Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptides (PACAP27 and PACAP38) Protect $CD4^+CD8^+$ Thymocytes From Glucocorticoid-Induced Apoptosis

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In the present study, the effects of vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptides, PACAP27 and PACAP38, in a concentration range from 10^{-13} to 10^{-6} mol/L were studied in vitro on the spontaneous and dexamethasone (DEX)-induced apoptosis in rat thymocytes. The results show that VIP and both PACAPs inhibit significantly and in a similar way the DNA fragmentation characteristic of glucocorticoid-induced apoptosis and increase the cell survival of thymocytes, with a maximal effect observed at 10^{-8} to 10^{-9} mol/L. This study showed the ability of the VIP-receptor (VIP-R) antagonist [N-Ac-Tyr^1,D-Phe^2]-GRF(1-29) amide to partially reverse the inhibitory effect of VIP and both PACAPs on DEX-induced apoptosis, providing evidence for a specific VIP1-R-mediated response and supporting the involvement of a single receptor for the three neuropeptides. Phenotypic analysis showed that VIP, PACAP27, and PACAP38 protect predominantly $CD4^+CD8^+$ thymocytes from glucocorticoid-induced apoptosis. These findings suggest that these neuropeptides could be involved in intrathymic T-cell maturation.

To Elvira Garrido in memoriam

APOPTOSIS OR PROGRAMMED cell death is an essential physiologic process in the differentiation and maintenance of thymocyte populations. It has been described during thymus ontogeny as in the deletion of autoreactive T-cell clones[1] and in response to a specific signal, ie, thymocyte stimulation with antibodies to CD3/T-cell receptor (TCR) complex,[2,3] exposition to glucocorticoids,[4-7] gamma-irradiation,[8] treatment with calcium ionophores,[9,10] and presentation of a specific antigen.[11,12] The mechanisms of thymocyte apoptosis induced by TCR activation or treatment with glucocorticoids are similar and represent one of the best model systems to study apoptosis. Glucocorticoid induction is characterized by morphologic changes, such as condensation of nuclear chromatin, blebbing of cell surface, and transient increase in cytoplasmic density,[13-15] and is preceded by fragmentation of genomic DNA at the linker region between nucleosomes.[4,5,8] This chromatin fragmentation results from the activation of an endogenous calcium-dependent endonuclease and is not observed when the cells are killed by other methods such as heating, antibody plus complement, or chemically induced membrane damage.[5]

Vasoactive intestinal peptide (VIP) is a neuropeptide that has been implicated in mechanisms related to proliferation and maturation of the nervous and immune systems. It has been involved in the regulation of neuronal survival, as well as in the maturation and maintenance of different nerve cells populations.[16-18] Moreover, in the immune system, VIP inhibits the proliferation of thymocytes and peripheral T cells.[19,20] Furthermore, we have recently shown that VIP and VIP1-receptor (VIP1-R) genes are expressed in double-($CD4^+CD8^+$) and single-positive ($CD4^+CD8^-$, $CD4^-CD8^+$) thymocyte populations, whereas double-negative thymocytes lack that expression.[20a] The effects on T-cell proliferation and the differential expression of CD4 and CD8 surface glycoproteins suggest a role for VIP in the differentiation, activation, and/or proliferation of T cells.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a recently described neuropeptide[21] of the VIP/glucagon/secretin family that exists in two amidated forms, PACAP27 and PACAP38. Althought studies on the effects of PACAP in the immune system are scarce, it has been shown that it inhibits the mitogen-stimulated proliferation of splenocytes[22] and decreases the mobility of thymocytes and splenocytes.[23]

To gain insight into the putative role of VIP and PACAP in the physiology of the immune system, we investigated the effect of these two signal molecules, shared by nervous and immune systems, on glucocorticoid-induced apoptosis. Our results show that both neuropeptides protect $CD4^+CD8^+$ thymocytes from dexamethasone (DEX)-induced apoptosis through the specific VIP1-R.

MATERIALS AND METHODS

Animals. Male Wistar rats (Iffa Credo, Lyon, France) weighing 250 to 300 g were maintained at constant temperature (23°C ± 2°C) on a 12-hour light/dark cycle and 50% ± 5% humidity.

Antibodies and reagents. Fluorescein isothiocyanate (FITC)-conjugated mouse antirat CD4 (clone OX38) and phycoerythrin (PE)-conjugated mouse antirat CD8 (clone OX8) antibodies were obtained from PharMingen (San Diego, CA). VIP and VIP-antagonist [N-Ac-Tyr^1, D-Phe^2]-GRF(1-29) amide were purchased from Cambridge Research BioChemicals (Wilmington, DE). PACAP27 and PACAP38 were obtained from Novabiochem (Postfach, Switzerland). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), diphenylamine (DPA), propidium iodide (PI), and 9α-fluoro, 16α-methylprednisolone (DEX) were purchased from Sigma (St Louis, MO).

Thymocyte preparation and culture. Thymus cell suspensions were obtained by gently pressing through a stainless-steel wire mesh (Sigma) in RPMI 1640 medium (GIBCO BRL Laboratories, Grand

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Island, NY). Thymocytes were cultured in RPMI supplemented with 5% heat-inactivated fetal calf serum (FCS; GIBCO BRL), containing 0.01 mol/L HEPES buffer, 1 mmol/L pyruvate, 0.1 mol/L nonessential amino acids, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin, in 24-well flat-bottom plates (Corning Glass, Corning, NY) at 2×10^6 cells per well in a final volume of 1.5 mL and incubated at 37°C in a moist 5% CO_2 atmosphere. In all cases, thymocytes were stimulated with 2.5 μg/mL Concanavaline A (ConA; Sigma). DEX was added to the cell culture at a final concentration of 1 μmol/L. To examine the effects of VIP, PACAP38, and PACAP27 on DEX-induced apoptosis, thymocytes were cultured simultaneously with each neuropeptide and DEX. To evaluate the effects of the three neuropeptides on spontaneous apoptosis, cells were cultured with medium alone (controls), or with VIP, PACAP38, or PACAP27. At the indicated time points, the cell suspension was transferred to 1.5-mL polypropylene tubes for one of the apoptosis assays described later.

Assessment of cell viability and morphology. Cell viability was assessed by trypan blue exclusion and loss of mitochondrial function with MTT-staining methods.[24] For MTT staining, 100 μL of culture was placed in one well of a 96-well tissue culture plate (Corning) and 10 μL of MTT solution (2.5mg/mL H_2O) was added. After incubation at 37°C for 4 hours, 100 μL of acid-isopropanol (0.04*N* HCl in isopropanol) was added and mixed by gently pipetting, and OD 560 nm was assessed on an enzyme-linked immunosorbent assay (ELISA) reader.

