Prolactin stimulates cell proliferation through a long form of prolactin receptor and K^+ channel activation

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PRL (prolactin) has been implicated in the proliferation and differentiation of numerous tissues, including the prostate gland. However, the PRL-R (PRL receptor) signal transduction pathway, leading to the stimulation of cell proliferation, remains unclear and has yet to be mapped. The present study was undertaken to develop a clear understanding of the mechanisms involved in this pathway and, in particular, to determine the role of K^+ channels. We used androgen-sensitive prostate cancer (LNCaP) cells whose proliferation is known to be stimulated by PRL. Reverse transcriptase PCR analysis showed that LNCaP cells express a long form of PRL-R, but do not produce its intermediate isoform. Patch-clamp techniques showed that the application of 5 nM PRL increased both the macroscopic K^+ current amplitude and the single K^+-channel open probability. This single-channel activity increase was reduced by the tyrosine kinase inhibitors genistein, herbimycin A and lavandustine A, thereby indicating that tyrosine kinase phosphorylation is required in PRL-induced K^+ channel stimulation. PRL enhances $p59^{fyn}$ phosphorylation by a factor of 2 after a 10 min application in culture. In addition, where an anti-$p59^{fyn}$ antibody is present in the patch pipette, PRL no longer increases K^+ current amplitude. Furthermore, the PRL-stimulated proliferation is inhibited by the K^+ channel inhibitors α-dendrotoxin and tetraethylammonium. Thus, as K^+ channels are known to be involved in LNCaP cell proliferation, we suggest that K^+ channel modulation by PRL, via $p59^{fyn}$ pathway, is the primary ionic event in PRL signal transduction, triggering cell proliferation.

Key words: LNCaP cell, patch-clamp, potassium channel, prostate adenocarcinoma, tyrosine kinase.

INTRODUCTION

Due to the constant increase in life expectancy, benign prostate hyperplasia and prostate cancer have become very common diseases. Prostate cancer is now the primary cause of death induced by cancer among men. The growth, differentiation and apoptosis of prostate cells are mainly regulated by androgens. For this reason, the main treatments of prostate tumours consist of inhibiting the synthesis or the action of androgens. Unfortunately, most of the time, the tumour volume initially decreases, but malignant cells continue to divide.

It is clear that other non-androgenic factors interfere with these phenomena. We and others have previously shown that PRL (prolactin) is one of the non-steroidal factors assumed to be involved both in prostate cell proliferation [1] and in the development of BPH (benign prostate hyperplasia) and prostate cancer [1–5]. The role of PRL in prostate physiopathology becomes more important with age. Indeed, PRL levels increase with age [6], whereas testosterone levels decrease [7]. Furthermore, it has been shown that the prostate itself synthesizes PRL [1], which may act via the PRL receptor located on the apical side of prostatic acinar epithelial cells. This could lead to a paracrine and/or autocrine PRL-induced stimulation of prostatic cell proliferation.

Considerable progress has been made in understanding the mechanisms of intracellular signalling (for a review see [8]). At the molecular level, PRL induces the homodimerization of the PRL-R (PRL receptor), which lacks intrinsic enzymic activity and then goes on to activate associated kinases and other signalling factors [9]. Most of the data concerning the first events in intracellular PRL signal transduction were obtained in the PRL-dependent rat T-lymphoma cell line Nb_2 and in CHO (Chinese hamster ovary) cells stably transfected with the PRL-R cDNA [10,11]. $p59^{fyn}$ [10] and $p120^{jak2}$ [12,13] are two protein tyrosine kinases found in association with PRL-R. PRL-R dimerization also induces the GRB2 (growth factor receptor-bound protein 2)/SOS (son of sevenless guanine nucleotide exchange factor)/Ras/Raf/MEK [MAPK (mitogen-activated protein kinase)/extracellular-signal-regulated kinase kinase]/MAPK signalling cascade which plays an important role in cell cycle progression and tumorization [14,15]. Furthermore, in a series of studies using patch-clamp and microfluorimetric techniques, we have shown previously that the activation of ion channels is also one of the primary events in PRL-R signal transduction [11,16]. However, various mechanisms of numerous physiological functions of PRL remain unclear. While the diversity of PRL functions is, in part, mediated by a variety of signalling cascades, other functions may also be attributed to the PRL-R isoforms observed in different tissues. Three isoforms of PRL-R, which differ in their intracellular domains, have been reported in human cells: the long form, considered for many years as the only one in human cells [17], the intermediate form [18] and the short form [19]. However, the physiological functions of these isoforms have not

Abbreviations used: PRL, prolactin; PRL-R, prolactin receptor; DTX, α-dendrotoxin; TEA, tetraethylammonium; DHT, 5α-dihydrotestosterone; RT, reverse transcriptase; CHO, Chinese hamster ovary; ATP[S], adenosine 5′-[γ-thio]triphosphate.

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been sufficiently studied. As PRL has a mitogenic action on various cell models [20,21] and especially in prostate cancer cells [22], it is important to identify both the form of PRL-R and those signal transduction mechanisms involved in this process.

To study PRL-R signal transduction, we used the androgen-sensitive LNCaP cell line derived from a lymph node of a subject with metastatic carcinoma of the prostate [23]. LNCaP cells are currently used as a model for studies of prostate cancer cell growth [24,25]. We have previously identified and characterized a new type of voltage-activated Ca^{2+}-inhibited K^+ channel in LNCaP cells [26,27], and demonstrated that these K^+ channels are involved in cell proliferation control [26]. However, the nature of the receptor expressed in the LNCaP cells, PRL-R signal transduction and the involvement of the K^+ channel in this transduction have yet to be elucidated.

