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*J Immunol* 2000; 164:1200-1210; 
doi: 10.4049/jimmunol.164.3.1200

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Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Inhibit Antigen-Induced Apoptosis of Mature T Lymphocytes by Inhibiting Fas Ligand Expression

Mario Delgado*† and Doina Ganea**

Aptosis in T and B lymphocytes is a major element controlling the immune response. The Ag-induced cell death (AICD) in T cells is a main mechanism for maintaining peripheral tolerance and for limiting an ongoing immune response. AICD is initiated by Ag re-engagement of the TCR and is mediated through Fas/Fas ligand (FasL) interactions. Vasoactive intestinal peptide (VIP) and the structurally related pituitary adenylate cyclase-activating polypeptide (PACAP) are two multifunctional neuropeptides present in the lymphoid microenvironment that act primarily as anti-inflammatory agents. In the present study we investigated whether VIP and PACAP affect AICD in mature peripheral T cells and T cell hybridomas. VIP and PACAP reduce in a dose-dependent manner anti-CD3-induced apoptosis in Con A/IL-2-pretreated peripheral T cells and the murine T hybridomas 2B4.11 and A1.1. A functional study demonstrates that the inhibition of AICD is achieved through the inhibition of activation-induced FasL expression at protein and mRNA levels. VIP/PACAP-mediated inhibition of both AICD and FasL expression is mediated through the specific receptors VPAC1 and VPAC2. Of obvious biological significance is the fact that VIP and PACAP prevent Ag-induced clonal deletion of CD4+ T cells, but not that of CD8+ T cells. By affecting FasL expression, VIP and PACAP may play a physiological role in both the generation of memory T cells and the inhibition of FasL-mediated T cell cytotoxicity. The Journal of Immunology, 2000, 164: 1200–1210.

Lymphocyte apoptosis is recognized as a major element in the control of the immune response. T lymphocytes undergo apoptosis in response to a variety of stimuli, including antigenic stimulation, withdrawal of growth factors, glucocorticoids, irradiation, or cytoxic agents (1, 2). The Ag-induced cell death (AICD)1 in mature T cells is thought to be a major mechanism for the maintenance of peripheral tolerance and for limiting an ongoing immune response (3).

Upon encountering the Ag, resting T cells are activated and enter a proliferation stage. T cells are resistant to apoptosis following initial TCR engagement, but become highly susceptible to apoptosis during the ensuing cell cycling (4–6). In the absence of further antigenic stimulation, passive apoptosis through lymphokine withdrawal eliminates the cycling cells. In the presence of Ag, cycling T cells reactivated through the TCR undergo active apoptosis, i.e., AICD (1). In contrast to passive apoptosis, AICD is mediated by Fas (CD95, Apo-1)/FasL and TNF/TNFR interactions (7–9). The Fas/FasL interactions appear to play a primary role in CD4+ T cell AICD, whereas TNF/TNFR interactions are essential for CD8+ T cell AICD (10). The expression of Fas and FasL on CD4+ T cells varies with the activation stage. In contrast to Fas, which is expressed in resting T cells and is up-regulated following activation (11), FasL is expressed only in TCR-activated T cells (12). The physiological significance of Fas/FasL-mediated apoptosis for immune homeostasis and normal immune functions is illustrated by the massive lymphoproliferation and autoimmunity occurring in gld and lpr mice, which are homozygous for genetic defects in FasL and Fas, respectively (13–15).

Vasoactive intestinal peptide (VIP) and the structurally related peptide, the pituitary adenylate cyclase-activating polypeptide (PACAP), are two neuropeptides present in the immune microenvironment (16, 17) that elicit a broad spectrum of biological functions, including actions on natural and acquired immunity (17–19). VIP and PACAP bind to specific receptors expressed on immune cells (20–24) and inhibit the production of proinflammatory cytokines, such as IL-2, TNF-α, IL-12, and IL-6, in activated T cells and macrophages (18, 25, 26). In addition, VIP and PACAP promote the differentiation of CD4+ T cells into Th2 effectors, presumably through their effect on macrophage B7.2 expression (27, 28).

At the present time the effects of VIP and PACAP on Ag-induced apoptosis in mature T cells are not known. Therefore, in the present study we investigated whether VIP and PACAP affect AICD in mature peripheral T cells and T cell hybridomas. The data presented here demonstrate that both neuropeptides reduce Ag-induced apoptosis in vitro and in vivo. This inhibitory activity is mediated through the inhibition of activation-induced FasL expression. To our knowledge, this is the first report describing the role...
of a neutrophil present in lymphoid microenvironment on the Ag-induced apoptosis of mature T cells.

Materials and Methods

Methods and cell culture
Female 6- to 8-wk-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A1.1 and 2B4.11 have been previously described (9, 29, 30). L1210 (leukemia cell line) and L1210-Fas+ (L1210 transfected with fas) cells were provided by Dr. P. Golstein (31). The cells were cultured in RPMI 1640 supplemented with 10% FCS (Life Technologies, Grand Island, NY), 10 mM HEPES buffer, 1 mM sodium pyruvate, 0.1 M nonessential amino acids, 2 mM glutamine, 50 μM ME, 100 U/ml penicillin, and 10 μg/ml streptomycin (complete medium).

Reagents
Synthetic VIP and PACAP38 were purchased from Novabiochem (Laufelfingen, Switzerland). The VPAC1 antagonist [Ac-His3,\(^{\circ}\)-d-Phe-\(^{\circ}\)-Trp13, R10-L7]-VIP(3-7)-GRF(8-27) and the VPAC1 agonist [K8, R10-L7]-VIP(1-7)-GRF(8-27) were donated by Dr. Patrick Robertbere (Université Libre de Bruxelles, Brussels, Belgium). The VPAC2 agonist Ro 25-1553 Ac-[Glu8,Lys12,Nle17,Ala19,Asp25,Leu26,Lys27,28,Gly20,30, Thr11]-VIP cyclic[21–25] was a gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PAC1 agonist maxadilan was a gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). The PAC1 antagonist PACAP38 was obtained from Peninsula Laboratories (Belmont, CA). Annexin V-FITC, and mAbs to murine Fas (CD95, J02), Fas Ligand (Fasl, Vg8 [F23.1], Vg6 [RR4-7], TNF-c, CD3e (145-2C11), and FITC-conjugated anti-mouse CD4 and CD8 mAbs were purchased from PharMingen (San Diego, CA). Propidium iodide, MTI, diphenylamine (DPA), proteinase K, and Staphylococcus aureus enterotoxin B (SEB) were purchased from Sigma (St. Louis, MO), and [\(^{3}H\)]Tdr was obtained from ICN Pharmaceuticals (Costa Mesa, CA).