Morphologic changes characteristic of apoptosis (nuclear condensation and vacuolation) were assessed by cytospin preparations of thymocytes stained with hematoxylin-eosin and observed under light microscopy (400× magnification).

Agarose gel electrophoresis DNA fragmentation assay. DNA was prepared for gel electrophoresis as described.[25] Cultured thymocytes were centrifuged at 750 × *g* for 10 minutes, resuspended in 400 μL hypotonic lysis buffer (10 mmol/L Tris/HCl, 1 mmol/L EDTA [pH 8.0], and 0.2% Triton X-100), incubated on ice for 20 minutes, and centrifuged for 15 minutes at 13,800*g*. The supernatants that contained small DNA fragments were separated immediately from the pellets that contained large uncut chromatin.

DNA fragments were precipitated with 0.5 mol/L NaCl and 1 vol of isopropanol at −20°C overnight. The samples were centrifuged at 13,800*g* for 20 minutes at 4°C, and the pellet was washed with 70% ethanol and then allowed to dry at room temperature. The DNA was resuspended with TE solution (10 mmol/L Tris/HCl, 1 mmol/L EDTA [pH 7.4]) and loading buffer (50% glycerol, 10% saturated bromophenol blue, and 1% xylene cyanol in Tris-acetate EDTA [TAE] buffer) and was incubated at 37°C for 20 minutes. The electrophoresis was performed on 2% agarose slab gels for 2 hours with TAE as running buffer. Each line represents 10^6 cell equivalents. DNA ladders were visualized by staining with ethidium bromide and UV illumination. Gels were photographed with a Polaroid camera (Polaroid Corp, Cambridge, MA). λ DNA/*Hin*dIII was used as a molecular weight marker.

Quantification of DNA fragmentation. DNA fragmentation was assayed using the diphenylamine reaction, modified from Burton.[26] The pellets that contained uncut DNA were resuspended with 200 μL of hypotonic lysis buffer plus perchloric acid (0.5 mol/L). The supernatants that contained DNA fragments were treated with 2 vol of a solution containing 0.088 mol/L DPA, 98% vol/vol glacial acetic acid, 1.5% vol/vol sulfuric acid, and 0.5% vol/vol of 1.6% acetaldehyde solution, and the samples stored at 4°C for 48 hours. The colorimetric reaction was spectrophotometrically measured at 560 nm with an ELISA reader. The percentage of DNA fragmentation was expressed by the following formula: [OD_{560} of supernatant/(OD_{560} of supernatant + OD_{560} of pellet)] × 100.

DNA labeling technique and flow cytometry analysis. Cells were harvested from the plates and centrifuged at 750*g* for 5 minutes. The pellets were fixed in cold 70% ethanol for 5 minutes at room temperature, washed with PBS/0.1% bovine serum albumin (BSA), and resuspended in 0.25 mL of RNase solution (500 U/mL RNase A in 1.12% sodium citrate [pH 8.4] plus 0.1% Triton X-100). After 30 minutes at 37°C, the reaction was stopped by addition of 0.25 mL hypotonic fluorochrome solution containing 50 μg/mL PI in 1.12% sodium citrate (pH 8.4), and 0.1% Triton X-100, and the mixture was incubated for 30 minutes in the dark at room temperature.[27] The cells were maintained on ice until the PI-fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Debris with low foward scatter was excluded before analysis. The percentage of apoptotic cells (subdiploid DNA peak in the DNA fluorescent histogram) was calculated using specific FACScan research software (Becton Dickinson).

In some experiments, cultured thymocytes were stained by incubation with both PE-labeled mouse antirat CD4 and FITC-labeled mouse antirat CD8 monoclonal antibodies (dilution 1/50 in Tris/HCl pH 7.4) for 1 hour at 4°C. Cells were washed three times to remove the unbound fluorochrome and kept in the dark at 4°C until analysis. Cell-surface fluorescence was analyzed on a FACScan flow cytometer with foward- and side-scatter gates set to exclude nonviable cells.

Statistical analysis. All values are expressed as the mean ± SD of the number of experiments performed in duplicate, as indicated in the corresponding tables and figures. Comparisons between groups were made using Student's *t* test followed by Scheffe's F-test, with $P < .05$ as the minimum significant level.

RESULTS

In the present report, we show the effects of VIP and PACAPs on spontaneous and DEX-induced apoptosis in rat thymocytes using different methodologic approaches. Cell viability was determined by means of trypan blue test and measuring the loss of mitochondrial function with tetrazolium assay (MTT); apoptotic cell death was confirmed by conventional morphologic methods. DNA fragmentation was examined by agarose gel electrophoresis and its quantification performed using DPA reaction. Moreover, we measured the percentage of apoptotic cells with PI by flow cytometry.

VIP, PACAP38, and PACAP27 partly inhibit thymocyte spontaneous apoptosis. To determine the effects of VIP and PACAPs on spontaneous apoptosis and cell death, we first cultured freshly isolated thymocytes for different times and in the presence or in the absence of 10^{-8} mol/L VIP, PACAP27, or PACAP38 and assessed cell viability and DNA fragmentation. Thymocytes cultured with medium alone (controls) showed a progressive increase in spontaneous apoptosis measured by means of DNA fragmentation (DPA reaction) (Table 1) and PI staining with flow cytometric analysis (Table 2). VIP, PACAP27, and PACAP38 significantly decreased spontaneous DNA fragmentation at 4 to 72 hours of incubation compared with control cultures. Moreover, VIP and PACAP38 significantly increased cell viability at 16 to 72 hours (Table 1). Although PACAP27 shows higher cell viability than control values, no significant differences were observed. The electrophoresis performed on low–molecular weight DNA demonstrated that neuropeptide-treated thymocytes showed a slighty decreased sponta-

Table 1. Inhibitory Effect of VIP and PACAPs on Spontaneous Apoptosis of Rat Thymocytes

