ELSEVIER

European Journal of Pharmacology 335 (1997) 255–265

ejp

Opioid alkaloids and casomorphin peptides decrease the proliferation of prostatic cancer cell lines (LNCaP, PC3 and DU145) through a partial interaction with opioid receptors

Marilenna Kampa [a], Efstathia Bakogeorgou [a], Anastassia Hatzoglou [a,c], Athina Damianaki [a], Pierre-Marie Martin [b], Elias Castanas [a,b,*]

[a] *Laboratory of Experimental Endocrinology, University of Crete, School of Medicine and University Hospital, P.O. Box 1393, Heraklion GR-71110, Greece*

[b] *Laboratory of Experimental Cancerology, INSERM CJF 93-11, University of Aix-Marseille II, Marseille, France*

[c] *CJF-INSERM 95-02, Clamart, France*

Received 27 May 1997; revised 24 July 1997; accepted 29 July 1997

Abstract

Opioid agonists (ethylketocyclazocine, etorphine, [D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ala^2,*N*-Me–Phe^4–Gly-ol]enkephalin (DAGO), [D-Ser^2,Leu^5]enkephalin–Thr^6 (DSLET) and morphine were found to inhibit the proliferation of human prostate cancer cell lines (LNCaP, DU145, and PC3), in a dose-dependent manner. The 50% inhibitory concentrations (IC_{50}) were in the picomolar range. In many cases, this effect was antagonized by the general opioid antagonist, diprenorphine, indicating the existence of specific opioid binding sites. Saturation binding experiments with selective ligands and effectors showed no opioid sites on the LNCaP cell line, κ_1 and μ sites on the PC3 cell line, and κ_1, κ_3 and μ sites on the DU145 cell line. In other cases, the opioid effect was not antagonized by diprenorphine, indicating that the action of opioids might be mediated through other membrane receptors. Furthermore, casomorphin peptides, issued from bovine alpha- (alpha-casein-90-95 and alpha-casein-90-96) and beta-caseins (beta-casomorphin and beta-casomorphin-1-5), and human $alpha_{S1}$-casein ($alpha_{S1}$-casomorphin and $alpha_{S1}$-casomorphin amide) inhibited cell proliferation of human prostate cell lines, also by a mechanism partly involving opioid receptors. As opioid neurons can be found in the prostate gland, and casomorphin peptides might reach the gland through the general circulation, the above findings indicate a putative role of opioids in prostate cancer cell growth. © 1997 Elsevier Science B.V.

Keywords: Prostate cancer; Cell line, , LNCaP, DU145, PC3; Cell proliferation; δ-Opioid receptor; μ-Opioid receptor; κ-Opioid receptor; Receptor subtype; Casein; Casomorphin

1. Introduction

Prostatic cancer is the second most common cause of death from cancer in adult males and the leading cancer in men over 75. Both genetic and exogenous factors are probably important in prostate cancer development, but the specific causes remain poorly understood.

Early stage disease is treated by eliminating the action of endogenous androgens, either by the administration of antiandrogens or by castration (Hanks et al., 1993). Unfortunately, in most of the patients that experience disease progression, it is due to the development of an androgen-independent tumor. Although there has been much research on prostate cancer and the parameters involved in its growth, development and progression, only few parameters have yet been identified, including endocrine response, age and inheritance. In addition, an increasing number of studies indicate that dietary factors, including red meat and dairy products, perhaps through saturated fat, increase the risk for prostate cancer (see Giovannucci, 1995, for a review). More detailed studies however, show that the main risk factor related to an elevated risk for prostate cancer is saturated fatty acids and α-linolenic acid (Dupont et al., 1991; Le Marchand et al., 1994).

Histological prostate cancer is much more frequent than the clinical disease. Comparing the histology of prostate

* Corresponding author. Tel.: (30-81) 394-580; Fax: (30-81) 394-581; e-mail: castanas@med.uch.gr

sections from US veterans and those from other diseased people, it becomes obvious that only 1–2% of all histologically detected malignant foci will develop to clinically relevant cancer (Carter et al., 1990a; Carter and Coffey, 1990; Boring et al., 1991). It is concluded that either prostate cancer is a multistep disease, or that factors exist in the prostate gland that control malignant cell proliferation (Carter et al., 1990b). Of these, neuropeptides are considered to influence cancer cell replication and growth (Gkonos et al., 1995; Noordzij et al., 1995). Opioid peptides, and opiergic neurons were also found in the prostate gland (Krieger et al., 1984; Tainio, 1995). In the present work, we have investigated the role of opioid agonists in prostate cell proliferation. We have used three established cell lines, namely LNCaP, PC3 and DU145, with different hormone sensitivity and expression of functional steroid hormone receptors. We now report that opioid alkaloids and peptides, as well as casomorphins, including alpha$_{S1}$-casomorphin, recently isolated from human alpha$_{S1}$-casein by our group (Kampa et al., 1996), decrease cell proliferation, acting, in most cases, through opioid binding sites. Nevertheless, the interaction of opioid receptor agonists with opioid receptors cannot explain the whole spectrum of their antiproliferative activity.

2. Materials and methods

2.1. Cell cultures

Three human prostate cancer cell lines, LNCaP, PC3 and DU145 with different receptor profile for a series of hormones and growth factors were used:

– LNCaP cells were established from a lymph node metastatic lesion of human prostatic adenocarcinoma (Horoszewicz et al., 1983). It is a steroid hormone sensitive cell line, containing high-affinity androgen (Horoszewicz et al., 1983) and estrogen receptors (Morphy et al., 1980). Functional differentiation of the normal prostate is preserved and cells produce prostatic acid phosphatase and the prostatic specific antigen (Papsidero et al., 1981).

– The DU145 cell line is derived from a cerebral metastasis of an epithelial prostate cancer. They are hormone-insensitive, not expressing androgen receptors and therefore are not stimulated by androgens (Stone et al., 1978).

– Finally, the PC3 cell line is an epithelial cell line derived from a bone marrow metastasis of a prostate adenocarcinoma with low differentiation. It is insensitive to the action of androgens even though it possesses an androgen receptor. The loss of sensitivity to androgens is due to a functional deficiency of the androgen receptor (Kaighn et al., 1979).

The three cell lines LNCaP, PC3 and DU145, were obtained at passages 53, 60 and 65, respectively. Cells were routinely grown in RPMI medium, supplemented with 10% heat inactivated fetal calf serum. They were cultured at 37°C, in a humidified atmosphere of 5% CO_2 in air.

2.2. Cell growth conditions

Cells were plated in 24-well ELISA plates, at an initial density of 2×10^4 cells, with 1.0 ml medium per well. All drugs were added to cultures one day after seeding (designated as day 0), in order to ensure uniform attachment of cells at the start of the experiments, with the exception of the LNCaP cell line in which drugs were added two days after seeding. Cells were grown for a total of 4 days for DU145 and PC3 cells and 6 days for LNCaP, with daily change of the medium containing opioid drugs. All drugs were dissolved from ethanolic stock solutions, shortly before use, in growth medium (added volume, 10 μl of drug per 10 ml of medium).

Cell growth was measured with the tetrazolium salt assay (Mosmann, 1973). Cells were incubated for 4 h at 37°C with the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), and metabolically active cells reduced the dye to purple formazan. Dark blue crystals were dissolved in propanol. Absorbance was measured at 570 nm and compared against a standard curve for known numbers of cells. All experiments were performed in triplate, a minimum of three times.

Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) and Student's *t*-test, in which the residual variance of the ANOVA was used as a common estimate of the standard error. This procedure is validated by the fact that the variance of the individual point sets was rather homogeneous. For sigmoidal fitting, we used automatic initial estimates, and used a one-or-two site model, according to the significance level of the estimated curve. Sigmoidal fitting was done with the Origin v 4.1 package (MicroCal, Northampton, MA, USA).

