Pituitary Adenylyl Cyclase-activating Polypeptide Prevents Induced Cell Death in Retinal Tissue through Activation of Cyclic AMP-dependent Protein Kinase*

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Multiple neuroactive substances are secreted by neurons and/or glial cells and modulate the sensitivity to cell death. In the developing retina, it has been shown that increased intracellular levels of cAMP protect cells from degeneration. We tested the hypothesis that the neuroactive peptide pituitary adenylyl cyclase-activating polypeptide (PACAP) has neuroprotective effects upon the developing rat retina. PACAP38 prevented anisomycin-induced cell death in the neuroblastic layer (NBL) of retinal explants, and complete inhibition of induced cell death was obtained with 1 μM. A similar protective effect was observed with PACAP27 and with the specific PAC1 receptor agonist maxadilan but not with glucagon. Photoreceptor cell death induced by thapsigargin was also prevented by PACAP38. The neuroprotective effect of PACAP38 upon the NBL could be reverted by the competitive PACAP receptor antagonist PACAP6–38 and by the specific PAC1 receptor antagonist Maxd.4. Molecular and immunohistochemical analysis demonstrated PAC1 receptors, and treatment with PACAP38 induced phospho-cAMP-response element-binding protein immunoreactivity in the anisomyein-sensitive undifferentiated postmitotic cells within the NBL. PACAP38 produced an increase in cAMP but not inositol triphosphate, and treatment with the cAMP-dependent protein kinase inhibitor Rp-cAMPS blocked the protective effect of PACAP38. The results indicate that activation of PAC1 receptors by PACAP38 modulates cell death in the developing retina through the intracellular cAMP/cAMP-dependent protein kinase pathway.

Developmental cell death is a major event in neurogenesis, controlled by various secreted molecules, many of which play distinct roles in the mature nervous system. Identification of neuroprotective molecules is relevant both for embryogenesis as well as for studies of neurodegenerative diseases because the modes of cell death and many upstream control pathways appear to be conserved among both normal and pathological conditions (1). Classically, neuroprotection is attributed to neurotrophic proteins such as the neurotrophin family of growth factors (for a review, see Refs. 2 and 3), but many lines of evidence support the involvement of both classical neurotransmitters and neuropeptides in the control of cell death (4, 5).

The retina of newborn rats is composed of two cellular strata separated by the inner plexiform layer. The innermost cellular stratum is the ganglion cell layer, the long axons of which form the optic nerve. On the opposite side of the inner plexiform layer, the outer cellular stratum contains a few rows of early developing amacrine cells in the inner nuclear layer. The remainder of the outer stratum constitutes the neuroblastic layer (NBL), which corresponds to the ventricular zone, in which high proliferative activity persists postnatally (6). In addition to the proliferating neuroblasts, the NBL in newborn rats contains undifferentiated postmitotic cells that are migrating toward their final destinations across the depth of the retinal tissue as well as a row of regularly spaced, early differentiating horizontal cells. At about 4 days after birth, the outer plexiform layer separates the neuroblastic layer from an outer nuclear layer, which progressively concentrates at the outermost retinal tier the cell bodies of the photoreceptors (7). More than 95% of the latter are of the rhodopsin-containing rod type.

Evolution into the multilayered structure of the mature retina is accompanied by a wave of naturally occurring cell death that shapes the final cell populations, similar to other areas of the central nervous system. The period of naturally occurring cell death is closely associated with both neuronal differentiation and the encounter of both target and afferent partners and tends to precede the establishment of the mature morphology of synaptic connections.

Reproducible timing and a close relation with the stage of maturation of the various cell populations imply tight regulation of sensitivity to programmed cell death. Indeed, a host of extracellular molecules that affect programmed cell death were identified in experimental models of chemically or otherwise induced cell death in the developing retina (8–12). Among those, we showed that dopamine protects the postmitotic undifferentiated retinal cells from degeneration induced by inhibition of protein synthesis through an increase in intracellular cAMP (13).