	Relative Cell Viability				DNA Fragmentation (%)			
Time (hours)	Control	VIP	PACAP38	PACAP27	Control	VIP	PACAP38	PACAP27
0	493 ± 51				7 ± 1			
4	438 ± 38	449 ± 28	469 ± 53	464 ± 55	12 ± 2	9 ± 1†	10 ± 1†	10 ± 1†
8	455 ± 17	468 ± 13	467 ± 14	465 ± 18	21 ± 7	14 ± 1*	15 ± 2*	14 ± 1*
12	501 ± 52	524 ± 69	517 ± 83	522 ± 72	25 ± 2	18 ± 2‡	20 ± 3‡	22 ± 2†
16	477 ± 32	548 ± 58*	530 ± 50*	520 ± 48	31 ± 5	22 ± 3†	23 ± 2†	25 ± 1*
20	470 ± 48	529 ± 36*	526 ± 41*	521 ± 46	34 ± 5	24 ± 3†	24 ± 3‡	27 ± 2†
24	413 ± 33	451 ± 26*	545 ± 38*	445 ± 35	39 ± 4	26 ± 2‡	28 ± 4‡	28 ± 2‡
48	506 ± 47	604 ± 14†	552 ± 35	549 ± 17	48 ± 3	41 ± 2‡	44 ± 4*	43 ± 3*
72	613 ± 36	678 ± 14†	685 ± 13*	659 ± 12	49 ± 1	41 ± 5†	43 ± 3‡	42 ± 3‡

ConA-stimulated thymocytes were incubated with medium (control), VIP 10^{-8} mol/L, PACAP27 10^{-8} mol/L, or PACAP38 10^{-8} mol/L for different times. Relative cell viability was assessed by the loss of mitochondrial function with tetrazolium assay (MTT), and percentage of DNA fragmentation was assessed by DPA reaction as described in the Materials and Methods. The OD_{560nm} values of relative cell viability were multiplied by 1,000. Results are the mean ± SD of 8 experiments performed in duplicate. *P* values are with respect to control samples.

* $P < .05$.

† $P < .01$.

‡ $P < .001$.

neous DNA fragmentation (Fig 1). Moreover, the percentages of apoptotic cells determined by PI staining were lower in the cultures incubated with VIP and both PACAPs than in the control cultures, with the decrease being significant at 16, 20, and 24 hours for VIP-treated samples, at 16 hours for PACAP38, and at 16 and 20 hours for PACAP27 (Table 2). When thymocytes were cultured with VIP or PACAPs, no cells with nuclear condensation typical of apoptosis were observed in hematoxylin-eosin–stained cytospin preparations until 16 hours of treatment. However, thymocytes with condensed chromatin were detected after 8 hours in control cultures (data not shown).

Effect of VIP, PACAP38, and PACAP27 on glucocorticoid-induced apoptosis of thymocytes. Since VIP and PACAPs seemed to decrease spontaneous apoptosis of rat thymocytes in culture, we studied the effects of the three neuropeptides on glucocorticoid-induced apoptosis. Thymocytes were cultured for different times with 10^{-6} mol/L DEX, a glucocorticoid analog, in the presence or in the absence of 10^{-8} mol/L VIP, PACAP27, or PACAP38; the relative cell viability and percentage of DNA fragmentation were then evaluated. We first studied the sequence of events during DEX-induced apoptosis in rat thymocytes (Fig 2). We observed that loss of cell viability in thymocytes treated with DEX began at approximately 8 hours of incubation (Fig 2A), whereas DNA fragmentation began at 4 hours, as determined by quantification of DPA reaction (Fig 2B) and by the appearance of a DNA ladder on agarose gels (data not shown). Therefore, DNA fragmentation precedes cell death in this system. The three neuropeptides increase cell viability (Fig 2A) and decrease the percentage of DNA fragmentation (Fig 2B) compared with the thymocytes cultured with DEX alone, which showed similar values to control cultures. The maximum inhibitory effect was observed after 12 hours of treatment. Moreover, the three neuropeptides partly decreased chromatin fragmentation in oligonucleosome-sized (Fig 1).

Table 2. VIP and PACAPs Decrease Percentage of Spontaneous Apoptotic Thymocytes

	Treatment			
Time (hours)	None	VIP	PACAP38	PACAP27
0	1.15 ± 0.29			
12	42.60 ± 11.30	35.88 ± 7.51	35.38 ± 6.88	42.51 ± 6.94
16	57.62 ± 2.49	41.99 ± 7.06*	43.46 ± 8.14*	45.07 ± 7.06*
20	59.80 ± 5.62	49.06 ± 2.92*	51.78 ± 2.16	47.00 ± 1.39*
24	59.14 ± 5.79	50.46 ± 1.07*	55.34 ± 3.05	52.03 ± 1.13

Cells were cultured with medium (control), VIP 10^{-8} mol/L, PACAP38 10^{-8} mol/L, or PACAP27 10^{-8} mol/L. Thymocytes were stimulated with ConA (2.5 μg/mL). After different incubation times, the hypodiploid DNA peak was analyzed by PI staining in a flow cytometer. Results represent the mean ± SD of 3 experiments performed in duplicate.

* $P < .05$ with respect to control values.

It has been stablished that during DEX-induced apoptosis, thymocytes show a sequence of morphologic changes.[13,14] These changes include a decrease in cell size and an increase in cell density, cytoplasmic vacuolization, and nuclear condensation. Incubation with VIP, PACAP38, and PACAP27 decreases the number of thymocytes with apoptotic morphology. The morphologic changes induced by DEX precede the decrease in cell viability assessed by trypan blue exclusion, and this decrease was significantly prevented by the three neuropeptides after 12 to 72 hours in culture (data not shown). We evaluated the percentage of apoptotic cells by measuring hypodiploid DNA content using PI staining by flow cytometry, and the presence of VIP or PACAPs in DEX-treated thymocyte cultures significantly decreased the percentage of apoptotic cells. The maximum inhibitory effect was seen after 12 hours of culture (Fig 3).

The inhibitory effect of VIP and PACAPs on DEX-induced apoptosis of thymocytes was dose-dependent. The three neuropeptides (10^{-12} to 10^{-6} mol/L) inhibit in a similar way the percentage of DNA fragmentation. The maximal effects were observed at a concentration of 10^{-8} mol/L (Fig 4B). A similar dose-dependent response was observed in the stimulation of cell viability (Fig 4A) and in the decrease of