2.3. Opioid binding conditions

Ligand binding assays in whole prostate cancer cells were performed as described in Hatzoglou et al. (1996a,b) for breast cancer cell lines, after a brief acidification. Briefly, cells were washed with 2 ml phosphate buffered saline, and incubated for 3 min with 0.5 ml of glycine buffer (50 mM, pH 3.0), at 4°C. Afterwards, the glycine buffer was removed, the cells were washed twice with 3 ml phosphate-buffered saline, and processed for opioid binding immediately. Initial experiments with breast cancer (Hatzoglou et al., 1994, 1995a,b, 1996a,b), as well as with prostate cancer cell lines showed that this brief acidification did not modify the viability of the cells, and that the pH returned to 7.4 after the first wash with phosphate-buffered saline. For saturation binding experi-

ments in whole cells, about 10^6 cells/well were used. Before binding, the cells were washed twice with 2 ml of phosphate-buffered saline. Binding was performed in phosphate-buffered saline, in a total volume of 0.4 ml, containing radioactive opioid, without (total binding) or with (non-specific binding) a thousand-fold molar excess of the same unlabelled opioid and effectors (see below). At least 8–10 points, with different concentrations of radiolabelled opioid (varying from 1 to 50 nM), were assayed in duplicate. The cells were incubated for 2 h at room temperature (18–22°C). Experiments on opioid binding in the breast cell lines (Hatzoglou et al., 1994, 1995a,b, 1996a,b) and prostate cell line showed that equilibrium was attained for all ligands used (not shown). At the end of the incubation period, the unbound radioactivity was eliminated by washing the cells twice with 2 ml cold buffer. Cells were removed from plates with 0.4 ml 2 M NaOH, and mixed with 4 ml scintillation cocktail (SigmaFluor, Sigma, St. Louis, MO, USA). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard), with 60% efficiency for tritium. Binding was repeated at least three times (in duplicate). Non-specific binding under the conditions described ranged from 20 to 36%. The results were analyzed with the Origin (MicroCal) v. 4.1 package, using equations described by Munson and Rodbard (1980).

2.4. Specific conditions for differential detection of various types of opioid binding sites

The conditions used for the differential detection of various opioid sites were described in previous studies from our group (Castanas et al., 1984, 1985a,b; Hatzoglou et al., 1995a, 1996a). Briefly, delta opioid sites were detected with tritiated [D-Penicilamine^2,D-Penicilamine^5]enkephalin (DPDPE). Mu sites were detected with the selective ligand, [D-Ala^2, *N*-Me–Phe^4–Gly-ol]-Enkephalin (DAGO). The interaction of tritiated ethylketocyclazocine with the κ_1 site was calculated by performing the binding in the presence of 5 μM [D-Ala^2,D-Leu^5]enkephalin (DADLE). Under these conditions, we and others have found that DADLE masks delta, μ and κ_2 sites, on which ethylketocyclazocine could bind with high affinity (Castanas et al., 1985a,b). Finally, diprenorphine binding (reacting mainly with δ, μ, κ_2 and κ_3 sites at nanomolar concentrations) was performed under the same conditions as for ethylketocyclazocine. The interaction of the ligand with the κ_3 site was estimated, in the presence of 5 μM DADLE (see Castanas et al., 1985a, for a detailed description; Castanas et al., 1985b).

2.5. Radiochemicals and chemicals

[^{3}H]ethylketocyclazocine (spec. act. 18 Ci/mmol) was bought from New England Nuclear (Boston, MA, USA). [^{3}H]diprenorphine (spec. act. 29 Ci/mmol), [^{3}H]DAGO (spec. act. 60 Ci/mmol) and [^{3}H]DPDPE (spec. act. 28 Ci/mmol) were from Amersham (Amersham, UK).

Unlabelled DAGO, DSLET and DADLE were from Sigma. Ethylketocyclazocine was a gift from Sterling–Winthrop. Diprenorphine and etorphine were from Reckitt and Colman. Morphine and naloxone were from Francopia. All casomorphin peptides (with the exception of alpha$_{S1}$-casomorphin which was synthesized in our laboratory) and all chemicals were purchased from Sigma.

3. Results

3.1. Cell viability

After plating, we assayed the number and the viability of cells for at least 4 days of culture for PC3 and DU145 cells and 6 days for LNCaP. We found that about 85–90% of the total number of cells were attached after 24 h. Cell

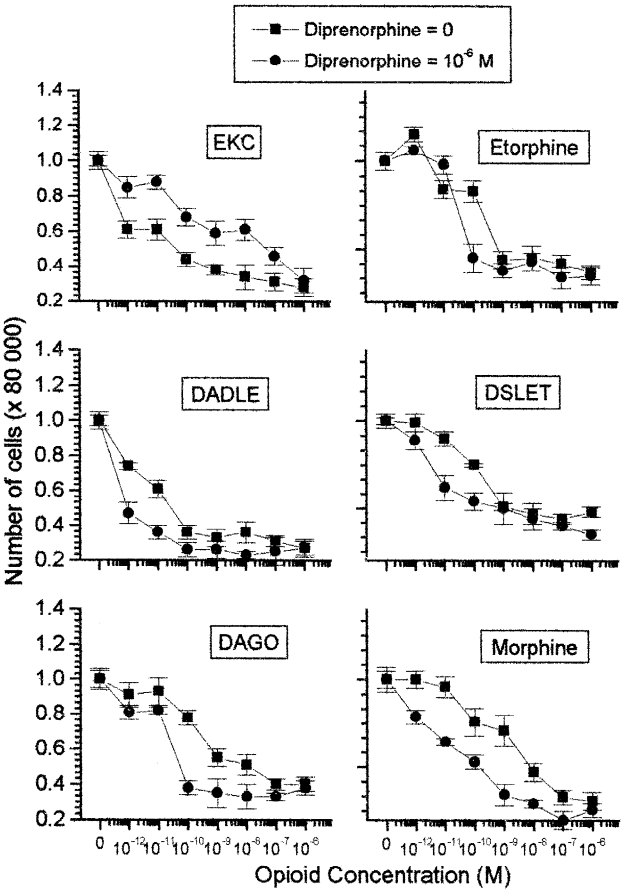

Fig. 1. Effect of opioid agonists on cell proliferation of the LNCaP cell line. Cells were incubated in the presence of the indicated concentrations of opioids (EKC = ethylketocyclazocine; DADLE = [D-Ala^2,D-Leu^5]enkephalin; DSLET = [D-Ser^2,Leu^5]enkephalin, Thr^6; DAGO = [D-Ala^2, *N*-Me–Phe^4–Gly-ol]–enkephalin) for 6 days, in the absence (squares) or in the presence (circles) of 10^{-6} M diprenorphine. Cell growth was estimated by the tetrazolium salt assay, as described in Section 2. Data presented are the means ± S.E. from three experiments in triplicate.