The pituitary adenyl cyclase-activating polypeptide (PACAP) is a neuroactive peptide of the secretin/glucacon/vasoactive intestinal peptide (VIP) superfamily. PACAP was first isolated for its ability to induce the production of cAMP in

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1 The abbreviations used are: NBL, neuroblastic layer; PACAP, pituitary adenyl cyclase-activating polypeptide; PKA, cAMP-dependent protein kinase; CREB, cAMP-response element-biding protein; VIP, vasoactive intestinal peptide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; IP3, inositol triphosphate; BrdUrd, 5-bromo-2′-deoxyuridine; Rp-cAMPS, adenosine 3′,5′-cyclic monophosphorothioate; Rp-isomer, 6-CI-PB, (±)-6-chloro-7,8-dihydro-1-phenyl-2,3,4,5-tetrahydro-1H-benzazepine; p, postnatal.
PAC1 receptor isoforms (29). The PACAP precursor molecule is post-translationally processed into two biologically active products, PACAP38 and PACAP27 (14, 15), which share high amino acid homology with VIP. In the nervous system, PACAP has been associated with proliferation (16), differentiation (17, 18), and cell survival (5, 19, 21, 22).

Both PACAP and VIP act on receptors described pharmacologically as either type I (PACAP-specific) or type II (non-discriminative) (23–25). Molecular cloning revealed three distinct receptors: PAC1, VPAC1, and VPAC2, of which the PAC1 receptor is selective for PACAP. The present study was designed to examine the effects of PACAP on induced cell death in the developing retina and the mechanisms that mediate its effects on retinal cell survival.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture medium, fetal calf serum, and Trizol were from Invitrogen. Anisomycin was from Sigma. PACAP38 was from Peninsula Laboratories, San Carlos, CA. U73122, verapamil, nifedipine, and U73342 cAMPS were from Calbiochem. First-strand cDNA kit was from Amersham Biosciences. PACAP27 was kindly provided by Dr. Ethan Lerner from Harvard Medical School. Apoptag Tunel kit was from Intergen, Purchase, NY, and the antibody CM1 for activated caspase-3 was kindly provided by Dr. Anu Srivinasan (Idun Pharmaceuticals).

**Tissue Culture and Histology**—All experimental procedures with animals were approved by the Committee on Animal Experimentation of the Institute of Biophysics Carlos Chagas Filho, based on the currently accepted international rules. Retinæ were excised from the eyes of either 2-day-old or 6-day-old Lister hooded rat pups killed by instantaneous decapitation, and explants of ~1 mm² were maintained in an orbital shaker at 70–90 rpm in basal medium of Eagle supplemented with 5% fetal calf serum and 20 mM HEPES at pH 7.4 for 22 h except when noted. At the end of each experiment, the explants were fixed by immersion in 4% paraformaldehyde in sodium phosphate buffer, pH 7.4, for 2 h and then cryoprotected in 30% sucrose in phosphate buffer. Transverse 10-μm-thick sections through the retinal tissue were cut at −20°C in a cryostat and stained either with neutral red (postnatal day 2 [P2] explants) or with a monoclonal antibody to the rod photoreceptor pigment rhodopsin (P6). Although there is a clear centro-peripheral gradient of development in the rat retina, previous work showed that retinal explants respond to the induction of cell death upon exposure to either a given cell type or cells at a given stage of differentiation (i.e. either proliferating or postmitotic) are the same in both central and peripheral locations (26).

In the current experiments, usually eight explants were cut from each retina, and explants from at least six distinct retinæ were pooled irrespective of retinal location in each experimental group.