In the present study we show that only the long form of the human PRL-R is expressed in LNCaP cells. Furthermore, using whole-cell and single-channel configurations of patch-clamp techniques, we demonstrate both that PRL stimulates K^+ channel activity by increasing channel open probability and that the activation of tyrosine kinase is required for this mechanism to operate. In addition, we show that PRL increases the phosphorylation both of its receptor and of $p59^{fyn}$. We also show that LNCaP cell proliferation stimulated by PRL is partially inhibited by the K^+ channel inhibitors DTX (α-dendrotoxin) and TEA (tetraethylammonium).

Since K^+ channels are involved in the control of cell proliferation in LNCaP cells, we suggest that the K^+ channel modulation by PRL via the tyrosine kinase pathway is one of the primary ionic events in PRL signal transduction, which induces cell proliferation.

MATERIALS AND METHODS

Cell culture

LNCaP from the American Type Culture Collection were grown in RPMI 1640 (Biowhittaker, Fontenay sous Bois, France) supplemented with 5 mM L-glutamine (Sigma, L'Isle d'Abeau, France), and 10 % fetal bovine serum (Seromed; Poly-Labo, Strasbourg, France). Cells were used for a maximum of 12 passages. Cells were plated at a density of 20 000 cells/cm^2. The culture medium also contained 50 000 i.u./l penicillin and 50 mg/l streptomycin. Cells were routinely grown in 50 ml flasks (Nunc; Poly-labo) and kept at 37 °C in a humidified incubator in an air/CO_2 (95:5) atmosphere.We used RPMI 1640 without Phenol Red and charcoal-treated fetal calf serum in androgen-free conditions. The cell culture medium was renewed three times a week.

Electrophysiology

The whole-cell and single-channel modes of the patch-clamp technique were employed. The technique used has been described in detail in previous publications [16,27].

Cells were first allowed to settle in Petri dishes and were then placed at the opening of a 250 μm-inner diameter capillary for extracellular perfusions. The cells under investigation were continuously perfused with control or test solutions.

Recording solutions

The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 5 mM glucose and 10 mM Hepes. The osmolarity of the external salt solution was adjusted to 300–310 mosmol $\cdot l^{-1}$ with sucrose, and the pH adjusted to 7.3 ± 0.1 with NaOH. The recording pipette in whole-cell and outside-out experiments was filled with an artificial intracellular saline containing 140 mM potassium glutamate, 1 mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM EGTA, 0.1 mM MgATP and 5 mM Hepes (pH 7.2 ± 0.01 with KOH), osmolarity 290 mosmol $\cdot l^{-1}$. For some experiments, we added antibodies to the recording pipette solution: a monoclonal IgG_1 antibody against $p59^{fyn}$ (0.1 %, anti-Fyn sc-434; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or a negative control monoclonal IgG_1 antibody (0.1 %, X 0931; Dakocytomation, Trappes, France). Free Ca^{2+} concentration of this solution was 0.1 μM (calculated using Maxc Software by Chris Patton, Hopkins Marine Station, Stanford University, Pacific Grove, CA, U.S.A.).

RT (reverse transcriptase) PCR analysis

Total RNA from the LNCaP human prostate tumour cell line, human placental tissue and human breast cancer cells (MCF-7) was isolated by the guanidinium thiocyanate/phenol/chloroform extraction procedure [28]. After DNase I treatment (1 unit/10 μl, 1 h at 25 °C; Life Technologies) to eliminate genomic DNA, 2.5 μg of total RNA was reverse transcribed into cDNA at 42 °C, using random hexamer primers (Perkin Elmer, Branchburg, NJ, U.S.A.) and Moloney murine leukaemia virus RT, in a total volume of 20 μl. To amplify the cDNA of the PRL-Rs by PCR, the primers were designed on the basis of established GenBank sequences with commercially available software (Genejockey II; Biosoft, Cambridge, U.K.). The oligonucleotide primers used to amplify the long PRL-R isoform in this study were designed from the human PRL-R cDNA sequence [17]. The sense primer was 5′-GACTATGAGGACTTGCTGGTGGAG-3′ (primer P1; nucleotides 916–939), encoding an upstream portion of the cytoplasmic domain of the human PRL-R and the antisense primer (5′-CACTTGCTTGATGTTGCAGTGAAG-3′; primer P2) was a 24-mer, encoding a downstream portion (nucleotides 1782–1805) of the human PRL-R cytoplasmic domain. The predicted size of the PCR-amplified product was 890 bp. The PCR primers used to amplify the RT-generated human long and intermediate PRL-R cDNAs were designed from the human PRL-R cDNA sequence [17]. The primers were: 5′-TGAGGACTTGCTGGTGGAGT-3′ (primer P3; nucleotides 921–940) and 5′-CTTGCTTGATGTTGCAGTGAAGTTG-3′ (primer P4; nucleotides 1779–1802). Primers P3 and P4 were used for the amplification of an 882 bp cDNA fragment of the cytoplasmic domain of the long human PRL-R isoform. Consequently, the amplification of a 311 bp cDNA fragment will reveal the expression of the putative intermediate PRL-R.

For the PCR amplifications, 1 μl of RT product was incubated with 0.2 mM of each dNTP, 50 pM of both forward and reverse oligonucleotide primers (Life Technologies), 1 unit of *Taq* Gold DNA polymerase (Perkin Elmer) in PCR buffer (10 mM Tris/HCl, pH 8.3), 50 mM KCl and 2.5 mM $MgCl_2$, in a final volume of 25 or 50 μl. Amplifications were carried out in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer) for 40 cycles. Samples were denatured at 94 °C for 45 s, annealed at 60 °C for 45 s and extended at 72 °C for 45 s. A final extension period at 72 °C for 10 min completed the amplification. To control the amplification of genomic DNA, PCRs were also performed on the non-reverse transcribed RNA (where the reverse transcriptase was omitted in the RT mix) of each sample. After amplification, 6 μl of each PCR product were electrophoresed through a 1.6 % agarose gel stained with ethidium bromide (0.5 μg/ml) and the RT-PCR products were visualized under UV light.