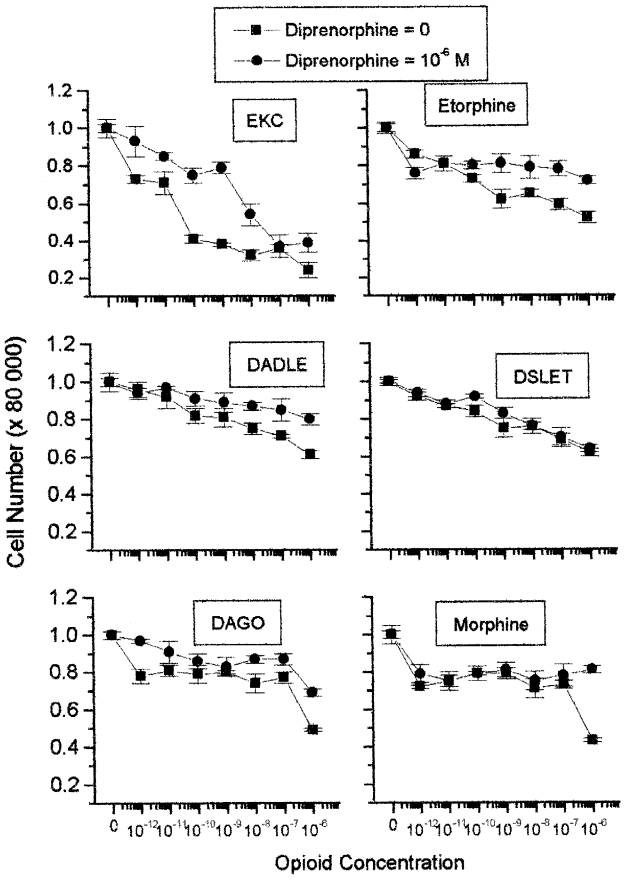

Fig. 2. Effect of opioid agonists on cell proliferation of the DU145 cell line. Cells were incubated in the presence of the indicated concentrations of opioids for 4 days, in the absence (squares) or in the presence (circles) of 10^{-6} M diprenorphine. Abbreviations are presented in Fig. 1. Data presented are the means ± S.E. from three experiments in triplicate.

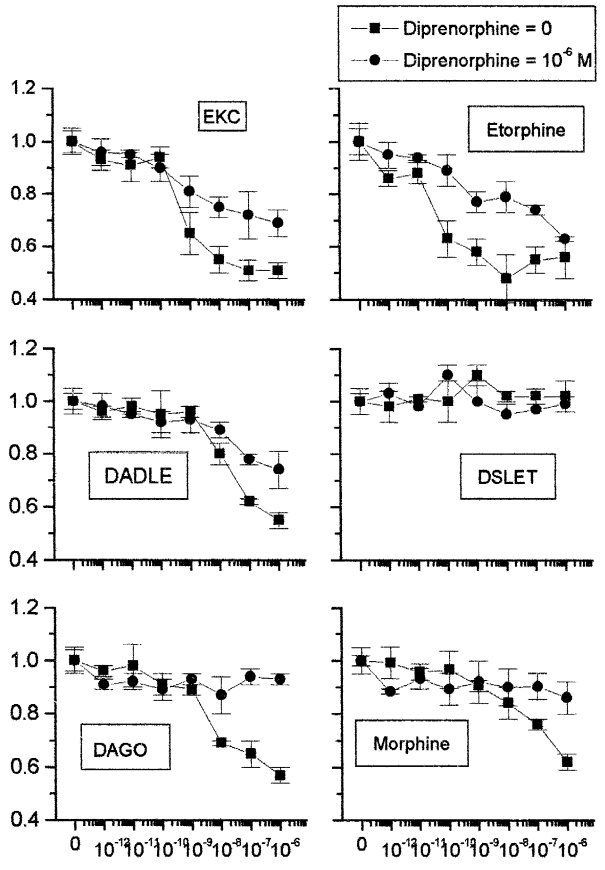

Fig. 3. Effect of opioid agonists on cell proliferation of the PC3 cell line. Cells were incubated in the presence of the indicated concentrations of opioids for 4 days, in the absence (squares) or in the presence (circles) of 10^{-6} M diprenorphine. Abbreviations are presented in Fig. 1. Data presented are the means ± S.E. from three experiments in triplicate.

viability was assayed routinely. Under our experimental conditions, and for the period of time studied, there was no apparent change of cell viability, under basal conditions, or after the addition of opioid drugs or casomorphin peptides. The cell number at day 4 for DU145 and PC3 cell lines and day 6 for LNCaP was about 80 000 since their proliferation doubling time was 34, 33 and 70–72 h, respectively.

Table 1
Effect of opioid agonists and antagonists on cell proliferation

Opioid		LNCaP		DU145		PC3	
		IC_{50} (nM)	max. inhibition (%)	IC_{50} (nM)	max. inhibition (%)	IC_{50} (nM)	max. inhibition (%)
EKC				0.0001	29		
		0.0037	72	0.1400	76	0.5990	49
	+ Diprenorphine	0.1400	68	0.0012	20		
				1.0400	61	0.5000	31
Etorphine		0.1200	63	0.0051	48	0.0340	44
	+ Diprenorphine	0.0350	65	0.0001	28	10.0000	37
DADLE		0.0007	73	0.6200	39	12.0000	45
	+ Diprenorphine	0.0003	73	0.2200	20	55.0000	26
DSLET		0.1100	52	0.0496	38	> 10 000	0
	+ Diprenorphine	0.0023	65	2.4900	36	> 10 000	0
Morphine		1.3000	70	0.0002	57	1700.0000	38
	+ Diprenorphine	0.0066	74	0.0023	19	> 10 000	9
DAGO		0.2700	60	0.0001	51	4.3000	43
	+ Diprenorphine	0.0420	62	0.0082	31	> 10 000	7

Table presents the IC_{50} of opioid agonists and antagonists calculated by sigmoidal fitting of data shown in Figs. 1–3.

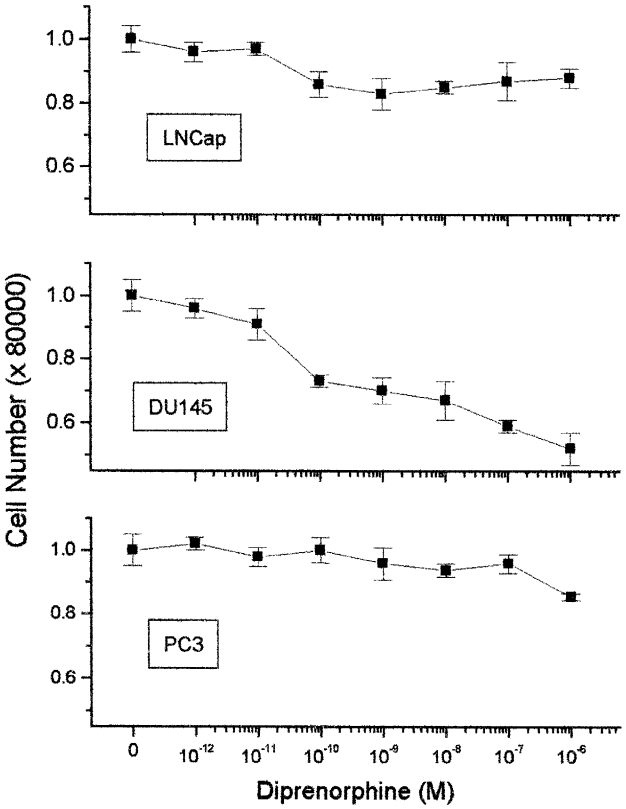

Fig. 4. Effect of the opioid antagonist, diprenorphine on the proliferation of three human prostate cell lines. Cells were incubated in the presence of the indicated concentrations of the opioid antagonist, diprenorphine, for 4 days (PC3 and DU145) and 6 days (LNCaP). Cell growth was estimated with the tetrazolium salt assay, as described in Section 2. Data presented are the means ± S.E. from three experiments in triplicate.

3.2. Effect of opioid receptor agonists and antagonists on cell proliferation

As indicated in Section 1, opioid receptor agonists decrease cell proliferation in various systems, including breast cancer cells. In order to examine the effect of opioids on cell proliferation, we chose the following opioid receptor agonists, presenting different affinities for each class of opioid receptors (Castanas et al., 1985a,b): DADLE (δ- and μ-opioid receptor agonist at nanomolar concentrations), DSLET (δ-opioid receptor agonist), ethylketocyclazocine (presenting, at nanomolar concentrations, a strong interaction with δ, μ, κ_1 and κ_2 opioid sites), etorphine (presenting a high affinity with δ, μ, κ_2 and κ_3 sites), and morphine as the prototype μ ligand. As shown in Figs. 1–3, all opioid agonists used produce dose-dependent inhibition of cell proliferation, as follows:

(a) In the LNCaP cell line, all opioids used produced a dose-dependent statistically significant ($P < 0.001$) inhibition of cell growth. The most potent opioid agonist was etorphine (producing significant inhibition at 10^{-11} M), followed by DADLE, ethylketocyclazocine, DAGO, DSLET and morphine (Fig. 1).