**Quantitative Measurement of Cell Death and Identification of Apoptosis**—Dead cells were recognized by their condensed homogeneously and deeply stained chromatin among normal neighboring cells when explant sections were stained with neutral red (27). Counts of pyknotic profiles were made at ×1000 magnification under oil immersion in three random fields of 0.0145 mm² within the neuroblastic layer, and at least three randomly selected explants were analyzed for each group in each experiment. Photoreceptor cell death was identified by the rounded and condensed morphology of rhodopsin-positive profiles within the outer nuclear layer, wherein normal photoreceptors present an elongated shape at that age (33). Counts were made at ×1000 magnification under oil immersion in three random fields of 0.0074 mm² within the outer nuclear layer. At least three randomly selected explants were analyzed for each group in each of two independent experiments. Analysis of variance followed by planned comparisons using Duncan’s multiple range test were done with an SPSSPC statistical package. The apoptotic form of cell death was selectively examined in some experiments by staining dead cell profiles with either the TUNEL technique using an Apoptag kit or the antibody CM1, which recognizes the activated form of caspase-3 (28).

**mRNA Analysis**—Total RNA was prepared from the retinas of rat pups at postnatal day 2 (P2) using Trizol (Invitrogen), and cDNA was synthesized and amplified using primers (5′-CACAGATTCCGCTTCTCTCC-3′, 5′-GGCATATCCCTATCTCTCTT-3′) that recognize a region from the carboxyl-terminal intracellular domain common to all PAC1 receptor isoforms (29).

**Labeling of Proliferating Cells**—Newborn rats (postnatal day 1) were anesthetized by hypothermia and received a series of three 5-bromo-2′-deoxyuridine (BrdUrd) injections (60 mg/kg of body weight at 0, 5, and 19 h) to label all proliferating cells, and experiments were performed 1 h after the last injection (28).

**Immunohistochemistry**—To locate the PAC1 receptor within retinal tissue, sections through the eye were immunostained using an affinity-purified rabbit anti-PAC1 antibody kindly provided by Dr. Victor May (University of Vermont, Vermont, ME) (29). For phospho-CREB immunohistochemistry, retinal explants from rats injected with BrdUrd were maintained in vitro for various intervals either with or without PACAP38, and sections were processed with a rabbit anti-phospho-CREB antibody (Cell Signaling Technology, Inc., Beverly, MA) and a monoclonal antibody for BrdUrd (Amersham Biosciences). Photoreceptor nuclei were stained with monoclonal antibody rhod2, kindly provided by Dr. Robert S. Molday, at 37°C overnight at 1:50. PAC1 and rhod2 immunoreactivity were developed with the appropriate rabbit or mouse horseradish peroxidase-ABC kits, respectively (Vector, Burlingame, CA) with diaminobenzidine as chromogen. Both BrdUrd and phospho-CREB immunoreactivity were developed with Alexa Fluor conjugate fluorescent antibodies from Molecular Probes (Eugene, OR) and analyzed in a Zeiss LSM310 confocal microscope.

**Measurement of Intracellular cAMP**—Cyclic AMP was quantitated according to the competitive binding assay of Gilman (30) as described previously (13). Briefly, retinas from P2 rats were preincubated for 10 min at 37°C in basmal medium Eagle’s buffered at pH 7.4, containing 0.5 mM CaCl₂ and 10 mM benzathine, and 100 μM ascorbic acid, and stimulated for 15 min with either PACAP38 or 6-CI-PB ([±]-6-chloro-7, 8-dihydroxy-1-phenyl-2, 3, 4,5-tetrahydro-1H-3-benzazepine), a 1,1-like agonist that increases intracellular cAMP used as a positive control. The reaction was stopped with trichloroacetic acid, and after centrifugation, the supernatant was passed through an ion-exchange resin column (Dowex 50) to remove trichloroacetic acid and other nucleotides. The sample obtained was then used in a competition assay with the regulatory subunit of PKA with the addition of a fixed, trace amount of [3H]cAMP.

**Measurement of Intracellular Ip**—Inositol triphosphate was assayed according to a modification (31) of the method described by Bertridge et al. (32). Retinal explants from P2 rats were preincubated for 8 h in inositol-free defined medium containing [3H]myoinositol. The cultures were then treated with either 10 mM PACAP38 or 500 μM kainate as a positive control for 15 min in 10 mM LiCl. The reaction was stopped by the addition of trichloroacetic acid, and following ether extraction, the supernatants were separated by ion-exchange chromatography (Dowex AG1-X8 resin, formate form; Bio-Rad). Inositol triphosphate (IP3) was quantified in a liquid scintillation analyzer.

