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VIP and PACAP are autocrine factors that protect the androgen-independent prostate cancer cell line PC-3 from apoptosis induced by serum withdrawal

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1 In the present study, we describe the expression of the neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as well as their receptors in PC-3 cells, a human prostate cancer cell line. In addition, we have investigated their role in apoptosis induced by serum starvation.

2 By RT–PCR and immunocytochemistry assays, we have demonstrated the production of VIP and PACAP in PC-3 cells.

3 We have demonstrated by RT–PCR and binding assays the expression of common PACAP/VIP ($VPAC_1$ and $VPAC_2$) receptors, but not PACAP-specific (PAC_1) receptors. The pharmacological profile of [^{125}I]-VIP binding assays was as follows: $VPAC_1$ antagonist = $VPAC_1$ agonist > VIP > $VPAC_2$ agonist (IC_{50} = 1.2, 1.5, 2.3 and 30 nM, respectively). In addition, both receptor subtypes are functional since VIP, PACAP-27 or $VPAC_1$ and $VPAC_2$ agonists all increased the intracellular levels of cAMP.

4 The expression of both peptides and their receptors is similar in serum-cultured and serum-deprived PC-3 cells. The treatment of serum-deprived PC-3 cells with exogenous VIP or PACAP-27 increases cell number and viability in a dose-dependent manner, as demonstrated by cellular counting and MTT assays. The increased cell survival is exerted through the $VPAC_1$ receptor, since a $VPAC_1$, but not $VPAC_2$, receptor agonist, mimics the effects and a $VPAC_1$ receptor antagonist blocks it. Moreover, VIP and PACAP-27 inhibit genomic DNA fragmentation in PC-3 cells triggered by serum starvation, and increase the immunoreactivity of the antiapoptotic protein bcl-2.

5 Our results suggest that VIP and PACAP are autocrine/paracrine factors that protect PC-3 cells from apoptosis through $VPAC_1$ receptors.
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Keywords: VIP; PACAP; apoptosis; prostate cancer; PC-3

Abbreviations: IC_{50}, concentration of ligand required for 50% inhibition of tracer binding; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; $VPAC_1$ agonist, [K^{15}, R^{16}, L^{27}] VIP (1–7)/GFR (8–27); $VPAC_1$ antagonist, acetyl-His^1[DPhe^2, K^{15}, R^{16}, L^{27}] VIP (1–7)/GFR (8–27); $VPAC_2$ agonist, RO 25-1553: acetyl-His^1[E^8, K^{12}, Nle^{17}, A^{19}, D^{25}, L^{26}, K^{27}, K^{28}, G^{29}, G^{30}, T^{31}] cyclo 21-25 VIP (2–24); $VPAC_2$ antagonist, myristoyl-[K^{12}]VIP (1–26)KKGGT

Introduction

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) are two pleiotropic and structurally related neuropeptides widely distributed in the central and peripheral nervous systems, including the male genital tract (Said, 1992; Vaudry *et al*., 2000). Both neuropeptides are involved in the proliferation and/or differentiation of various normal and cancer cell lines, and several studies have reported an effect of VIP and PACAP analogues on tumour growth in animal models, mediated by specific receptors (Reubi *et al*., 2000). The biological effects of VIP are mediated by at least two receptors, designated as $VPAC_1$ and $VPAC_2$ receptors (Harmar *et al*., 1998). Both VIP receptors can also be activated by the parent peptide PACAP, which recognises PACAP-selective receptors known as PAC_1 receptors. PAC_1, $VPAC_1$ and $VPAC_2$ receptors are coupled to adenylate cyclase (AC) via a Gs protein. They display distinct pharmacological characteristics and a different tissue distribution (Harmar *et al*., 1998). Recently, different selective agonists and antagonists discriminating these receptors have been described. The $VPAC_1$ agonist [K^{15}, R^{16}, L^{27}] VIP (1–7)/ GFR (8–27) and the $VPAC_1$ antagonist Acetyl-His^1[DPhe^2, K^{15}, R^{16}, L^{27}] VIP (1–7)/ GFR (8–27) are a highly selective for $VPAC_1$ receptors (Gourlet *et al*., 1997a, e). RO 25–1553 and myristoyl-[K^{12}]VIP (1–26)KKGGT are highly selective agonist and antagonist for $VPAC_2$ receptors, respectively (Gourlet *et al*., 1997d; Moreno *et al*., 2000). VIP receptors coupled to AC are present in rat ventral prostate epithelial cells (Carmena & Prieto, 1983;

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Prieto & Carmena, 1983; Juarranz *et al*., 1994) and correspond mainly to the $VPAC_1$ receptor subtype (Juarranz *et al*., 1999). This receptor subtype is also predominant in human prostate cancer tissue (Reubi *et al*., 2000) and in the androgen-dependent prostate cancer cell line LNCaP (Juarranz *et al*., 2001b).

Prostate cancer is composed of androgen-dependent and androgen-independent cells. It is well established that androgen ablation eliminates most androgen-dependent cancer cells by inducing apoptosis, but can rarely cure the patients because of the presence of androgen-independent cells and the emergence of apoptosis-resistant clones (Denmeade *et al*., 1996). Quite a large number of factors have now been identified in such tumours that may individually contribute to resistance to apoptosis in prostate cancer, including neuropeptides such as calcitonin, neurotensin and bombesin, which regulate the growth of prostate cancer cells, as well as their progression to hormone independence (Sehgal *et al*., 1994; Shah *et al*., 1994; Markwalder and Reubi, 1999). Furthermore, some of these neuropeptides have been described to modulate cell survival pathways in prostate cancer (Salido *et al*., 2000). In this regard, VIP stimulates rat prostatic epithelial cell proliferation and induces neuroendocrine differentiation in LNCaP cells (Juarranz *et al*., 2001a, b).

The aim of this study was to investigate whether VIP and PACAP act as autocrine/paracrine factors in PC-3 cells, to establish the expression pattern of VIP/PACAP receptors in these cells and to analyse the role of these two neuropeptides in apoptosis triggered by serum starvation of PC-3 cells during 4 days.