(b) In the DU145 cell line, only ethylketocyclazocine produced a potent biphasic inhibition ($P < 0.001$) of cell growth. All other opioids had minor effects (Fig. 2) (statistical significance ranging from $P < 0.03$ to $P < 0.04$).

(c) Finally, in the PC3 cell line, the most effective opioid agonist etorphine ($P < 0.001$), followed by ethylketocyclazocine, DAGO, DADLE and morphine, while DSLET did not show any notable inhibition of cell growth (Fig. 3).

All the above results are summarized in Table 1.

To establish an opioid receptor-mediated mechanism of action of opiates, three criteria should be fulfilled:

(1) It has to be dose-dependent. This was the case, as reported here.

(2) Opioid antagonists must reverse the effect of agonists. As shown in Figs. 1–3, the addition of diprenorphine produced different effects in the three cell lines:

(a) In LNCaP cells (Fig. 1), diprenorphine reversed the action of ethylketocyclazocine and etorphine, at concentrations lower than 5×10^{-9} M. At higher concentrations, the antagonistic effect of diprenorphine was blunted. On the other hand, potentiation of the action of DSLET, DADLE, DAGO and morphine was observed in the presence of 10^{-6} M diprenorphine. These results indicate that ethylketocyclazocine and etorphine might be at least 100 times more potent than diprenorphine. Furthermore, in the case of the enhanced action of other opioids, their antiproliferative effect cannot be mediated by opioid receptors, or conversely, endogenous opioids could mask opioid sites in this cell line.

(b) An antagonistic effect of diprenorphine, on cell proliferation of DU145 cells was observed only in the case of ethylketocyclazocine (Fig. 2). In all other cases, the

Table 2
Mean cpm ± SE from two different experiments in duplicate

Cell line	[^{3}H]EKC		[^{3}H]diprenorphine	
	without acidification	with acidification	without acidification	with acidification
LNCaP	0	0	0	0
DU145	171 ± 9	204 ± 22	150 ± 17	375 ± 15
PC3	262 ± 22	1505 ± 36	ND	ND

Cells (10^6/well) were incubated with 5 nM opioid agonists (ethylketocyclazocine and diprenorphine, approx. 100 000 cpm) for 2 h at room temperature, with or without 3 min acidification, as described in Section 2.
ND = not detected.

effect of other opioids was minimal, and diprenorphine had no effect.

(c) Finally, in the PC3 cell line (Fig. 3), diprenorphine antagonizes the effects of all opioids having an effect on cell proliferation.

The above results (especially those showing a potentiation of the action of opioids in the presence of the antagonist), indicate that opioid antagonists might have a per se inhibitory effect on cell proliferation, or conversely, that the observed effect of opioids might not be mediated through opioid receptors. We therefore assayed the effects of diprenorphine on cell proliferation. Our results are shown in Fig. 4. Diprenorphine had minor effects, at concentrations higher than 10^{-8} M, on the LNCaP cell line (a result which could explain the observations presented in Fig. 1, as we used the antagonist at 10^{-6} M), dose dependent inhibitory effects on the DU145 cell line, and no effect on the PC3 line.

(3) Opioid binding sites must be present on the cells (presenting an affinity compatible with the effect of opioids). We therefore tested for the presence of opioid binding sites on the three cell lines.

3.3. Identification of opioid binding sites in human prostatic cancer cell lines

In several tissues, the detection of binding sites is difficult, due to the coexistence of their endogenous ligand, which could mask these sites (Castanas et al., 1985a,b; Hatzoglou et al., 1996c). We have developed a specific technique, for such cases. This technique involves brief acidification of membranes or cells, eliminating the endogenous ligand, thus making possible the detection of the totality of binding sites (Hatzoglou et al., 1994, 1995a, 1996c). Table 2 shows the results of acidification on the binding of opioids in the three cell lines. As indicated, in the LNCaP cell line, no interaction of the opioid ligands was found, before or after acidification. In the DU145 and the PC3 cell line, on the contrary, enhancement of opioid binding was found when [^{3}H]diprenorphine and [^{3}H]ethylketocyclazocine (two almost universal opioid ligands, see Section 2) were the opioid ligands used, respectively. This result indicates that endogenous opioids must be synthesized and secreted by cells as was previously reported for prostatic tissue (Krieger et al., 1984). We have therefore performed the identification and characterization of opioid sites after acidification of cells.

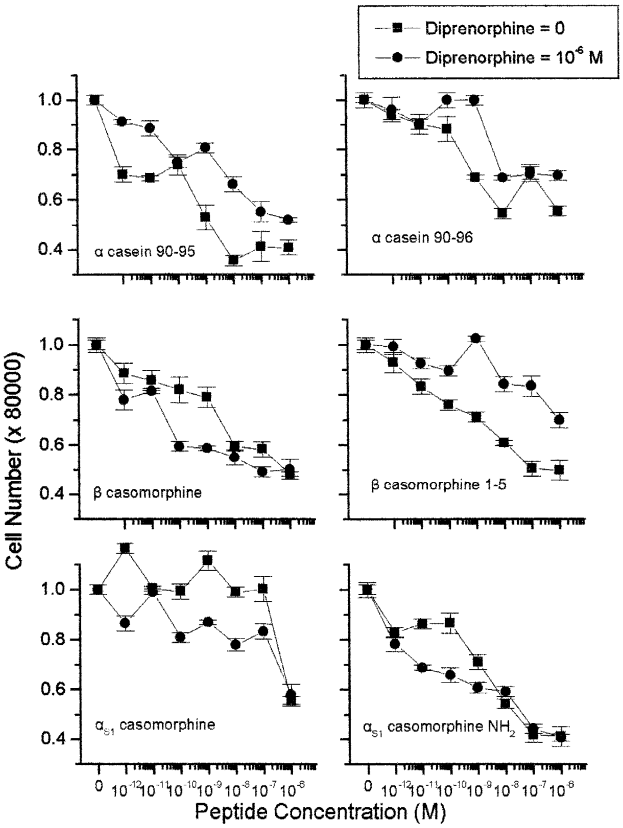

Fig. 5. Effect of casomorphin peptides on the proliferation of the LNCaP cells. Cells were incubated in the presence of the indicated concentrations of different casomorphins as shown, for 6 days, in the absence (squares) or in the presence (circles) of 10^{-6} M diprenorphine. Cell growth was estimated by the tetrazolium salt assay, as described in Section 2. Data presented are the means ± S.E. from three experiments in triplicate.