**RESULTS**

**PACAP38 Is a Potent Neuroprotective Agent in the Newborn Rat Retina**—We have shown previously that inhibition of protein synthesis by anisomycin induces cell death within the NBL of retinal explants from newborn rats (27). The sensitive cells could be rescued by the increase of intracellular cAMP levels (27). Therefore, we investigated whether PACAP38, a potent cAMP inducer, affected retinal cell survival. PACAP38 protected cells from death induced by anisomycin with maximum effect at 1 mM (Fig. 1, A–C). Retinal tissue treated with PACAP38 showed a very low density of pyknotic profiles, approximately the same as the density of control explants; part of these results, at least, are likely to be caused by slight mechanical damage to the tissue when preparing the explants. Protection against anisomycin-induced cell death was also observed with PACAP27 (data not shown). In contrast, glucagon, a peptide that belongs to the same family as PACAP, had no effect at a similar concentration range (Fig. 1C, inset).

We specifically tested whether apoptosis could be prevented by PACAP38. Adjacent sections from the same explants were stained with neutral red in Fig. 1B, with or without TUNEL procedure (Fig. 1D), or with an antibody that detects the activated form of caspase-3 (Fig. 1E). Treatment with PACAP38 led to a reduction in the number of degenerating profiles identified with all three methods (Fig. 1F). The lower number of degenerating profiles detected with either TUNEL or the CM1 antibody may reflect the simultaneous occurrence of other forms of cell death besides apoptosis. Nevertheless, the data...
show that PACAP38 is effective against the caspase-3-dependent, apoptotic form of cell death.

Previous work from our laboratory showed that thapsigargin selectively kills photoreceptors in the outer nuclear layer of retinal explants from 1-week-old rats (33). The degeneration of rhodopsin-containing photoreceptors was also prevented by PACAP38 (Fig. 2). Therefore, the protective effect of PACAP is not restricted to anisomycin-induced cell death.

Antagonists of the PAC1 Receptor Block the Protective Effect of PACAP38—To test whether the neuroprotective effect was mediated by interaction of PACAP38 with its receptors, we used the competitive PACAP receptor antagonist PACA6–38. Increasing amounts of PACA6–38 prevented, in a dose-dependent fashion, the action of PACAP38 upon anisomycin-induced cell death (Fig. 3A). The maximum inhibitory effect of PACA6–38 was observed at 1 μM. To further characterize PACAP receptors involved in neuroprotection, we used the specific PAC1 receptor antagonist Maxd.4, which was developed based on the sequence of the PAC1 receptor-specific agonist maxadilan, originally cloned from sandfly salivary glands (34–36). Treatment with increasing concentrations of Maxd.4 also prevented neuroprotection by PACAP38 (Fig. 3B) within a similar concentration range as that observed with PACA6–38. In agreement with these results, 1 nM maxadilan also prevented anisomycin-induced cell death, and this protective effect was blocked by Maxd.4 (data not shown).

PAC1 Receptor mRNA and Protein Are Present in Neonatal Rat Retina—The results obtained with both Maxd.4 and maxadilan suggested a major role for the PAC1 receptor on the neuroprotective effect of PACAP38 upon retinal cells. The presence of this receptor was then investigated both by reverse transcription-PCR and by immunohistochemistry with an anti-PAC1 antibody (Fig. 4). Using primers for the carboxyl-terminal intracellular domain of the PAC1 receptor (29), we amplified a product of the expected 449-bp size (Fig. 4A). Consistent with the latter result, immunoreactivity for the PAC1 receptor was found in all layers of the retina in P2 rat eyes, including the NBL (Fig. 4, B and C).

