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Effects of the luteinising hormone-releasing hormone (LH-RH) agonist leuprolide on adenylyl cyclase regulation through G-protein coupled receptors in rat ventral prostate

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Abstract

Luteinising hormone-releasing hormone (LH-RH) agonists are widely used for the therapy of advanced prostate cancer through the suppression of testosterone secretion. Furthermore, recent studies indicate the existence of prostate LH-RH receptors coupled to signalling pathways resulting in direct antiproliferative effects. In order to shed light on the mechanisms through which these compounds inhibit prostate cell growth, we investigated the effects of leuprolide (a LH-RH agonist) treatment of rats compared with the effects of surgical castration on the behaviour of G-protein coupled receptors acting through adenylyl cyclase in the ventral prostate. Important decreases of both plasma testosterone levels and ventral prostate weight were observed 5 weeks after subcutaneous (s.c.) injection of a leuprolide-depot preparation (1.5 mg/kg body weight (b.w.)) or 5 days after bilateral gonadectomy. However, leuprolide treatment increased the number of vasoactive intestinal peptide (VIP) receptors and the ability of this neuropeptide to stimulate adenylyl cyclase activity in prostate membranes, whereas surgical castration decreased both parameters. Moreover, leuprolide resulted in significant increases of prostate α_s and α_{i1-3} (but not α_{i1} and β) G-protein levels, while the four G-protein subunits were overexpressed after gonadectomy. The estimation of α_s and α_i activity by experiments with Gpp[NH]p and forskolin indicated a potentiation of the two arms of adenylyl cyclase regulation in leuprolide-treated rats. Present observations suggest that leuprolide treatment leads to an antimitogenic response by acting mainly through the activation of Gi proteins negatively coupled to adenylyl cyclase.

Keywords: LH-RH agonist; Prostate cancer; GPCRs; Adenylyl cyclase; VIP

1. Introduction

Prostate cancer has become the most commonly diagnosed malignancy in men and the second commonest cause of cancer death after lung neoplasms [1,2]. Many prostate cancers are unfortunately diagnosed at an advanced, disseminated stage with no possibility of cure by radical prostatectomy. Androgen ablation continues to be the standard approach for treatment of disseminated carcinoma of the prostate in the earliest, androgen-responsive stages of the tumour [3,4]. However, the disease overcomes androgen blockade in later stages and develops an androgen-unresponsive phenotype, with resistance to therapy, but presents a prominent involvement of locally synthesised growth factors in cell proliferation [5,6].

Androgen ablation blocks the androgen-receptor hormonal signalling cascade, resulting in down-regulation of androgen-responsive genes and leading to apoptosis [7,8]. The hormonal manipulation may involve the inhibition of androgen production (by surgical castration or the administration of luteinising hormone-releasing hormone, LH-RH agonists) or activity (by administration of antiandrogens). This therapy generally results in involution of prostate tumours, but the molecular mechanisms are incompletely understood.

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LH-RH agonists are widely used in the treatment of prostate cancer by taking advantage of their ability to block the activity of the pituitary–testicular axis and, hence, to reduce testosterone secretion [4,7]. During recent years, convincing evidence has been accumulated indicating that LH-RH is present in normal and tumoral extrapituitary tissues where it might exert an autocrine/paracrine role [9]. This appears to be the case in prostate cancer tissue since various studies in human prostate cancer cell lines (either androgen-dependent or androgen-independent) have shown that they express both LH-RH and LH-RH receptors and that LH-RH agonists inhibit cell proliferation [10,11]. Interestingly, at variance with the receptor of the gonadotrophs, prostate cancer LH-RH receptors seem to be coupled to $G\alpha_i$ protein-adenylyl cyclase rather than to $G\alpha_{q/11}$-phospholipase C [11].