Table 3 presents the results of the identification and

Table 3
Characterization of opioid binding sites in three human prostatic cell lines

	LNCaP	DU145	PC3	Detected sites
EKC		K_d 17.74 nM	K_d 3.13 nM	δ, μ, κ_1, κ_2
	0	37 200 sites/cell	12 100 sites/cell	
EKC + DADLE		K_d 16.8 nM	K_d 3.13 nM	κ_1
	0	36 000 sites/cell	11 200 sites/cell	
Diprenorphine		K_d 6.0 nM		δ, μ, κ_2, κ_3
	0	12 100 sites/cell	0	
Diprenorphine + DADLE		K_d 5.7 nM		κ_3
	0	11 500 sites/cell	0	
DAGO		K_d 0.19 nM	K_d 2.4 nM	μ
	0	650 sites/cell	950 sites/cell	
DPDPE	0	0	0	δ

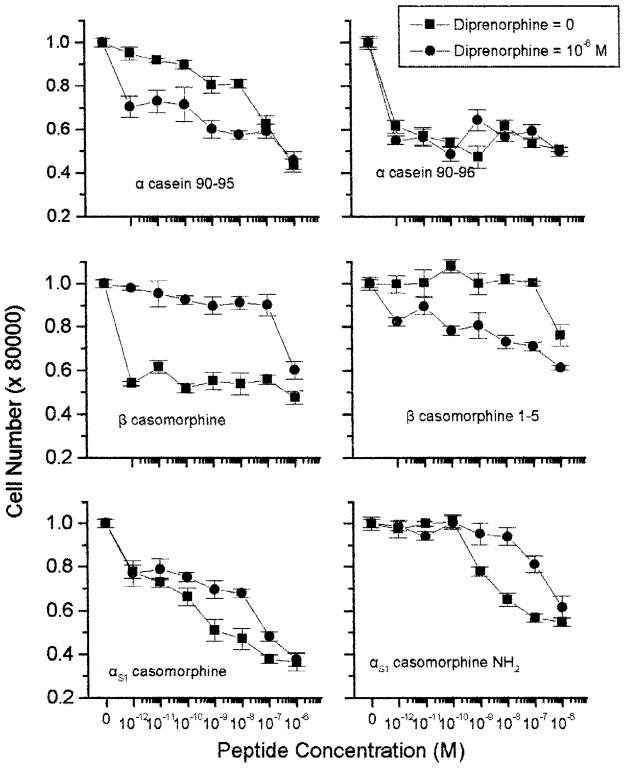

Fig. 6. Effect of casomorphin peptides on the proliferation of the DU145 cells. Cells were incubated in the presence of the indicated concentrations of different casomorphins as shown, for 4 days, in the absence (squares) or in the presence (circles) of 10^{-6} M diprenorphine. Cell growth was estimated with the tetrazolium salt assay, as described in Section 2. Data presented are the means ± S.E. from three experiments in triplicate.

characterization of opioid sites by ligand binding experiments. We found that:

(1) In the LNCaP cell line, none of the opioid ligands used, showed any specific opioid binding, indicating the absence of opioid binding sites. This result was further confirmed by testing opioid binding on cell membrane preparations, with or without acidification (not shown).

(2) In the DU145 cell line, [^{3}H]DPDPE show no specific binding, ruling out the existence of δ opioid sites. [^{3}H]ethylketocyclazocine showed the existence of a low-affinity binding site (K_d 17 nM), which presented a κ_1 selectivity, as it was insensitive to the addition of DADLE (36 000 sites/cell). Furthermore, a κ_3 selective opioid site was detected, as [^{3}H]diprenorphine specific binding was not modified by the addition of 5 μM DADLE (K_d 6 nM, 12 000 sites/cell). Finally, [^{3}H]DAGO identified a low concentration of high affinity μ opioid sites (K_d 0.19 nM, 650 sites/cell).

(3) Finally, in the PC3 cell line, only κ_1 (K_d 3.1 nM, 11 000 sites/cell) and μ (K_d 2.4 nM, 1000 sites/cell) were detected.

The above results confirm that the majority of opioid agonists used might act mainly through κ opioid receptors, in the case of DU145 and PC3 cell lines. On the contrary, the effect of opioids on the LNCaP cell line, is apparently not mediated through opioid sites.

3.4. Effect of casomorphin peptides on cell proliferation

In the present study, we have used six different casomorphin peptides. Two of these peptides (α-casein 90–95, –Arg–Thr–Leu–Gly–Tyr–Leu–, and 90–96, –Arg–Thr–Leu–Gly–Tyr–Leu–Glu–), are fragments of bovine α-casein (Loukas et al., 1983), while β-casomorphin (Tyr–Pro–Phe–Val–Glu–Pro–Ile) and β-casomorphin 1–5 (Tyr–Pro–Phe–Pro–Gly) derive from bovine beta-casein (Brantl et al., 1979; Lottspeich et al., 1980; Meisel, 1986).

Table 4
Effect of casomorphin peptides on cell proliferation

Opioid		LNCaP		DU145		PC3	
		IC_{50} (nM)	max. inhibition (%)	IC_{50} (nM)	max. inhibition (%)	IC_{50} (nM)	max. inhibition (%)
α-casein 90–95		0.0064	30				
		0.9400	59	137.0000	57	6.9200	47
	+ Diprenorphine	3.9000	48	0.0028	55	0.3700	39
α-casein 90–96		0.1900	40	0.0002	49	> 10 000.000	9
	+ Diprenorphine	3.8000	30	0.0002	50	0.1300	24
β-casomorphine		0.6200	52	0.0001	52	0.0960	30
	+ Diprenorphine	0.0038	50	> 1000.000	5	1700.0000	23
β-casomorphine 1–5		0.0650	50	1000.0000	0	0.1900	13
	+ Diprenorphine	0.3900	30	0.0520	39	5.2000	29
α_{S1}-casomorphine		1000.0000	45	0.0092	64	8.8000	19
	+ Diprenorphine			0.0021	31		
		46.0000	42	65.0000	63	39.0000	15
α_{S1}-casomorphine–NH_2		3.1000	59	1.2000	46	3.2000	36
	+ Diprenorphine	0.0021	59	420.0000	39	35.0000	23

Table presents the IC_{50} of opioid agonists and antagonists, calculated by sigmoidal fitting of data shown in Figs. 5–7. In cases in which two numbers are shown, fitting was better when performed on a two-site model.

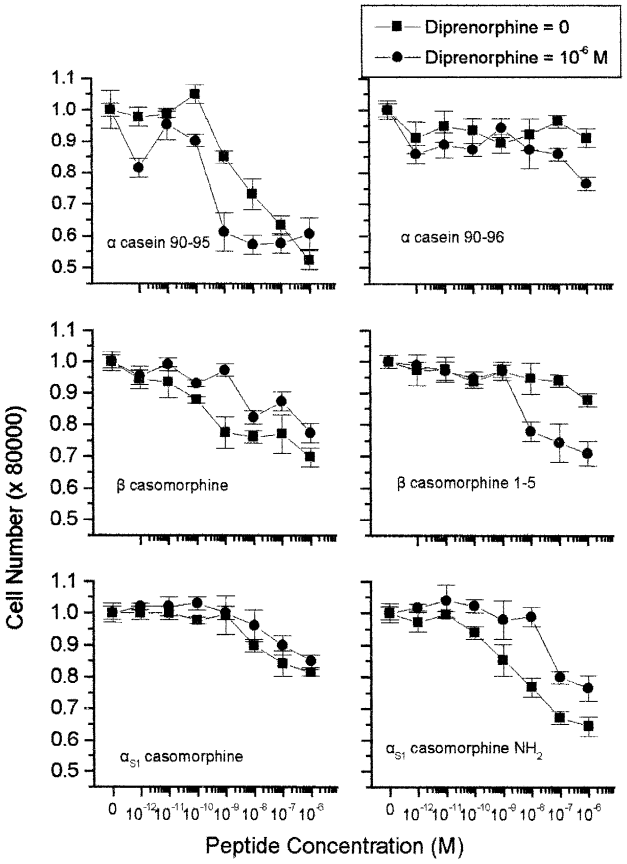

Fig. 7. Effect of casomorphin peptides on the proliferation of the PC3 cells. Cells were incubated in the presence of the indicated concentrations of different casomorphins as shown, for 4 days, in the absence (squares) or in the presence (circles) of 10^{-6} M diprenorphine. Cell growth was estimated with the tetrazolium salt assay, as described in Section 2. Data presented are the means ± S.E. from three experiments in triplicate.