Southern blot analysis

The specificity of the amplified PCR products was confirmed by Southern blot analysis, using a *Hin*dIII fragment (3.4 kb) of rabbit PRL-R cDNA as a probe [29]. The probe was radiolabelled using a random primer DNA labelling kit (Roche Diagnostics, Meylan, France) and [α-^{32}P]dCTP 3000 Ci/mmol (Amersham Biosciences, Saclay, France). After amplification by PCR, 10 μl of each sample were loaded on to a 1.6 % agarose gel stained with 0.5 μg/ml ethidium bromide, then transferred overnight to a Hybond N nylon membrane (Amersham Biosciences) in 10 × SSC buffer (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). Hybridization was performed overnight at 65 °C in 10 × SSC buffer containing 3×10^6 c.p.m./ml of the labelled PRL-R cDNA probe and 10^6 c.p.m. of the *Hae*III probe. After washes, the blot was exposed to Kodak film (X-Omat, Kodak AR film; Amersham Biosciences) at − 70 °C for 4 min.

Calcium measurements

The culture medium was replaced by the external solution (see the section on Recording solutions, above). When a calcium-free medium was required, $CaCl_2$ was omitted and replaced by equimolar $MgCl_2$. Dye loading was achieved by transferring the cells into standard Hanks balanced salt solution, containing 1 μM fura 2 acetoxymethyl ester (Calbiochem, Meudon, France) for 40 min at 37 °C, then rinsing three times with the same dye-free solution. Intracellular calcium was measured with an imaging system (Quanticell 900; Applied Imaging, Sunderland, U.K.). Fura 2 fluorescence was excited at 340 and 380 nm and the emitted fluorescence was measured at 510 nm. The cytosolic [Ca^{2+}] was calculated from the ratio of the fluorescence intensities for each of the excitation wavelengths (F340/F380) and from the Grynkiewicz equation [30].

LNCaP cells were cultured in an androgen-depleted medium to provide control conditions (see the section on Cell culture, above), to which we added either PRL (5 nM) or DHT (5α-dihydrotestosterone; 10 nM) or both of these two hormones.

Immunoprecipitation

After 10 min of PRL (5 nM) incubation, the cell culture medium was discarded and the flasks washed with 10 ml of iced 150 mM NaCl. Cellular proteins were then extracted with 1 ml of RIPA buffer (1 % Triton X-100, 1 % sodium deoxycholate, 0.15 M NaCl and 10 mM phosphate buffer) with a protease-inhibitor cocktail (Sigma) for 30 min at 4 °C. After scraping, any insoluble material was discarded by centrifugation at 10 000 ***g*** for 10 min and the amount of protein was assessed by the BCA method (Pierce Chemical Company, Rockford, IL, U.S.A.). Equal amounts of proteins were incubated for 2 h at 4 °C by rotation in primary antibodies (anti-Fyn sc-434) with added Protein A–Sepharose CL 4B beads in a phosphate buffer (20 mM NaH_2PO_4 and 150 mM NaCl, pH 8). After four washes in the same cold buffer, the beads were boiled in an SDS sample buffer and the immunoprecipitates were subjected to SDS/PAGE (8 % gels). Finally, the proteins were transfered on to a nitrocellulose membrane and subjected to Western blotting. After saturation in non-fat milk and overnight incubation with anti-Fyn or anti-phosphotyrosine PY99 (sc-7020; Santa Cruz Biotechnology) primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG for 1 h. Proteins were revealed using the enhanced chemiluminescence detection system West Pico (Pierce Chemical Company) according to the manufacturer's specifications. The membranes were exposed on Bio-Max film (Eastman Kodak Company, Rochester, NY, U.S.A.).

Proliferation assay

PRL and DTX (both 5 nM) and 4 mM TEA were added to the culture medium, which was renewed daily. After 7 days of treatment, the cells were removed from the Petri dishes using trypsin/EDTA (0.5 g/l and 0.2 g/l respectively; Sigma). To determine the number of viable cells, we used the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, U.S.A.), which uses MTS [3,4-(dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt] as the reagent.

Chemicals

PRL (o-PRL-19) was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, MD, U.S.A.). TEA and thapsigargin were obtained from Sigma. DTX was obtained from Latoxan, Valence, France.

Data analysis and statistics

Single-channel data were analysed as described previously [16,27]. The results are expressed as means ± S.D. where appropriate. The Student's t test was used for statistical comparison among means, and differences with $P < 0.05$ were considered significant.

RESULTS

LNCaP human prostate tumour cell line expresses a long form of PRL-R

We studied the expression of the PRL-Rs at the mRNA level in a total RNA extract from LNCaP cells, by RT-PCR using specific primers. As described in the Materials and methods section, each cDNA was subjected to PCR using oligonucleotide primers (P1 and P2) selected to amplify part of the cytoplasmic domain of the human long PRL-R. A single amplified product with the predicted size (890 bp) was detected in the LNCaP cell line and in the human placental tissue (Figure 1A), used in these studies as a positive control for the expression of the long human PRL-R [2,31]. To clarify the identity of this PRL-R mRNA amplified by RT-PCR, we performed a Southern blot analysis after agarose gel electrophoresis. As shown in Figure 1(B), each PCR product hybridized with the rabbit probe contained the entire nucleotide sequence of the PRL-R cDNA, confirming the identity of the long PRL-R PCR product amplified in LNCaP cells.

