Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Inhibit Expression of Fas Ligand in Activated T Lymphocytes by Regulating c-Myc, NF-κB, NF-AT, and Early Growth Factors 2/3

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Activation-induced cell death in T cells, a major mechanism for limiting an ongoing immune response, is initiated by Ag reengagement and mediated through Fas/Fas ligand interactions. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), two multifunctional neuropeptides, modulate innate and adaptive immunity. We reported previously that VIP/PACAP protect T cells from activation-induced cell death through down-regulation of Fas ligand (FasL). In this study, we investigate the molecular mechanisms involved in the protective effect of VIP and PACAP. VIP/PACAP reduce in a dose-dependent manner anti-CD3-induced apoptosis in 2B4.11 T cell hybridomas. The protective effect is mediated through the specific type 2 VIP receptor, and the cAMP/protein kinase A pathway. A functional study demonstrates that VIP/PACAP inhibit activation-induced FasL expression. VIP/PACAP inhibit the expression and/or DNA-binding activity of several transcriptional factors involved in FasL expression, i.e., c-myc, NF-κB, NF-ATp, and early growth factors (Egr) 2/3. The inhibition of NF-κB binding is due to the stabilization of IκB (inhibitory protein that dissociates from NF-κB), through the inhibition of IκB kinase α activity. Subsequently, p65 nuclear translocation is significantly reduced. The inhibition in NF-ATp binding results from a calcineurin-independent reduction in NF-ATp nuclear translocation. VIP/PACAP inhibit the expression of Egr2 and 3, but not of Egr1. The effects on the transcriptional factors are mediated through type 2 VIP receptor with cAMP as secondary messenger. The Journal of Immunology, 2001, 166: 1028–1040.

Materials and Methods

Reagents

Synthetic VIP and PACAP38 were purchased from Novabiochem (Laufelfingen, Switzerland). The PACAP receptor (PAC1)/type 2 VIP receptor (VPAC2) antagonist PACAP6–38 was obtained from Peninsula Laboratories (Belmont, CA). mAbs to murine FasL (CD95L, MFL3), and FasL regulatory element; FLRE, FasL regulatory element; IκB, inhibitory protein that dissociates from NF-κB; IKK, IκB kinase; MEKK, mitogen-activated protein kinase kinase; NF-ATp, ??; PAC1, PACAP receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, protein kinase A; VPAC, VIP receptor.
Calphostin C, PMA, ionomycin, MTI, cyclosporin A (CsA), protease inhibitors, dibutylryl cAMP (dbcAMP), and forskolin were purchased from Sigma (St. Louis, MO), and I-2-(2-p-bromocinnamylaminomethyl)-ethyl-5-isothiourea/nunolactam (H89) from ICN Pharmaceuticals (Costa Mesa, CA), I-2-BsO (inhibitory protein that dissociates from NF-kB) (1-317)-tagged fusion protein and Abs against c-Myc, p65, p50, I-β-BsO, I-κB kinase α (IKKα), phosphorylated I-β-BsO, NF-ATp, Egr1, Egr2, and Egr3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The VPAC1 antagonist [Ac-His1, p-Phe2, K7, R9, L17] VIP (3-7-GFRF) (8-27) and the VPAC1 agonist [K9, R17, L17] VIP (1-7-GRF) (8-27) were kindly donated by Dr. Patrick Robberecht (Université Libre de Bruxelles, Brussels, Belgium). The VPAC2 agonist Ro 25-1553 Ac-Glu6, Lys12, Nle17, Ala19, Asp26, Leu26, Lys27, 28, Gly29, Thr31-VPF (21-25) was a generous gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PAC1 agonist maxadilan was a generous gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PAC1 agonist maxadilan was a generous gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). The VPAC1, VPAC2, and PAC1 agonists and the VPAC1 antagonist have been previously described (13).

Cell lines
The murine T cell hybridoma 2B4.11 has been described previously (27, 28). L1210 (a leukemia cell line) and L1210-Fas+ (L1210 cells transfected with fas) cells were kindly provided by Dr. P. Golstein (Center d’Immunologie of the National Institute of the Research, Lattes, Centre National de la Recherche Scientifique, Marseille, France) (29). All cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Life Technologies), containing 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 10 μg/ml streptomycin (complete medium).

Induction of apoptosis
For induction of apoptosis, 2B4.11 cells (5 x 10^6/ml) were cultured in 96-well plates (Corning Glass, Corning, NY) with immobilized anti-CD3 mAb (1 μg/ml), or with PMA (10 ng/ml) and ionomycin (1 μg/ml), in the presence or absence of VIP, PACAP, VIP/PACAP receptor antagonists, and other agents, as described in text. Apoptosis was determined at 24 h, as described below.

Assessment of cell viability and apoptosis
Cell viability was assessed by trypan blue exclusion and loss of mitochondrial function with the MTT staining method. For MTT staining, 100 μl of culture was placed in one well of a 96-well tissue culture plate, and 10 μl of MTT solution (2.5 mg/ml) was added. After incubation at 37°C for 4 h, 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added and visualized under UV light after staining with ethidium bromide.

RNA extraction and Northern blot analysis
Northern blot analysis was performed according to standard methods. The 2B4.11 cells were prepared and stimulated as described above. At the various time points, 1 x 10^6 cells were harvested and total RNA was extracted by the acid guanidinium-phenol-chloroform method, electrophoresed on 1.2% agarose-formaldehyde gels, transferred to S&S Nytran membranes (Schleicher & Schuell, Keene, NJ), and cross-linked to the nylon membrane using UV light.