Induction of AICD in BALB/c lymph node T cells

Anti-CD3-induced apoptosis of Con A-stimulated T cell blasts was assessed as described previously (32). Briefly, BALB/c mouse lymph node cells (2 × 10\(^6\) cells/ml) were stimulated with 5 μg/ml Con A for 48 h, washed with 10 ng/ml of α-methyl mannoside (Sigma), and incubated in RPMI complete medium with 50 U/ml of IL-2 for 48 h. Viable T lymphocytes were purified by density centrifugation and resuspended in fresh RPMI complete medium supplemented with 2% heat-inactivated FCS. Fifty thousand T cells (>99% αβ T cells) were seeded into wells of flat-bottom 96-well plates (Corning, Corning, NY) precoated with different concentrations (0.1–20 μg/ml) of anti-CD3ε mAbs (145-2C11) in the presence or the absence of VIP, PACAP, and VIP/PACAP receptor agonists and antagonists, and incubated for different time periods. Apoptosis was determined as described below.

Activation-induced apoptosis of Ag-stimulated T cells was assessed as previously described (33). Briefly, purified naive αβ CD4\(^+\) T cells were isolated by passing BALB/c lymph node cells over nylon columns to remove B, adherent, and activated cells, followed by treatment with anti-CD8 and anti-heat-stable antigen (J11D) Abs plus complement. The purified viable B, adherent, and activated cells, followed by treatment with anti-CD8 and anti-heat-stable antigen (J11D) Abs plus complement. The purified viable T cells were cultured in the presence of the superantigen SEB (150 μg/ml) and 10 mM biotin-16-dUTP) for 30 min at 37°C. Washed cells (5 × 10\(^6\) cells/ml) were centrifuged at 750 g for 10 min, resuspended in 400 l of hypotonic lysis buffer B (10 mM Tris-HCl, 40 mM EDTA (pH 8.0), 0.8% sodium lauryl sarcosinate, and 0.5 mg/ml proteinase K), and incubated at 50°C for 4 h, followed by the addition of RNase to a final concentration of 0.2 mg/ml. After incubation at 37°C for another 48 h, viable T lymphocytes were purified by density gradient centrifugation and resuspended at 5 × 10\(^4\) cells/well with SEB (0.5 μg/ml) and irradiated APCs (5 × 10\(^3\) cells/well) in the presence of different concentrations of VIP or PACAP. At different times, apoptosis and FasL expression was determined as described below.

Induction of AICD in T cell hybridomas

2B4.11 and A1.1 cells (5 × 10\(^3\) cells/ml) were cultured in 96-well plates with different concentrations of immobilized anti-CD3 mAbs (0.1–20 μg/ml), with PMA (10 ng/ml) plus ionomycin (1 μg/ml), with dexamethasone (10\(^{-6}\) M), or with the DNA-damaging drug etoposide (10 μM) in the presence of the absence of VIP and PACAP. In some experiments, UV irradiation was used at 200 J/m\(^2\) to induce apoptosis.

In vivo Ag-induced apoptosis

BALB/c mice (groups of three) were injected i.p. with the superantigen SEB (500 μg/mouse), with or without VIP, PACAP, or VPAC1 and VPAC2 agonists (5 nmol/mouse), for 48 h, followed by two i.p. injections of SEB (150 μg/mouse) at 48-h intervals, with or without VIP, PACAP, or VPAC1 and VPAC2 agonists (5 nmol/ml). Spleen and mesenteric lymph node T cells (1 × 10\(^6\) cells/ml) were analyzed for the expression of V\(^{\beta}\) (responsive to SEB) and V\(^{\beta}\) (nonresponsive to SEB, used as control) by flow cytometry as described below.

Assessment of cell viability and morphology

Cell viability was assessed by trypan blue exclusion, and loss of mitochondrial function was assessed using the MTT-staining method. For MTT staining, 100 μl of cell culture containing 2.5 × 10\(^6\) cells was placed in the wells of 96-well tissue culture plates, and 10 μl of MTT solution (2.5 mg/ml) was added. After incubation at 37°C for 4 h, 100 μl of acid-isoopropanol (0.04 N HCl in isopropanol) was added and mixed gently with the cell suspension, and the OD\(^{550}\) was determined with an ELISA reader.

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

Measurements of apoptosis

Assessment of apoptotic cells.
In most experiments, apoptosis was assayed by staining with annexin V-FITC plus propidium iodide and FACS analysis as previously described (34, 35). Briefly, cells (5 × 10\(^6\) cells/ml) were harvested in ice-cold PBS. Blocking of nonspecific binding sites was performed by incubation with PBS containing 2% BSA, 0.01% NaN\(_3\), and 1% casein for 20 min on ice. Subsequently, the cells were washed with staining buffer (SB; containing 1% BSA in 50 mM HEPES buffer, pH 7.4) and fixed with 4% paraformaldehyde in SB for 10 min. The fixed cells were first labeled with annexin V-FITC (2.5 μg/ml HEPES buffer containing 150 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), and 1.8 mM CaCl\(_2\), pH 7.4) for 15 min on ice to determine phosphatidylserine exposure to the outer face of the cell membrane. After repeated washing with SB, the cells were permeabilized with 0.1% saponin, stained with propidium iodide (5 μg/ml) for 15 min, and washed again in SB containing 0.1% saponin. A total of 10,000 cells were analyzed by flow cytometry on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The percentage of apoptotic cells was determined based on their ability to bind annexin V and propidium iodide (35).

Agarose gel electrophoresis DNA fragmentation assay.
Cultured lymphocytes were centrifuged at 750 × g for 10 min, resuspended in 100 μl of hypotonic lysis buffer A (100 mM Tris-HCl, 40 mM EDTA (pH 8.0), 0.8% sodium lauryl sarcosinate, and 0.5 mg/ml proteinase K), and incubated at 50°C for 4 h, followed by the addition of RNase to a final concentration of 0.2 mg/ml. After incubation at 37°C for another 30 min, the resulting DNA fragments were precipitated with 0.5 mM NaCl and 1 vol of isopropanol at −20°C overnight. The samples were centrifuged at 14,000 × g for 20 min at 4°C, and the pellet was washed with 70% ethanol and allowed to dry at room temperature. The DNA resuspended in TE solution (10 mM Tris-HCl and 1 mM EDTA (pH 7.4)) was fractionated by agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

Quantification of DNA fragmentation.
DNA fragmentation was assayed using the DPA reaction. Cultured lymphocytes were centrifuged at 750 × g for 10 min, resuspended in 400 μl of hypotonic lysis buffer B (100 mM Tris-HCl, 40 mM EDTA (pH 8.0), and 0.2% TCA and 0.1% Triton X-100), washed with PBS containing 10 μg/ml 30% acetic acid and 0.5 mg/ml proteinase K, and incubated at 50°C for 4 h. The supernatants containing small DNA fragments were separated immediately from the pellets that contained large uncut chromatin. Both the pellets (resuspended in 200 μl of hypotonic lysis buffer) and 200 μl of supernatants were hydrolyzed in 0.5 N perchloric acid for 20 min at 68°C. After cooling to 4°C, the samples were treated with 2 vol of a solution containing 0.08% M. DNA was dissolved in 0.98% (v/v) glacial acetic acid, 1.5% (v/v) sulfuric acid, and 0.5% (v/v) of 1.6% aceotylacetate solution and stored at 4°C for 4 h. The colorimetric reaction was assessed spectrophotometrically at 560 nm in an ELISA.