Methods

Cell culture and membrane preparation

The androgen-independent human prostate cancer cell line PC-3 was obtained from American Type Culture Collection (ATCC) and routinely cultured in RPMI-1640 medium (Life Technologies, Barcelona, Spain) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (Life Technologies) and seeded at a density of 30,000–40,000 cells cm^{-2}. The culture medium was changed every 3 days. For PC-3-induced apoptosis, cells were allowed to attach to plates for 24 h and were subsequently washed twice with PBS. The medium was changed to serum-free RPMI-1640 and maintained in culture for 4 days. For membrane preparation, cells were harvested with a rubber policeman and pelleted by low-speed centrifugation. The supernatant was discarded and the cells lysed in 1 mM $NaHCO_3$ and immediately frozen in liquid nitrogen. After thawing, the lysate was first centrifuged at $800 \times g$ for 10 min. The supernatant was further centrifuged at $20{,}000 \times g$ for 15 min. The pellet was resuspended in 1 mM $NaHCO_3$ and used immediately as a crude membrane preparation. Membrane protein concentration was measured according to the method of Bradford.

RT–PCR assays

Total RNA was prepared from PC-3 cells using standard techniques. Total RNA (5 μg) was reverse-transcribed using 6 μg hexamer random primers and 200 U M-MLV retrotranscriptase (Life Technologies) in the buffer supplied with the enzyme supplemented with 10 mM dithiothreitol, 40 U RNasin (Promega, Madison, WI, U.S.A.), and 0.5 mM of deoxyribonucleotides (dNTPs). RT reaction (2 μl) then PCR-amplified with specific primers for VIP, PACAP and each VIP/PACAP receptor subtype. Primers for VIP were: sense, 5′-TAAAAGAAGACATTGACATGTTG-3′ and antisense, 5′-GAAGTTGTTTTCTTGAATTACTT-3′, which should give a PCR product of 470 bp; for PACAP: sense, 5′-AAACAAAG-GACGACGCCGATAG-3′ and antisense, 5′-AGACTCACT-GGGAAAGAATGC-3′, which should give a PCR product of 576 bp; for $VPAC_1$ receptor: sense, 5′-ATGTGCAGAT-GATCGAGGTG and antisense, 5′-TGTAGCCGGTCTTCA-CAGAA, which should give a PCR product of 324 bp; for $VPAC_2$ receptor: sense, 5′-TACAGAGCTTCTGAGGTCTC and antisense, 5′-TACTGCAGGAAGACCAGGC, which yield a PCR product of 529 bp; for PAC_1 receptor: sense, 5′-GCCTGTACCTCTTCACTCTGC and antisense, 5′-CTTTC-CCTTTTGCTGACATTC, which would be expected to produce a PCR product size of 450 bp for the null variant. The PCR conditions for VIP were: denaturation at 94°C for 3 min followed by 40 cycles of 50 s at 94°C, 50 s at 56°C and 1 min at 72°C and then a final cycle of 10 min at 72°C. The PCR conditions for PACAP were similar to those for VIP, except for the cycles: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. In the case of VIP/PACAP receptors, the PCR conditions were: denaturation at 94°C for 3 min, and 45 cycles of 30 s at 94°C, 45 s at 58°C and 45 s at 72°C and then a final cycle of 10 min at 72°C. The PCR products were analysed in 2% agarose gels.

Immunocytochemical staining

Cells were detached with trypsin/EDTA and pelleted by low-speed centrifugation. The resulting supernatant was discarded and the cells resuspended in PBS. Cell suspensions were centrifuged onto glass slides (9×10^4 cells per slide), dried overnight and then fixed for 10 min in acetone at −20°C. After drying for 2 h, slides were rinsed in PBS and then treated for 5 min with methanol/water/H_2O_2 in order to block endogenous peroxidase. Slides were again rinsed again in PBS and treated with normal rabbit and goat serum (for VIP or PACAP antibodies, respectively) to block background staining. Immunocytochemical demonstration of immunoreactive VIP (IR-VIP) and PACAP (IR-PACAP) was carried out by successive incubation with a monoclonal anti-VIP antibody (dilution 1 : 1000) or rabbit anti-PACAP antibody (dilution 1 : 4000) for 1 h, biotine-conjugated rabbit anti-mouse or goat anti-rabbit IgG (for IR-VIP and IR-PACAP, respectively), and subsequently incubated with streptavidin–peroxidase for 1 h followed by addition of the 3,3′-diaminobenzidine-tetrachloride (DAB) substrate and hydrogen peroxide in PBS. Methylene blue was used for counterstaining. Controls for the immunocytochemical studies were carried out by replacing the mouse anti-VIP or rabbit anti-PACAP with PBS and staining with the secondary antibodies according to the above protocol. The specificity for these antibodies was previously demonstrated (Wong *et al*., 1996; Abad *et al*., 2002). The mouse anti-VIP antibody was kindly supplied by Dr Helen Wong. The rabbit anti-PACAP antibody was a generous gift from Dr A. Arimura.

Binding assays

For identification of VIP/PACAP receptors, VIP and PACAP-27 were iodinated as described previously (Juarranz *et al*., 2001b). Membranes (10–20 μg protein) were incubated with [^{125}I]VIP or [^{125}I]PACAP-27 and increasing concentrations of unlabelled peptides. Nonspecific binding was defined as the residual binding in the presence of 1 μM VIP or PACAP-27, respectively (Neosystem, Strasburg, France). The selective agonists and antagonists for $VPAC_1$ and $VPAC_2$ receptors were kindly supplied by Dr P. Robberecht. [K^{15}, R^{16}, L^{27}] VIP (1–7)/ GFR (8–27) and acetyl-His^1[DPhe^2, K^{15}, R^{16}, L^{27}] VIP (1–7)/ GFR (8–27) were used as a $VPAC_1$ selective agonist and antagonist, respectively. RO 25–1553 and myristoyl-[K^{12}]VIP(1–26)KKGGT were used as $VPAC_2$ selective agonist and antagonist, respectively.