DNA fragmentation was assessed by agarose gel electrophoresis. After culture for the indicated times, 5 x 10^5 cells were harvested and centrifuged at 750 x g for 10 min, resuspended in 100 μl hypotonic lysis buffer A (10 mM Tris-HCl, 140 mM EDTA pH 7.4, 1 mM sodium laureth sulfate, and 0.5 mg/ml protease K), incubated at 50°C for 4 h, followed by the addition of RNase A 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 μl NaCl and 1 vol of isopropanol at −20°C over-night. The samples were centrifuged at 14,000 x g for 20 min at 4°C, and the pellets were washed with 70% ethanol and allowed to dry at room temperature. The DNA resuspended in TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was fractionated by agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

FACS analysis
The 2B4.11 T cells (1 x 10^6 cells/ml) stimulated in 96-well plates under conditions indicated in text were harvested in ice-cold RPMI complete medium and washed twice with 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, followed by anti-CD3 (1 μg/ml), FITC-MAb (1 μg/ml), and incubation at 4°C for 1 h. Isotyped-matched mouse Abs were used as controls, and IgG block (Sigma) was used to block the nonspecific binding of the mAb to FeR. The cells were washed and further stained with 2.5 μg/ml of FITC-conjugated goat F(ab')2 anti-hamster IgG (Sigma) for 30 min at 4°C. After extensive washing, cells were fixed in 1% buffered paraformaldehyde. Stained lymphocytes, gated according to scatter characteristics, were analyzed with a FACScan flow cytometer (Becton Dickinson). Fluorescence data are expressed as mean channel fluorescence and as percentage of positive cells after subtraction of background isotype-matched values.

Analysis of functional FasL expression
Activation-induced FasL expression on the anti-CD3-stimulated 2B4.11 T cells was assessed by determining the ability of these cells to cause lysis of Fas+ target cells, as described previously (20). Briefly, 2B4.11 T cells were activated with immobilized anti-CD3 mAbs in the presence or absence of VIP or PACAP (10−8 M) for 8 h to allow FasL expression. After washing twice, the 2B4.11 T cells were resuspended in complete medium, and added to the wells of a 96-well V-bottom microtiter plate (Corning) in graded dilutions to obtain the desired E:T ratio. The target cells, L1210 (wild type) or L1210-Fas+ cells (10^5/ml), were labeled for 2 h at 37°C with 150 μCi of sodium [3^5]Crjchomate (Amersham), washed three times with PBS/5% FCS, resuspended in complete medium, and added to the microtiter plates at a concentration of 1 x 10^3 cells/well. In other experiments, VIP and PACAP were added directly to the cocultures to determine their effects on Fas-mediated lysis. The plates were incubated overnight at 37°C and 5% CO2, then centrifuged, and a 100-μl aliquot of the supernatant was removed for measurement in a Beckman gamma 8000 counter (Beckman, Fullerton, CA). The percent lysis was determined as follows: % lysis = (E − S)/(M − S) x 100, in which E is the release from experimental samples, S is the spontaneous release, and M is the maximum release upon lysis with 10% SDS.

EMSA
Nuclear extracts were prepared by the mini-extraction procedure of Schreiber et al. (30) with slight modifications. The 2B4.11 cells were cultured at a density of 10^6 cells in six-well plates, stimulated with anti-CD3 as described above, washed twice with ice-cold PBS/0.1% BSA, and harvested. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT,
0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM Na3VO4. After 15 min on ice, Nonidet P-40 was added to a final 0.5% concentration. The tubes were gently vortexed for 15 s, and nuclei were sedimented and separated from cytosol by centrifugation at 12,000 × g for 40 s. Pelleted nuclei were washed once with 0.2 ml of ice-cold buffer A, and the soluble nuclear proteins were released by adding 0.1 ml of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 0.5 M PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM Na3VO4). After incubation for 30 min on ice, centrifugation at 14,000 rpm at 4°C, the supernatants containing the nuclear proteins were harvested, the protein concentration was determined by the Bradford method, and aliquots were stored at −80°C for later use in EMSAs.

Oligonucleotides corresponding to the proximal NF-κB site (−138/−128, 5′-GAGAAAGGGAAGGAGGTCTTTCTGAC-3′), NF-AT (−246/−229, 5′-GGGGGGAAGGAGGAGGTCTTTCTGAC-3′), and FasL regulatory element (FLRE) motif (−220/−205, 5′-AAGTGGATGTTGTCTTT-3′) of the murine FasL promoter, and to the NF-AT site (−134/−108, 5′-TAATGTTCATTGTTGAGGAGGAACTCC-3′) of the murine egr3 promoter were synthesized and annealed. Aliquots of 50 ng of the double-stranded oligonucleotides were end labeled with [γ-32P]ATP using T4 polynucleotide kinase. For EMSAs with hybridoma nuclear extracts, 20,000–50,000 cpm of double-stranded oligonucleotides, corresponding to −0.5 ng, were used for each reaction. The binding reaction mixtures (15 μl) consisted of: 0.5–1 ng DNA probe, 5 μg nuclear extract, 2 μg poly(dI-dC)/poly(dI-dC), and binding buffer (50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol, and 10 mM Tris-HCl, pH 7.5). The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. Samples were loaded onto 4% nondenaturing polyacrylamide gels, electrophoresed in TBE buffer (50 mM Tris, 75 mM KCl, 3 mM MgCl2, 0.5 mM PMSF, 0.5 mM Pefabloc, 10 mM Tris, pH 7.5, 0.38 M glycine, and 2 mM EDTA) at 100 V, followed by transfer to Whatman paper, drying under vacuum at 80°C, and autoradiography. In competition and Ab supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific Ab (1 μg) or competing cold oligonucleotide (50-fold excess) before the addition of the labeled probe.

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

Total RNA was isolated from unstimulated and anti-CD3-stimulated 2B4.11 T cells (1 × 106 cells) using the Ultraspec RNA reagent (Biotecx, Houston, TX), as recommended by the manufacturer. Two micrograms of total RNA were reverse transcribed in the presence of 20 U Moloney murine leukemia virus reverse transcription, 40 U RNasin, 1 μM M-mercaptoethanol, and 1 μg of random primers, 0.5 mM dNTPs, 3 μg BSA, and Moloney murine leukemia virus reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2) in a total volume of 30 μl at 37°C for 1 h.