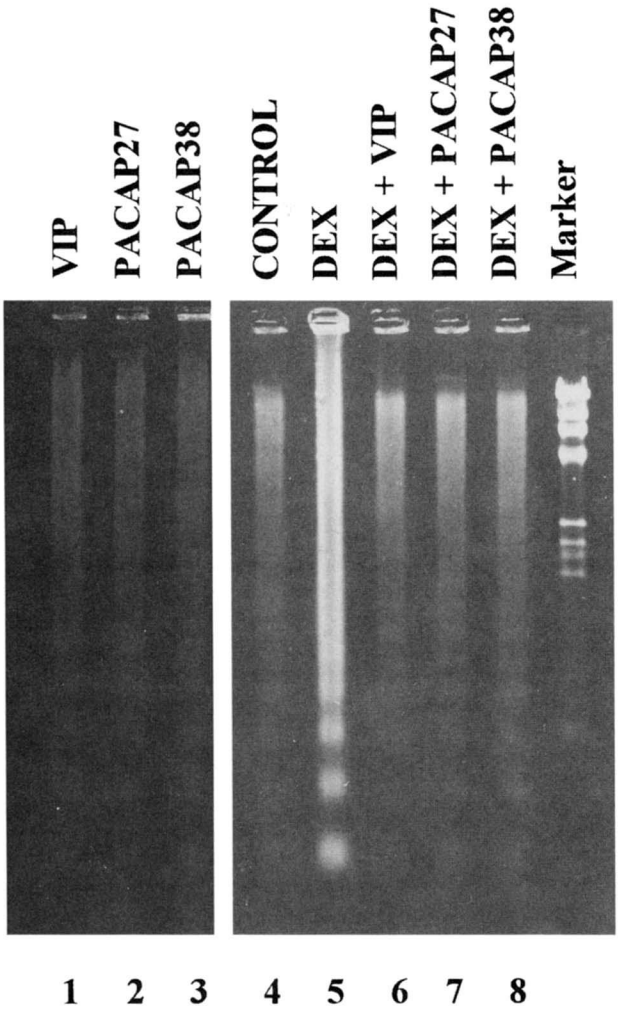

Fig 1. Agarose gel electrophoresis of DNA extracted from rat thymocytes stimulated with ConA (2.5 μg/mL) after 12 hours of culture in the presence of VIP 10^{-8} mol/L (lane 1), PACAP27 10^{-8} mol/L (lane 2), PACAP38 10^{-8} mol/L (lane 3), medium (lane 4), DEX 10^{-6} mol/L (lane 5), DEX 10^{-6} mol/L plus VIP 10^{-8} mol/L (lane 6), DEX 10^{-6} mol/L plus PACAP27 10^{-8} mol/L (lane 7), and DEX 10^{-6} mol/L plus PACAP38 10^{-8} mol/L (lane 8). Molecular weight DNA markers were included. This result is a representative example of 5 experiments.

DNA cleavage (Fig 4C). Since the highest inhibition of DNA fragmentation was obtained at a neuropeptide concentration of 10^{-8} mol/L after 12 hours of culture, we used these conditions in the following experiments.

Specific suppression by VIP and PACAP of DEX-induced apoptosis of thymocytes through VIP-R. To elucidate whether the inhibitory effect of these neuropeptides was VIP-R–mediated or nonspecific, we investigated the ability of the (N-Ac-Tyr^1, D-Phe^2)GRF(1-29)-NH_2, a VIP-R antagonist,[28] to reverse VIP and PACAPs effects on glucocorticoid-induced apoptosis in thymocytes. We studied the effect on DNA fragmentation assayed by DPA reaction and gel electrophoresis by incubating increasing concentrations of the VIP-R antagonist in the presence of VIP or PACAP (10^{-8} mol/L). As shown in Fig 5, the inhibitory effect of the three neuropeptides on DNA fragmentation (Fig 5B) was progressively abolished by simultaneous incubation with increasing concentrations of VIP-R antagonist, which indicates dose-dependent inhibition. Moreover, this VIP antagonist effect was observed in the maintenance of cell viability by VIP and PACAP (Fig 5A). The lower VIP antagonist concentration used (10^{-8} mol/L) was ineffective in reducing the effect on cell viability of VIP and PACAP, and a concentration of 10^{-5} mol/L produced an effect that gave rise to a culture that showed no significant difference with respect to DEX-treated cultures. Incubation with VIP antagonist alone at concentrations of 10^{-5} to 10^{-8} mol/L had no significant effect on either cell viability or DNA fragmentation of thymocytes treated with DEX (data not shown).

VIP and PACAP peptides prevent reduction of $CD4^+CD8^+$ thymocytes after DEX treatment. To examine whether VIP and PACAP prevented the apoptosis of a specific thymocyte subpopulation, we studied the effects of neu-

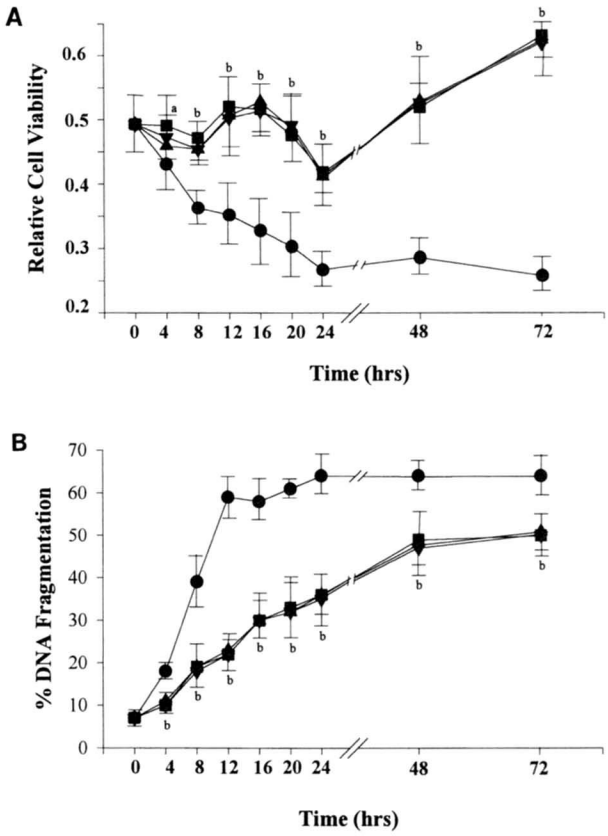

Fig 2. Time course of inhibition of DEX-induced apoptosis in thymocyte cultures by VIP and PACAPs. ConA-stimulated thymocytes were incubated with DEX 10^{-6} mol/L (●), DEX plus VIP 10^{-8} mol/L (■), DEX plus PACAP38 10^{-8} mol/L (▲), or DEX plus PACAP27 10^{-8} mol/L (▼), and assessed at different times for cell viability or DNA fragmentation. (A) Viability assessed by MTT. (B) Percent of DNA fragmentation assessed by DPA reaction. Results are the mean ± SD of 8 separate experiments performed in duplicate. $^aP < .05$ and $^bP < .001$ *v* DEX-treated samples.

