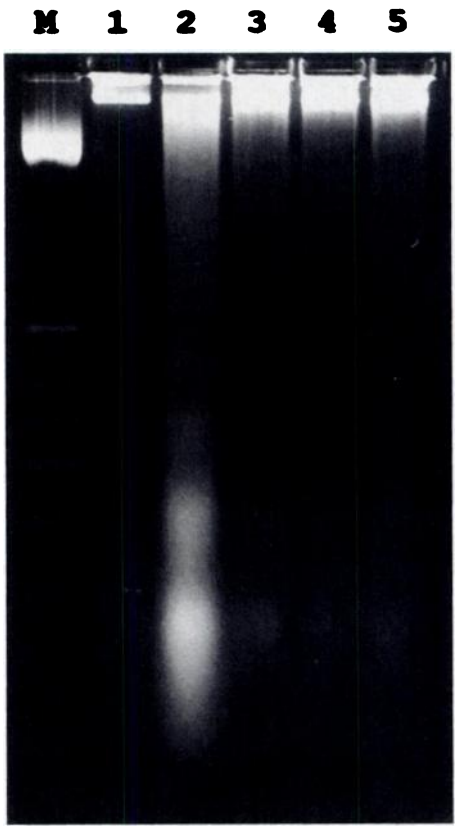

Fig. 4. Gel electrophoresis of DNA extracted from cultured cerebellar granule cells at 2 DIV. *Lane 1*, cultures grown in the presence of FCS. *Lanes 2–5*, cultures grown in the absence of FCS and treated with vehicle, PACAP27 (100 nM), PACAP38 (100 nM), and VIP (1 μM), respectively. *M*, DNA standard lane (100-bp ladder).

PACAP38 significantly reduced the development of apoptosis induced by the absence of FCS, as assessed both morphologically (Figs. 2C and 3) and biochemically (Figs. 4 and 5). The protective effect of PACAP27 and PACAP38 was already present at picomolar concentrations and maximal (~50% reduction) at 100 nM (Figs. 3 and 5). The action of PACAP 27 and PACAP38 was mimicked by forskolin (10 μM) but not by carbamylcholine (1 mM) (Fig. 3), whereas VIP was effective only at 1 μM (Figs. 4 and 5).

PACAP stimulation prevents apoptosis induced by low K^+ in cultured cerebellar granule cells. In cultured cerebellar granule cells, apoptosis can be triggered by lowering the concentration of extracellular potassium to 10 mM since their plating (23) or allowing neurons to fully develop in standard medium for 6–7 days and then shifting to medium containing 5 mM K^+ (21, 22). We used both experimental paradigms to investigate the ability of PACAP to prevent apoptosis induced by low K^+.

The addition of 100 nM PACAP38 at 1, 3, and 5 DIV, almost completely prevented apoptosis of cerebellar granule cells grown in medium containing 10 mM K^+ and 10% FCS for 6 days. This was assessed in sister cultures either morphologically, through fluorescent microscopy with the nuclear dye Hoechst 33258 (Fig. 6 and Table 1), or biochemically, through measurement of LDH release into the medium (Table 1) and DNA fragmentation (data not shown). PACAP treatment was also effective in preventing apoptosis of cerebellar neurons grown in serum-free medium containing 5 mM K^+ for 72 hr after being allowed to develop for 6 days in standard medium (Table 1).

Discussion

Previous studies have demonstrated the functional expression of type I PACAP receptors coupled to cAMP accumulation and polyphosphoinositide hydrolysis in cultured cerebellar granule cells (17–19). To date, the cDNAs of five type I PACAP receptors have been isolated and shown to originate from a common gene through alternative splicing (10–15). Because of the structural homology and pharmacological similarity of the type I PACAP receptors, the ligand binding probes currently available do not distinguish among the various isoforms. To investigate the expression of type I PACAP receptor isoforms in cultured cerebellar granule cell, we therefore analyzed their respective mRNAs with the use of RT-PCR. This analysis revealed the mRNA expression of the PACAP-R and PACAP-R-hop variant at 1 DIV. We cannot rule out the possibility that the PACAP-receptor mRNAs detected by RT-PCR derive from other neurons or non-neuronal cells present in the culture. However, this possibility appears unlikely because (i) primary cultures of cerebellar granule cells represent a highly homogeneous population of neurons (~95%) (24) and (ii) the presence of type I PACAP binding sites on cerebellar granule cells has been specifically shown by autoradiography both *in vivo* at postnatal day 8 and *in vitro* at 1 DIV (17, 20).

One- or two-receptor models were previously envisioned to account for the activation of adenylyl cyclase and phospholipase C elicited by PACAP in cerebellar granule cells (18, 19). Our data suggest that PACAP-induced cAMP and inositol phosphate formation in cerebellar granule cells is mediated by two receptors, the PACAP-R and PACAP-R-hop variants, coupled to adenylate cyclase and phospholipase C.