Finally, α_{S1}-casomorphin (Tyr–Val–Pro–Phe–Pro) and its amide, proposed by our group as very potent opioid peptides (Kampa et al., 1996) are derived from human alpha$_{S1}$-casein.

Figs. 5–7 and Table 4 show the effects of casomorphin peptides on cell proliferation. In details we have found:

(1) In the LNCaP cell line, α-casein-derived peptides (90–95 and 90–96), as well as β-casomorphin 1–5, produce a dose-dependent and (partially) reversible inhibition of cell growth. On the contrary, β-casomorphin, and α_{S1}-casomorphin amide produce a dose-dependent inhibition of cell growth, which was potentiated by the addition of the antagonist. Finally, α_{S1}-casomorphin had no effect at concentrations from 10^{-12} to 10^{-7} M.

(2) In the DU145 cell line, β-casomorphin, α_{S1}-casomorphin and α_{S1}-casomorphin amide, produce a reversible inhibition of cell growth. α-casein 90–96 produce an inhibition of cell proliferation, not reversed by diprenorphine, while the antiproliferative action of α-casein 90–95 and β-casomorphin 1–5 was enhanced by the antagonist, indicating the involvement of other receptors, different from opioid sites.

(3) Finally, in the PC3 cell line, only the antiproliferative action of β-casomorphin, α_{S1}-casomorphin and its amide was reversed by diprenorphine, while the action of other casomorphins was null (α-casein 90–96) or enhanced by the addition of the antagonist.

The above results are summarized in Table 4.

4. Discussion

Various neuropeptides have been found in the human prostate, including somatostatin, neuropeptide Y, vasoactive intestinal polypeptide, and substance P (Gu et al., 1983). These peptides are proposed to be neuromediators, playing a possibly important role in prostate physiology. Nevertheless, opioid peptide involvement in prostate tumors remains controversial. Indeed Gu et al. (1983) have reported no enkephalin immunoreactivity in sections of human prostate, while Krieger et al. (1984) have found β-endorphin, as well as other POMC-derived peptides in rodent prostate, and Tainio has shown Leu–enkephalin innervation of the adult human organ (Tainio, 1995). In fetal prostate, a tissue possibly related to the neoplastic organ, Jen and Dixon (1995) showed by immunohistochemistry, that Met- and Leu–enkephalin-specific nerve fibers, associated with smooth muscle bundles, were detectable between weeks 13 and 26 of gestation in the human. In a detailed immunohistochemical study, Crowe et al. (1991) found many neuropeptides, including Met- and Leu–enkephalin in autonomic ganglia, which are very abundant in all regions of the human prostate. Peptidergic neurons have been proposed as possible regulators of tumor growth (Noordzij et al., 1995), influencing cancer cell replication (Gkonos et al., 1995). Finally, it was reported that the opioid-receptor agonist bremazocine, by a central action, inhibited the growth of the transplanted rat tumor, Dunning R3327H, which did not contain specific opioid receptors agonist (Reubi, 1985), while opioid receptors of the δ-, μ-, and κ-type have been characterized in one case of embryonal prostate rabdomyosarcoma (Zagon et al., 1987), which did not contain Met–enkephalin or β-endorphin immunoreactivity. In other systems, sharing similar hormone dependence, as is breast cells, we (Hatzoglou et al., 1996a,b) and others (Maneckjee et al., 1990) have shown that opioid agonists decrease cell growth, interacting with opioid (Maneckjee et al., 1990; Hatzoglou et al., 1996a) and somatostatin receptors (Hatzoglou et al., 1995b). Furthermore, casomorphins, peptides derived from the limited proteolysis of α- and β-bovine or human caseins, interact with both opioid and somatostatin receptors, and decrease cell proliferation (Hatzoglou et al., 1996b; Kampa et al., 1996).

In view of the above results, we have now investigated, the possible implication of opioids in the proliferation of three prostate cancer cell lines. Our results, presented in Figs. 1–4 and Table 1, show that, in most cases, opioids decrease cell growth. Nevertheless, there were distinct patterns for each opioid inhibition in the three cell lines, in relation to the existence and the affinity of opioids for their binding sites. Three conditions should be fulfilled in order to demonstrate a receptor-mediated action:

(1) A dose-dependent action. This was observed for the great majority of opioid agonists used. Furthermore, in most cases, the observed effect was obtained at low concentrations, comparable with the affinity of opioids for the binding sites detected (compare Tables 1 and 3).

(2) Reversal of this action by the addition of an excess of opioid antagonists. In the case of the three prostate cell lines used, diprenorphine presented two distinct patterns: either it reversed opioid action, indicating an opioid site-related phenomenon, or it potentiated the agonist effect, indicating mediation of the opioid action through other membrane receptors on which opioid agonists and antagonists could bind. Indeed, as found for breast, some opioids could act through somatostatin receptors (Hatzoglou et al., 1995b) which could be found on the surface of cells. It was reported that prostate cells also possess somatostatin binding sites (Tatoud et al., 1995) which play an inhibitory role in cell proliferation. We are investigating a possible somatostatin–opioid interaction on prostate cell lines. Finally, opioid antagonists may have a specific inhibitory effect on cell proliferation, as shown in Fig. 4 and report for other experimental tumors or cell lines (Aylsworth et al., 1979; Zagon and McLaughlin, 1981, 1983; Kikuchi et al., 1987; Hatzoglou et al., 1996a).

(3) Specific opioid binding sites could be found on the membrane of cells. As indicated in Table 3, opioid sites were found in most cases. It is interesting that opioid-related inhibition of cell growth is achieved at concentrations comparable with the affinity of opioid binding sites. Another interesting finding presented in Table 2, is that, in order to identify opioid binding sites, we had to acidify the cells. As reported previously (Hatzoglou et al., 1994, 1995a, 1996c), this acidification can dissociate endogenous ligands from their binding sites, permitting detection of the totality of binding sites. Possible production of endogenous opioids by prostate cell lines in culture is now under investigation.

Analysis of the results shown in Figs. 1–4 and Tables 1 and 3, in relation with the prerequisites presented above for a receptor-mediated action, shows the following:

(1) In the LNCaP cell line, the only opioid whose action might be mediated by opioid receptors could be ethylketocyclazocine. Indeed, only the ethylketocyclazocine antiproliferative effect is partially reversed by diprenorphine. In all other cases (etorphine, DADLE, DSLET, DAGO and morphine) this action is either not affected by the antagonist, or is further potentiated. Detection of opioid binding sites, on the contrary, did not reveal δ, μ, or κ (κ_1, κ_2 and κ_3) opioid sites on membranes or whole cells. This apparent controversy is not due to saturation of existing opioid receptors by endogenous opioids, as acidification of cells or membranes did not produce different results (see Table 2). A possible explanation might be the interaction of opioid agonists and antagonists with another site different from the classical opioid receptor, such as for example the σ (Vilner et al., 1995) or the ζ site (Zagon et al., 1989), or even the 'orphan' receptor described recently (Heiber et al., 1995). All opioids tested were active on this cell line. The most potent agents inhibiting cell proliferation were ethylketocyclazocine and DADLE, followed by morphine, etorphine, DAGO and DSLET. We are currently testing cross-interactions of opioids with other membrane receptors.

(2) In the DU145 line, μ, κ_1 and κ_3 opioid sites were found. In contrast, no δ or κ_2 receptors were detected (Table 3). This result was reflected by the ability of opioids to inhibit cell growth. Indeed, all opioids (ethylketocyclazocine, etorphine, DADLE, morphine and DAGO) which act on these opioid receptors (Castanas et al., 1985a,b), inhibited in a dose-dependent and reversible manner cell proliferation. On the contrary, DSLET, an agonist of the δ-opioid site did not inhibit cell growth reversibly. The antiproliferative effect of this agonist might be mediated through other sites. The most active opioid-receptor agonist was ethylketocyclazocine, followed distantly by etorphine, DADLE and DSLET. It should be noted that opioids are much more active on the LNCaP than on DU145 cell line (see Table 1).