The cDNA were amplified with specific primers. Amplification with β-actin primers was used as a control. The primers for VPAC1, VPAC2, and PAC1 receptors have been described before (9, 13), and have the following sequence: VPAC1 sense, 5′-CCCTCTCTCCTGAGGGAAGGAGGACTTT-3′, and antisense, 5′-CCTGACACCTACCATTAGAGGAAAGCAG-3′; VPAC2 sense, 5′-GTCAAGGACAGGTCTTACTACTCC-3′, and antisense, 5′-CTGTACACTGACCGTGTACTGACTCC-3′; and antisense, 5′-CATGCAGATGTGGGAAAGGAACT-3′; β-actin sense, 5′-GATGTTGTTGTGATGCTGAGGG-3′, and antisense, 5′-GCTTATTGCGATATGATGACT-3′. The expected sizes for the amplified fragments are: 450 bp for VPAC1, 572 bp for VPAC2, 317 bp for PAC1, and 660 bp for PAC2. One representative experiment of three is shown.
IKKα immunoprecipitation and kinase assay

Cell lysates were prepared from $2 \times 10^6$ cells in 200 μl lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 5 mM NaF, 10 mM p-nitrophenyl phosphate, and 1 mM Na$_2$VO$_4$). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysate was cleared by centrifugation at $13,000 \times g$ for 3 min, and the supernatant was stored at $-80^\circ$C. Endogenous IKKα was immunoprecipitated from 150–250 μg of cell lysate by incubation with 0.5 μg of anti-IKKα Ab for 2 h at 4°C. The immune complexes were collected by incubation with protein A/G-Sepharose beads (Sigma) for 45 min at 4°C. The beads were extensively washed with lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, and 0.1% Triton X-100), twice with kinase buffer (20 mM MOPS, pH 7.6, 2 mM EGTA, 10 mM MgCl$_2$, 1 mM DTT, 0.1% Triton X-100, 1 mM p-nitrophenyl phosphate, and 1 mM Na$_2$VO$_4$). The pelleted beads were resuspended in 30 μl kinase buffer with 15 μM ATP, 10 μCi of [$\gamma^32$P]ATP (3000 Ci/mmol), containing 5 μg of rl-xB.α. The kinase reaction was performed at 30°C for 30 min, and stopped by the addition of 15 μl of 2× SDS sample buffer. Samples were boiled for 5 min and subjected to 9% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (Schleicher-Schuell), followed by autoradiography. The IKKα kinase activity was determined by incorporation of $^32$P into its substrate (rl-xBα-tagged fusion protein). The phosphorylated proteins were quantitated by a PhosphorImager. Expression of the IKKα was verified by immunoblotting of cell lysates, as described above.

Calcineurin assay

The calcineurin (CaN) phosphatase activity was assayed in whole cellular protein extracts by using the Biomol Green Cellular Calcineurin Assay Kit Plus (Biomol Research Laboratories, Plymouth Meeting, PA), according to the manufacturer’s instructions.

Results

VIP and PACAP decrease AICD in T cell hybridomas

As previously reported (27, 28, 31, 32), the 2B4.11 T cell hybridoma undergoes apoptosis following cross-linking of the TCR/CD3 complex by immobilized anti-CD3 Abs, or following activation with PMA plus ionomycin (Fig. 1A). The presence of VIP and PACAP during TCR stimulation decreases apoptosis at all times assayed (Fig. 1A). The neuropeptides inhibit the DNA fragmentation characteristic of TCR-induced apoptosis in a dose-dependent manner (Fig. 1B). CsA, previously shown to inhibit AICD (33), was used as positive control (Fig. 1B).

The effect of VIP and PACAP is mediated through the VPAC2 receptor

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 (34). As reported for several T cell lines (13, 35), the 2B4.11 cells express only VPAC2 (Fig. 2). The involvement of VPAC2 in the effect of VIP/PACAP on AICD was established by using specific agonists and antagonists. The VPAC2 agonist, but not the VPAC1 or the PAC1 agonist, inhibited anti-CD3-induced apoptosis of 2B4.11 cells (Fig. 2B). In addition, PACAP$_{6,38}$, an antagonist specific for PAC1 and to a lesser degree for VPAC2, but not the specific VPAC1 antagonist (13), reversed the effects of VIP and PACAP (Fig. 2C). These results indicate that VIP and PACAP exert their action through VPAC2.

VIP and PACAP inhibit activation-induced FasL expression

AICD in T cell hybridomas proceeds via expression of FasL and subsequent Fas/FasL interaction. We have recently reported that VIP and PACAP reduce FasL expression in activated peripheral T cells (13). In this study, 2B4.11 T cells were stimulated with immobilized anti-CD3 Abs in the presence or absence of VIP or PACAP, and the expression of FasL was assayed at the protein and mRNA level by flow cytometry and Northern blot, respectively. Activation-induced expression of FasL was greatly reduced in a time- and dose-dependent manner by VIP and PACAP, with an

FIGURE 2. Inhibition of AICD by VIP/PACAP is mediated through VPAC2. A. Expression of VPAC1, VPAC2, and PAC1 mRNA in 2B4.11 cells. Total RNA extracted from unstimulated and anti-CD3-stimulated (12 h) 2B4.11 cells ($2 \times 10^5$ cells) was subjected to RT-PCR with specific primers for VPAC1, VPAC2, and PAC1, as described in Materials and Methods. Reactions without cDNA served as negative control (not shown). One representative experiment of two is shown. B. Comparative effects of VIP/PACAP agonists in anti-CD3-induced apoptosis. The 2B4.11 cells ($5 \times 10^5$ cells) were activated with immobilized anti-CD3 mAbs (1 μg/ml) in the presence or absence of different concentrations of maximadilan (PAC1 agonist), Ro 25-1553 (VPAC2 agonist), and [K$^{13}$, R$^{16}$, L$^{37}$]VIP (1–7)-GRF (8–27) (VPAC1 agonist). Apoptosis (24 h) and cell viability (48 h) were assessed by annexin V/propidium iodide staining and MTT assay, respectively. The percentage of cell loss was calculated as: (1 - OD$_{560}$ of stimulated cells/OD$_{560}$ of unstimulated cells) × 100%. Each result is the mean ± SD of four separate experiments performed in duplicate. C. Effect of VPAC antagonists. The 2B4.11 cells ($5 \times 10^5$ cells) were activated with immobilized anti-CD3 mAbs, and treated simultaneously with VIP or PACAP (10$^{-8}$ M), and different concentrations of a VPAC1 antagonist, [Ac-His (1), n-Phe (2), K$^{15}$, R$^{16}$, L$^{37}$]VIP (3–7)-GRF (8–27), or a PAC1/VPAC2 antagonist (PACAP$_{6,38}$). Apoptosis (24 h) and cell viability (48 h) were assessed by MTT and TUNEL assay, respectively. Incubation with antagonists alone did not show any effect on anti-CD3-induced apoptosis (not shown). Each result is the mean ± SD of three separate experiments performed in duplicate.
almost complete inhibition in the concentration range of $10^{-8}$–$10^{-6}$ M (Fig. 3A).