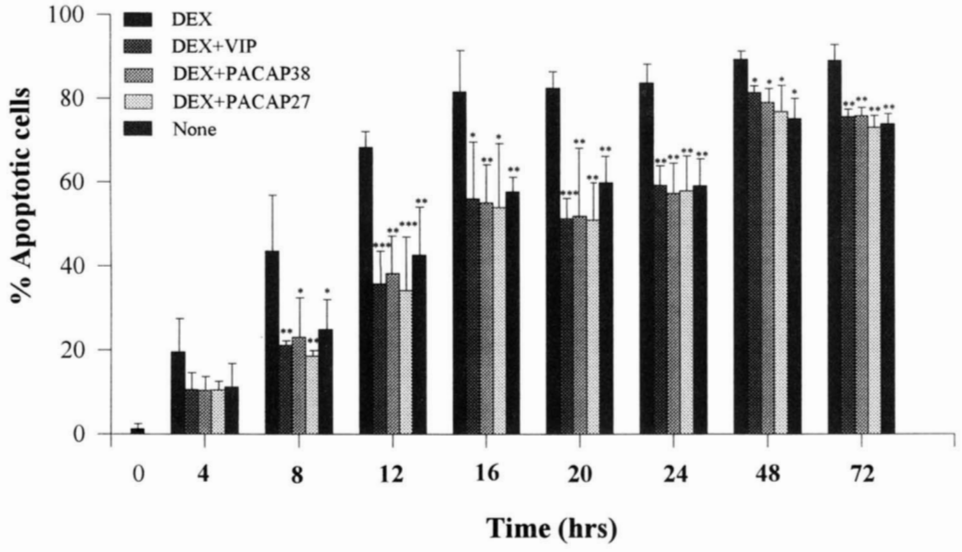

Fig 3. VIP and PACAP decrease the percentage of apoptotic thymocytes induced by DEX treatment. ConA-stimulated thymocytes were incubated with medium (None), DEX 10^{-6} mol/L, DEX plus VIP 10^{-8} mol/L, DEX plus PACAP38 10^{-8} mol/L, and DEX plus PACAP27 10^{-8} mol/L for different times, and the percentage of apoptotic nuclei (hypodiploid DNA peak) was measured by flow cytometry analysis of PI-stained thymocyte nuclei. The results are the mean ± SD of 3 experiments performed in duplicate. *$P < .05$, **$P < .01$, and ***$P < .001$ with respect to DEX-treated samples.

ropeptides on the four main thymocyte subsets defined by the expression of CD4 and CD8 glycoproteins. Thymocytes were cultured with 10^{-6} mol/L DEX in the presence or absence of 10^{-8} mol/L VIP, PACAP27, or PACAP38. After 12 hours in culture, cells were harvested and stained with fluorescently labeled anti-CD4 and anti-CD8 antibodies and analyzed with a flow cytometer. Our results clearly indicate a loss of double-positive cells ($CD4^+CD8^+$) in cultures treated with DEX (Table 3). Both the percentage and yield of double-positive cells in the thymocyte population decreased to the minimum at 24 hours, then gradually recovered. There was also a significant increase in the proportion and absolute numbers of $CD4^+CD8^-$ single-positive thymocytes. Incubation with VIP or PACAP prevents the loss of double-positive thymocytes induced by DEX treatment, with the maximum effect at 24 hours of incubation (Table 3). The results were comparable to control values without DEX.

DISCUSSION

Increasing recent evidence suggests that VIP plays a role in cell survival in different systems. VIP has been found to prevent the neurotoxic effect of the envelope glycoprotein 120 of human immune deficiency virus (HIV),[17] to increase the survival of cultured neurons,[16,29,30] and to act as a differentiating agent in the human neuroblastoma cell line SK-N-SH.[31] Moreover, VIP protects mouse and human thymocytes exposed to a cytolytic dose of the synthetic glucocorticoid prednisolone in vitro.[32]

In this report, we show the effects of VIP and the structurally related neuropeptides PACAP27 and PACAP38 on spontaneous and DEX-induced apoptosis in rat thymocytes. We have found that VIP and both PACAPs inhibit, at least in part, spontaneous apoptosis in rat thymocytes. The sequence of events during glucocorticoid-induced apoptosis in thymocytes is well established. It has been described that DNA fragmentation precedes cell death during apoptosis induced in target cells by cytotoxic T lymphocytes,[33] in thymocytes treated with glucocorticoids,[5] and in T-cell hybridoma activated with anti-CD3 antibodies.[34] In accordance with this, we have found that treatment of thymocytes with DEX induces degradation of chromatin DNA (assayed by DPA reaction) during the first 4 to 12 hours. When analyzed by agarose gel electrophoresis, the pattern of DNA fragmentation observed in DEX-treated thymocytes indicated that endogenous endonuclease, which produces DNA fragmentation, had been activated by this treatment. In addition, DNA degradation was observed before the appearance of apoptotic cells (measured by PI staining and light microscopy) and loss of cell viability (evaluated by tetrazolium assay). This death program induced by DEX in rat thymocytes was completely inhibited by incubation with VIP, PACAP38, or PACAP27. The three neuropeptides prevented DNA degradation and loss of cell viability induced by treatment with DEX, with a maximum effect at 12 hours of incubation. Furthermore, they reduced the number of apoptotic cells and the morphologic changes induced by the treatment of thymocytes with this glucocorticoid. These effects were found in a concentration-dependent manner within a wide range of concentrations, from 10^{-6} to 10^{-12} mol/L, with the maximum effect being observed at 10^{-8} to 10^{-9} mol/L. This is a dose range at which VIP and PACAPs have been shown to produce effects in other systems. The Kd of the high-affinity binding sites described for VIP and PACAPs[22,35] corresponds to neuropeptide concentrations (10^{-8} to 10^{-9} mol/L) that showed the highest inhibitory effect in our study, which suggests that these high-affinity binding sites may mediate the inhibitory effect of these neuropeptides on glucocorticoid-induced apoptosis in rat thymocytes. The lower effect observed at high neuropeptide concentrations might account for a process of cell desensitization by internalization of neuropeptide receptors with sequestration and/or downregulation of their receptors.[36] In addition, the protective effect of VIP and both PACAPs appears to decrease with time. This most probably is due to the spontaneous