The Journal of Immunology 1201
The RNA extraction and Northern blot analysis

Northern blot analysis was performed according to standard methods. BALB/c lymph node T cells were prepared and stimulated as described above. At various time points, 1 × 10^6 cells were harvested, and total RNA was extracted by the acid guanidinium-phenol-chloroform method, ethotropolysed on 1.2% agarose-formaldehyde gels, transferred to Nytran membranes (Schleicher & Schuell, Keene, NH), and cross-linked to the nitrocellulose membranes using an UV light.

The probes for murine Fas, FasL, and GADPH were generated by RT-PCR as described previously (4) using the following primers: Fas, 5′-GACCCGAATACCAAGTGCAAGTG-3′ and 5′-GTTGGCCCTCTT GATGTTATTTTT-3′; FasL, 5′-TCACCAACAAAGCTTTAACAGTA-3′ and 5′-TCACTTTCCTTCCTCATTAGCA-3′; and GADPH, 5′-TCCTGGACCCACCAACCCCTTAC-3′ and 5′-GTCAGACCTTGAT GACCTGGCC-3′. Oligonucleotides were end labeled with [γ-32P]ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL) using T4 polynucleotide kinase. The RNA-containing membranes were prehybridized for 16 h at 42°C and then hybridized at 60°C for 16 h with the appropriate probes. The membranes were washed twice in 2× SSC containing 0.1% SDS at room temperature (20 min each time), once at 37°C for 20 min, and once in 0.1× SSC containing 0.1% SDS at 50°C. The prehybridization and hybridization buffers were purchased from 5′ Prime-3 Prime (Boulder, CO). The membranes were exposed to x-ray films (Eastman Kodak, Rochester, NY). Signal quantitation was performed in a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

FACS analysis

BALB/c lymph node T cells or 2B4.11 cells (1 × 10^6 cells/ml) prepared and stimulated in 96-well plates were harvested in ice-cold RPMI complete medium and washed twice in lysis PBS containing 0.1% sodium azide plus 2% heat-inactivated FCS (wash buffer). The cells were incubated in wash buffer containing 2.5 μg/ml normal mouse Ig for 15 min, after which anti-Fas (Jo2), anti-FasL (MFL3), anti-Fas (Jo2), anti-FasL (MFL3), or FasL (RR4-7) Abs were added (2.5 μg/ml, final concentration), followed by incubation at 4°C for 1 h. Isotype-matched Abs were used as controls, and IgG2b block (purchased from Sigma) was used to block the nonspecific binding to Fc receptors. The cells were washed and further stained with 2.5 μg/ml of FITC-conjugated goat F(ab′)2 anti-hamster IgG (for Fas and FasL), FITC-conjugated goat F(ab′)2 anti- rat IgG (for FasL), or FITC-conjugated goat F(ab′)2 anti-mouse IgG (for FasL) (Sigma), for 30 min at 4°C. After extensive washing, the cells were fixed in 1% buffered paraformaldehyde. Stained lymphocytes, gated according to forward and side scatter characteristics, were analyzed on a FACScan flow cytometer (Becton Dickinson). Samples in which isotype-matched Ab was used instead of specific Ab were used as negative controls to determine the proper region or window setting. For analysis, cells stained between channel 36 and channel 1023 were scored as positive. This region contained <4% of the cells in negative controls. Fluorescence data were expressed as the mean channel fluorescence (MCF), and as the percentage of positive cells after subtraction of background isotype-matched values.

RT-PCR for the detection of VPAC1, VPAC2, and PAC1 mRNA

Total RNA was isolated from Con A/IL-2-stimulated lymph node T cells (1 × 10^7 cells) using the Ultraspec RNA reagent (Biotech, Houston, TX) as recommended by the manufacturer. Two micrograms of total RNA was reverse transcribed in the presence of 200 U of Moloney murine leukemia virus reverse transcriptase, 40 U of RNasin, 1 μl of dNTPs, 1 μl of random primers, 0.5 μl of RNaseH, and 5 μl of reaction buffer. The cDNA was subjected to RT-PCR in the presence of 0.5 U of Taq polymerase, 1 μM sense and antisense primers, 0.2 mM dNTPs, and polymerase buffer (50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 20 mM (NH4)2SO4, and 50 μg/ml BSA). The PCR conditions were denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and primer extension at 72°C for 90 s for 35 cycles. The PCR products were size separated on 2% agarose gels and visualized by UV light.

Assessment of AICD in CD4+ and CD8+ T cells

Lymph node cells were stimulated with 5 μg/ml of Con A for 48 h, washed with 10 μg/ml of α-methyl mannoside, and incubated in RPMI complete medium with 50 U/ml of IL-2 for 48 h. Viable T lymphocytes were purified by density gradient centrifugation and resuspended in fresh RPMI complete medium. CD4+ and CD8+ T cells were positively selected after incubation with magnetic Dynabeads conjugated with anti-CD4 and anti-CD8 mAbs (Dynal, Lake Success, NY) and separation in an external magnetic field according to the manufacturer’s instructions. The CD4+ and CD8+ T cells (5 × 10^5 cells/well) were incubated for 48 h in wells that were either uncoated or coated with 10 μg/ml anti-CD3 mAb in the presence or the absence of VIP or PACAP, and the number of viable cells was determined by trypan blue. In addition, FasL expression in the CD4+ and CD8+ T cells was assessed by flow cytometry.

Alternatively, unseparated Con A/IL-2 blasts were exposed to immobilized anti-CD3 in the presence or the absence of VIP/PACAP and stained 48 h later with anti-CD4 and anti-CD8 mAbs. The numbers of viable CD4+ and CD8+ T cells were determined by FACS analysis following plating for viable cells with propidium iodide as previously described (32). In some samples, anti-TNF-α mAb (10 μg/ml) was added to the anti-CD3-stimulated cultures.

Analysis of functional FasL expression

Activation-induced FasL expression in anti-CD3 stimulated T lymphocytes was assessed by determining the ability of these cells to cause DNA fragmentation in Fas+ target cells as described previously (29). Briefly, Con A/IL-2-stimulated lymph node T cells or 2B4.11 cells were activated with immobilized anti-CD3 mAbs in the presence or the absence of different concentrations of VIP or PACAP and cultured for 8 h to allow FasL expression. The cells were washed twice and incubated for another 8 h with [3H]TdR-labeled FasL+ L1210 (wt) or FasL– L1210 target cells (2 × 10^5 cells/well). The L1210 and L1210-FasL+ cells were labeled for 2 h with 5 μCi/ml [3H]TdR (79 Ci/mmol; Amersham) in RPMI containing 5% FCS. In other experiments, VIP and PACAP were added 8 h after plating on immobilized anti-CD3. [3H]TdR-labeled unfragmented high m.w. DNA was harvested on glass-fiber filters (Pharmacia, Piscataway, NJ) and counted in a liquid scintillation counter. The DNA fragmentation was calculated as follows: %DNA fragmentation = 100 × (cpm control group – cpm experimental group/cpm control group) × 100. No DNA fragmentation was observed when target cells were cultured with anti-CD3 mAb in the absence of activated T cells or with T cells in the absence of anti-CD3 mAb.