Measurement of cAMP accumulation

PC-3 cells were seeded in 24-multiwell plates (60,000 cells per well). After 24 h, cells were washed with 1 ml of serum-free medium and treated with 1 μCi of [2-H^3]adenine for 2 h in a serum-free medium. The cells were then incubated for 10 min at 37°C in HBS buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 4.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose, 1 mM IBMX and 0.1 mg ml^{-1} BSA) and subsequently for 15 min with increasing concentrations of the peptides tested. After incubation period, the medium was aspirated and the reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid at 4°C. After scraping and centrifugation, cAMP levels in the supernatant were measured as described previously (Salomon *et al*., 1974).

Cell growth assays

PC-3 cells were seeded in 24-well plates at $1.5 \times 10^{4.}$ cells per well and allowed to attach for 24 h in RPMI-1640 medium with 10% FBS and 1% antibiotic. The following day, the medium was removed, and the cells were cultured in serum-free medium with or without increasing concentrations of VIP, PACAP-27, the $VPAC_1$ selective agonist or $VPAC_2$ selective antagonist. The medium and peptide were renewed every 48 h. After 4 days, cells in each well were trypsinised, resuspended in Fast-Flow Beckton Dickinson Isotone™ solution and counted in a Casy® Model DT counter.

Cell viability measurement: MTT assay

Cell survival was estimated using a microculture tetrazolium assay. This assay measures the reduction of substrate [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) to a dark blue formazan product by mitochondrial dehydrogenases in living cells. Briefly, 50 μl of 2 mg ml^{-1} MTT (Sigma, Alcobendas, Madrid) was added to each well. After 3 h at 37°C in darkness, 0.01 N HCl containing 10% SDS was added to dissolve the formazan product. Absorbance at 540 nm was measured in the plate reader (Bio-Tek Instruments, ELX 800).

Genomic DNA fragmentation

PC-3 cells were seeded in 10 cm diameter dishes with complete medium. After 24 h, the medium was replaced by complete medium (control cells), or serum-free medium with or without 0.1 μM VIP. The medium and peptide were renewed every 48 h. After 4 days, cells were detached and centrifuged. The pellet was resuspended in 450 μl of ice-cold lysis buffer (5 mM Tris-HCl, pH 7.4, 20 mM EDTA, 0.5% Triton X-100) for 20 min at 4°C. The chromatin was pelleted by centrifugation at $20{,}000 \times g$ for 30 min. The supernatant (fragmented DNA) was removed and incubated with 4.5 μl of 20 mg ml^{-1} Proteinase K (Sigma) at 60°C for 2 h and then at 37°C overnight. DNA was extracted with phenol/chloroform/isoamylic alcohol. DNA was precipitated with ethanol, adding glycogen as a carrier, and then resuspended in TE buffer (10 mM Tris-HCl, pH = 8.0, 1 mM EDTA) supplemented with 2 μg ml^{-1} RNase A, and incubated overnight at 37°C. Samples (20 μl) were electrophoresed through a 1.2% agarose gel.

Western blotting of Bcl-2 and caspase-3 proteins

For Western blotting of Bcl-2 and caspase-3, cells were collected, rinsed twice with cold PBS and pelleted. Cells were then briefly sonicated in lysis buffer A (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsuflonyl fluoride, 10 μg ml^{-1} aprotinin, and 10 μg ml^{-1} leupeptin). Total fraction was used for Bcl-2 immunodetection, whereas cytosolic fractions were separated by centrifugation of cell extracts for 30 min at $50{,}000 \times g$ and used for procaspase-3 immunodetection. Equal amounts of protein (30 μg for caspase-3 and 80 μg for Bcl-2) were subjected to SDS–PAGE and blotted on a nitrocellulose membrane (BioTrace® NT, Pall Corporation, VWR International). Membranes were blocked with Tris-buffered saline (pH 7.6) containing 5% nonfat dry milk and 0.05% Tween-20 and then incubated with mouse anti-caspase-3 (1 : 1000; Transduction Laboratorie, BD Biosciences), or rabbit anti-Bcl-2 (1 : 250; Calbiochem). For detection, horseradish peroxidase conjugated secondary antibody was used. Proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Pierce). The membranes were then stripped and reprobed with mouse antiactin (1 : 10,000; Oncogene), used as a control for loading. Densitometric analyses of protein bands from scanned ray films were performed using Scion Image software (Scion Corporation, MD, U.S.A.) and the values were normalised against the intensity of actin.

Statistical analysis

The results are expressed either as the mean ± s.e.m. or as representative experiments. When appropriate, statistical significance was assessed comparing data from those obtained with starved cells using the Student's *t*-test. The level of significance was regarded as $P < 0.05$.

Results

Presence of VIP and PACAP in PC-3 cells

The expression of mRNA coding for VIP and PACAP in PC-3 cells was measured by RT–PCR amplification. A single DNA band was observed in both control and starved cells, at 470 bp for VIP and 576 bp for PACAP (Figure 1). Similar PCR products amplification was observed for both peptides in all