The rodent prostate gland is widely utilised as an *in vivo* model system to study the molecular mechanisms involved in androgen regulation of cellular apoptosis, as well as to understand prostate disease states and their potential treatments [12,13]. The present study was undertaken in order to better characterise the molecular mechanisms involved in prostate cell proliferation by considering normal cell growth instead of tumour growth. To address this issue, we tested the effects of *in vivo* treatment with leuprolide (a LH-RH agonist) on the behaviour of G-protein coupled receptors acting through adenylyl cyclase in rat ventral prostate. To this purpose, we considered the vasoactive intestinal peptide (VIP) receptor/effector system that has been widely characterised in both rat [14,15] and human [16,17] prostate. The same signal transduction parameters were evaluated in involuting prostate after surgical castration.

2. Materials and methods

2.1. Materials

The LH-RH agonist leuprolide (des-Gly^{10},[D-Leu^6]-LH-RH ethylamide) was kindly donated by Abbott Laboratories, S.A. (Madrid, Spain) as a depot preparation (Procrin Depot®) of microcapsules of leuprolide acetate in poly(DL-lactide-co-glycolide). VIP was purchased from Neosystem (Strasbourg, France). [^{125}I]-VIP was prepared at a specific activity of approximately 250 Ci/g [14]. Specific antisera for G-protein subunits (α_s, α_{i1}, α_{i1-3} and β) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Forskolin, bovine serum albumin (BSA), phenyl methylsulphonylfluoride (PMSF), 3′-isobutyl-1-methylxanthine (IBMX), guanyl-5′-yl-imisotriphosphate (Gpp[NH]p), and protein markers and other chemicals for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were provided by Sigma (Alcobendas, Spain).

2.2. Animals and preparation of prostate membranes

Male Wistar rats (obtained from the Laboratory Animal Services Centre of the Alcalá University) of approximately 350 g body weight were anaesthetised and subjected to bilateral orchidectomy by the scrotal route. The castrated rats were kept in cages with chow and water *ad libitum* for 5 days before sacrifice. Another group of rats was subcutaneously (s.c.) injected with a single dose of leuprolide (1.5 mg/kg body weight) and maintained as above for 5 weeks. Intact age-matched rats were used as controls. The animals were sacrificed by decapitation, and the ventral prostate was immediately removed. As previously reported [18], the dissected tissue was homogenised in 25 mM triethanolamine–HCl buffer (pH 7.5) containing 0.25 mM sucrose, 0.1 mM PMSF, 0.5 mM ethyldiamine tetra acetic acid (EDTA) and 0.1 mg/ml bacitracin. After centrifugation at 600*g* for 10 min at 4°C, the pellet was discarded and the supernatant was centrifuged again at 25 000*g* for 30 min at 4°C. Prostate membranes were stored at −80°C until use. Protein was measured using BSA as a standard.

2.3. VIP binding

The VIP radioreceptor assay was carried out as described elsewhere [16] by using [^{125}I]-VIP as the specific ligand. Briefly, displacement curves were performed by incubating 0.25-ml cell membrane aliquot with [^{125}I]-VIP (45 pM) either in the absence or presence of increasing concentrations of VIP (10^{-11}–10^{-7} M). Non-specific binding was determined in the presence of 1 μM VIP and represented approximately 1% of total radioactivity added. The incubation was performed at 15°C for 60 min. The LIGAND computerised curve-fitting program [19] was used to determine the types of receptor binding, the maximal binding capacity (B_{max}) of the receptors and the dissociation constant (K_d) values.

2.4. Adenylyl cyclase

As previously described [16], prostate membranes were incubated in 0.1 ml 25 mM triethanolamine–HCl buffer (pH 7.4) containing 1 mM IBMX, 2 mM EDTA, 5 mM $MgSO_4$, 1 mg/ml bacitracin, 1.5 mM adenosine triphosphate (ATP) and an ATP-regenerating system in the absence or presence of increasing concentrations of test substances. After incubation for 10 min at 37°C, the reaction was terminated by boiling for 3 min and refrigeration, followed by the addition of 0.2 ml of an alumina slurry (0.25 g/ml in triethanolamine–HCl buffer, pH 7.4) and centrifugation. The supernatant was taken for cyclic AMP measurement.