The autocrine T cell suicide mediated by FasL/Fas interaction was assessed as previously described (7). Briefly, single 2B4.11 cells were distributed into 60-well Terasaki microplates (Nunc, Roskilde, Denmark; two plates per experimental group) using an autologous device unit of FACS (FACS VANTAGE, Becton Dickinson). Terasaki plates were previously coated with anti-CD3 mAbs (1 μg/ml) or medium as described above. Cells were incubated in 10-μl cultures in the presence or the absence of VIP or PACAP (10−8 M) and inspected by microscope at a 24-h interval for 4 days. Wells containing a cell remaining at the single-cell stage were scored as growth-negative wells (apoptosis positive), and wells with cells progressing from the single-cell to the multiple-cell stage were scored as growth-positive wells (apoptosis negative). The microscopic analysis and scoring were performed in a blind fashion.

Statistical analysis

All values are expressed as the mean ± SD of the indicated number of experiments performed in duplicate, as shown in the corresponding figures. Comparisons between groups were made using Student’s t test followed by Scheffe’s F test, with p < 0.05 as the minimum significant level.

Results

VIP and PACAP decrease AICD in mature T cells

To investigate the roles of VIP and PACAP in AICD, we used an in vitro model of apoptosis following TCR reengagement in cycling T cells (5, 32). Resting lymph node T cells activated with

reader. The percentage of DNA fragmentation was expressed by the following formula: [OD_{500} of supernatant (OD_{500} of supernatant + OD_{500} of pellet)] × 100.
sis is a representative example of three experiments. SD of three separate experiments performed in duplicate. Gel electrophoresis (DNA fragmentation) or by gel electrophoresis. Each result is the mean fragmentation was determined by using the DPA assay (percentage of viability was assessed by the annexin V/propidium iodide method, and the DNA viability was determined by MTT assay, the percentage of apoptotic cells plates (unstimulated) were used as control. Forty-eight hours later the cell different concentrations of VIP or PACAP. Cells cultured in uncoated concentrations of immobilized anti-CD3 mAbs in the presence or the absence of VIP or PACAP. Cells cultured in uncoated plates (unstimulated) were used as control. After 24 h apoptosis was determined by the TUNEL assay. Cell viability was determined by the MTT assay after 48 h of incubation. Each result is the mean ± SD of four separated experiments performed in duplicate. The dotted line represents anti-CD3-stimulated cells in the absence of VIP or PACAP. After 24 h, apoptosis was determined by the TUNEL assay. Cell viability was determined by the MTT assay after 48 h of incubation. Cells cultured in uncoated plates (unstimulated) were used as a control (cell viability, 1.8 ± 0.2; % apoptosis, 6 ± 1%). Each result is the mean ± SD of four separate experiments performed in duplicate. *, p < 0.001 with respect to samples treated with anti-CD3 alone.

Con A followed by IL-2 treatment and restimulation with different concentrations of immobilized anti-CD3 Abs undergo apoptosis, and the presence of VIP and PACAP during restimulation leads to a dramatic decrease in apoptosis at all time points and for all anti-CD3 concentrations (Fig. 1, A and B). The inhibitory effect of VIP and PACAP is dose dependent, already apparent at a concentration of 0.1 nM, and becoming maximal at 10 nM (Fig. 1C). In addition, treatment with either neuropeptide inhibits the DNA fragmentation characteristic of TCR-induced apoptosis (Fig. 1C). Moreover, microscopic inspection of hematoxylin-eosin-stained cytospin preparations showed that both neuropeptides decreased the number of T cells with morphologic changes characteristic of activation-induced apoptosis, such as increased cell density, cytoplasmatic vacuolization, and condensed chromatin (not shown).

Because T hybridomas are proliferating cells, direct exposure to immobilized anti-CD3 Abs induces apoptosis (7, 9, 29). The murine T cell hybridomas 2B4.11 and A1.1 have been extensively used in AICD studies (9, 29, 30, 36, 37). Similar to activated, proliferating mature T cells, the 2B4.11 and A1.1 T cell hybridomas undergo apoptosis following cross-linking of the TCR/CD3 complex, and VIP and PACAP dramatically decrease cell loss and apoptosis (Fig. 2A). Next we studied the effect of VIP and PACAP on apoptosis induced by other agents (36, 38–42). VIP and PACAP inhibited apoptosis induced by PMA plus ionomycin, a treatment that mimics TCR stimulation, by the topoisomerase II inhibitor etoposide, and by UV irradiation (Fig. 2B).

**FIGURE 1.** VIP and PACAP decrease AICD in restimulated Con A blasts. A, Time course. Con A/IL-2-stimulated lymph node T cells (5 × 10⁶ cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml; 200 μl/well) in the presence or the absence of VIP or PACAP (10⁻⁸ M) and assessed at different times for cell viability or apoptosis. Cell viability was assessed by the MTT assay. Apoptosis was analyzed by the TUNEL assay. Cells cultured in uncoated plates (unstimulated) were used as control. B, Effect of VIP/PACAP at different doses of anti-CD3. Con A/IL-2-stimulated lymph node T cells (5 × 10⁶ cells) were activated with different concentrations of immobilized anti-CD3 mAbs in the presence or the absence of VIP or PACAP (10⁻⁸ M). After 48 h, apoptosis was determined by the TUNEL assay, and cell viability was assessed by the MTT assay. The percentage of cell loss was calculated as: (1 – OD₅₆₀ of stimulated cells/OD₅₆₀ of unstimulated cells) × 100%. C, Dose-response curve. Con A/IL-2-stimulated lymph node T cells (5 × 10⁶ cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of different concentrations of VIP or PACAP. Cells cultured in uncoated plates (unstimulated) were used as control. Forty-eight hours later the cell viability was determined by MTT assay, the percentage of apoptotic cells was assessed by the annexin V/propidium iodide method, and the DNA fragmentation was determined by using the DPA assay (percentage of DNA fragmentation) or by gel electrophoresis. Each result is the mean ± SD of three separate experiments performed in duplicate. Gel electrophoresis is a representative example of three experiments. *, p < 0.001 with respect to control values with anti-CD3 alone.
VIP and PACAP failed to inhibit dexamethasone-induced apoptosis in 2B4.11 cells (Fig. 2B).