In order to study expression of the intermediate PRL-R isoform in LNCaP cells, we designed primers P3 and P4, selected to amplify the intermediate and the long PRL-R isoforms. As it has been shown that breast cancer cells express the intermediate PRL-R isoform [18], we used the human breast cancer cell line (MCF-7 cells) as a posititive control for the expression of this isoform. As shown in Figure 1(C), the primers amplified only a 882 bp PCR product, corresponding to long PRL-R in LNCaP cells. In conclusion, LNCaP cells were not observed to express the intermediate PRL-R isoform. In the same experiments performed on the RT-generated cDNA from MCF-7 cells, we observed two expected PCR products of 882 and 311 bp respectively, corresponding to the long and intermediate PRL-Rs.

Effect of PRL on K^+ current

To investigate the action of PRL on K^+ conductance, LNCaP cells were voltage-clamped at − 60 mV, which is close to the

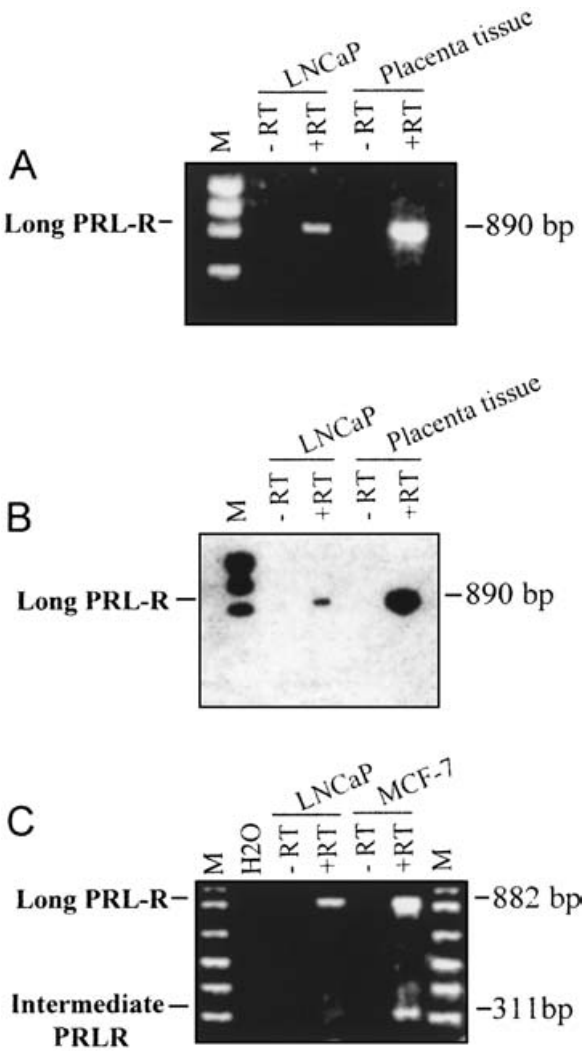

Figure 1 RT-PCR analysis of the PRL-R mRNA expression in LNCaP cells

(**A**) Expression of the long PRL-R isoform in LNCaP cells. The agarose gel electrophoretic pattern of RT-PCR products using primers P1 and P2. The total RNA of each sample was isolated, reverse transcribed and amplified using oligonucleotides, as described in the Materials and methods section. The predicted size of the amplified product was 890 bp. RT-PCR reactions were separated on a 1.6 % agarose gel. The lanes contain PCR products obtained either from RT-generated (+ RT) cDNA from LNCaP cell line and placental tissue, or from RNAs incubated without reverse transcriptase (− RT) and used as negative controls. A standard DNA size marker was also run (lane M). (**B**) Southern blot analysis of the PRL-R PCR product using a rabbit PRL-R cDNA probe. The PCR products of the samples described in (**A**) were analysed by the method of Southern, using the entire rabbit PRL-R encoding nucleotide sequence as a cDNA probe. (**C**) The agarose gel electrophoretic pattern of RT-PCR products using primers P3 and P4. The procedures are as described in (**A**). The PCR was performed by using either RT-generated (+ RT) cDNA from LNCaP cell line and MCF-7 cells or from RNAs incubated without reverse transcriptase (− RT; negative controls). To control for the external contamination of the samples in PCRs, water was used instead of cDNA (H_2O). The predicted sizes of the amplified products were 882 bp for the long isoform and 311 bp for the intermediate isoform. MCF-7 cells expressed both the long and intermediate PRL-R isoforms, whereas LNCaP cells expressed only the long isoform.

mean value of the resting membrane potential [26]. Sustained K^+ outward currents were obtained by stepping up the membrane potential from the holding potential to +60 mV for 200 ms. The extracellular perfusion of PRL (5 nM) induced an increase in the K^+ current amplitude in 32 out of 40 cells (Figure 2A). The amplitude of the steady-state K^+ current increased by 35 ± 11 % of the control ($n = 32$). Figure 2(B) shows the time course of the increase in the K^+ current by PRL (5 nM). The peak effect was reached, in this instance, in 11 min (mean 8.92 ± 1.10, $n = 6$) with an incomplete return to control levels after 10 min of washing (results not shown). The current/voltage curve obtained under control conditions (Figure 2C, ■) and after PRL application (Figure 2C, ●) shows that PRL induced current potentiation, whatever the potential studied. The increase in K^+ current by PRL was completely inhibited by 4 mM TEA ($n = 7$) or 5 nM DTX ($n = 3$; results not shown), two potassium channel blockers that were shown previously to inhibit K^+ channels in LNCaP cells [26,27]. To study the possible participation of tyrosine kinase in PRL-induced K^+ current activation, we used genistein, a broad-spectrum protein tyrosine kinase inhibitor, isolated from vegetables [32,33], at a concentration previously shown to inhibit ion channels regulated by protein tyrosine kinases [11]. Following PRL, a genistein application (50 μM) caused a progressive and complete inhibition of the K^+ current stimulated by PRL (Figure 2D, $n = 7$), thus suggesting the involvement of tyrosine kinase in PRL-induced K^+ current stimulation.