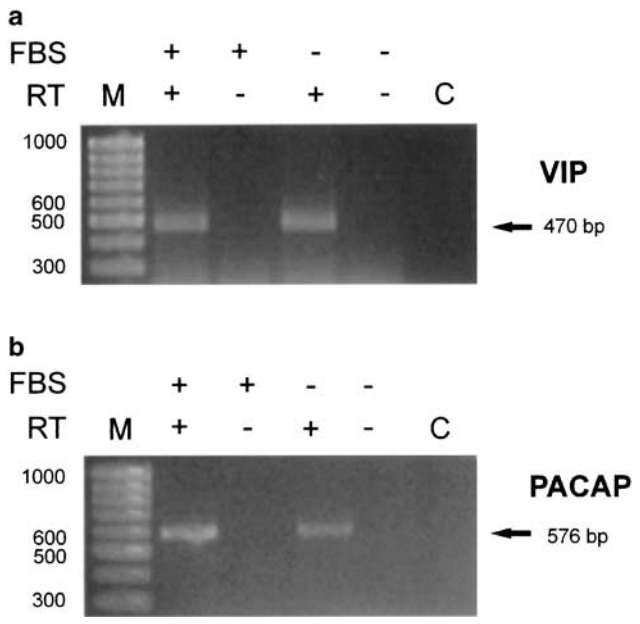

Figure 1 VIP and PACAP mRNA expression in control and serum-deprived PC-3 cells. RT–PCR was performed with total RNA from control (+FBS) and serumdeprived (-FBS) PC-3 cells as described in the Methods section. Reverse transcriptase was omitted in the control experiments (RT−). The sise of the amplified fragments were 470 bp for VIP (a) and 576 bp for PACAP (b). Lane M: 100 bp ladder. Lane C: PCR performed without cDNA. A representative experiment of three others is shown.

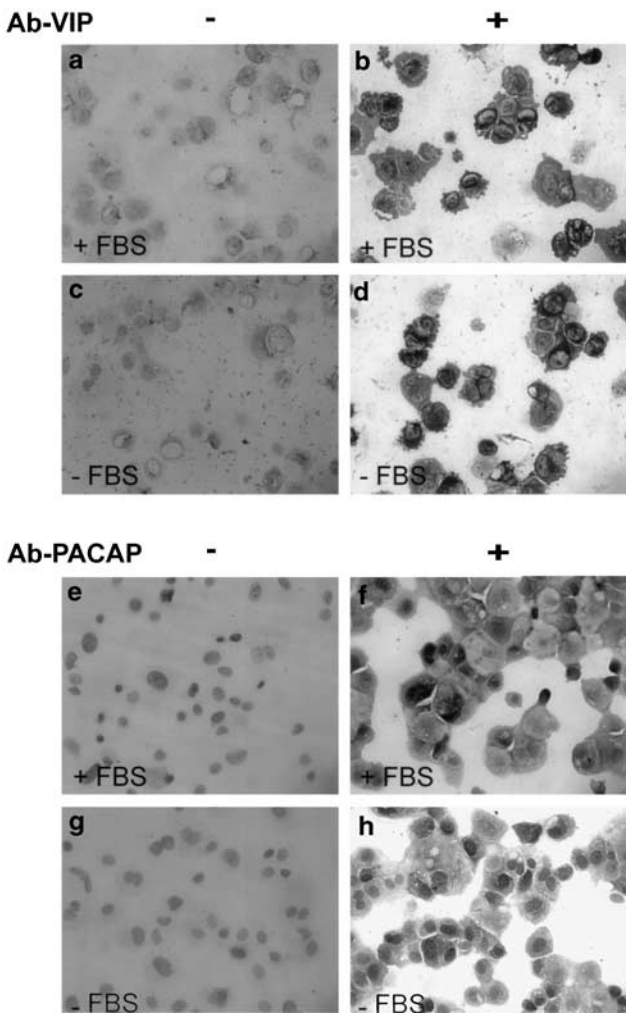

Figure 2 Immunocytochemical analysis of VIP and PACAP expression in control and serum-deprived PC-3 cells. VIP immunoreactive cells were detected in control (b) (×40) and serum-deprived (d) (×40) PC-3 cells. Negative controls of immunostaining were performed in the absence of monoclonal anti-VIP antibody (a, c). PACAP immunoreactive cells were detected in control (f) (×40) and serumdeprived (h) (×40) PC-3 cells. Negative controls of immunostaining were performed in the absence of polyclonal anti-PACAP antibody (e, g). A representative experiment of three others is shown.

situations. These PCR products correspond to the predicted size for PCR amplification of VIP and PACAP. Furthermore, both sequences were verified by sequencing the PCR products. Having demonstrated the expression of VIP and PACAP mRNAs in PC-3 cells, we next studied the production of these neuropeptides in cell suspensions by immunocytochemical methods. Immunoreactive VIP and PACAP were detected in cytocentrifuge preparations from control and serum-deprived PC-3 cells (Figure 2b, d, f and h). In both situations, the reaction products were spread throughout the cytoplasm and appeared in secretory vesicles as well. In addition, negative controls were performed by treating the cytocentrifuge preparations with PBS instead of the primary antibody (Figure 2a, c, e and g).

Expression of VIP/PACAP receptors in PC-3 cells

Figure 3 shows the expression of mRNA for PAC_1, $VPAC_1$ and $VPAC_2$ receptors in control and serum-deprived PC-3 cells. As a control, the corresponding pcDNA3-cDNAs for each VIP/PACAP receptor subtype were amplified. Amplification of the RT reactions exhibited bands of 324 and 529 bp corresponding to $VPAC_1$ and $VPAC_2$, receptors, whereas no product amplification was found for the PAC_1 receptor. This pattern of VIP/PACAP receptor expression was similar in serum-cultured (Figure 3a) and serum-deprived (Figure 3b) PC-3 cells. In order to discard genomic DNA amplification, the same PCR reactions were carried out omitting the RT reaction. In this case, no product amplification was detected. We next identified the VIP/PACAP receptor proteins by analysing the binding of [^{125}I]PACAP-27 and [^{125}I]VIP to PC-3 membranes (Figure 4). Competition curves for [^{125}I]PACAP-27 binding by PACAP-27 and VIP showed IC_{50} values of 3.0 and 4.5 nM, respectively, indicating that common VIP/PACAP receptors are expressed in PC-3 membranes (Figure 4a). This result is in accordance with the absence of expression of PAC_1 receptor mRNA in PC-3 cells, demonstrated by the RT–PCR approach. $VPAC_1$ and $VPAC_2$ receptor expression was evaluated by the capacity of selected agonists to compete with [^{125}I]VIP for binding. The order of potency of each peptide tested was as follows: $VPAC_1$ antagonist (IC_{50} = 1.2 nM) = $VPAC_1$ agonist (IC_{50} = 1.5 nM) > VIP (IC_{50} = 2.3 nM) > $VPAC_2$ agonist (IC_{50} = 30 nM). These results suggest that the majority of binding sites expressed in PC-3 cell membranes were $VPAC_1$ receptors, although $VPAC_2$ receptors are also present (Figure 4b).