Table 1
Effect of leuprolide treatment or surgical castration on rat ventral prostate weight and plasma testosterone levels

Groups	Ventral prostate weight (g)	Plasma testosterone (ng/ml)	*n*
Control	0.69±0.03	3.33±0.53	26
Leuprolide	0.29±0.02	0.21±0.02	44
Castrated	0.28±0.04	0.03±0.01	10

2.5. Western blot analysis of G-protein subunits

The experiments were carried out as described [16]. Briefly, prostate membranes were solubilised in 60 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol, 0.001% bromophenol blue and 3% SDS. Proteins were subjected to SDS-PAGE and then to electrophoretic transfer to nitrocellulose filters. Thereafter, G-protein subunits were immunodetected with specific antisera for α_s, α_{i1}, α_{i1-3} and β subunits.

2.6. Statistical analysis

The results are expressed as the mean±standard error of the mean (SEM) of experiments that were repeated at least three times. When appropriate, the data were analysed according to Dunnett's test after one-way ANOVA. The level of significance was set at $P < 0.05$.

3. Results

Table 1 shows the ventral prostate weight and the plasma testosterone concentration (as measured by a radioimmunoassay technique) in the three groups of rats studied. The weight of the gland decreased to approximately 40% of control 5 days after gonadectomy, and the same occurred 5 weeks after leuprolide administration. Extremely low plasma levels of testosterone were detected in castrated (1% of control) and leuprolide-treated (6% of control) rats.

In order to analyse if the LH-RH agonist leuprolide modifies the levels and/or the extent of coupling of the components of G-protein coupled receptor (GPCR)/effector systems in prostate plasma membranes, we chose the VIP receptor/effector system that acts through adenylyl cyclase stimulation [14–18]. As shown in Fig. 1, increasing concentrations of unlabelled VIP resulted in competitive inhibition of [^{125}I]-VIP binding to rat prostate membranes. Interestingly, castration decreased VIP binding values while leuprolide administration increased them compared with control conditions. These competitive inhibition data were next analysed by Scatchard transformation using the least squares curve-fitting program LIGAND [19]. The results were consistent with a two binding sites model and they suggested that the observed differences are exclusively due to changes in the number of both classes of VIP binding sites (174 and 42% of controls for the high-affinity site, and 192 and 52% of controls for the low-affinity site for leuprolide-treated and castrated rats, respectively), without modifications in the corresponding affinities (Table 2).

Table 2
Effect of leuprolide treatment or surgical castration on stoichiometric parameters of VIP receptors in rat ventral prostate membranes

Group	High affinity		Low affinity	
	K_d	B_{max}	K_d	B_{max}
Control	0.68±0.15	0.19±0.04	25.3±5.1	4.32±0.67
Leuprolide	0.62±0.06	0.33±0.03*	17.8±3.2	8.28±1.62*
Castrated	0.78±0.27	0.08±0.01**	28.4±1.0	2.25±1.00*

Equilibrium dissociation constants (Kd, nM) and maximal binding capacities (Bmax, pmol VIP bound/mg protein) (±SEM). The data were calculated by scatchard analysis of equilibrium binding data (Fig. 1) using the LIGAND program [19]. *$P = < 0.05$ versus control values.

Fig. 2 shows the functional coupling of VIP receptors to adenylyl cyclase in rat prostate membranes. The results were compatible with those obtained in the study of VIP binding since leuprolide treatment increased the ability of VIP to stimulate the enzyme activity. On the contrary, castration led to a decrease of VIP effect at this level, compared with control animals. The observed changes could be explained in terms of different effi-