To determine whether the inhibition of AICD correlates with the decrease in FasL expression induced by VIP and PACAP, we used a functional assay. The 2B4.11 T cells were incubated with immobilized anti-CD3 Abs for 8 h to induce FasL expression, in the presence or absence of VIP or PACAP. The anti-CD3-treated 2B4.11 cells were incubated with $^{51}$Cr-labeled Fas-bearing target cells (L1210-Fas$^+$ cells). In the absence of CD3 cross-linking, no functional FasL is expressed, as indicated by the lack of Fas$^+$-target lysis (Fig. 3B, unstimulated). In contrast, 2B4.11 cells treated with anti-CD3 Abs lysed the Fas$^+$, but not the Fas$^-$ (L1210-wild type) targets (Fig. 3B). VIP and PACAP added at the initiation of anti-CD3 treatment (time 0) inhibit in a dose-dependent fashion the lysis of L1210-Fas$^+$ cells (Fig. 3B). In contrast, the addition of VIP or PACAP to cocultures of L1210-Fas$^+$ target cells and activated 2B4.11 cells (8 h after activation) failed to inhibit lysis in Fas$^+$ targets (Fig. 3C). This suggests that VIP and PACAP inhibit AICD by preventing FasL expression and not by blocking the Fas signaling pathway.

cAMP as a second messenger for the inhibition of AICD and FasL expression by VIP and PACAP

VPAC2 induces intracellular cAMP as a secondary messenger (reviewed in Ref. 36). Therefore, we investigated the role of cAMP in the inhibition of AICD. First, we determined the effects of forskolin (a cAMP-inducing agent) and dbcAMP (a cAMP analogue) on FasL expression and apoptosis in 2B4.11 T cells. Similar to VIP and PACAP, forskolin and dbcAMP inhibit both FasL expression and apoptosis in anti-CD3-stimulated 2B4.11 cells (Fig. 4A). The role of cAMP as second messenger is supported by the fact that the protein kinase A (PKA) inhibitor H89 reverses in a dose-dependent manner the inhibitory effects of VIP and PACAP (Fig. 4B). In contrast, calphostin C, a PKC inhibitor, does not reverse the inhibitory effects of the two neuropeptides (Fig. 4B). These results

FIGURE 3. VIP and PACAP inhibit FasL functional expression. A, 2B4.11 cells ($5 \times 10^6$ cells for flow cytometry; $2 \times 10^7$ cells for Northern blot) were activated with anti-CD3 mAbs (1 µg/ml), in the presence or absence of different concentrations of VIP or PACAP ($10^{-8}$ M for flow cytometry assay). Expression of FasL protein was analyzed by flow cytometry at the indicated time points (16 h for flow cytometry profiles). The gates (dotted lines) were set based on staining with unrelated isotype control Abs (flow cytometry profiles). Data are representative of four similar experiments. Time curve results expressed as mean channel fluorescence (MCF) are the mean ± SD of four independent experiments performed in duplicate. FasL mRNA expression (6 h) was analyzed by Northern blot analysis (inset, $10^{-8}$ M VIP and PACAP). Results are expressed in arbitrary densitometric units normalized for the expression of GAPDH in each sample (mean ± SD of three separate experiments). B, VIP and PACAP decrease FasL-mediated cytotoxicity of Fas-bearing cells by blocking expression of FasL in each sample (mean ± SD of three separate experiments). B, VIP and PACAP decrease FasL-mediated cytotoxicity of Fas-bearing cells by blocking expression of FasL. The 2B4.11 cells ($5 \times 10^6$ cells) were activated at time 0 with anti-CD3 mAbs (1 µg/ml), in the presence or absence of different concentrations of VIP or PACAP ($10^{-8}$ M for left panel). Cells were cultured for 8 h to allow FasL expression, harvested, washed twice, and incubated with $^{51}$Cr-labeled L1210-Fas$^+$ cells ($1 \times 10^4$ cells) in graded dilutions to obtain various E:T ratio (10:1 for right panels). Percentage of lysis after overnight incubation was assessed by the percentage of $^{51}$Cr release. Each result is the mean ± SD of three independent experiments performed in duplicate. C, In a parallel experiment, 2B4.11 cells ($5 \times 10^6$ cells) were activated with anti-CD3 mAb, and VIP or PACAP was added at different concentrations 8 h later at the time of setting up the cocultures with activated 2B4.11 cells, and $^{51}$Cr-labeled target cells (L1210 and L1210-Fas$^+$ cells, $2 \times 10^4$) were mixed ($t = +8$ h). Percentage of lysis after overnight incubation was assessed by the percentage of $^{51}$Cr release. Each result is the mean ± SD of three independent experiments performed in duplicate.
suggested that the inhibitory effects of VIP and PACAP in both AICD and FasL expression are mediated through increases in intracellular cAMP.