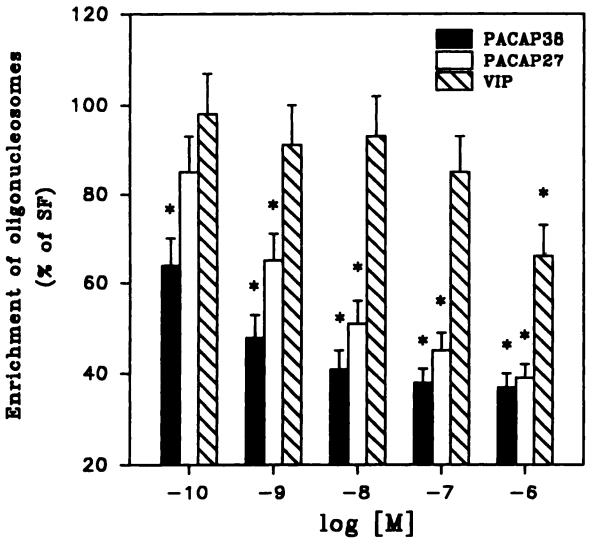

Fig. 5. Effect of PACAP and VIP on oligonucleosome formation in cerebellar granule cells grown in the absence of serum. The effects of increasing concentrations of PACAP27, PACAP38, and VIP on oligonucleosome formation were measured in cultured cerebellar granule cells grown in the absence of FCS. Values (mean ± standard error) are expressed as percentages of control cultures grown in the absence of serum and were calculated from 6–10 determinations in two different experiments. Absolute values were 234 ± 28 and 750 ± 62 munits of absorbance (A_{405nm}/A_{490nm})/1300 cell equivalents in cultures grown in the presence of absence of serum, respectively. *, $p < 0.05$ (Student's *t* test).

Although these receptor variants are able to stimulate simultaneously both transduction pathways in transfection experiments (14), this possibility must be verified for the native PACAP receptors in cerebellar granule cells.

Several lines of evidence suggest that PACAP acts as a neurotrophic factor: PACAP was shown to promote neurite outgrowth in neuroblastoma and pheochromocytoma cells (34–36), to stimulate cell proliferation in various tumoral cell lines (37, 38), and to prevent naturally programmed neuronal cell death in motor neurons from the chick embryo (39). The expression of PACAP receptors in rat cerebellum during neonatal life is temporally related to periods of increased growth, consolidation of synaptic connections, and high synaptic plasticity (17, 20, 27, 40). In the cerebellum of 4-day-old rats, high densities of PACAP binding sites have been observed in the developing medulla and in the external granule layer, a germinative matrix that generates most of cerebellar interneurons. From P8 to P25, PACAP binding sites gradually vanish in the external granule layer in parallel with the involution of the germinal layer and appear in the internal granule layer (17, 20). We recently described a switch in the splicing pattern of the PACAP receptor gene during postnatal development of the cerebellum (27). The expression of the PACAP-R-hop and PACAP-R variants occurs during early and late postnatal development, respectively. The expression of the PACAP-R-hop variant is temporally related to the PACAP stimulation of cAMP and inositol monophosphate formation, whereas the expression of the PACAP-R is correlated with the activation of adenylate cyclase only. Based on these observations, the expression of PACAP receptors during histogenesis of the cerebellum has been involved in functions such as survival, proliferation, differentiation, and migration of cerebellar interneurons.