PAC1 Receptors Expressed in the Neuroblastic Layer Are Functionally Active—We examined whether cells from the NBL responded to PACAP38 by testing for the induction of CREB phosphorylation. CREB is a transcriptional factor that may be activated by multiple stimuli, including intracellular CAMP (for a review, see Ref. 37). Retinal explants were maintained for 2 h without stimulus, and then either PACAP38 or vehicle was added for 5, 20, or 60 min. Nuclei labeled for phospho-CREB were already detected at 5 min of treatment with PACAP38 in the neuroblastic layer (data not shown), but labeling was more intense after 20 min of incubation (Fig. 5A, red), consistent with the detection of the PAC1 receptor (Fig. 4). In contrast, sections from explants maintained in control medium showed almost no detectable labeling for phospho-CREB within the NBL (Fig. 5C).

Phospho-CREB labeling of cells located in the outer half of the NBL was particularly strong. Cells within the NBL that degenerate following treatment with anisomycin are mainly located in the outer half of the neuroblastic layer and were identified as undifferentiated postmitotic cells because they neither incorporate BrdUrd following serial injections designed as to label the maximum possible number of proliferating neuroblasts nor can they be stained with various antibodies to retinal cell differentiation markers (26). We tested whether postmitotic cells were phospho-CREB-positive by prelabeling proliferating cells with BrdUrd injections identical to those used in our previous study (26). Confocal microscopic examination of sections from explants treated with PACAP38 (Fig. 5) showed that a large number of cells unlabeled for BrdUrd within the NBL (in green) were strongly labeled with phospho-CREB (in red). These data suggest that PACAP38 may affect directly the cells that are sensitive to cell death induced by the blockade of protein synthesis.

The cAMP/PKA Pathway, but Not Activation of Phospholipase C, Is Required for Neuroprotection by PACAP38—The various PAC1 receptor isoforms can trigger several signal transduction mechanisms, including the activation of phospholipase C, adenyl cyclase, or the modulation of voltage-dependent L-type Ca\(^{2+}\) channels. We tested whether one or more of these pathways were associated with the neuroprotective effect of PACAP38.

No IP\(_3\) production was found in PACAP38-stimulated retinal explants (Fig. 6A). The PLC inhibitor, U73122, also failed to prevent the neuroprotective effect of PACAP38 upon anisomycin-induced cell death (Fig. 6B). At the concentration range tested, U73122 had no effect by itself (data not shown). The selective L-type Ca\(^{2+}\) channel inhibitors verapamil (30 μM) and nifedipine (10 μM) also did not prevent the neuroprotective effect of PACAP (data not shown). In contrast, PACAP38 at 1 and 10 nM induced a 2.5-fold and 6-fold increase in cAMP levels, respectively (Fig. 7A), similar to the dopamine D1-like receptor agonist 6-CI-PB, which was used as a positive control (13). Moreover, the PKA inhibitor, R\(_8\)-cAMPS, completely reverted the neuroprotective effect of PACAP38 (Fig. 7B). These results clearly show a requirement of the cAMP/PKA signaling pathway for PACAP38-induced neuroprotection.

**DISCUSSION**

This investigation showed that PACAP counteracts the induction of retinal cell death. Activation of PAC1 receptors expressed in the NBL of the neonatal rat retina resulted in...
production of cAMP and consequent activation of PKA, whereas a PKA inhibitor prevented the neuroprotective effect. PACAP38 also led to phosphorylation of CREB in the retinal tissue. In addition, PACAP38 counteracted photoreceptor cell death induced by thapsigargin. The detection of PACAP-induced effects upon both recent postmitotic cells of the neuroblastic layer as well as upon rhodopsin-containing photoreceptors suggest that both undifferentiated and differentiated cells may be subject to neuroprotection by PACAP.

Both PACAP and VIP act on the same receptors, which were pharmacologically classified as types I and II by their relative affinity for the peptides. Subsequent molecular characterization showed three genes that encoded G-protein-coupled receptors responsive to these peptides with great functional heterogeneity (23–25, 38). The PAC1 receptor is considered a PACAP-specific receptor and may present splicing variants that differ in the intracellular signaling pathways activated, which include adenylyl cyclase, phospholipase C, or modulation of L-type calcium channels (24, 38, 39).