(3) In the PC3 line, we have detected only κ_1, and μ opioid sites (Table 3), explaining the reversible inhibition of cell growth by ethylketocyclazocine, etorphine, DADLE, DAGO and morphine (Fig. 3). On the contrary, DSLET, acting on δ-opioid sites, does not inhibit cell growth at all.

It is interesting to compare the results found for the three cell lines: As indicated above, the LNCaP cell line possesses active androgen receptors, PC3 has a defective steroid receptor, while DU145 has no steroid receptors. The same pattern was found for the ability of opioid-receptor agonists to inhibit cell growth. The spectrum of opioid receptors found on cells follows the reverse pattern: Greater diversity of opioid sites was found in the most undifferentiated DU145 line, followed by PC3 and LNCaP. It is therefore tempting to speculate that the emergence of opioid receptors is accompanied by a more undifferentiated and aggressive phenotype. Conversely, we have recently found that, in breast cells, there is an interaction of opioids and steroid receptors (Panagiotou et al., submitted). Our results could therefore be explained if such a relation also exists in the prostate. This is currently being investigated in our laboratory.

In addition to possible local production of opioids in the prostate, as indicated above, these substances could reach

the gland either from locally infiltrating cells (Kita et al., 1992) or through the general circulation. In this latter case, β-endorphin, which is the only endogenous opioid with a sufficiently long half-life (Scholar et al., 1987), could be the endogenous ligand of the opioid sites found. In addition, other food-derived peptides with opioid activity could also reach the prostate. Of these, casomorphins are possible candidates. Indeed, casomorphin peptides were found to cross the intestinal barrier, and immunoreactive casomorphin peptides were detected in the blood after milk ingestion (Tome et al., 1987; Mahe et al., 1989; Nyberg et al., 1989). We have recently reported that, in breast cells, casomorphin peptides can decrease growth by an interaction mainly with opioid receptors (Hatzoglou et al., 1996b). Therefore, in view of a possible physiological significance of casomorphin peptides in prostate cancer control, we have tested their effect on cell growth. Our results are presented in Figs. 5–7 and Table 4.

Comparing the effects of opioids presented in Tables 1 and 4, it is obvious that casomorphin peptides have less pronounced effects than opioid alkaloids. This could be due to a possible degradation of peptides in the culture medium, to the masking of opioid sites by endogenously produced opioid substances, or to a decreased affinity of the peptides for opioid sites. This latter could not be confirmed by our results. As indicated, the antiproliferative effect of opioid alkaloids and casomorphin peptides is exerted at very low concentrations, indicating that full receptor occupancy might not be necessary for completion of the biological effect. Taking into consideration the opioid sites detected in the cell lines (Table 3), as well as the reported casomorphin selectivity (Hatzoglou et al., 1996b; Kampa et al., 1996), we propose that, in both the DU145 and PC3 cell lines, the interaction of casomorphins might be exerted through κ_1 and κ_3 sites, respectively. On the contrary, the effect of α-casomorphins and β-casomorphin 1-5, might be exerted through other sites, different from opioid receptors.

As indicated above, major peptidergic innervation, including opiergic nerves was found in the normal prostate. These peptidergic neurons are proposed to exert, in addition to their neurotransmitter action, a possible role in tumor regulation (Noordzij et al., 1995), influencing cancer cell growth (Gkonos et al., 1995). The results of the present study could therefore provide evidence for a putative role of local or circulating opioids (casomorphins) in the growth of prostate cancer cells.

Acknowledgements

Work partially supported by the University of Crete Research committee, Hellenic Anticancer Society, General Secretariat of Research and Technology (GGET), Ministry of Health (KESY), and Varelas SA grants.

References

Aylsworth, C.F., Hodson, C.A., Meites, J., 1979. Opiate antagonists can inhibit mammary tumor growth in rats. Proc. Soc. Exp. Biol. Med. 161, 18–20.

Boring, C.C., Squires, T.S., Tong, T., 1991. Cancer statistics, 1991. CA Cancer J. Clin. 41, 19–36.

Brantl, V., Teschemacher, H., Henschen, A., Lottspeich, F., 1979. Novel opioid peptides derived from casein (β-casomorphins): I. Isolation from bovine casein peptone. Hoppe-Seyler's Z. Physiol. Chem. 360, 1211–1216.

Carter, H.B., Coffey, D.S., 1990. The prostate: An increasing medical problem. Prostate 16, 39–48.

Carter, B.S., Carter, H.B., Isaacs, J.T., 1990a. Epidemiologic evidence regarding predisposing factors to prostate cancer. Prostate 16, 187–197.

Carter, H.B., Piantadosi, S., Isaacs, J.T., 1990b. Clinical evidence for and implications of the multistep development of prostate cancer. J. Urol. 143, 742–746.

Castanas, E., Giraud, P., Audigier, Y., Drissi, R., Boudouresque, F., Conte-Devolx, B., Oliver, C., 1984. Adrenal medullary opiate receptors: Pharmacological characterization in bovine adrenal medulla and a human pheochromocytoma. Mol. Pharmacol. 25, 38–45.

Castanas, E., Bourhim, N., Giraud, P., Boudouresque, F., Cantau, P., Oliver, C., 1985a. Interaction of opiates with opioid binding sites in the bovine adrenal medulla: I Interaction with delta and mu sites. J. Neurochem. 45, 677–687.

Castanas, E., Bourhim, N., Giraud, P., Boudouresque, F., Cantau, P., Oliver, C., 1985b. Interaction of opiates with opioid binding sites in the bovine adrenal medulla: II Interaction with kappa sites. J. Neurochem. 45, 688–699.

Crowe, R., Chapple, C.R., Burnstock, G., 1991. The human prostategland: A histochemical and immunohistochemical study of neuropeptodes, serotonin, dopamine beta-hydroxylase and acetylcholinesterase in autonomic nerves ganglia. Br. J. Urol. 68, 53–61.

Dupont, J., White, P.J., Feldman, E.B., 1991. Saturated and hydrogenated fats in food in relation to health. J. Am. Coll. Nutr. 10, 577–592.

Giovannucci, E., 1995. Epidemiologic characteristics of prostate cancer. Cancer 75, 1766–1777.

Gkonos, P.J., Krongrad, A., Roos, B.A., 1995. Neuroendocrine peptides in the prostate. Urol. Res. 23, 81–87.

Gu, J., Polak, J.M., Probert, L., Islam, K.N., Marangos, P.J., Mina, S., Adrian, T.E., McGregor, G.P., O'Shaughnessy, D.J., Bloom, S.R., 1983. Peptidergic innervation of the human male genital tract. J. Urol. 130, 386–391.

Hanks, G.E., Myers, C.E., Scardino, P.T., 1993. Cancer of the prostate. In: DeVita, V.T., Hellman, S., Rosenberg, S.A. (Eds.), Cancer: Principle and Practice of Oncology. J.B. Lippincott, Philadelphia, PA.

Hatzoglou, A., Romain, S., Dalapascha, H., Castanas, E., 1994. Acidification reveals a greater number of epidermal growth factor receptors in human membrane preparations. Clin. Chim. Acta 227, 97–109.

Hatzoglou, A., Gravanis, A., Zoumakis, E., Margioris, A., Castanas, E., 1995a. Identification and characterization of opioid binding sites present in the Ishikawa human endometrial adenocarcinoma cell line. J. Clin. Endocrinol. Metab. 80, 418–423.

Hatzoglou, A., Ouafik, L.H., Bakogeorgou, E., Thermos, K., Castanas, E., 1995b. Morphine cross-reacts with somatostatin receptor SSTR2 in the T47D human breast cancer cell line, and decreases cell proliferation. Cancer Res. 65, 5632–5636.