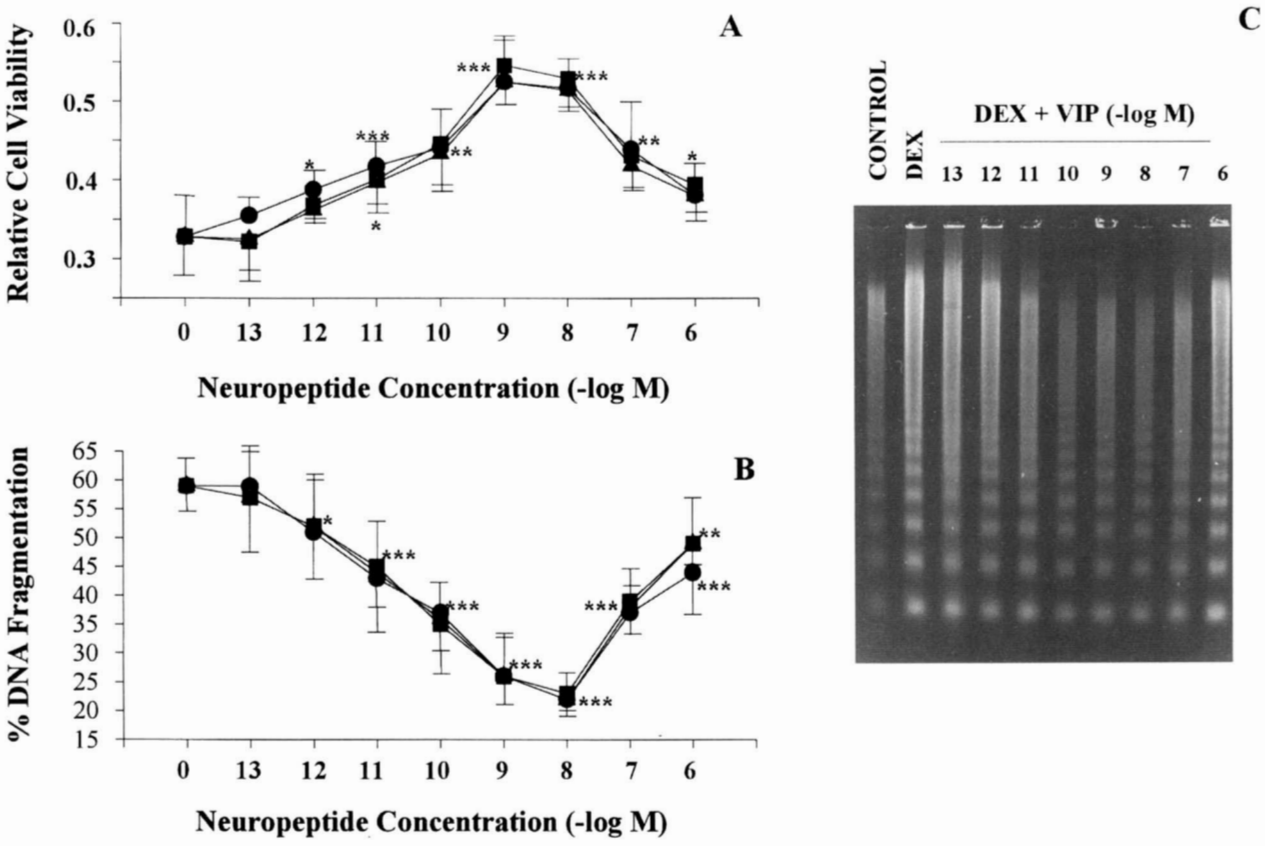

Fig 4. Curve-dose effect of VIP and PACAP on DEX-induced apoptosis of rat thymocytes. Cells were treated with DEX 10^{-6} mol/L in the presence or in the absence of different concentrations of VIP (●), PACAP38 (■), or PACAP27 (▲). In all cases, thymocytes were stimulated with ConA (2.5 μg/mL). (A) Cells were assessed for cell viability using MTT after 16 hours of incubation. (B) DNA fragmentation was assessed by DPA reaction after 12 hours of incubation. Results are the mean ± SD of 8 experiments performed in duplicate. *$P < .05$, **$P < .01$, and ***$P < .001$ with respect to DEX-treated samples. (C) Gel electrophoresis of cleaved DNA extracted from thymocytes treated with medium (controls), DEX 10^{-6} mol/L, and DEX 10^{-6} mol/L plus different concentrations of VIP. The result is an example representative of 3 experiments. Similar results were observed for PACAP38 and PACAP27.

hydrolysis of VIP in neutral solution[37] or to the capacity of lymphocytes to degrade VIP by endopeptidase activity.[38]

Studies that have involved VIP as a immunoregulatory peptide have been performed mainly in peripheral lymphoid organs, including lymphocyte adhesion and traffic,[39-41] inhibition of proliferation of peripheral T cells and thymocytes,[18,42-46] inhibition of interleukin (IL)-2 and IL-4 production in stimulated peripheral T-cell cultures,[18,19,45-48] modulation of immunoglobulin synthesis,[49] and modulation of natural-killer and macrophage activity.[50-53] The immunologic actions of VIP are exerted through specific receptors that have been described on T and B lymphocytes[54,55] and human peripheral blood lymphocytes.[35,54] Regarding PACAP, little is known about its effect on the immune system. PACAP has been recently shown to inhibit the mitogen-stimulated proliferation of murine splenocytes[22] and to decrease the mobility of rat splenic lymphocytes.[23] However, studies of VIP and/or PACAP effects on thymocytes are scarce. Although few binding sites for VIP have been described in murine thymocytes,[42] a recent report showed that VIP receptor is expressed in rat thymocytes.[55] Recently, it has been found that VIP inhibits both IL-2 and IL-4 production, as well as the proliferation of murine thymocytes stimulated through the TCR/CD3 complex.[19] Furthermore, VIP inhibits rat thymocyte mobility.[23] PACAP binding sites have not been described in rat thymus[56]; however, an inhibitory effect of PACAP on chemotaxis of rat thymocytes has been described.[23]

One of the most interesting points raised by our results is that VIP and PACAP appear to inhibit proliferation, as well as inhibit apoptosis. This apparent paradox has also been described for inducers of cell proliferation like c-*myc* and E1A that also act as potent inducers of apoptosis.[57] Like c-*myc*, VIP and PACAP might regulate one set of genes whose products inhibit either proliferation or apoptosis depending on downstream signals. In addition, it has been described that VIP inhibits c-*myc* expression in human gastric carcinoma cells.[58] Therefore, another possibility could be that the inhibition of cell proliferation and apoptosis by VIP could be due to a possible inhibition of c-*myc* expression by this neuropeptide. Nevertheless, it is well established that in the mouse cell system, differentiation and proliferation are in-