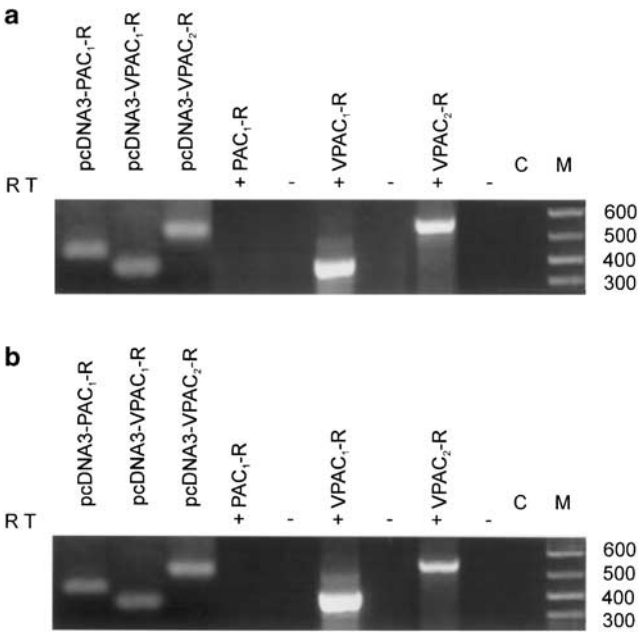

Figure 3 VIP/PACAP receptor mRNA expression in PC-3 cells. RT–PCR was performed with total RNA from control (a) and serum-deprived (b) PC-3 cells as described in the Methods section. Reverse transcriptase was omitted in the control experiments (RT−). As positive controls for PAC_1, $VPAC_1$ and $VPAC_2$ receptor amplification, a PCR was performed with the corresponding pcDNA3-cDNAs. The amplified fragment sises were 450 bp for PAC_1, 324 bp for $VPAC_1$ and 529 bp for $VPAC_2$. Lane M: 100 bp ladder. Lane C: PCR performed without DNA. A representative experiment of three others is shown.

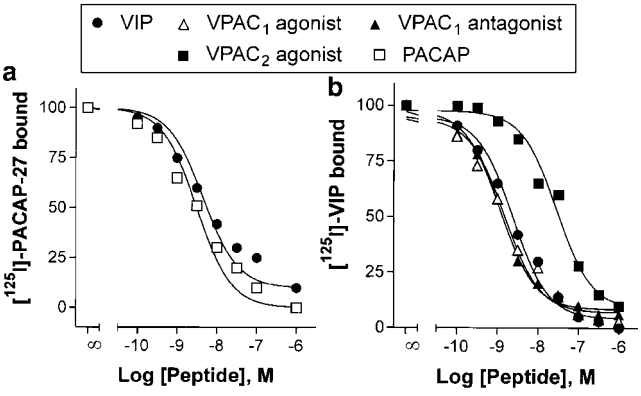

Figure 4 Competitive inhibition of [^{125}I]PACAP-27 (a) and [^{125}I]-VIP (b) binding to control PC-3 membranes. Binding competitors were VIP, PACAP-27, $VPAC_1$ agonist, $VPAC_1$ antagonist and $VPAC_2$ agonist. Nonspecific binding was determined in the presence of 1 μM unlabelled PACAP-27 (a) and VIP (b). Results are the mean of four separate experiments performed in duplicate and the standard deviation (s.d.) was less than 0.1 log unit in all cases ($\pm$25% of the mean value, or less).

Functionality of VIP/PACAP receptors in PC-3 cells

The functional properties of VIP/PACAP receptors were assayed by measuring the intracellular cAMP levels in PC-3 cells (Table 1). VIP and PACAP-27 increased cAMP levels with the same potency ($ED_{50} = 5$ nM) and efficacy, which indicates a common receptor for both peptides. $VPAC_1$ and $VPAC_2$ agonists were also able to stimulate the enzyme with an $ED_{50} = 23.7$ and 1.4 nM, respectively, indicating that both VIP receptors present in these cells are coupled to the adenylate cyclase system. In serum-deprived cells, intracellular cAMP levels were also measured (Table 1). VIP, PACAP-27, $VPAC_1$ and $VPAC_2$ agonists showed a similar potency ($ED_{50} = 3.3$, 1.5, 1.3 and 6.2 nM, respectively). These results indicate that both receptors ($VPAC_1$ and $VPAC_2$) expressed in PC3 cells are also functional in the serum-deprived cells.

Effect of VIP, PACAP-27, $VPAC_1$ and $VPAC_2$ agonists on the survival of PC-3 cells after serum withdrawal

Figure 5 shows the effect of VIP, PACAP-27, the $VPAC_1$ agonist or the $VPAC_2$ agonist on the growth of starved PC-3 cells. Cells grown in the absence of trophic factors reduced their survival to 59% compared with controls. However, VIP, PACAP-27 and the $VPAC_1$ agonist (but not the $VPAC_2$ agonist) were able to increase starved PC-3 survival up to 77%. The effects of VIP, PACAP-27 and the $VPAC_1$ agonist were dose dependent, with ED_{50} values of 5, 1 and 1 nM, respectively, differences being significant from starved PC-3 cells for doses of 100 nM neuropeptide or $VPAC_1$ agonist. None of the $VPAC_2$ agonist concentrations used had any effect on PC-3 cell survival. This observation is consistent with MTT measurement (Figure 6) showing that VIP, PACAP-27 and the $VPAC_1$ agonist (but not $VPAC_2$ agonist) are able to increase the survival of PC-3 cells grown in the absence of trophic factors from 30 to 45% (Figure 6a). When the serum-deprived PC-3 cells were grown in the presence of VIP and the specific $VPAC_1$ receptor antagonist, no increase in survival was detected, whereas the specific $VPAC_2$ receptor antagonist was unable to block the effect produced by VIP (Figure 6b).