PRL activates K^+ channels by increasing their open probability

Series of outside-out patch-clamp experiments were carried out to investigate the effect of PRL on K^+ channels in LNCaP cells. PRL (5 nM) stimulated K^+ channel activity in 73 % (11 of 15) of patches examined (Figure 3A). The amplitude histograms (Figure 3B) for K^+ channels in controls (mean, 7.12 pA) and in the presence of PRL (mean, 7.09 pA) showed that PRL does not activate additional conductances. PRL (5 nM) caused an increase in the open probability of the studied channels (Figure 3C), showing the half-maximum increase of the open probability to occur within 4.2 ± 1.4 min. The open probability of the channel after the addition of PRL was not constant, but oscillated between lower and higher open probability values (Figure 3C). Moreover, in the presence of TEA (4 mM) or DTX (5 nM), PRL (5 nM) failed to stimulate K^+ channel activity, thereby indicating that PRL activated the DTX-sensitive voltage-dependent K^+ channels as well as any other type of outward channel (Figure 3D). PRL was unable to stimulate K^+ channel activity when ATP was absent from the internal solution. PRL was also ineffective in the presence of 0.1 mM ATP[S] (adenosine 5′-[γ-thio]triphosphate, a non-hydrolysable analogue of ATP; results not shown), demonstrating that kinase phosphorylation is needed for channel stimulation by PRL.

Several tyrosine kinase inhibitors (genistein, herbimycin A and lavendustin A) were used to confirm the involvement of the tyrosine kinase in PRL-induced K^+ channel stimulation. Following PRL, a bath application of genistein (50 μM) caused a progressive and complete inhibition of K^+ channel open probability (Figures 3A, 3C and 3D, $n = 7$ out of 9 patches). The inhibition of K^+ channel open probability by genistein at this concentration was always complete within 14 ± 3 min (Figure 3C). After genistein was washed out, K^+ channel activity stimulated by PRL gradually recovered, indicating that the depression was reversible. The onset of genistein action depended on the concentration and was slower (about 8 min) for low (5–10 μM) than for high genistein concentrations (3 ± 1 min for 50 μM, $n = 6$). Genistin (100 μM), an inactive analogue of genistein that lacks protein tyrosine kinase inhibitory activity [33], had no effect on K^+ channel open probability either in control or in PRL-stimulated LNCaP cells ($n = 3$). Furthermore, the amplitude histogram (Figure 3B) for K^+ channels in the presence of genistein (mean, 7.34 pA) confirms that genistein inhibited the activity of the K^+ channels stimulated by PRL. We also tested two structurally distinct protein tyrosine kinase inhibitors, herbimycin A [34] and lavendustin A

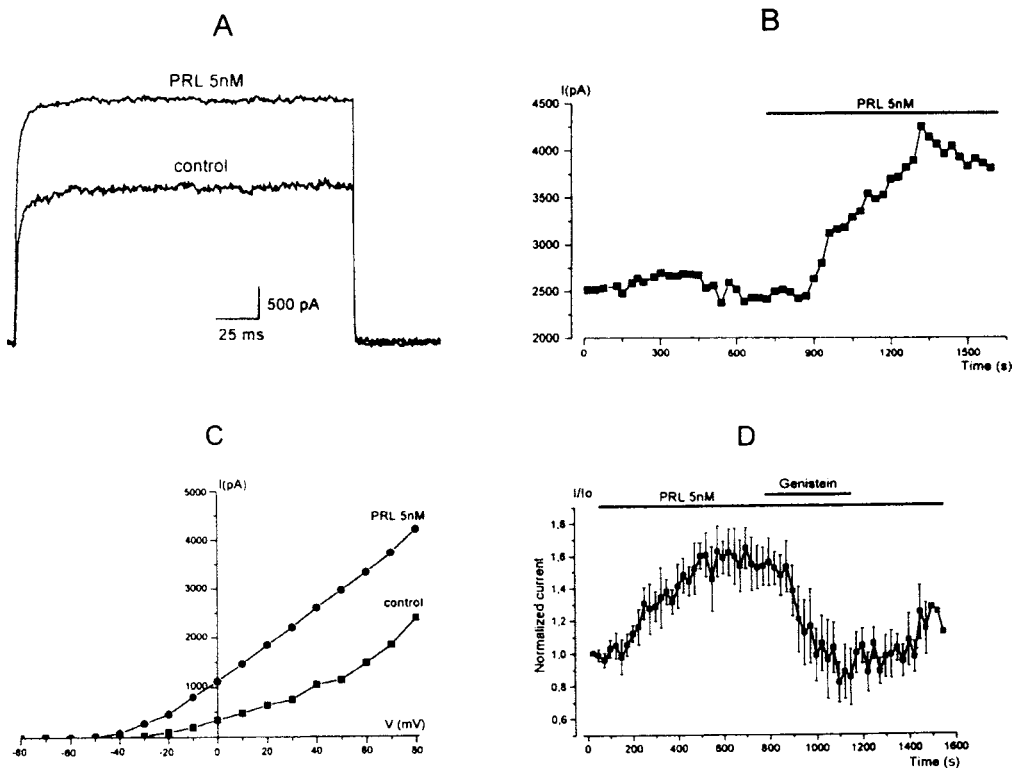

Figure 2 Effects of PRL on whole K^+ current

(**A**) An example of the effect of PRL on K^+ current recorded before and after 11 min of PRL application. The cell was depolarized from a holding potential -60 to $+60$ mV for 200 ms. (**B**) Time course of the effect of PRL (5 nM) on the K^+ current. (**C**) The potentiation effect of PRL (5 nM) on the current/voltage relationships of K^+ current under control conditions (■) and after application of PRL (5 nM; ●). (**D**) The time course of the K^+ current stimulated by PRL with subsequent inhibition by the protein kinase inhibitor genistein (50 μM; $n=7$).