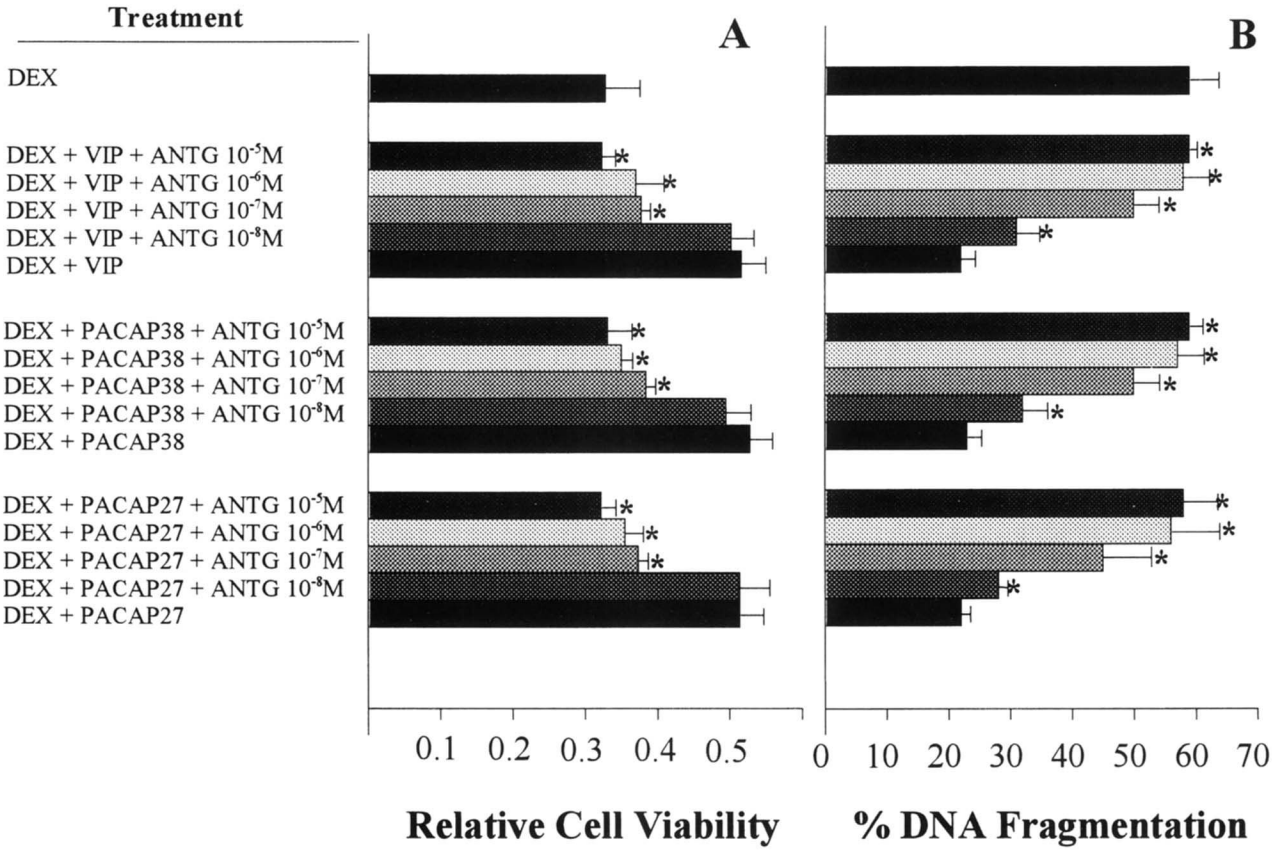

Fig 5. Effect of VIP-R antagonist (N-Ac-Tyr^1, D-Phe^2)GRF(1-29)-NH_2 on the protective activity of VIP (10^{-8} mol/L) and PACAP (10^{-8} mol/L) against apoptosis induced by DEX 10^{-6} mol/L in rat thymocytes stimulated with ConA (2.5 μg/mL) assessed by cell viability after 16 hours of incubation (A) and percentage of DNA fragmentation after 12 hours of incubation (B). Cell viability and percentage of DNA fragmentation in control cultures without DEX treatment were 0.480 ± 0.036 and 27 ± 3, respectively. The percentage of DNA fragmentation was 28 ± 4, 25 ± 2, 25 ± 3, and 27 ± 3 for controls treated with VIP antagonist 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mol/L alone, respectively. The results are the mean ± SD of 8 experiments performed in duplicate. *$P < .001$ with respect to neuropeptide-treated samples.

versely related. Differentiation programs are initiated as cell proliferation decreases and growth arrest occurs.[59] Thymocyte rescue from negative selection by inhibition of apoptosis is one of the first events of thymocyte differentiation. Since VIP inhibits thymocyte proliferation and apoptosis, it suggests that this neuropeptide could use both effects to promote thymocyte development.

VIP and PACAP have been previously shown to have similar affinities for both VIP1-R and VIP2-R.[60,61] In addition to these two receptors, a third highly specific PACAP receptor (PACAP-R type I) is expressed in certain cell types.[62] To examine the specificity of the neuropeptide effects observed on glucocorticoid-induced apoptosis in thymocytes, as well as to provide additional evidence for the receptor-mediated nature of the response, we investigated the ability of (N-Ac-Tyr^1, D-Phe^2)-GRF(1-29)-NH_2, a VIP receptor antagonist,[28] to attenuate the inhibitory effect of the neuropeptides on DEX-induced apoptosis. This VIP-R antagonist partially reversed, in a dose-dependent manner, the inhibitory effect of VIP, PACAP27, and PACAP38 on this system. Similar results were previously obtained for mobility inhibition[23] and IL-10 production in spleen cells and thymocytes (manuscript submitted). These results provide evidence for a specific VIP-R–mediated response and support the involvement of a single receptor. Moreover, the similarity in the dose-response curves for VIP and PACAP inhibition of DEX-induced apoptosis suggests that VIP and PACAPs exert their effects through the same VIP-R. The specificity of this VIP antagonist for VIP1-R[63] suggests the involvement of VIP1-R in the inhibitory activity of VIP and PACAPs on DEX-induced apoptosis. This hypothesis is in agreement with previous reports[22,23] that suggested VIP and PACAPs bind to the same or similar binding sites to exert their immune functions.