Hatzoglou, A., Bakogeorgou, E., Castanas, E., 1996a. The antiproliferative effect of opioid agonists on the T47D human breast cancer cell line, is partially mediated through opioid receptors. Eur. J. Pharmacol. 296, 199–207.

Hatzoglou, A., Bakogeorgou, E., Hatzoglou, C., Martin, P.-M., Castanas, E., 1996b. Antiproliferative and Receptor binding properties of α- and β-casomorphin peptides, in the T47D human breast cancer cell line. Eur. J. Pharmacol. 310, 217–223.

Hatzoglou, A., Margioris, A., Bakogeorgou, E., Gravanis, A., Castanas, E., 1996c. Identification, characterization and localisation of corticotropin-releasing hormone receptors in human placenta. Life Sci. 59, 1871–1879.

Heiber, M., Docherty, J.M., Shah, G., Nguyen, T., Cheng, R., Heng, H.H., Marchese, A., Tsui, L.C., Shi, X., George, S.R., 1995. Isolation of three novel human genes encoding G protein-coupled receptors. DNA Cell Biol. 14, 25–35.

Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Ming Chu, T., Mirand, E.A., Murphy, G.P., 1983. LNCaP model of human prostatic carcinoma. Cancer Res. 43, 1809–1818.

Jen, P.Y., Dixon, J.S., 1995. Development of peptide-containing nerves in the human fetal prostate gland. J. Anat. 187, 169–179.

Kaighn, M.E., Naayan, K.S., Ohnuki, Y., Lechner, J.F., Jones, L.W., 1979. Establishment and characterisation of a human prostatic carcinoma cell line (PC3). Invest. Urol. 17, 16–23.

Kampa, M., Loukas, S., Hatzoglou, A., Martin, P., Martin, P.-M., astanas, E., 1996. Identification of a novel opioid peptide (Tyr–Val–Pro–Phe–Pro) derived from human alphaS1 casein (αS1-casomorphin, and αS1-casomorphin-amide). Biochem. J. 319, 903–908.

Kikuchi, Y., Kita, T., Miyauchi, M., Kizawa, I., Oomori, K., Kato, K., 1987. Effects of naloxone on human ovarian cancer cell growth in vitro and in vivo. Jpn. J. Cancer Res. 78, 519–525.

Kita, T., Kikushi, Y., Ooomori, K., Nagata, I., 1992. Effects of opioid peptides on the tumoricidal activity of spleen cells from nude mice with or without tumors. Cancer Detect. Prev. 16, 211–214.

Krieger, D.T., Margioris, A.N., Liotta, A.S., Shaha, C., Gerandai, I., Pintar, J., Bardin, C.W., 1984. Pro-opiomelanocortin-derived peptides in the rodent male reproductive tract. In: Delitala, G., Motta, M., Serio, M. (Eds.), Opioid Modulation of Endocrine Function. Raven Press, New York, NY, pp. 223–235.

Le Marchand, L., Kolonel, L.N., Wilkens, L.R., Myers, B.C., Hirohata, T., 1994. Animal fat consumption and prostate cancer: A prospective study in Hawaii. Epidemiology 5, 276–282.

Lottspeich, F., Henchen, A., Brantl, V., Teschemacher, A., 1980. Novel opioid peptides derived from casein (β-casomorphins): III. Synthetic peptides corresponding to components of bovine casein peptone. Hoppe-Seyler's Z. Physiol. Chem. 361, 1835–1839.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R.A., Klee, W.A., 1983. Opioid activities and structures of α-casein-derived exorphins. Biochemistry 22, 4567–4573.

Mahe, S., Tome, D., Dumontier, A.M., Desjeux, J.F., 1989. Absorption of intact beta-casomorphins (beta-CM) in rabbit ileum in vitro. Reprod. Nutr. Dev. 29, 725–733.

Maneckjee, R., Biswas, R., Vonderhaar, B.K., 1990. Binding of opioids to human MCF-7 breast cancer cells, and their effects on growth. Cancer Res. 50, 2234–2238.

Meisel, H., 1986. Chemical characterisation and opioid activity of an exorphin isolated from in vivo digests of casein. FEBS Lett. 196, 223–227.

Morphy, J.B., Emmott, R.C., Hicks, L.L., Walsh, P.C., 1980. Estrogen receptors in the human prostate, seminal vesicle, epididymis, testis, and genital skin: A marker for estrogen responsive tissues. J. Clin. Endocrinol. Metab. 50, 938–948.

Mosmann, T., 1973. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 53–63.

Munson, P.J., Rodbard, D., 1980. LIGAND: A versatile computerized approach to characterization of ligand-binding systems. Anal. Biochem. 107, 220–239.

Noordzij, M.A., van Steenbrugge, G.J., van der Kwast, T.h., Schroder, F.H., 1995. Neuroendocrine cells in the normal, hyperplastic and neoplastic prostate. Urol. Res. 22, 333–341.

Nyberg, F., Lieberman, H., Lindstrom, L.H., Lirenas, S., Koch, G., Terenius, L., 1989. Immunoreactive beta casomorphin-8 in cerebrospinal fluid from pregnant and lactating women: Correlation with plasma levels. J. Clin. Endocrinol. Metab. 68, 283–289.

Papsidero, L.D., Kuriyama, M., Wang, M.C., Horoszwicz, J.S., Leong, S.S., Valenzuela, L., Murphy, G.P., Chu, T.M., 1981. Prostate antigen: A marker for human prostate epithelial cells. J. Natl. Cancer Inst. 66, 37–42.

Reubi, J.C., 1985. Central nervous system-mediated growth inhibition of a rat prostate carcinoma by an opioid. J. Endocrinol. 107, 247–250.

Scholar, E.M., Violi, L., Hexum, T.D., 1987. The antimetastatic effect of enkephalin-like peptides. Cancer Lett. 35, 133–138.

Stone, K.R., Mickey, R.R., Wuderly, H., Mickey, J.H., Paulson, D.F., 1978. Isolation of a human prostate carcinoma cell line (DU 145). Int. J. Cancer 21, 274–281.

Tainio, H., 1995. Peptidergic innervation of the human prostate, seminal vesicle and vas deferens. Acta Histochem. 97, 113–119.

Tatoud, R., Degeorges, A., Prevost, G., Hoepffner, J.L., Gauville, C., Millot, G., Thomas, F., Calvo, F., 1995. Somatostatin receptors in prostate tissues and derived cell cultures, and the in vitro growth inhibitory effect of BIM-23014 analog. Mol. Cell. Endocrinol. 113, 195–204.

Tome, D., Dumontier, A.M., Hautefeuille, M., Desjeux, J.F., 1987. Opiate activity and transepithelial passage of intact beta-casomorphins in rabbit ileum. Am. J. Physiol. 253, G737–744.

Vilner, B.J., John, C.S., Bowen, W.D., 1995. Sigma-1 and sogma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. Cancer Res. 55, 408–413.

Zagon, I.S., McLaughlin, P.J., 1981. Naloxone prolongs the survival time of mice treated with neuroblastoma. Life Sci. 28, 1095–1102.

Zagon, I.S., McLaughlin, P.J., 1983. Naltrexone modulates tumor response in mice with neuroblastoma. Science 221, 671–673.

Zagon, I.S., McLaughlin, P.J., Goodman, S.R., Rhodes, R.E., 1987. Opioid receptors and endogenous opioids in diverse human and animal cancers. J. Natl. Cancer Inst. 79, 1059–1065.

Zagon, I.S., Goodman, S.R., McLaughlin, P.J., 1989. Characterization of zeta (ζ): A new opioid receptor involved in growth. Brain Res. 482, 297–305.