Inhibition of AICD is mediated through both VPAC1 and VPAC2

The immunological actions of VIP and PACAP are exerted through a family of VIP/PACAP receptors, i.e., VPAC1 and VPAC2, which exhibit similar affinities for the two neuropeptides, and PAC1, which exhibits a 300- to 1000-fold higher affinity for PACAP than for VIP (43). Con A/IL-2-stimulated lymph node T cells (5 x 10^6 cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of different concentrations of maxadilan (PAC1 agonist), Ro 25-1555 (VPAC2 agonist), and [K^13,R^16,L^27]VIP[1–7]-GRF[8–27] (VPAC1 agonist). After 48-h incubation, cell viability and percentage of apoptotic cells were determined by the MTT assay and annexin V/propidium iodide staining, respectively. Cells cultured in uncoated plates (unstimulated) were used as a control. *, p < 0.001 with respect to control values with anti-CD3 alone. C, Effect of VIP/PACAP receptor antagonists. Con A/IL-2-stimulated lymph node T cells (5 x 10^6 cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml) and treated simultaneously with VIP or PACAP (10^-8 M), and different concentrations of a VPAC1 antagonist, [Ac-His^1,p-Phe^2,K^13,R^16,L^27]VIP[3–7]-GRF[8–27], or a PAC1/VPAC2 antagonist (PACAP_6-38). After 48 h, apoptosis was determined by the TUNEL assay, and cell viability was assessed by the MTT assay. The percentage of cell loss was calculated as: (1 – OD_{560} of stimulated cells/OD_{560} of unstimulated cells) x 100%. Incubation with antagonists alone did not affect anti-CD3-induced apoptosis (not shown). Each result is the mean ± SD of three separate experiments performed in duplicate.

FIGURE 3. VPAC1 and VPAC2 mediate the effect of VIP/PACAP on AICD in Con A blasts. A, Expression of VPAC1, VPAC2 and PAC1 mRNA in Con A/IL-2-stimulated T cells. Total RNA extracted from Con A/IL-2-stimulated lymph node T cells (2 x 10^7 cells) was subjected to RT-PCR with specific primers for VPAC1, VPAC2, PAC1, and β-actin as described in Materials and Methods. One representative experiment of two is shown. B, Comparative effects of VIP/PACAP receptor agonists. Con A/IL-2-stimulated lymph node T cells (5 x 10^6 cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of different concentrations of maxadilan (PAC1 agonist), Ro 25-1555 (VPAC2 agonist), and [K^13,R^16,L^27]VIP[1–7]-GRF[8–27] (VPAC1 agonist). After 48-h incubation, cell viability and percentage of apoptotic cells were determined by the MTT assay and annexin V/propidium iodide staining, respectively. Cells cultured in uncoated plates (unstimulated) were used as a control. *, p < 0.001 with respect to control values with anti-CD3 alone.

FIGURE 4. VIP and PACAP inhibit anti-CD3-induced FasL expression in Con A blasts. A and B, Con A/IL-2-stimulated lymph node T cells (5 x 10^6 cells) were incubated with medium alone (unstimulated) or activated with immobilized anti-CD3 mAbs (10 μg/ml) for 16 h (A) or for different times (B). Expression of FasL and Fas was analyzed by flow cytometry. The gates (dashed lines) were set based on staining with unrelated isotype control Abs (A). Data are representative of four similar experiments. The results in B are expressed as MCF and are the mean ± SD of four independent experiments performed in duplicate. C, Dose-response curve. Con A/IL-2-stimulated lymph node T cells (5 x 10^6 cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of different concentrations of VIP or PACAP. Cells cultured in uncoated plates (unstimulated) were used as a control. After 16 h, Fasl and Fas expressions were analyzed by flow cytometry. Results are the mean ± SD of three independent experiments performed in duplicate. D and E, VIP and PACAP inhibit FasL expression at the mRNA level. Con A/IL-2-stimulated lymph node T cells (5 x 10^6 cells) were incubated with medium alone (unstimulated) or activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of different concentrations of VIP or PACAP. Cells cultured in uncoated plates (unstimulated) were used as a control. After 2 h for Fas, 6 h for Fasl.

Results in E are expressed in arbitrary densitometric units normalized for the expression of GAPDH. One representative experiment of three is shown. *, p < 0.001 with respect to cultures treated with anti-CD3 alone.
VIP and PACAP inhibit anti-CD3-induced FasL expression

It has been previously demonstrated that AICD in mature T cells and T cell hybridomas proceeds via expression of FasL and subsequent Fas/FasL interaction (4, 29, 32, 38). Therefore, we envisioned three nonexclusive possibilities for the mechanism of inhibition of AICD by VIP and PACAP: inhibition of FasL expression, inhibition of Fas expression, and/or inhibition of the Fas signaling pathways.

To determine whether VIP and PACAP affect Fas and FasL expression, Con A/IL-2-stimulated lymph node T cells and 2B4.11 hybridomas were stimulated with immobilized anti-CD3 Abs in the presence or the absence of VIP or PACAP, and the expression of Fas and FasL was assayed by flow cytometry and Northern blots. Low levels of Fas are expressed constitutively on murine peripheral T cells and increase following anti-CD3 activation. FasL expression is induced by activation (Fig. 4A). Maximum FasL mRNA expression occurs 4 h, and maximum surface FasL protein expression occurs 12–24 h after anti-CD3 activation (Fig. 4, B and E). VIP and PACAP only slightly decrease Fas expression. In contrast, the activation-induced expression of mRNA and protein FasL is greatly reduced by VIP and PACAP at all time points and in a dose-dependent manner (Fig. 4). Similar results were obtained with the 2B4.11 T cell hybridoma (results not shown).

Similar to the inhibitory effect observed in AICD, FasL protein and mRNA expression in restimulated Con A blasts was inhibited by VPAC1 and VPAC2 agonists, but not by PAC1 agonists (Fig. 5, A and B), and the VPAC1 and VPAC2 antagonists reversed the effect of VIP/PACAP (Fig. 5C). In contrast, in the 2B4.11 hybridoma, which expresses only VPAC2, the VPAC2 agonist, but not the VPAC1 or PAC1 agonists, inhibited anti-CD3-induced FasL expression, and PACAP6–38, but not the VPAC1 antagonist, reversed the effect of VIP/PACAP (Fig. 5D). These results indicate that the inhibitory effect of VIP and PACAP on AICD correlates with the reduction in FasL expression.

To substantiate that the reduction of FasL expression by VIP/PACAP has functional significance, we stimulated 2B4.11 hybridoma cells with immobilized anti-CD3 for 8 h in the presence or the absence of VIP/PACAP. Based on previous Northern and Western blots, this time interval is enough for the expression of FasL. The 2B4.11 cells harvested after 8-h incubation with the immobilized PACAP6–38. FasL expression was analyzed by flow cytometry after 16-h culture. Incubation with antagonists alone did not shown any effect on anti-CD3-induced apoptosis and FasL expression (not shown). Each result is the mean ± SD of three separate experiments performed in duplicate. ∗, p < 0.001 with respect to control values with anti-CD3 alone.