Effect of VIP on DNA fragmentation in PC-3 cells

The increase in the survival of serum-deprived PC-3 cells treated with VIP may be due to a decrease of apoptosis in the cells. Apoptosis is characterised by a number of morphological and biochemical events such as nuclear DNA fragmentation, membrane blebbing or increase in the expression of some proapoptotic proteins. PC-3 cells showed nuclear DNA fragmentation after 4 days of growth in the absence of trophic factors (Figure 7). When 0.1 μM of VIP was added to the culture medium, a decreased nuclear DNA fragmentation was observed, suggesting a protective role for VIP from apoptosis induced by serum withdrawal in PC-3 cells.

Effect of VIP on the expression of caspase-3 and Bcl-2 in PC-3 cells

Figure 8 shown the Western blot analysis for caspase-3 and Bcl-2 analysis in PC-3 cells. Results revealed the presence of a 32-kDa band corresponding to the zymogen form of caspase-3 in control PC-3 cells that decreases after serum withdrawal. The treatment of PC-3 cells with 0.1 μM VIP or PACAP-27, increased the procaspase-3 immunoreactive band (Figure 8a). Diminished levels of procaspase-3 (which has a low proteolytic activity) have been associated with increased activity of caspase-3 (Munshi *et al*., 2001). Western blot analysis of bcl-2 revealed a 26-kDa band whose intensity is reduced in serum-deprived cells, whereas treatment with 0.1 μM VIP or PACAP-

Table 1 *cAMP production in PC3 cells*

	Control cells (+FBS)		*Serum-deprived cells (−FBS)*	
	cAMP production[a]	ED_{50} (nM)	*cAMP production*[a]	ED_{50} (nM)
VIP (1 μM)	100.0	5.3	100.0	3.3
PACAP-27 (1 μM)	97.3	5.2	100.0	1.5
$VPAC_1$ agonist (1 μM)	60.5	23.7	65.7	1.3
$VPAC_2$ agonist (1 μM)	81.2	1.4	82.9	6.2

[a]Results are the mean of two to five separate experiments performed in triplicate and the standard deviation (s.d.) was always lower than $\pm 10\%$ for agonists cAMP production and below 0.1 log units for ED_{50} values. Data are expressed as percentage of maximum: that produced by (1 μM) VIP corresponding to two-fold basal value.

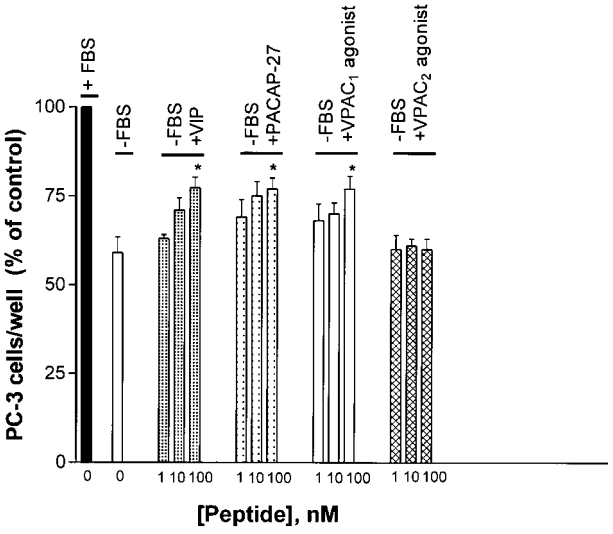

Figure 5 Effect of increasing concentrations of VIP, PACAP-27, $VPAC_1$ agonist and $VPAC_2$ agonist on the growth of PC-3 cells. Quantitation of cell number present in the well after 4 days of treatment was carried out as described in the Methods section, in the presence or absence of FBS. Results are the percentage cell number compared to control (cells grown in the presence of FBS, 158.000 cells per well), and derived from four independent experiments performed in triplicate. $^*P < 0.05$.