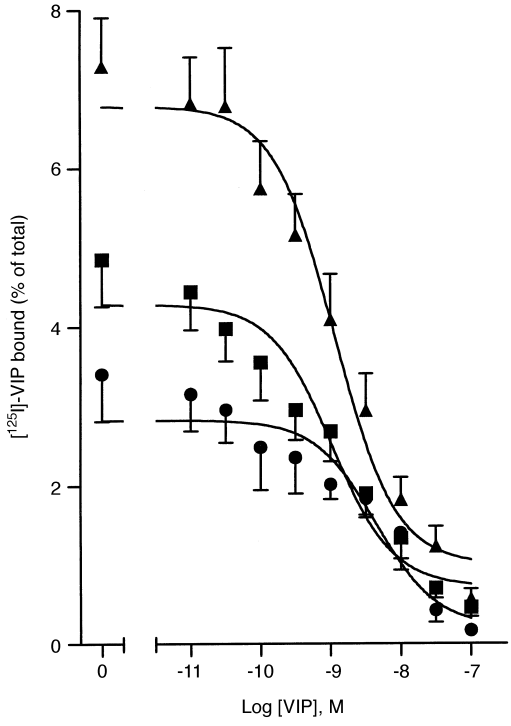

Fig. 1. Competitive inhibition of [^{125}I]-VIP binding by unlabelled VIP in rat prostate membranes. Results are mean±standard error of the mean (SEM) of five determinations, performed in triplicate, and correspond to control (■), leuprolide-treated (▲) and castrated (●) rats.

cacies, since the maximally effective VIP dose elicited responses of 156 and 25% of controls in leuprolide-treated and castrated rats, respectively. No differences could be detected in basal levels (108.9±14.9, 124.5±18.3 and 113.7±24.8 pmol cAMP/min/mg protein in control, leuprolide-treated and castrated rats, respectively).

The function of G_s proteins can be inferred from the observed stimulatory effect of VIP on adenylyl cyclase activity. On the other hand, G_i function was similarly assessed by determining the ability of low Gpp[NH]p concentrations to inhibit forskolin-stimulated enzyme activity [20]. Fig. 3 shows that prostate membranes from control, leuprolide-treated or castrated rats yielded a characteristic biphasic curve for adenylyl cyclase activity at a fixed dose of the diterpene forskolin and increasing concentrations of the nucleotide (10^{-12} and 10^{-4} M). There were not significant differences between the patterns of control and castrated rats. However, leuprolide treatment resulted in a significant decrease of the enzyme activity at low nucleotide doses between 10^{-12}–10^{-9} M (corresponding to G_i activation) whereas a significant increase was reached at high Gpp[NH]p concentrations between 10^{-5} and 10^{-4} M (through G_s activation).

Figs. 4–7 present the data of immunoblots obtained with antisera to different G-protein subunits in membranes from control, leuprolide-treated and castrated rats. Each figure includes a representative immunoblotting and the summarised quantitation of the relative intensity of the bands by densitometric analysis. Fig. 4 indicates that the α_s subunit was expressed in the three groups and that both leuprolide and castration resulted in a significant increase of α_s expression. The expression pattern of α_{i1-3} was similar to that of α_s (Fig. 5). However, the specific study of α_{i1} expression indicated only a significant increase after castration (Fig. 6). This significant increase following castration only was also observed for the β subunit levels (Fig. 7).

4. Discussion

The present study contributes to the convincing evidence that has been accumulated during recent years on how LH-RH affects extrapituitary tissues [9,21]. Furthermore, our results add to the knowledge on the molecular mechanism of action of leuprolide, a LH-RH agonist that is widely used in the treatment of prostate carcinoma on the basis of its ability to suppress testosterone secretion through the downregulation of the pituitary–testicular axis [4,7].

As compared with controls, we found that *in vivo* leuprolide treatment of rats (5 weeks after a single s.c. dose of 1.5 mg/kg body weight) resulted in decreases of both: (a) ventral prostate weight, and (b) plasma testosterone levels, as well as in increases (in prostate