**VIP and PACAP inhibit c-myc expression in activated T cell hybridomas**

Fasl expression during AICD requires the activation of several transcription factors. Previous studies indicated that c-myc regulates the expression of FasL and subsequent apoptosis in some systems, including T cells. To determine whether VIP and PACAP regulate c-myc expression in T cell hybridomas, we analyzed the expression of c-myc by Northern and Western blots. VIP and PACAP inhibited anti-CD3-induced c-myc expression at both protein and mRNA levels (Fig. 5).

**VIP and PACAP inhibit NF-κB binding to the FasL promoter**

Although the FasL promoter contains a complex array of transcription factors. We investigated whether VIP/PACAP affect NF-κB nuclear translocation by EMSA. Treatment of 2B4.11 cells with anti-CD3 Abs led to strong NF-κB binding, and VIP and PACAP inhibited this binding (Fig. 6A, left panel). The specificity of the NF-κB binding was evident by the complete displacement with a 50-fold excess of unlabeled homologous oligonucleotide (NF-κB) (Fig. 6A, middle panels). In contrast, a 50-fold excess of unlabeled nonhomologous oligonucleotide (NF-AT) had no effect (Fig. 6A, middle panels). Ab supershift experiments indicated the presence of both p50 and p65 in the NF-κB complex, whereas no supershift was observed with an irrelevant Ab (anti-NF-ATp) (Fig. 6A, right panel).

**VIP and PACAP inhibit activation-dependent phosphorylation of I-κBα and nuclear translocation of the p65 subunit of NF-κB**

In unstimulated T cells, NF-κB is sequestered in the cytoplasm complexed to I-κB (reviewed in Ref. 38). Stimulation through TCR/CD3 results in the phosphorylation and proteolytic degradation of I-κB, and subsequent translocation of NF-κB to the nucleus. To determine whether VIP and PACAP act on I-κBα, we examined the cytoplasmic levels of I-κBα by Western blot. In anti-CD3-stimulated cells, the time-dependent I-κBα degradation is apparent (Fig. 6, B and C). The decrease in cytoplasmic I-κBα levels is paralleled by an increase in I-κBα phosphorylation (Fig. 6C, lower panel). Treatment with VIP or PACAP blocks the activation-mediated phosphorylation and subsequent degradation of I-κBα (Fig. 6, B and C).

Because NF-κB activation involves its nuclear translocation, we measured the levels of p65 protein in cytoplasm and nucleus. As expected, anti-CD3 treatment decreased the level of p65 in cytoplasm and increased the p65 levels in the nucleus (Fig. 6B). Treatment with VIP or PACAP abolished the change in nuclear and cytoplasmic p65 levels (Fig. 6B). This indicates that VIP and PACAP inhibit the activation-induced nuclear translocation of p65, which is consistent with the inhibition of I-κBα phosphorylation and degradation. Neither anti-CD3 treatment by itself, nor in combination with VIP/PACAP, affected nuclear and cytoplasmic transcription factors.

**FIGURE 4.** cAMP as a second messenger for the effect of VIP and PACAP on AICD and FasL expression. A, Effects of various cAMP-inducing agents. The 2B4.11 cells (5 × 10^4 cells) were activated anti-CD3 mAbs in the presence or absence of different concentrations of forskolin (FK) or dbcAMP. B, Comparative effects of calphostin C (a PKC inhibitor) and H89 (a PKA inhibitor) on the effect of VIP and PACAP. The 2B4.11 cells (5 × 10^4 cells) were treated with anti-CD3 mAbs and VIP or PACAP (10^-8 M), in the presence or absence of calphostin C (100 nM), or different concentrations of H89 (0.1–100 nM). Apoptosis (24 h) and cell viability (48 h) were assessed by TUNEL and MTT assay, respectively. Fasl expression (16 h) was analyzed by flow cytometry. MCF, mean channel fluorescence. The dotted lines (B) represent control values from cultures stimulated with anti-CD3 alone. Results are the mean ± SD of four independent experiments performed in duplicate.

**FIGURE 5.** VIP and PACAP inhibit activation-induced c-myc expression. The 2B4.11 cells (2 × 10^5 cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP or PACAP (10^-8 M). A, Expression of c-myc and GAPDH mRNA was analyzed by Northern blot analysis at the indicated time points (upper panels, 2 h). Results are expressed in arbitrary densitometric units (mean ± SD of three experiments). B, After 4-h incubation, the expression of c-Myc protein was analyzed by Western blot. One representative experiment of three is shown.
p50 levels (Fig. 6B). The equal levels of p50 also serve as loading controls (Fig. 6B).

Because phosphorylation of I-κB by the I-κB kinases (IKK) is crucial for NF-κB activation, we investigated whether VIP and PACAP regulate IKK activity by using an in vitro kinase assay. Stimulation of 2B4.11 cells with anti-CD3 Abs results in a time-dependent increase in IKK activity (Fig. 6D). VIP and PACAP inhibit activation-induced IKK activity (Fig. 6D).

**FIGURE 6.** VIP and PACAP inhibit NF-κB nuclear translocation and DNA binding by inhibiting phosphorylation of I-κBα. **A,** VIP and PACAP inhibit NF-κB binding to the κB1 site of the FasL promoter. The 2B4.11 cells (2 × 10⁷ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP or PACAP (10⁻⁸ M). Nuclear extracts (2 h) were prepared and NF-κB binding was assessed by EMSA using a radiolabeled oligonucleotide containing the κB1 site of the murine FasL promoter. Middle panels, Specificity was assessed by the addition of 50-fold excess unlabeled homologous (κB1) or nonhomologous (NF-AT) oligonucleotides to nuclear extracts (Comp). Right panel, Identification of the proteins bound to the NF-κB site using supershift analysis. Nuclear extracts were incubated with polyclonal Abs (1 μg) against p65, p50, or NF-ATp for 20 min before adding the probe. Arrows indicate the supershifted p50- and p65-specific bands. Similar results were observed in three independent experiments. B and C, Effect of VIP and PACAP on activation-induced phosphorylation/degradation of I-κBα and on levels of p65 and p50. The 2B4.11 cells (2 × 10⁷ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP or PACAP (10⁻⁸ M). Cytosolic and nuclear proteins (1 h) were extracted, and Western blot analysis was performed for I-κBα in cytoplasmic fraction, and for p50 and p65 in cytoplasmic as well as nuclear extracts (B). At different times, the expression of I-κBα and phosphorylated I-κBα was analyzed by Western blot in the cytosolic fraction (C). One representative experiment of three is shown. D, VIP and PACAP inhibit activation-induced IKKα activity. The 2B4.11 cells (2 × 10⁷ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP or PACAP (10⁻⁸ M) for different time periods (10 min for blots in left panel). Kinase activity of IKKα was assayed in an in vitro kinase assay, as described in Materials and Methods. Right panel, IKKα activity is expressed as arbitrary densitometric units using a PhosphorImager. Data represent the mean of three independent assays. As control, the amounts of IKKα were determined by immunoblotting with anti-IKKα Ab (left panel).