**FIGURE 5.** VIP/PACAP inhibit FasL expression in Con A blasts (A–C) and 2B4.11 T cell hybridoma (D) through specific receptors. A, Comparative effects of VIP/PACAP receptor agonists on anti-CD3-induced cell surface Fas and FasL expression. B, Comparative effects of VIP/PACAP receptor agonists on anti-CD3-induced Fas and FasL, mRNA expression. Con A/IL-2-stimulated lymph node T cells (5 × 10^6 cells for A and 2 × 10^7 cells for B) were incubated with medium (unstimulated) or activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of different concentrations (A) of maxadilan (PAC1 agonist), Ro 25-1553 (VPAC2 agonist), or [K^{3–7},R^{16},L^{27}]VIP[3–7]-GRF[8–27] (VPAC1 agonist). In B, the concentration of VIP/PACAP agonists was 10^{-8} M. After 16 h, surface FasL was measured by FACS analysis (A). Results are the mean ± SD of three independent experiments performed in duplicate. After 6 h (B), expression of FasL mRNA was analyzed by Northern blot. One representative experiment of three is shown. C, Effect of VIP/PACAP receptor antagonists. Con A/IL-2-stimulated lymph node T cells (5 × 10^6 cells) were activated with immobilized anti-CD3 mAbs (10 μg/ml), and treated simultaneously with VIP or PACAP (10^{-8} M) and different concentrations of a VPAC1 antagonist, [Ac-His(D-Phe)^{2},K^{15},R^{16},L^{27}]VIP[3–7]-GRF[8–27], a PAC1/V PAC2 antagonist (PACAP6–38), and VPAC1 agonist. After 16 h, FasL expression was assessed by flow cytometry. The dotted line represents control values from cultures incubated with anti-CD3 alone. Incubation with antagonists alone did not show any effect on FasL expression. Each result is the mean ± SD of three separate experiments performed in duplicate. D, Effect of VIP/PACAP receptor agonists and antagonists on anti-CD3-induced apoptosis of 2B4.11 cells. 2B4.11 cells (5 × 10^6 cells) were activated with plate-bound anti-CD3 mAbs (1 μg/ml) in the presence or the absence of different concentrations of immobilized anti-CD3 mAbs (10 μg/ml), and treated simultaneously with VIP or PACAP (10^{-8} M) and different concentrations of a VPAC1 antagonist, [Ac-His^{3},D-Phe^{2},K^{15},R^{16},L^{27}]VIP[3–7]-GRF[8–27], or a PAC1/VPAC2 antagonist (PACAP6–38). FasL expression was analyzed by flow cytometry after 16-h culture (Fig. 4A). Maximum FasL mRNA expression occurs 4 h, and maximum surface FasL protein expression occurs 12–24 h after anti-CD3 activation (Fig. 4, B and E). VIP and PACAP only slightly decrease Fas expression. In contrast, the activation-induced expression of mRNA and protein FasL is greatly reduced by VIP and PACAP at all time points and in a dose-dependent manner (Fig. 4). Similar results were obtained with the 2B4.11 T cell hybridoma (results not shown).

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To substantiate that the reduction of FasL expression by VIP/PACAP has functional significance, we stimulated 2B4.11 hybridoma cells with immobilized anti-CD3 for 8 h in the presence or the absence of VIP/PACAP. Based on previous Northern and Western blots, this time interval is enough for the expression of FasL. The 2B4.11 cells harvested after 8-h incubation with the immobilized PACAP6–38. FasL expression was analyzed by flow cytometry after 16-h culture. Incubation with antagonists alone did not shown any effect on anti-CD3-induced apoptosis and FasL expression (not shown). Each result is the mean ± SD of three separate experiments performed in duplicate. ∗, p < 0.001 with respect to control values with anti-CD3 alone.
Each result is the mean ± SD of three independent experiments performed in duplicate. A, B4.11 cells (5 × 10^6 cells) were activated at time zero with immobilized anti-CD3 mAbs (1 μg/ml) in the presence of the absence of different concentrations of VIP or PACAP. Cells were cultured for 8 h to allow FasL expression, harvested, washed twice, and incubated for an additional 8 h with [3 H]TdR-labeled L1210 (wt) or L1210-Fas-transfected L1210 cells (2 × 10^6 cells). The percentage of DNA fragmentation was calculated as described in Materials and Methods. Each result is the mean ± SD of three independent experiments performed in duplicate, *, p < 0.001 with respect to cultures treated with anti-CD3 alone. B, In a parallel experiment, different concentrations of VIP or PACAP were added after the 8-h activation of B4.11 cells with anti-CD3 mAbs (8 h). Effector and target cells were incubated for an additional 8 h in the presence or the absence of VIP or PACAP, and the percentage of DNA fragmentation was calculated as described in Materials and Methods. Each result is the mean ± SD of three independent experiments performed in duplicate. C, VIP and PACAP do not block soluble recombinant FasL-induced apoptosis. B4.11 cells (5 × 10^6 cells) were treated with different doses of soluble recombinant human FasL (sFasL) in the absence (control) or the presence of VIP or PACAP (10 ^-8 M). Fifteen minutes after the addition of sFasL, anti-Flag M2 Ab (1 μg/ml) was added to cross-link sFasL. After 12-h incubation, the percentage of apoptosis was assessed by the TUNEL assay. Each result is the mean ± SD of three separate experiments performed in duplicate.

VIP and PACAP inhibit activation-induced apoptosis by modulating FasL expression and function, but do not block signaling through the Fas receptor. A, B4.11 cells (5 × 10^6 cells) were activated with immobilized anti-CD3 mAbs (1 μg/ml) in the presence or the absence of different concentrations of VIP or PACAP. Cells were cultured for 8 h to allow FasL expression, harvested, washed twice, and incubated for an additional 8 h with [3 H]TdR-labeled L1210 (wt) or L1210-Fas-transfected L1210 cells (2 × 10^6 cells). The percentage of DNA fragmentation was calculated as described in Materials and Methods. Each result is the mean ± SD of three independent experiments performed in duplicate. A, B4.11 cells (5 × 10^6 cells) were treated with medium (unstimulated) or activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of VIP or PACAP (10 ^-8 M). After 16 h, the expression of FasL was analyzed by flow cytometry. After 48-h incubation, the number of viable cells was counted using the trypan blue assay. Each result is the mean ± SD of three independent experiments performed in duplicate. B, Con A/IL-2-stimulated lymph node T cells (5 × 10^6 cells) were incubated with medium (unstimulated) or activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of VIP (10 ^-8 M), PACAP (10 ^-8 M), and/or anti-TNF-α mAb (10 μg/ml). After 48-h culture, the cells were stained with FITC-labeled anti-CD4 or anti-CD8 mAbs and propidium iodide, and the numbers of viable CD4^+ or CD8^+ T cells were determined by flow cytometry. Results are the mean ± SD of three independent experiments performed in duplicate.