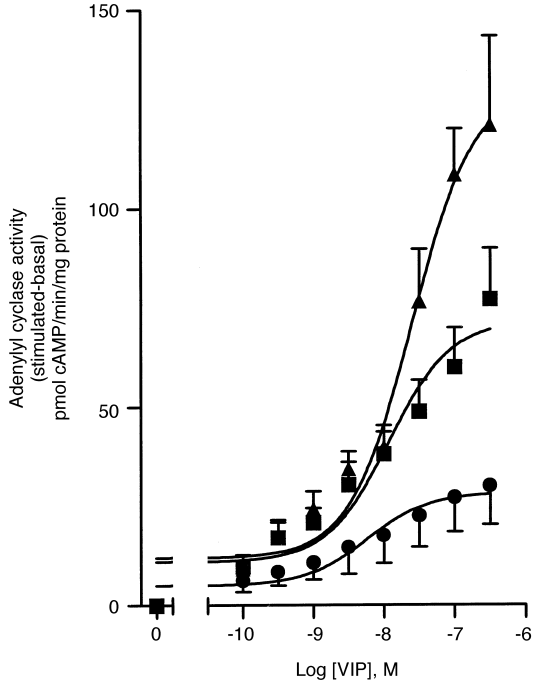

Fig. 2. VIP stimulation of adenylyl cyclase activity in rat prostate membranes. Results are mean±SEM of five determinations, performed in triplicate, and correspond to control (■), leuprolide-treated (▲) and castrated (●) rats.

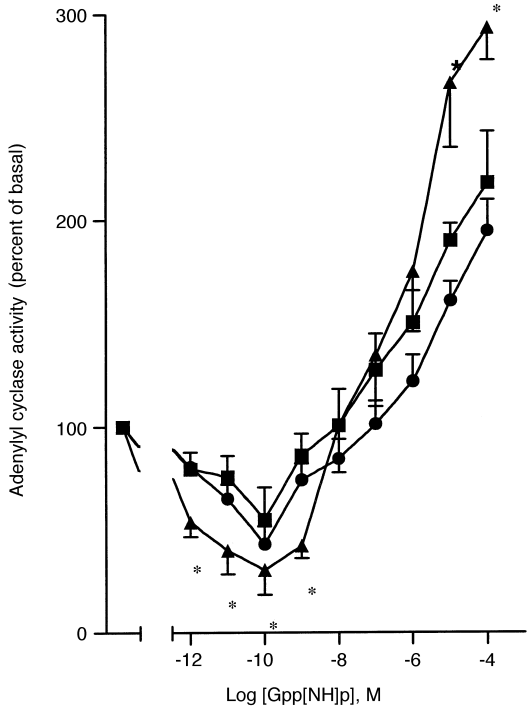

Fig. 3. Effect of low and high doses of Gpp[NH]p on forskolin (1 μM) stimulated adenylyl cyclase activity in prostate membranes. Results are mean±SEM of four determinations, performed in triplicate, and correspond to control (■), leuprolide-treated (▲) and castrated (●) rats. $*P < 0.05$ versus control.

plasma membranes) of: (c) the number of high- and low-affinity VIP receptors, (d) the VIP ability on adenylyl cyclase stimulation through the α_s G-protein subunit, (e) the α_i and α_s activation at a fixed dose of forskolin and low and high Gpp[NH]p concentrations, respectively, and (f) the levels of various G-protein subunits (α_s and α_{i1-3}, but not α_{i1} and β). These results obtained after chemical androgen ablation were rather different from the observations made after surgical castration (5 days after gonadectomy), as shown mainly by decreases of the number of both high- and low-affinity VIP receptors, as well as of the ability of the neuropeptide on adenylyl cyclase stimulation, together with a generalised increase in the expression of all the G-protein subunits tested.

The action of androgens in the physiology, development and growth of the prostate gland is well documented [3–8]. Since the early 1940s, androgen ablation has been the cornerstone of treatment for metastatic prostate cancer [7,8]. Here we used two models of androgen suppression (leuprolide treatment and surgical castration) that resulted, as expected, in residual levels of circulating testosterone, as well as in prostate regression that was conceivably due to inhibition of cell growth and an increasing rate of cell apoptosis [7,8,10,11,22]. In this context, some of the results observed in the present work may be partially related to cell biological changes after castration or leuprolide therapy. In fact, some changes in the epithelial to stromal ratio or differences of epithelial cell subtypes arise during androgen ablation procedures [12,13]. These features can be explained by the remarkable cellular composition of the rat ventral prostate that consists overwhelmingly (up to 85% cell population) of differentiated tall columnar secretory epithelium, the prostate cell type most dependent on androgenic steroids. Thus, androgen withdrawal leads to massive apoptosis of this epithelial cell fraction, whereas the basal cells remain essentially present. On the other hand, it should be noted that we worked with normal rats with essentially quiescent, low-proliferating prostate and not with cancer or other hyperproliferative conditions of the gland.