VIP and PACAP inhibit activation-induced nuclear translocation of NF-ATp and its binding to the FasL promoter

NF-AT was reported to play a critical role in FasL up-regulation following TCR stimulation (19, 22, 29, 33). Induction of FasL mRNA is prevented by CsA, an immunosuppressive drug that inhibits CaN activity. CaN dephosphorylates NF-AT and induces its nuclear translocation. NF-ATp has been directly implicated in FasL transcription by
as competitors (Fig. 7A). To determine whether VIP and PACAP inhibit the nuclear translocation of NF-ATp, we performed Western blots with nuclear and cytoplasmic extracts (Fig. 7B). As expected, in unstimulated 2B4.11 cells NF-ATp was located preferentially in the cytoplasm (Fig. 7B). However, anti-CD3 stimulation resulted in the reduction of cytoplasmic NF-ATp, and the increase in nuclear NF-ATp (Fig. 7B). VIP and PACAP inhibit NF-ATp nuclear translocation, and block the decrease in cytoplasmic NF-ATp at all times assayed (Fig. 7B). However, the inhibition of NF-AT nuclear translocation was not due to an inhibition of CaN, because VIP and PACAP did not affect CaN activity (Fig. 7C). As expected, CsA led to a dramatic decrease in CaN activity (Fig. 7C).

FIGURE 7. VIP and PACAP decrease NF-ATp nuclear translocation and DNA binding. A, VIP and PACAP inhibit NF-AT binding. The 2B4.11 cells (2 × 10⁶ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP (10⁻⁸ M), PACAP (10⁻⁸ M), or CsA (100 ng/ml). Nuclear extracts (3 h) were prepared and NF-AT binding was assessed by EMSA using a radiolabeled oligonucleotide containing the NF-AT site of the murine FasL promoter. Lower arrow indicates the supershifted NF-ATp-specific bands. Similar results were observed in three independent experiments. B, VIP and PACAP inhibit activation-induced NF-ATp nuclear translocation. The 2B4.11 cells (2 × 10⁶ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP (10⁻⁸ M), PACAP (10⁻⁸ M), or CsA (100 ng/ml). At different times (1 h for left panels), cytosolic and nuclear proteins were extracted, and Western blot analysis was performed for NF-ATp. One representative experiment of three is shown. C, VIP and PACAP do not affect CaN activity. The 2B4.11 cells (2 × 10⁶ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP (10⁻⁸ M), PACAP (10⁻⁸ M), or CsA (100 ng/ml) for 30 min. Cytosolic proteins were extracted and CaN activity was assayed, as described in Materials and Methods. Results are the mean ± SD of three different experiments performed in duplicate.

VIP and PACAP inhibit Egr2 and Egr3 expression and their subsequent binding to the FLRE site of the FasL promoter

Mittelstadt and Ashwell (25, 26) identified a FLRE in the FasL promoter, which confers the majority of TCR-inducible FasL promoter activity in 2B4.11 cells and in human T cell blasts. FLRE is a binding site for Egr1, Egr2, and Egr3, members of the early growth-response family of transcriptional factors. The effect of VIP and PACAP on Egr1, 2, and 3 binding to the FLRE site was assessed by EMSA. As previously described (25, 26), nuclear extracts from unstimulated 2B4.11 T cells exhibit only a nonspecific band (ns) (Fig. 8A, lane 1). In contrast, extracts from anti-CD3-stimulated cells exhibit two specific bands, a major, lower mobility band (corresponding to Egr1 and Egr2 binding) (25, 26), and a minor, higher mobility band (corresponding to Egr3 binding) (25, 26) (Fig. 8A, lane 2). VIP and PACAP, as well as CsA, prevent the appearance of the minor band, without obvious effects on the major band (Fig. 8A, lanes 3–5).

The composition of the FLRE-binding complexes was determined by supershift assays. In anti-CD3-stimulated cells, the major band is supershifted primarily by anti-Egr1, and to a lesser extent by anti-Egr2, whereas the minor band is supershifted completely by anti-Egr3 (Fig. 8B). No supershift was observed with anti-NF-ATp (an irrelevant Ab). Following VIP or PACAP treatment, the minor band disappears, and the major band is supershifted completely by anti-Egr1 (no supershift is evident for anti-Egr2) (Fig. 8B).
obtained for CsA treatment (Fig. 8B). Therefore, the FLRE-binding complexes in stimulated cells consist of Egr1, 2, and 3, and treatment with VIP or PACAP leads to complexes consisting only of Egr1.

Next we investigated whether VIP and PACAP regulate Egr1, Egr2, and Egr3 expression. The 2B4.11 cells were stimulated with immobilized anti-CD3 Abs in the presence or absence of VIP, PACAP, or CsA, and expression of the three Egr proteins was detected by Western blot. The Egr1 protein is constitutively expressed, and neither VIP/PACAP nor CsA affects its expression. However, Egr2 and Egr3 protein levels were increased after anti-CD3 stimulation, and VIP, PACAP, and CsA dramatically reduced both Egr2 and Egr3 (Fig. 8C). The inhibitory effect on Egr2 and Egr3 was exerted at the mRNA level, because VIP, PACAP, and CsA reduced both egr2 and egr3 steady state mRNA levels (Fig. 8C).