Immature double-positive thymocytes ($CD4^+CD8^+$) have been shown to be particularly sensitive to apoptotic death produced by treatment with glucocorticoids,[4,5,64-67] but the mechanism by which this process occurs has not yet been elucidated. In agreement with this, our results demonstrated that $CD4^+CD8^+$ thymocytes are the main target for DEX. VIP and both PACAPs selectively rescue this thymocyte subpopulation from glucocorticoid-induced apoptosis. This

Table 3. VIP and PACAPs Protect Double-Positive Thymocytes From DEX-Induced Apoptosis

Time (h)	Treatment	% Living Cells	Thymocyte Subpopulations (%)				No. of Cells (× 10^6/mL)			
			$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^+$	$CD4^+CD8^-$
0	None	99 ± 1	1.0 ± 0.1	84.2 ± 0.4	5.9 ± 0.2	8.9 ± 0.2	0.5 ± 0.1	42.1 ± 0.2	3.0 ± 0.1	5.9 ± 0.2
12	None	88 ± 6	1.8 ± 0.2	80.2 ± 0.3	8.4 ± 0.1	9.7 ± 0.2	1.9 ± 0.2	79.2 ± 0.3	8.3 ± 0.1	9.6 ± 0.2
	DEX	83 ± 6	5.1 ± 0.2	58.4 ± 1.3	10.6 ± 1.4	25.9 ± 0.8	2.9 ± 0.1	33.5 ± 0.7	6.1 ± 0.8	14.9 ± 0.4
	DEX + VIP	94 ± 4	1.8 ± 0.2	82.3 ± 0.7	7.0 ± 0.8	8.9 ± 0.2	1.8 ± 0.2	81.3 ± 0.7	6.9 ± 0.8	8.8 ± 0.2
	DEX + PACAP38	94 ± 3	1.7 ± 0.1	80.6 ± 1.0	7.8 ± 0.8	9.8 ± 0.2	1.8 ± 0.1	79.7 ± 1.2	7.8 ± 0.8	9.6 ± 0.3
	DEX + PACAP27	93 ± 5	1.7 ± 0.1	81.3 ± 0.3	7.6 ± 0.2	9.5 ± 0.1	1.5 ± 0.3	71.4 ± 7.5	6.7 ± 0.7	8.4 ± 0.9
24	None	76 ± 4	1.2 ± 0.1	60.7 ± 0.8	24.9 ± 0.1	13.2 ± 0.8	1.6 ± 0.1	82.7 ± 1.1	34.0 ± 0.1	18.0 ± 1.1
	DEX	53 ± 5	3.2 ± 0.6	37.1 ± 1.7	24.4 ± 2.2	35.3 ± 1.5	2.6 ± 0.4	29.5 ± 1.4	19.3 ± 1.8	28.0 ± 1.2
	DEX + VIP	76 ± 4	1.1 ± 0.1	66.4 ± 3.1	21.3 ± 1.5	11.2 ± 4.1	1.5 ± 0.1	90.5 ± 4.4	29.0 ± 2.0	15.2 ± 5.5
	DEX + PACAP38	77 ± 4	1.0 ± 0.1	62.2 ± 0.9	21.7 ± 1.4	15.1 ± 0.5	1.4 ± 0.2	84.9 ± 1.3	29.5 ± 1.8	20.5 ± 0.6
	DEX + PACAP27	77 ± 2	0.9 ± 0.1	65.1 ± 1.0	20.1 ± 1.0	13.9 ± 0.3	1.3 ± 0.2	88.7 ± 1.4	27.4 ± 1.3	19.0 ± 0.5
48	None	65 ± 8	4.0 ± 1.3	45.3 ± 3.9	30.1 ± 0.8	21.0 ± 1.4	5.0 ± 1.6	55.8 ± 4.8	37.1 ± 0.9	25.9 ± 1.7
	DEX	41 ± 7	1.9 ± 0.5	52.4 ± 1.6	29.5 ± 1.1	16.2 ± 0.5	1.5 ± 0.4	41.3 ± 1.2	23.2 ± 0.9	12.7 ± 0.4
	DEX + VIP	62 ± 5	3.2 ± 2.0	48.2 ± 5.4	30.2 ± 1.0	18.7 ± 3.6	4.3 ± 2.7	64.6 ± 7.2	40.5 ± 1.4	25.1 ± 4.8
	DEX + PACAP38	65 ± 7	3.3 ± 1.8	48.7 ± 5.9	28.8 ± 1.8	19.3 ± 2.5	4.1 ± 2.1	63.9 ± 8.1	38.2 ± 2.7	25.4 ± 3.4
	DEX + PACAP27	63 ± 3	3.4 ± 1.9	48.7 ± 4.8	29.1 ± 2.2	19.2 ± 1.5	3.9 ± 2.5	63.7 ± 6.3	37.1 ± 1.1	25.0 ± 2.1

Thymocytes were cultured with medium (none), or medium with DEX 10^{-6} mol/L in the presence or absence of VIP 10^{-8} mol/L, PACAP38 10^{-8} mol/L, or PACAP27 10^{-8} mol/L for different times, and yields and phenotypes of harvested thymocytes were determined. In all cases, thymocytes were stimulated with ConA (2.5 μg/mL). The cell suspensions were double-labeled with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibody and the percentage of cell subpopulation was analyzed by FACScan. The yield of thymocyte subpopulation was determined by multiplying the number of thymocytes per milliliter harvested from culture by the percentage of thymocyte subpopulation. Cell viability was determined by trypan blue exclusion. The results are the mean ± SD of 3 experiments performed in duplicate.

finding is in accordance with recent experiments performed in our laboratory that demonstrated expression of VIP-R by rat $CD4^+CD8^+$ thymocytes.[20a]

Furthermore, it has been described that VIP and PACAP are constituents of the thymic microenviroment. VIPergic fibers have been found in thymus,[68] and VIP immunoreactivity,[69] as well as VIP gene expression,[70] has been characterized in rat thymocytes. Moreover, we have recently reported VIP mRNA expression in both double-positive and single-positive rat thymocytes (manuscript submitted). PACAP immunoreactivity was also found in lymphoid cells from rat thymus.[71]

In the present report, we show for the first time that microenvironment neuropeptides and their receptors are involved in the rescue of thymocytes from apoptosis. These results suggest that the binding of VIP and PACAP to VIP-R, as in interleukins, triggers a signaling pathway responsible for the aquisition of the resistance of the mature T cells to the glucocorticoid drugs. Additional experiments are required to elucidate the molecular mechanisms involved in this process. These findings, together with evidence for the presence of VIP and PACAP and the expression of VIP-R during the early stages of thymocyte development in the mouse, lead to the conclusion that these neuropeptides could be involved in intrathymic T-cell maturation.

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