[33], at concentrations shown to inhibit ion channels associated with protein tyrosine kinases in electrophysiological studies [35]. As with genistein, neither herbimycin A (1.5 μM; $n=4$ out of 5 patches) nor lavendustin A (10 μM; $n=3$ out of 3 patches) depressed K^+ channel activity in control conditions. However, they inhibited K^+ channel open probability increase by PRL, without affecting single-channel conductance ($n=7$ out of 8 patches, Figure 3D). Since protein tyrosine kinase inhibitors at high concentrations are known to be able to inhibit not only tyrosine kinase, but also protein kinase A and protein kinase C in some cell types [33], we therefore checked the putative involvement of these kinases in the PRL effect on K^+ channel activity. We tested 1 mM 8-bromo-cAMP, 2 μM forskolin and 10 nM PMA on K^+ channel activity and on the stimulated effect of PRL. All of these drugs were ineffective (results not shown) on PRL actions.

PRL does not modify the cytosolic Ca^{2+} concentration

We also studied whether PRL modifies the cytosolic Ca^{2+} concentration in LNCaP. Intracellular Ca^{2+} measurements were carried out after exposure of fura-2-loaded LNCaP cells to 5 nM PRL and 0.1 μM thapsigargin, a specific sarco-/endoplasmic reticulum Ca^{2+}-ATPase inhibitor. This inhibitor is classically used as a pharmacological tool to study the mechanisms involved in Ca^{2+} homoeostasis of various cell models [36,37] and in particular of LNCaP cells [38,39]. We observed that PRL (0.5–50 nM) did not affect the intracellular Ca^{2+}, either by Ca^{2+} mobilization from intracellular stores or by an induction of extracellular Ca^{2+} entry ($n=37$). We went on to study the long-term effect (18 h) of PRL on the intracellular Ca^{2+} present in these cells. Given that the LNCaP cells are androgen-sensitive and that androgens are able to modulate PRL signal transduction, we checked this long-term effect of PRL in the androgen-depleted medium and in cells treated with 10 nM DHT, 5 nM PRL or these two hormones together. The cytosolic calcium concentration was 105 ± 20 nM ($n=15$) in control conditions, 110 ± 12 nM ($n=18$) in PRL-treated cells, 100 ± 14 nM ($n=13$) in the presence of DHT and 125 ± 17 mM ($n=21$) with the two hormones. It was observed that neither PRL nor DHT, either alone or in combination, had an effect on cytosolic Ca^{2+} concentration in LNCaP cells.

PRL enhances $p59^{fyn}$ phosphorylation which is involved in K^+ channel stimulation

We demonstrated that the increase in single channel activity is reduced by tyrosine kinase inhibitors, such as genistein, herbimycin A and lavendustin A. It is known that PRL-R is associated with $p59^{fyn}$ [10]. Moreover, it has been demonstrated that $p59^{fyn}$ is involved in K^+ channel stimulation [40,41]. We therefore investigated the effects of PRL on the total amount of $p59^{fyn}$ and on its phosphorylation. Using the immunoprecipitation technique, we showed that the total amount of $p59^{fyn}$ is similar to that of the control conditions 10 min after the addition of 5 nM PRL to the cell culture (Figure 4A), whereas $p59^{fyn}$ phosphorylation is increased 2-fold (Figure 4B).

To confirm that $p59^{fyn}$ phosphorylation is involved in PRL signal transduction leading to K^+ channel activation, we used an antibody which abolishes the $p59^{fyn}$ function (Figure 4C). Using