VIP is a neuropeptide highly expressed in the prostate and thought to be involved in cell function, proliferation and differentiation in normal and pathological conditions when expressed at high levels [14–18,20,23]. The

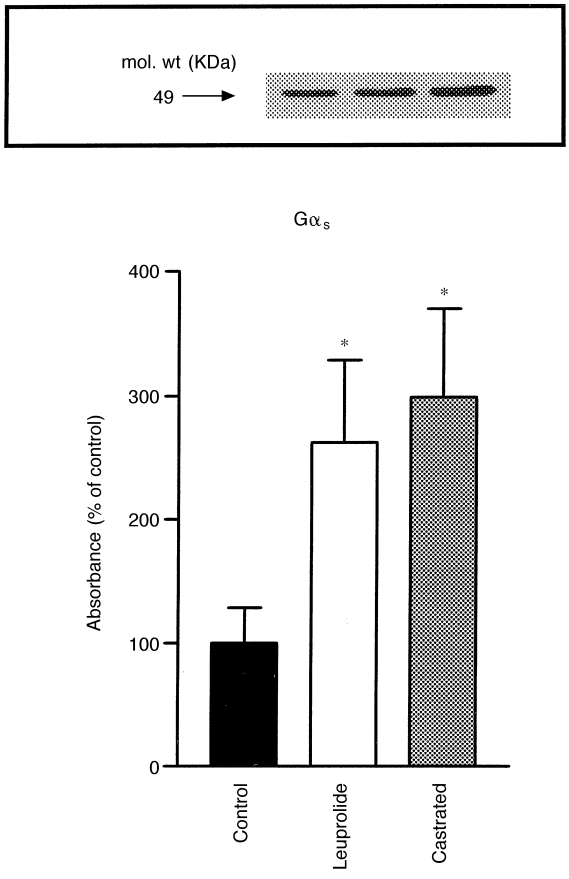

Fig. 4. Immunoblotting of G-protein α_s subunits. Prostate membranes from control, leuprolide-treated and castrated rats were used. Top: autoradiograph for a representative experiment including reference protein size markers. Bottom: videodensitometry quantification, mean ±SEM of four experiments. $^*P < 0.05$ versus control.

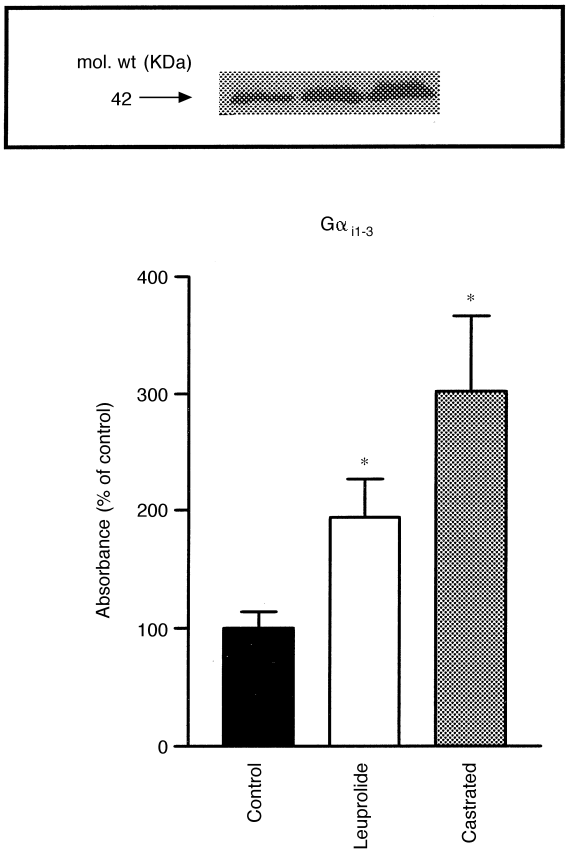

Fig. 5. Immunoblotting of G-protein α_{i1-3} subunits. Prostate membranes from control, leuprolide-treated and castrated rats were used. Top: autoradiograph for a representative experiment including reference protein size markers. Bottom: videodensitometry quantification, mean±SEM of four experiments. $^*P < 0.05$ versus control.