VIP and PACAP preferentially protect CD4^+ T cells from AICD. A, Immunomagnetic sorted populations of CD4^+ and CD8^+ T cells were activated with immobilized anti-CD3 mAbs (10 μg/ml), in the presence or the absence of VIP or PACAP (10 ^-8 M). After 16 h, the expression of FasL was analyzed by flow cytometry. After 48-h incubation, the number of viable cells was counted using the trypan blue assay. Each result is the mean ± SD of three independent experiments performed in duplicate. B, Con A/IL-2-stimulated lymph node T cells (5 × 10^6 cells) were incubated with medium (unstimulated) or activated with immobilized anti-CD3 mAbs (10 μg/ml) in the presence or the absence of VIP (10 ^-8 M), PACAP (10 ^-8 M), and/or anti-TNF-α mAb (10 μg/ml). After 48-h culture, the cells were stained with FITC-labeled anti-CD4 or anti-CD8 mAbs and propidium iodide, and the numbers of viable CD4^+ or CD8^+ T cells were determined by flow cytometry. Results are the mean ± SD of three independent experiments performed in duplicate.

VIP and PACAP preferentially protect CD4^+ T cells from AICD

Both CD4^+ and CD8^+ T cells are deleted by activation-induced apoptosis (32), and we had previously shown that both CD4^+ and CD8^+ T cells express similar levels of VPAC1 and VPAC2 receptors (21). We examined whether VIP or PACAP prevent AICD in both CD4^+ and CD8^+ T cells. Con A blasts were separated into CD4^+ and CD8^+ T cells before restimulation. Treatment with VIP or PACAP preferentially prevented CD4^+ T cell apoptosis (Fig. 7A). The protective effect correlated with the VIP/PACAP inhibition of activation-induced FasL expression (Fig. 7A). In contrast, although VIP and PACAP reduced anti-CD3-induced FasL expression in CD8^+ T cells, the neuropeptides did not significantly protect CD8^+ T cells from AICD (Fig. 7A).

It has been reported that CD4^+ T cells undergo AICD primarily through FasL/Fas interactions, whereas CD8^+ T cells respond primarily to TNF/TNFR and, to a lesser degree, to FasL/Fas interactions (32). Therefore, blockade of both the TNF and FasL pathways is required to abrogate cell death in CD8^+ T cells, whereas blockade of FasL is sufficient to prevent AICD in CD4^+ T cells. Indeed, treatment with anti-TNF mAbs significantly blocked TCR-induced apoptosis of CD8^+ T cells, but was incapable of preventing apoptosis of most CD4^+ T cells (Fig. 7B). In addition, CD8^+ T cell apoptosis was completely abrogated by the simultaneous treatment with anti-TNF Abs and VIP or PACAP (Fig. 7B). This is in contrast to the CD4^+ T cells, where anti-TNF Abs did not add to the protective effect of VIP or PACAP (Fig. 7B).
The effects of VIP and PACAP on Ag-induced T cell apoptosis were investigated in vitro and in vivo. The in vitro model consists of BALB/c lymph node CD4+ T cells stimulated with SEB in the presence of syngeneic APCs, cultured in the presence of IL-2, and restimulated with SEB and irradiated APCs. VIP and PACAP are present during the restimulation phase. VIP and PACAP reduce Ag-induced cell death of T cells in vitro and in vivo.

VIP and PACAP decrease autocrine suicide in T cell hybridomas

It has been reported that TCR-induced apoptosis in T cells could occur as a single-hit event and does not necessarily require cell interactions or accessory cells, i.e., autocrine apoptosis. To investigate whether VIP and PACAP inhibit autocrine apoptosis, we assessed the apoptosis of Single 2B4.11 cells plated in Terasaki microwells coated with anti-CD3 Abs, as previously described. Because assessment of single-cell apoptosis by microscopic inspection is not objective, we determined the fraction of 2B4.11 cells incapable of progressing beyond the single-cell stage. Apoptosis of cells remaining at a single-cell stage after 4 days of culture was confirmed by microscopic inspection of cellular morphology (apoptosis was evident from membrane blebbing and chromatin condensation). The fraction of single anti-CD3-stimulated T cell hybridomas undergoing apoptosis increased significantly compared with that in control cultures (uncoated plates), and VIP and PACAP prevented the apoptosis of single 2B4.11 cells to a large degree (Fig. 9).

Discussion

A significant function of the constantly renewing hematopoietic system consists in the maintenance of homeostasis. Clonal expansion of Ag-specific lymphocytes leads to the generation of specific effector cells and is followed by the removal of excess cells. Depending on the presence or the absence of the Ag, T cells are eliminated through two different apoptotic processes, i.e., passive and active apoptosis. Passive or growth factor withdrawal apoptosis occurs in the absence of further antigenic stimulation and eliminates T cells at the end of an immune response. Active agonists reduced the Ag-induced apoptosis of VB8+ T cells (Fig. 8B). These results confirm the protective activity of VIP and PACAP against AICD, both in vitro and in vivo. The higher efficiency of the two agonists compared with that of the natural peptides is probably due to their increased stability in vivo.
apoptosis or AICD limits the number of T cells in the presence of persistent antigenic stimulation, and requires TCR re-engagement. Experiments performed in vitro and in vivo led to a model of AICD in which apoptosis follows a three-step process: Ag stimulation and production of cytokines, cell cycle progression, and Ag receptor re-engagement, with mature T cell death occurring upon antigenic restimulation of previously activated and cycling T cells (5, 50, 51). The present study demonstrates that VIP and PACAP, two multifunctional neuropeptides present in the lymphoid organs (16, 17, 52), protect CD4+ T cells from AICD through the down-regulation of FasL expression. The inhibition is dose dependent within a wide range of neuropeptide concentrations (10^{-10}–10^{-7} M), with maximum effects being observed at 10^{-8} M. VIP and PACAP modulate several immunological functions within the same dose range (17–19, 25, 26).

T lymphocytes were previously shown to express VPAC1 and VPAC2 mRNA and both high and low affinity VIP/PACAP binding sites (20–22), and several T cell lines were reported to express only VPAC2 mRNA (53). Here we confirm the expression of VPAC1 and VPAC2, but not PAC1, on Con A/IL-2-stimulated T cells and the presence of VPAC2 on 2B4.11 and A1.1 T cell hybridomas. Our agonist studies indicate that VPAC1 and/or VPAC2 mediate the protective effect of VIP/PACAP. This conclusion is supported by the effects of VPAC1 and VPAC2 antagonists. A similar involvement of both receptors has been reported for the inhibition of IL-2 and IL-10 production in anti-CD3-stimulated naive T cells (54) and for lymphocyte chemotaxis (55). Both VPAC1 and VPAC2 are coupled primarily to the adenylate cyclase pathway (20), and preliminary experiments indicate that cAMP functions as the major secondary messenger that mediates the protective effect of VIP/PACAP against AICD.4