Because induction of Egr2 and Egr3 was sensitive to CsA (25, 26) (Fig. 8C), the expression of the egr2 and egr3 might be dependent on NF-AT. In fact, a transcriptionally active NF-AT site was identified in the egr3 promoter (31). Therefore, we investigated whether VIP and PACAP regulate NF-AT binding to the egr3 promoter by using the NF-AT site from the egr3 promoter as a specific probe. Similar to CsA, VIP and PACAP inhibit the binding to the egr3/NF-AT site (Fig. 8D, left panel). The binding specificity was confirmed by using homologous (NF-AT) or nonhomologous (NF-κB) oligonucleotides as competitors (Fig. 8D, middle panel). Supershift assays indicate that the complex binding to the egr3/NF-AT site consists of NF-AT. An irrelevant Ab (anti-p65) was used as control (Fig. 8D, right panel). These results suggest that the inhibition of Egr3, and possibly Egr2, by VIP/PACAP is mediated, at least partially, through the inhibition of NF-AT.

Involvement of VPAC2 and cAMP/PKA in the inhibitory effects of VIP and PACAP on c-myc, NF-κB, NF-AT, and Egr2/3

Because the inhibitory effect of VIP/PACAP on FasL expression and subsequent AICD in 2B4.11 cells is mediated through VPAC2 and cAMP, we determined the effects of PACAP6–38, a VPAC2 antagonist, and of the PKA inhibitor H89 on the changes induced by VIP/PACAP on c-myc, NF-κB, NF-AT, and Egr2/3. The receptor antagonist and the PKA inhibitor reversed the effect of VIP on c-myc, NF-κB, NF-AT, and Egr2/3. The receptor antagonist and the PKA inhibitor reversed the effect of VIP on c-myc, NF-κB, NF-AT, and Egr2/3. The receptor antagonist and the PKA inhibitor reversed the effect of VIP on c-myc, NF-κB, NF-AT, and Egr2/3. The receptor antagonist and the PKA inhibitor reversed the effect of VIP on c-myc, NF-κB, NF-AT, and Egr2/3. The receptor antagonist and the PKA inhibitor reversed the effect of VIP on c-myc, NF-κB, NF-AT, and Egr2/3.

FIGURE 8. VIP and PACAP inhibit Egr2 and Egr3 expression and their subsequent binding to the FLRE site. The 2B4.11 cells (2 × 10⁷ cells) were incubated with medium alone (unstimulated), or activated with anti-CD3 mAbs in the presence or absence of VIP (10⁻⁶ M), PACAP (10⁻⁸ M), or CsA (100 ng/ml). A and B. Nuclear extracts (3 h) were prepared and incubated with the end-labeled oligonucleotide corresponding to the FLRE region (16-mer) of the murine FasL promoter. Bands corresponding to Egr1, Egr2, Egr3, and a nonspecific (ns) band are indicated. B. Identification of the proteins bound to the FLRE site using supershift analysis. Nuclear extracts were incubated with polyclonal Abs (1 µg) against Egr1, Egr2, Egr3, Egr1 plus Egr2, Egr1 plus Egr3, or NF-ATp for 20 min before adding the probe. Arrows indicate the supershifted Egr1-, Egr2-, and Egr3-specific bands. Similar results were observed in three independent experiments. C. The expression of Egr1, Egr2, and Egr3 mRNA and protein was analyzed by Northern blot (2 h) and Western blot (3 h). One representative experiment of three is shown. D. Nuclear extracts (90 min) were prepared and incubated with the end-labeled oligonucleotide containing the NF-AT site of the murine Egr3 promoter. Middle panel. Specificity was assessed by the addition of 50-fold excess of unlabeled homologous (NF-AT) or nonhomologous (NF-κB) oligonucleotides to nuclear extracts (Comp). Right panel. Identification of the proteins bound to the FLRE site using supershift analysis. Nuclear extracts were incubated with polyclonal Abs (1 µg) against p65/p50 (NF-κB) or NF-κB in 20 min before adding the probe. Arrow indicates the supershifted NF-AT-specific band. Similar results were observed in three independent experiments.
We reported previously that two neuropeptides, VIP and PACAP, protect peripheral T cells from AICD in vitro and in vivo, through the inhibition of FasL expression (13). In this study, we investigated the molecular mechanisms involved in the VIP/PACAP regulation of FasL expression in T cell hybridomas. We established that the protective effect against AICD and the inhibition of FasL expression are mediated through the specific VPAC2 receptor and involve cAMP as the secondary messenger. Electromobility shift and supershift assays indicate that VIP/PACAP reduce the specific DNA binding of several transcriptional factors, i.e., NF-κB, NF-AT, and Egr2 and 3. VIP/PACAP block the nuclear translocation of both NF-κB and NF-ATp, and inhibit the expression of c- myc, Egr2, and Egr3. The effects of VIP/PACAP were mimicked by forskolin, and reversed by the PKA inhibitor H89 and by the VPAC2 receptor antagonist, confirming the involvement of VPAC2 and of intracellular cAMP as mediators for the regulation of FasL expression by VIP and PACAP (Fig. 10).

Although the exact mechanism by which c- myc regulates FasL expression is not clear, c- myc is required for AICD in T cells (16, 18, 40). Recently, the TCR-induced activity of the FasL promoter was shown to require functional Myc-Max heterodimers, even in the absence of specific Myc-binding motifs in the FasL promoter (41). Our studies indicate that VIP/PACAP inhibit c- myc expression in the TCR-stimulated 2B4.11 hybridoma, at both protein and mRNA level through a cAMP-dependent pathway. The relationship between cAMP and c- myc expression has been previously documented (42, 43). However, the intracellular pathways connecting the cAMP/PKA to c- myc expression in T cells are not understood. In a transformed myeloid cell line, cAMP was shown to induce the sequestration of E2F1, a transcriptional factor required for the expression of c- myc, and to prevent G1 cell cycle progression through the down-regulation of cyclin D1 and cdk4 (42).