VIP receptor/effector system was studied in this work as a qualified tool to follow the behaviour of GPCRs and adenylyl cyclase activity after treatment with the LH-RH agonist leuprolide. In agreement with previous results, surgical castration resulted in an important impairment of this receptor/effector system [24], that is conceivably a consequence of androgen withdrawal on prostate protein synthesis resulting in the observed low density of both high- and low-affinity VIP receptors. In contrast, *in vivo* administration of leuprolide clearly modulated, in a positive manner, the number of both classes of VIP receptors, as well as the efficacy of the neuropeptide on adenylyl cyclase stimulation (as estimated at the maximally effective concentrations). An explanation for this is not immediately apparent. Several genes have been described that are upregulated in the prostate after castration [22], which is presumably not the case for VIP receptors in the rapid and intensive antiproliferative activity studied (5 days after gonadectomy). However, selective gene upregulation could result in the observed overexpression of VIP receptors in the slow but sustained, inhibition of cell growth that is developed during the 5 weeks following leuprolide administration. Whatever the mechanism, it is known that the cAMP pathway interacts with growth factor signalling through inhibitory links to the extracellular signal-regulated kinase (ERK) cascade [25], which could result in the reported antiproliferative effects of cAMP on some prostate cancer cell lines [26], as well as in the present observations in normal rat prostate. The subject is unclear since cAMP has also been shown to mediate proliferative effects in other prostate cancer cell lines [27,28]. In any case, additional studies on other peptides engaged in adenylyl cyclase regulation are required in order to establish if the present observations are specific for VIP or represent a more generalised feature of the GPCRs/adenylyl cyclase family.

The consideration of the two arms (stimulatory and inhibitory) of the adenylyl cyclase pathway led us to perform an accepted test of both G_s and G_i function; the observation of the potentiating effect of high Gpp[NH]p doses and the inhibitory effect of low concentrations of this nucleotide on forskolin-stimulated adenylyl cyclase [20]. Leuprolide treatment resulted in a significant exacerbation of both stimulatory and inhibitory episodes, which could be related to changes in either Gs, and G_i levels or phosphorylation of G-protein subunits as proposed elsewhere [16,29].

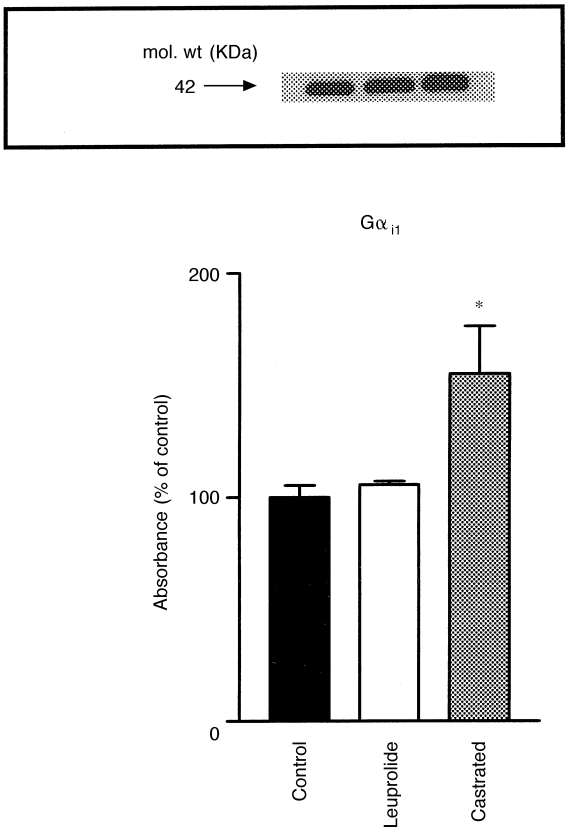

Fig. 6. Immunoblotting of G-protein α_{i1} subunits. Prostate membranes from control, leuprolide-treated and castrated rats were used. Top: autoradiograph for a representative experiment including reference protein size markers. Bottom: videodensitometry quantification, mean±SEM of four experiments. *$P < 0.05$ versus control.