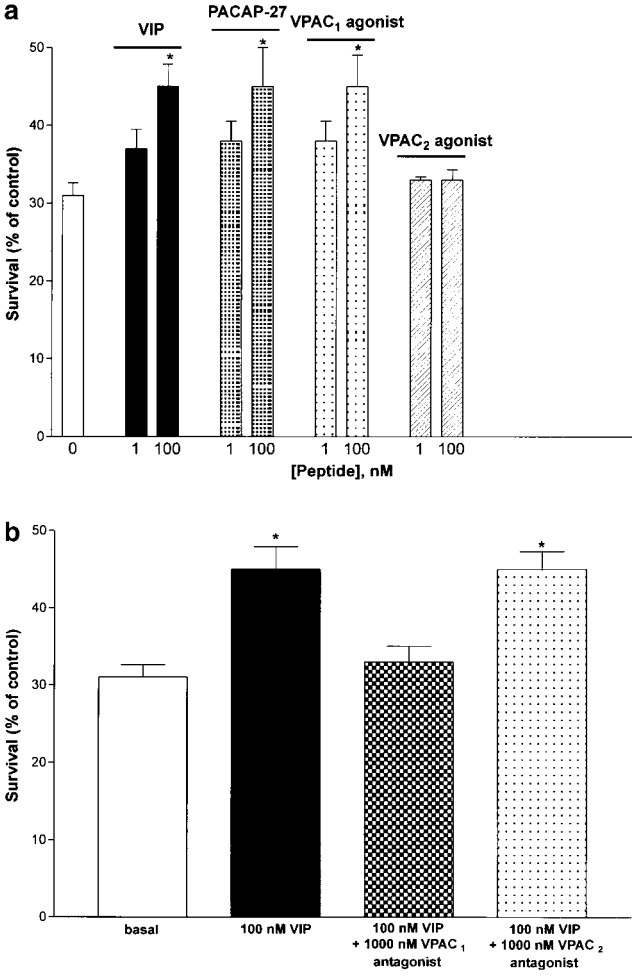

Figure 6 Effect of VIP, PACAP-27, $VPAC_1$ agonist, $VPAC_2$ agonist, $VPAC_1$ antagonist and $VPAC_2$ antagonist on the survival of PC-3 cells grown in the absence of trophic factors. Quantitation of live cells using MTT assays was performed as described in the Methods section. Results are the percentage of the absorbance (570–630 nm) compared to controls (cells grown in the presence of FBS, $A_{570-630} = 0.485 \pm 0.023$) and represent the mean of three separate experiments performed in triplicate. (a) Effect of increasing concentrations of VIP, PACAP-27, $VPAC_1$ agonist and $VPAC_2$ agonist on PC-3 survival in the absence of trophic factors. (b) Effect of 0.1 μM of $VPAC_1$ or $VPAC_2$ antagonists on VIP-increased survival of PC-3 cells grown in the absence of trophic factors. $^*P < 0.05$.

27 also increased the levels of this antiapoptotic protein (Figure 8b). Although neither VIP nor PACAP-27 was able to increase the levels of procaspase-3 and Bcl-2 to control values, we observed that both peptides increased the intensity of the immunoreactive band for procaspase- 3 and Bcl-2 to about 30 and 50%, respectively.

Discussion

In the prostate, androgens play a major role in supporting the maintenance of normal growth and function. However, this gland also contains neuropeptides located either in nerve terminals or in neuroendocrine cells, which can also play a regulatory role in the pathophysiology of the prostate (Gkonos *et al*., 1995). This is of great importance regarding prostate cancer, since neuropeptides may influence the behaviour of the tumours. In this sense, the neuroendocrine differentiation is a marker of poor prognosis in prostate cancer and may correlate with development of an androgen-resistant state (Bonkhoff *et al*., 1995). Prostate cancer is composed of androgen-dependent and -independent cancer cells and the emergence of apoptosis-resistant clones (Denmeade *et al*., 1996). One of the best studied neuropeptides present in the prostate is VIP, which stimulates rat prostatic epithelial cell proliferation and

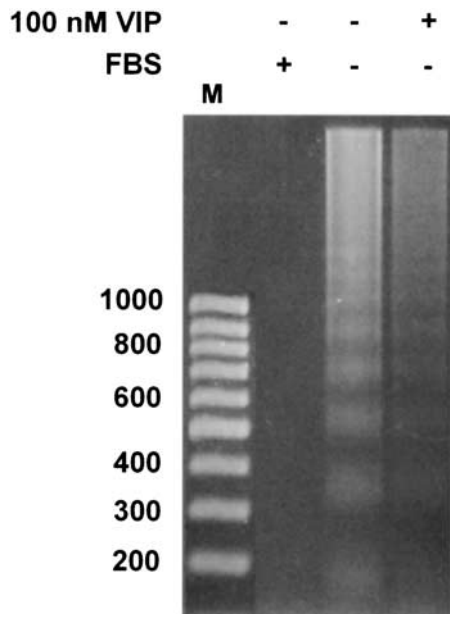

Figure 7 Effect of VIP on genomic DNA fragmentation in PC-3 cells. Fragmented DNA from control and serum-deprived PC-3 cells were isolated as described in the Methods section. DNA fragmentation was observed after growth factor withdrawal. Cells treated with 0.1 μM VIP show a reduction in DNA fragmentation. A representative experiment of three others is shown.

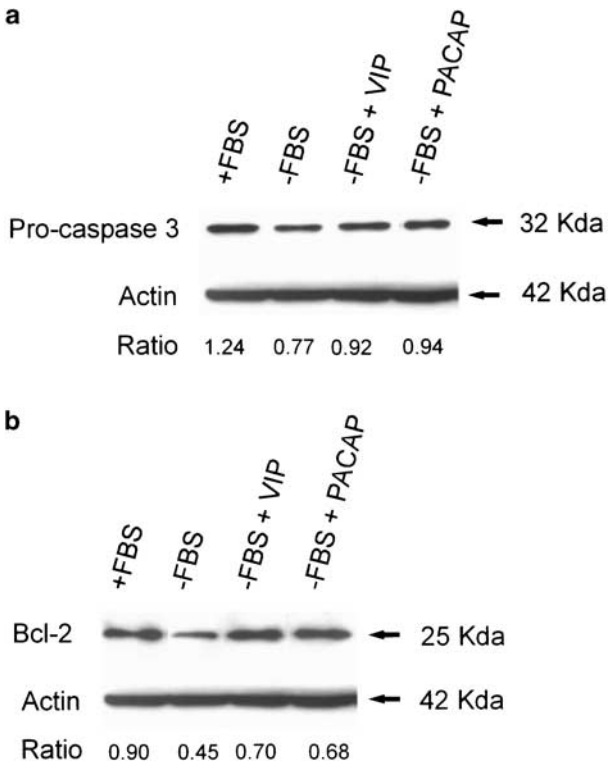

Figure 8 Western blot analysis of procaspase-3 (a) and Bcl-2 (b) protein expression in PC-3 cells. Cell lysates from control and serum-deprived cells treated or not with 0.1 μM VIP or PACAP were prepared and electrophoresed as described in the Methods section. A decrease in the procaspase 3 (a) and bcl-2 (b) proteins were observed in serum-deprived PC-3 cells. VIP and PACAP treatment of serum-deprived PC-3 cells increase the expression of procaspase 3 and bcl-2. Actin was used as a loading control and the ratio of procaspase-3 to bcl-2/actin is shown. A representative experiment of three others is shown.