Ag restimulation of activated/proliferating CD4+ T cells leads to Fas up-regulation, FasL induction, FasL/Fas interactions, and subsequent apoptotic death (1). Evidence from lpr and gld mice, deficient in functional Fas and FasL, respectively, suggested that Fas/FasL interactions are crucial for the regulation of T cell proliferation in vivo. Several reports using antagonists of Fas/FasL interaction, such as Fas-Fc fusion proteins or a neutralizing anti-Fas mAb, further demonstrated the direct involvement of these molecules in AICD of mature peripheral T cells (7–9, 29, 30). In this study we concluded that VIP and PACAP decrease AICD through the selective inhibition of FasL expression. This was demonstrated by analysis of FasL protein and mRNA expression and by functional assays. VIP and PACAP inhibit activation-induced FasL expression at both protein and mRNA levels in anti-CD3-restimulated Con A blasts and T cell hybridomas without significantly affecting Fas expression. The down-regulation of FasL expression correlates with the VIP/PACAP-induced decrease in AICD. Indeed, a functional study indicates that VIP and PACAP reduce FasL expression in T cell hybridomas and directly affect the capacity of these cells to induce apoptosis of Fas-bearing targets. In addition, once FasL is expressed or added as a soluble protein to Fas-bearing cells, VIP and PACAP cannot prevent apoptosis. These results suggest strongly that the inhibitory effect of the two neuropeptides on AICD is mediated through the inhibition of FasL expression. At the present time, VIP and PACAP are the only neuropeptides reported to inhibit FasL expression and Ag-induced cell death in T cells. The only other reported neuroendocrine factor affecting FasL expression is prolactin, which was shown to stimulate FasL expression on T cells and subsequent apoptosis in luteal cells (56).

The differential regulation of Fas and FasL expression by VIP and PACAP was previously reported for other inhibitors of AICD, such as TGF-β1, cyclosporin A, glucocorticoids, and retinoic acid analogues, which inhibit FasL, but not Fas expression in naive T cells and T cell hybridomas (30, 57–59). Therefore, our data support the idea that Fas expression is regulated in T cells in a fundamentally different way from its ligand. The inhibition of FasL expression by VIP/PACAP is probably the reason for the protective effect not only against TCR-induced apoptosis, but also against UV- and etoposide-induced apoptosis, because both stress- and radiation-induced cell death appear to involve FasL/Fas interactions (38, 60). Also, the lack of VIP/PACAP protective effect on glucocorticoid-induced apoptosis can be explained by the fact that glucocorticoids induce apoptosis independent of FasL/Fas signaling (39).

Ag restimulation induces apoptosis in both activated CD4+ and CD8+ T cells. However, CD4+ T cells are more prone to Fas/FasL-mediated apoptosis, whereas CD8+ T cells undergo apoptosis mostly through TNFR triggering and to a lesser degree through Fas signaling (32). Our data indicate that VIP and PACAP preferentially prevent the deletion of activated CD4+ T cells and that this effect is mediated through the inhibition of FasL expression. Although both neuropeptides down-regulate activation-induced FasL expression, they do not significantly abrogate activation-induced apoptosis in CD8+ T cells, whereas anti-TNF Abs have a significant protective effect. These data suggest that VIP and PACAP do not affect the TNF/TNF signaling pathway. Although VIP and PACAP do not play a significant role in the survival of CD8+ T cells following TCR stimulation, the inhibitory effect on FasL expression might be particularly relevant for the CD8+ T cell-mediated cytotoxicity of Fas-bearing target cells (31, 61).

Of obvious biological significance is the fact that the in vitro effect of VIP and PACAP on Ag-induced apoptosis was reproduced in vivo. VIP, PACAP, and the VPAC1 and VPAC2 agonists significantly prevented clonal deletion induced by the administration of the superantigen SEB. It remains to be established whether the inhibition of AICD in vivo correlates with a reduction in FasL expression.

The presence of neuropeptides such as VIP has been demonstrated in both the lymphoid innervation and immune cells (16, 17), and antigenic stimulation in vivo and in vitro was shown to induce VIP release (52, 62). Because neuropeptides are notoriously short-lived in the extracellular environment, and the major targets of VIP/PACAP are activated immune cells, the expectation is that VIP/PACAP will be released at a later time point during the immune response. This is indeed the case in vivo, when VIP is released a few days later than substance P, a proinflammatory neuropeptide, in a model of lung inflammation (62). As VIP and PACAP exert their immunological functions through receptors expressed on a variety of immune competent cells, both neuropeptides could play important roles as endogenous modulators of the immune responses. VIP and PACAP were originally described as negative regulators of both activated T cells and macrophages (17–19, 25, 26). Because AICD can be viewed as an anti-inflammatory process, VIP and PACAP were expected to stimulate activation-induced cell death. However, the effects of these pluripotent neuropeptides are more complex, depending on the cell type, differentiation stage, and overall activation state, and therefore, a more accurate description of the physiological role of VIP and PACAP is their participation in the control of immune homeostasis. Indeed, with regard to T cell differentiation, we have recently reported that VIP and PACAP contribute to a preferential Th2 response (27).

4 M. Delgado and D. Ganea. VIP and PACAP inhibit expression of Fas ligand in activated T lymphocytes by regulating c-Myc, NF-κB, NF-AT and Egr2/3 factors. Submitted for publication.
Also, although VIP/PACAP act as deactivators for stimulated macrophages (25, 26), they induce B7.2 expression and IL-6 production in resting macrophages (28, 63).

Therefore in view of these findings, what could be the physiologic significance of the anti-apoptotic effect of VIP and PACAP? During an immune response, mechanisms must operate not only to destroy no longer needed or even potentially damaging T cells, but also to allow the survival of a small number of activated T cells that differentiate into memory cells. Although all CD4+ T cells express Fas, naive T cells are apoptosis resistant and gradually switch to an apoptosis-sensitive phenotype during the proliferative stage following activation (1). Through their effect on FasL expression, VIP and PACAP might favor the local generation of memory T cells, allowing the surviving cells to switch back from an apoptosis-sensitive to an apoptosis-resistant phenotype. In addition, inhibition of the Fas/FasL-dependent cell-mediated cytotoxicity is another area of possible physiologic relevance for VIP/PACAP, especially since Fas/FasL-dependent cytotoxicity against autologous and bystander targets occurs in several organ-specific autoimmune and inflammatory diseases (64).

Acknowledgments

We thank Dr. Patrick Robberecht (Universite Libre de Bruxelles, Brussels, Belgium) for the VPAC1 agonist and antagonist, Drs. David Bolin and Ann Welton (Hoffmann-La Roche, Nutley, NJ) for the VPAC2 agonist Ro 25-1553, Dr. Ethan Lerner (Massachusetts General Hospital, Charlestown, MA) for the PAC1 agonist maxadilan, and Dr. Pierre Golstein (Centre d’Immunologie, Institut National de la Santé et Recherche Médicale-Centre National de la Recherche Scientifique, Marseille, France) for L1210 and L1210-Fas+ cells.

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