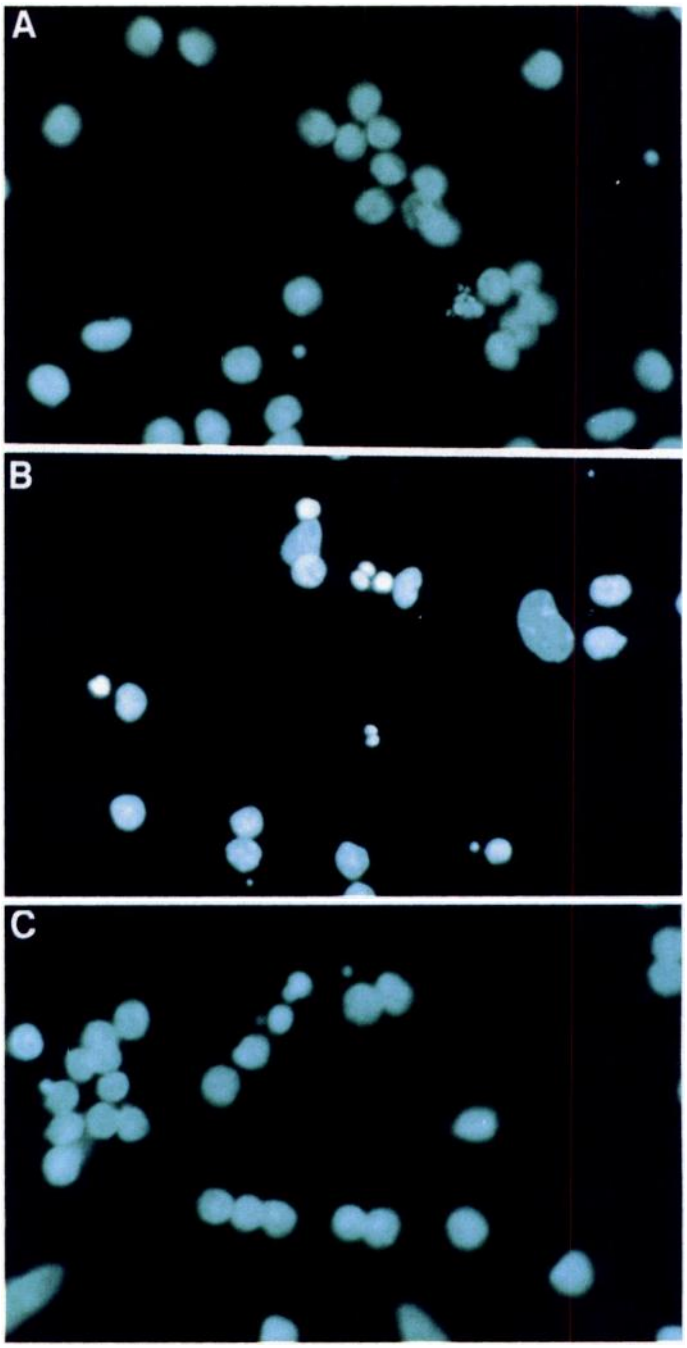

Fig. 6. Photomicrographs of cultured cerebellar granule cells stained with the fluorescent nuclear dye Hoechst 33258. Cells were grown in the presence of 10% FCS and 25 mM KCl (A), 10 mM KCl (B), or 10 mM KCl and 100 nM PACAP38 (C). Staining and microscopic analysis were performed in 6-DIV cultures. Cells were viewed at 40× magnification.

In the current study, we examined the effect of PACAP receptor activation and apoptotic neuronal death in primary cultures of differentiating or fully developed cerebellar granule cells, according to the models described previously (21–23). Exposure of cerebellar granule cells to PACAP27 and PACAP38 significantly reduced in a dose-dependent manner the development of apoptosis induced by the absence of FCS or by low K^+, as assessed both morphologically and biochemically. The fact that VIP treatment exhibited significant effects on neuronal survival only at 1 μM suggests that the

TABLE 1

PACAP prevents apoptosis of cultured cerebellar granule neurons grown in low K^+

Apoptosis was induced by (A) growing cells for 6 days in 10% FCS and 10 mM KCl (K10) or (B) shifting cells from standard medium to serum-free medium containing 5 mM KCl at 6 DIV. Apoptotic neurons were revealed with the use of Hoechst 33258 staining. LDH values refer to milliunits of absorbance/min. Values are mean ± standard error of four determinations.

Culture conditions	Apoptotic neurons	LDH release
	% total population	*munits absorbance*
A		
FCS + K25	8 ± 3	28 ± 3
FCS + K10	32 ± 5	56 ± 5
FCS + K10 + PACAP38	12 ± 3[a]	27 ± 4[a]
B		
K25	11 ± 2	31 ± 4
K5	30 ± 3	58 ± 3
K5 + PACAP38	14 ± 4[b]	28 ± 5[b]

[a] $p < 0.01$ versus FCS + K10 (Student's *t* test).
[b] $p < 0.01$ versus K5 (Student's *t* test).

neuroprotective effect of PACAP results from activation of type I PACAP receptors. Although PACAP activates a dual signal transduction mechanism in cultured cerebellar granule cells (17–19), its neuroprotective action is probably mediated via stimulation of adenylate cyclase because it was mimicked by forskolin but not by carbamylcholine, agents that stimulate adenylate cyclase and phospholipase C, respectively. However, the possibility that both signal transducing mechanisms cooperate in reducing apoptosis induced by serum deprivation cannot be ruled out.

Extensive neuronal death occurs during the normal development of the nervous system. This programmed neuronal death is often controlled by survival-promoting signals from other cells (41) and serves to match neuronal number to target size and to rid the nervous system of inappropriate connections. In the developing cerebellum, a loss of granule cells between postnatal days 5 and 9 is thought to regulate the granule cell to Purkinje cell stoichiometry (42). Indeed, the number of granule cells in the adult is directly proportional to the number of Purkinje cells, indicating the existence of factors that regulate the survival of granule cells. Based on the selective localization of PACAP-like immunoreactivity in Purkinje cells (43) and its neuroprotective effect *in vitro*, PACAP receptor activation may represent one of the endogenous mechanisms that regulate survival of cerebellar granule cells *in vivo*.

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