induces neuroendocrine cell differentiation in LNCaP cells (Juarranz *et al*., 2001a, b). The first aim of this study was to identify whether VIP and PACAP act as autocrine and/or paracrine factors in an androgen-independent prostate cancer cell line, PC-3. We have demonstrated the presence of both peptides by RT–PCR studies and immunocytochemical analysis. These results are in line with a previous study on nude mice bearing PC-3 human androgen-independent prostate carcinoma (Plonowski *et al*., 2002). The presence of VIP has been described in autonomic nerves surrounding the human prostate acini (Tainio, 1995) and, very recently, we have described the expression and distribution of PACAP in the epithelium layer of normal and carcinomatous human prostate (García-Fernández *et al*., 2002). On the other hand, $VPAC_1$ and $VPAC_2$ receptors, but not PAC_1 receptors, are present in PC-3 cells. RT–PCR studies as well as functional studies using a specific agonist and antagonist for each receptor subtype, peptide binding and adenylate cyclase stimulation, show that both VIP receptors are present in PC-3 cells. This is in accord with previous studies in rat prostate, human cancer prostate, prostate LNCaP cells and PC-3 cells (Juarranz *et al*., 1999; Reubi *et al*., 2000; Juarranz *et al*., 2001b; Plonowski *et al*., 2002). In contrast with the present results, Gkonos *et al*. have reported that PC-3 cells do not express $VPAC_1$ receptors, whereas PC-3 cells stably expressing the androgen receptor (PC-3/AR) do, although no regulation of the $VPAC_1$ receptor by androgens was observed (Gkonos *et al*., 2000). This discrepancy could be due to differences related to cell batch. Altogether, these results suggest that VIP and PACAP act, in addition with the well-described paracrine mechanism, as an autocrine factor in human prostate cancer.

The second aim of this study was to establish the role of both neuropeptides in this androgen-independent prostatic cancer cell line, since VIP is related to proliferation, neuroendocrine differentiation and migration in other prostatic cancer cell lines (Jongsma *et al*., 2000; Juarranz *et al*., 2001b; Nagakawa *et al*., 2001). In order to test this, PC-3 cells were grown in the absence of trophic factors in order to induce an apoptotic process (Tang *et al*., 1998). This cell status did not modify either the production of VIP and PACAP in PC-3 cells or the expression and functionality of their receptors. VIP, PACAP-27 and [K^{15}, R^{16}, L^{27}] VIP (1–7)/ GFR (8–27), the specific $VPAC_1$ agonist, were able to increase the survival of PC-3 cells grown in the absence of trophic factors, whereas RO-25-1553, the specific $VPAC_2$ agonist, was unable to do so, which suggests that the receptor involved in this effect is the $VPAC_1$ receptor. These results are in agreement with MTT studies since the specific $VPAC_1$ antagonist, but not the specific $VPAC_2$ antagonist, was able to block the increased cell survival induced by VIP. In this regard, another VIP antagonist (JV-1-53, a GH-RH analogue) can inhibit the growth of androgen-independent prostate tumours in nude mice (Plonowski *et al*., 2002).

The increased survival of PC-3 cells (grown in medium lacking trophic factors) induced by VIP, PACAP and the $VPAC_1$ agonist, may be associated with a decrease in the apoptotic process of the cells. The present results show that VIP reduces DNA fragmentation, increases the expression levels of some antiapoptotic proteins, such as Bcl-2, and decreases the expression of proapoptotic proteins such as

caspase-3 in this androgen-independent prostate cancer cell line. The resistance to apoptosis is clearly related with prostate cancer progression, since androgen ablation in patients with prostate cancer eliminates most androgen-dependent cancer cells by inducing apoptosis, but can rarely cure the patients due to the presence of androgen-independent cells and the emergence of apoptosis-resistant clones (Denmeade *et al*., 1996). In this regard, our results are interesting since PC-3, an androgen-independent cancer cell line, is characterised by a high expression of antiapoptotic proteins and a low expression of proapoptotic proteins (Tang *et al*., 1998); when we induced apoptosis in PC-3 cells by serum withdrawal, VIP and PACAP-27 treatments were able to modulate the expression levels of antiapoptotic and proapoptotic proteins (Bcl-2 and caspase-3) in addition to inhibiting DNA fragmentation. Caspase-3 makes an essential contribution to cell death; all cell types examined from caspase-3-defective mice fail to display some typical hallmarks of apoptosis (i.e., DNA fragmentation); moreover, activation of procaspase-3 is triggered, in part, by activated upstream caspases such as caspase-9, whose activity is regulated by the bcl-2 protein (Budihardjo *et al*., 1999). Thus, the increased levels of inactive caspase-3 and the partial inhibition from apoptosis exerted by VIP and PACAP could be associated with the observed increase of Bcl-2 immunoreactivity induced by both peptides in serum-deprived PC-3 cells. The protective effect of VIP and PACAP from cell death has been described in other systems such as cerebellar granule cells or pituitary adenoma cells (Vaudry *et al*., 2000). This effect has been reported for other neuropeptides as well, such as bombesin or calcitonin in a prostate cancer cell line (Salido *et al*., 2000). Recently, Sumitomo *et al*. (2001) have described a mechanism that could explain the survival effect exerted by G-protein-coupled receptor agonists, such as bombesin and endothelin-1. They observed that these neuropeptides stimulate ligand-independent activation of the IGF-I receptor, which results in the activation of Akt, a serine-threonin kinase that mediates cell survival. Moreover, they observed that neutral neuroendopeptidase 24.11 inhibits the survival effect exerted by bombesin and endothelin-1 in prostate cancer cells. Interestingly, VIP and PACAP-27 are substrates of this metalloprotease (Gourlet *et al*., 1997b), which is absent in PC-3 cells.

Our results extend previous observations showing that the neuropeptides VIP and PACAP play an important role in prostate cancer development, especially in the androgen-independent status, since they are able to stimulate the neurocrine differentiation of LNCaP cells grown in the absence of androgens (Juarranz *et al*., 2001b) and inhibit induced cell death in PC-3 cells (present study). Our data suggest that VIP/PACAP antagonists can inhibit the growth of androgen-independent prostate cancer cells by abrogating the autocrine/paracrine mitogenic stimuli of VIP and PACAP, which could be potentially beneficial for prostate cancer therapy.

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