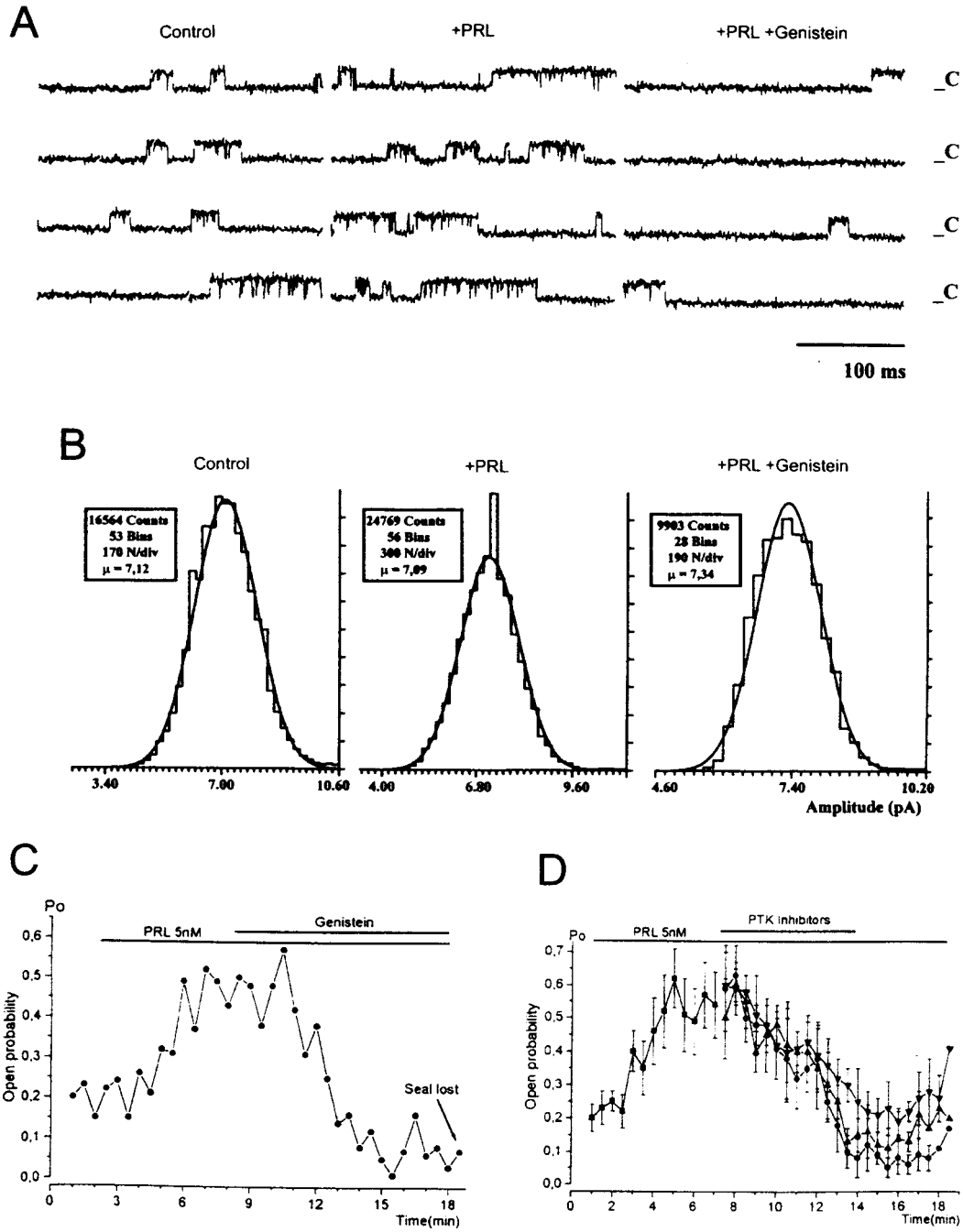

Figure 3 Modulation of K^+ single-channel activity by PRL and its inhibition by genistein

(**A**) Representative recordings of single K^+ currents in a cell-free patch in the outside-out configuration. Recordings are shown for the control solution, 6 min after the application of 5 nM PRL, and 8 min after the subsequent application of 50 μM of genistein, respectively. C indicates the current records when the channel is in the closed configuration. (**B**) Amplitude histograms for single K^+ channel conductance for control, in the presence of 5 nM PRL and in the presence of 50 μM genistein. (**C**) The time course of the open probability of the K^+ channels in the control and in the presence of 5 nM PRL and 50 μM genistein. (**D**) Time course of the effects of protein tyrosine kinase inhibitors on K^+ channel open probability stimulated by PRL (■). The protein tyrosine kinase inhibitors used in these experiments after PRL stimulation were genistein (▲; 50 μM), lavendustin A (▼; 10 μM) and herbimycin A (●; 1.5 μM).

a whole-cell configuration, we waited for 25 min to allow the antibody to diffuse into the cells. Under such conditions, PRL (5 nM) failed to enhance the K^+ current ($n = 7$). A similar increase in K^+ current by 5 nM PRL ($48.7 \pm 14.7\%$; $n = 8$) was obtained with a negative control antibody in the patch pipette to that observed under conditions without antibody in the patch pipette ($35 \pm 11\%$; $n = 32$).

Involvement of K^+ channels in the stimulation of LNCaP cell proliferation by PRL

PRL stimulates both human benign prostatic and human cancer prostatic epithelial cell proliferation [22]. In contrast, in a previous study, we demonstrated that K^+ channel inhibition by K^+ channel blockers, such as TEA, in human prostate cancer LNCaP cells

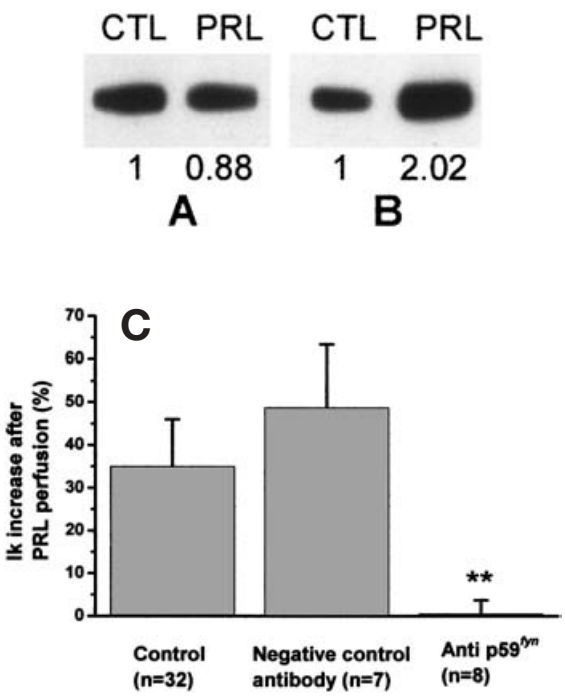

Figure 4 Effects of PRL on p59 *fyn* synthesis and phosphorylation

Immunoprecipitation of p59 *fyn* before (CTL) and after (PRL) 10 min of PRL (5 nM) application. (**A**) Total amount of p59 *fyn*. (**B**) p59 *fyn* phosphorylation. (**C**) Mean (± S.E.M.) increase in K^+ current either after PRL (5 nM) perfusion in control conditions or after 25 min diffusion through the patch pipette of a negative control antibody (0.01 % in the pipette solution) or an antibody against p59 *fyn* (0.01 % in the pipette solution). **Significantly different from the control value ($P < 0.001$).