In contrast with other regions of the central and peripheral nervous system, functional studies of PACAP peptides and their receptors in the developing retina are rare (42). Both PACAP as well as the mRNA and immunoreactivity for the PACAP receptor have been described in the retina of adult rats (47–49). Production of cAMP following activation of PACAP receptors was also described in the retinae of some mammalian species (47–49). In the present study, the PAC1 receptor was located in the developing retina and shown to be functional as well as participating in a neuroprotective signaling pathway. This is supported by the experiments showing that the specific antagonist Maxd.4 (35) reverted (Fig. 3B), whereas the specific agonist maxadilan (34, 36) reproduced the neuroprotective effect of PACAP. Nonetheless, a contribution of VPAC1 and VPAC2 receptors cannot be discarded.

Previous work in our laboratory has taken advantage of...
In conclusion, the overall data demonstrate that PACAP-induced activation of the cAMP/PKA pathway through the PAC1 receptor lowers the sensitivity of retinal cells to induced cell death. The results add PACAP to a growing list of intrinsic modulators of sensitivity to cell death within the developing central nervous system. Studies of the kinetics of neurodegenerative cell loss suggested that cell death in inherited neurodegenerations, rather than being caused by being cumulated damage, may be due to single catastrophic events imposed on an altered homeostatic state (20). The present data suggest that neurotrophies such as PACAP help to maintain retinal cells in a steady state removed from apoptosis execution pathways and may therefore be relevant for the control of inherited retinal dystrophies.

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FIG. 6. Phospholipase C pathway is not responsible for the neuroprotective effect of PACAP38. A, retinal explants treated either with 10 nM PACAP38 or with 500 μM kainate (KA) as a positive control. Production of IP3 is shown in the vertical axis. Data are from one representative experiment out of three experiments with similar results, each performed in duplicate. CTR, control. B, effect of the phospholipase C inhibitor U73122 in explants incubated with anisomycin plus PACAP38. Data are the means ± S.E. pooled from two experiments with three independent explants each. *, p < 0.01 versus anisomycin plus 10 nM PACAP38.

FIG. 7. The cAMP/PKA pathway is required for modulation of anisomycin-induced cell death by PACAP38. A, relative levels of cAMP in retinal explants treated with either 1 or 10 nM PACAP38. The levels in control explants were set to 1, and the dopamine D1-like receptor agonist 6-Ci-PB (100 μM) was used as a positive control. The data represent the mean ± S.E. of four experiments with at least two replicates per group. B, effect of the PKA inhibitor Rp-cAMPS (100 μM) in explants treated with 1 μg/ml anisomycin plus 1 nM PACAP38. The rate of cell death is expressed as pyknotic profiles (PKF) per mm2 in the NBL, and data represent the means ± S.E. pooled from three experiments with three independent explants each. *, p < 0.01 versus anisomycin plus 1 nM PACAP38.

retinal explants in culture to study the sensitivity of developing nervous tissue to cell death (for a review, see Refs. 40 and 41). It was established that treatment of retinal explants with inhibitors of protein synthesis induces cell death in undifferentiated postmitotic cells (26, 27) and that an increase in intracellular cAMP levels, as well as dopamine-induced activation of a D1-like receptor (13), protected the neuroblastic layer from anisomycin-induced cell death.

We tested the roles of PACAP signaling pathways in the neuroprotective effect. Activation of phospholipase C does not appear to be involved because neither was IP3 production decreased following incubation of retinal tissue with PACAP at a maximally effective concentration (Fig. 6A) nor did treatment with the PLC inhibitor, U73122, prevent the effect (Fig. 6B). A role of L-type calcium channels (39) was also ruled out in the present conditions. On the other hand, the PACAP-induced activation of PKA was required for neuroprotection (Fig. 7).
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