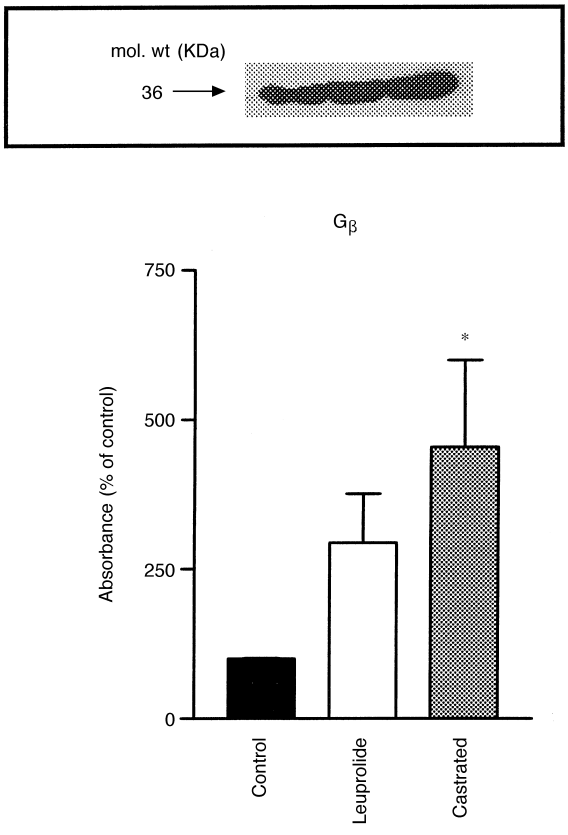

Fig. 7. Immunoblotting of G-protein β subunits. Prostate membranes from control, leuprolide-treated and castrated rats were used. Top: autoradiograph for a representative experiment including reference protein size markers. Bottom: videodensitometry quantification, mean ±SEM of four experiments. *$P < 0.05$ versus control.

The α subunits, as well as the βγ-subunits, of G proteins regulate several critical pathways involved in cell proliferation, differentiation and apoptosis [30]. Immunoblot studies indicated that the pattern of G-protein expression after leuprolide treatment was characterised by high levels of α_s and α_{i1-3} subunits, whereas those of α_{i1} and β were not significantly modified compared with control values. It should be noted that we could not detect α_{i2} expression by means of a specific antibody (data not shown). Increased gene expression, but also high stability of the proteins or their corresponding mRNAs may be responsible for the high levels of α_s and α_{i1-3}, an aspect deserving further investigation [22,31]. The coexistence of increased expression of α_s and α_i units has been also demonstrated in some pathophysiological states such as toxic thyroid adenomas [32]. The high expression of α_{i3} may be responsible for an increased capacity of inhibitory hormones to reduce cAMP production. In any case, cells probably require relatively low levels of G proteins for transducing hormonal signals and it is their quality that determines the generation of defined intracellular effectors [33].

In summary, our results suggest that leuprolide treatment has a dual ability to potentiate both stimulatory and inhibitory processes on adenylyl cyclase activity, which will depend on the nature of the agonists (i.e. VIP in the present study) available at the corresponding prostate membrane GPCRs. If these features are a consequence of androgen deprivation or are related to a direct effect of the LH-RH agonist on prostate cells remains to be established. However, the first possibility is unlikely since our results on surgically castrated animals did not offer superimposable patterns for the parameters studied. Whereas experiments on leuprolide effects on primary cultures of prostate cells are lacking, it is interesting to note that various reports demonstrate the presence of LH-RH receptors and the direct antiproliferative effect of LH-RH agonists on prostate tumours [10,11,34,35]. The action of LH-RH in prostate cancer has been associated with the α_i/cAMP signalling system [11] and to the inhibition of epidermal growth factor (EGF)-induced mitogen-activated protein (MAP)-kinase activity [34]. Our observations in normal rat prostate suggest that leuprolide could behave as an inhibitory factor exerting its antimitogenic action predominantly through the activation of G_i proteins negatively coupled to adenylyl cyclase.

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