Studies with the FasL promoter have established the presence of several sites important in transcription, i.e., NF-κB (21, 23, 24), NF-AT (19, 20), AP-1 (37), and Egr2/3 specific binding sites (25, 26). The relative functional importance of these sites might differ among species. For example, mutations in the Erg binding site of the human FasL promoter abolish its activity (26), whereas resulting only in 50–60% reduction for the murine promoter (37). However, mutations in both the Egr- and the κB1-binding sites silence the murine FasL promoter (37). These results suggest that NF-κB and Egr2/3 are the most important transcriptional factors for the expression of murine FasL.

Our results indicate that VIP and PACAP reduce NF-κB binding to the κB1 site, and that the inhibitory effect is mediated through VPAC2 and intracellular cAMP. The inhibition of NF-κB binding is associated with a reduction in p65 nuclear translocation. p65 is retained in the cytoplasm due to the stabilization of I-κB. As previously demonstrated for macrophages (44, 45), VIP and PACAP inhibit I-κBα phosphorylation. This is accomplished through an inhibitory effect on the IKKα, a kinase specific for I-κB. Although the link between cAMP and IKKα is not elucidated, one possibility is that mitogen-activated protein kinase kinase 1 (MEKK1), which was shown to synergize with NF-κB-inducing kinase in the activation of IKKα and β (46, 47), is inhibited by increased levels of intracellular cAMP. We reported recently that VIP and PACAP inhibit MEKK1 in macrophages through a cAMP-dependent mechanism (48). In addition, increases in cAMP lead to cAMP regulatory element binding protein (CREB) phosphorylation and nuclear translocation. CREB has a high avidity for the CREB-binding protein (CBP), a nuclear cofactor required for NF-κB transcriptional activity (49). Because CBP is present in limiting amounts, its sequestration by CREB leads to a reduction in the transcriptional activity of NF-κB.

A major mechanism for the maintenance of immune homeostasis is the AICD that affects activated and proliferating T cells upon restimulation through the TCR. AICD, particularly in CD4+ T cells, is mediated through Fas/FasL interactions, leading to the activation of the intracellular Fas/Fas-associated death domain protein/caspase cascade, and ultimately to the apoptotic death of the Fas-bearing target. In contrast to Fas, which is expressed constitutively in peripheral T cells, and is up-regulated upon activation, FasL expression is induced following TCR stimulation (reviewed in Refs. 14 and 15). Regulation of FasL expression has significant physiological consequences, and therefore, is of considerable interest.
Our results indicate that VIP and PACAP also inhibit NF-AT binding, and NF-ATp translocation to the nucleus, and that the effects on NF-AT are mediated through VPAC2 and intracellular cAMP. FasL expression was reported to be sensitive to CsA, and NF-AT sites were indeed identified in the FasL promoter (19, 20). NF-ATp nuclear translocation and transcriptional activity depends on its dephosphorylation by CaN (50). Therefore, CaN represents one of the potential targets for the inhibitory effect of VIP/PACAP on NF-ATp translocation. However, in contrast to CsA, VIP and PACAP did not affect CaN activity. The cAMP-dependent pathway initiated through VPAC2 by VIP and PACAP could affect NF-AT activity in several ways. A direct PKA-mediated phosphorylation of NF-AT, which results in a decrease in transcriptional activity, was reported recently (51). In addition, previous reports indicated the generation of cAMP-dependent transcriptional inhibitors for NF-AT (52). Finally, our results indicate that VIP and PACAP inhibit Erg2 and 3 binding to the FLRE site, and inhibit the expression of the inducible Egr2 and 3 factors at protein and mRNA level. In contrast, Egr1, which is constitutively expressed in the 2B4.11 cells, is not affected by VIP or PACAP. Egr2 and 3 emerged recently as probably the most important transcriptional factors for FasL expression (25, 26). Induction of both Erg2 and 3 is CsA sensitive, suggesting that NF-AT participates in the expression of the erg2/3 genes (26). Therefore, the effect of VIP/PACAP on Egr2/3 synthesis is probably mediated, at least partially, through the reduction in NF-AT translocation. However, at the present time, a direct connection between cAMP-dependent events and Erg2/3 expression cannot be eliminated.

Although neuropeptides such as VIP and PACAP were initially characterized as antiinflammatory agents, a more accurate functional description is as endogenous modulators of the immune response. During an immune response, mechanisms must be in place to allow the survival of a small number of activated T cells and their differentiation into

**FIGURE 10.** Model for the inhibitory effect of VIP/PACAP on activation-induced FasL gene expression in T cells. Binding of VIP to VPAC2 activates the cAMP/PKA signaling pathway, which in turn down-regulates several transduction factors that are essentials for the gene expression of FasL after TCR/CD3 stimulation. Inhibition of NF-ATp nuclear translocation results in the inhibition of two different signaling pathways, a decrease in the direct binding of NF-ATp to the NF-AT site of the FasL promoter, and an inhibition in the NF-AT-dependent expression of Egr2 and Egr3, which are required for FasL expression. In contrast, VIP/PACAP inhibit IκB phosphorylation/degradation by IKK possibly through the effect on MEKK1. This prevents the subsequent nuclear translocation and binding of NF-κB to the FasL promoter. In addition, VIP/PACAP decrease activation-induced c-Myc expression. Inhibition of all these transcriptional factors results in the reduction of activation-induced FasL gene expression.
memory cells. Naïve T cells, although Fas−, are apoptosis resis-
tant, and upon activation switch to an apoptosis-sensitive pheno-
type during the proliferative stage (14). Through the down-regu-
lation of FasL expression, VIP and PACAP might favor the local
generation of apoptosis-resistant memory T cells. Another physi-
ological relevance for the inhibition of FasL expression by VIP
and PACAP is the effect on Fas/FasL-dependent cell-mediated
lytotoxicity, especially in organ-specific autoimmune diseases in
which Fas/FasL-dependent cytotoxicity against bystander targets
contributes to tissue destruction.

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