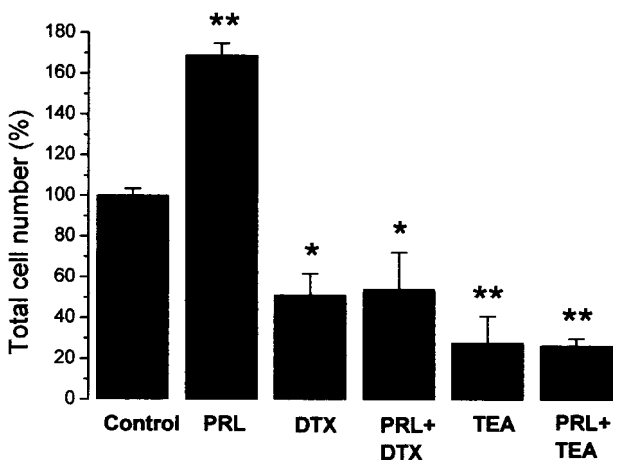

Figure 5 Proliferation assay

Total cell number after various treatments for 7 days in control conditions and after the addition of PRL (5 nM), DTX (5 nM), PRL + DTX, TEA (4 mM) or PRL + TEA to the culture medium. Means ± S.E.M. are shown. Significantly different from the control value: **$P < 0.001$, *$P < 0.05$.

inhibited cell proliferation [26]. These channels are present in LNCaP cells under control culture conditions and are known to be involved in the control of the cell proliferation. We therefore determined whether K^+ channels are involved in the stimulatory effect of PRL on the proliferation of LNCaP cells. Figure 5 shows that 5 nM PRL (during the exposure time used in previous studies) induces the growth-stimulatory effect on LNCaP cells (168.8 ± 5.9 %). In the presence of 5 nM DTX, the proliferation rate was 51.1 ± 10.6 %. In the presence of 4 mM TEA, the cell growth was reduced by 72.2 ± 13.2 %. The mitogenic effect of PRL was not observed when the K^+ channels were inhibited by DTX or by TEA. Alteration in cell-growth kinetics by DTX or TEA was not due to cytotoxicity, as the percentage of cells, excluding Trypan Blue, was not affected by the incubation of cells with these K^+ channel inhibitors. Neither was the increase in the total number of cells induced by PRL due to a decrease in apoptosis, since the level of apoptosis in LNCaP cells (measured using Hoechst staining) was lower than 0.1 % under both control conditions and in PRL-treated cells (results not shown).

DISCUSSION

The present study demonstrates that (i) PRL stimulates androgen-sensitive human cancer prostate cell proliferation through a long form of PRL-R, (ii) PRL stimulates K^+ channels by increasing the open probability via the activation of a tyrosine kinase pathway and (iii) K^+ channels are involved in the growth-stimulatory effect of PRL. It may consequently be seen that the activation of K^+ channels by PRL is one of the primary ionic events in PRL-R signal transduction leading to proliferation.

Two PRL-R isoforms have been identified and characterized in humans: a 85–90 kDa long PRL-R form, cloned from the liver [17], and a 50 kDa intermediate PRL-R form cloned from the human breast cancer line T47D [18]. Recently Ruffion et al. [19] demonstrated that human cancerous prostatic cell lines LNCaP, DU145 and PC3 express both the short and the long forms of PRL-R. Our RT-PCR experiments have shown the expression of the long form of PRL-R in LNCaP cells. We have demonstrated that the intermediate isoform of PRL-R is not expressed in LNCaP cells. PRL transduction only occurs after homodimerization or heterodimerization of the intermediate or long form of PRL-R (for a review see [20]). As the intracytoplasmic part of the receptor which transduces the signal is absent from the short PRL-R isoform, homodimerization or heterodimerization of the short PRL-R isoform with the long or intermediate isoforms is ineffective. In conclusion, PRL-induced K^+ channel activation and cell proliferation was only shown to occur after long PRL-R isoform dimerization. The tissue expression of both long and intermediate forms of PRL-R mRNA varies greatly [18] and this variability of expression could represent a physiological mechanism for tissue regulation, which is a specific activity of the PRL-R complex. Kline et al. [18], using Ba/F3 cells transfected with the long and intermediate isoforms of PRL-R, have suggested that fundamental signalling differences exist between the two human PRL-R isoforms, as cells transfected with the long PRL-R demonstrated robust proliferation whereas the intermediate-PRL-R transfectants exhibited no proliferation at physiological concentrations of PRL. The authors suggest that this could be explained by the differences in proximal PRL-R signalling. The activation of ion channels is one of these primary proximal events in PRL-R signal transduction [11,16,42,43]. Using CHO cells stably transfected with the long form of the PRL-R, we have previously shown that K^+ channels are closely associated with tyrosine kinase and PRL-R [11]. It is therefore probable that ion channels play an important role in the stimulatory effect of PRL on cell proliferation. In various cell models, these ion channels, and particularly K^+ channels, have been suggested as playing an important role both in cell proliferation control [44,45] and several diseases (for a review, see [46,47]).

The role of K^+ channels has been explained either by their possible influence on the intracellular Ca^{2+} concentration [48], the membrane potential [49] or on the modulation of cell volume [44]. Moreover, Pardo et al. [50,51] clearly showed the direct role of *EAG* (ether à go-go) K^+ channels in the control of the cell cycle and/or cell proliferation, thereby providing evidence for the oncogenic potential of K^+ channels. Indeed, according to these authors, an inhibition by antisense of *EAG* expression in several cancer cell lines induced a significant reduction in cell proliferation. Moreover, they also showed that the expression of *EAG* channels favours tumour progression, when *EAG*-transfected cells are injected into immune-depressed mice. The therapeutic exploitation of K^+ channels as new drug targets is consequently becoming more widespread [47].

Our previous studies showed that human prostate cancer cells LNCaP express a new type of outward voltage-activated Ca^{2+}-inhibited K^+ channel [26,27,52]. Pharmacological evidence indicates that these K^+ channels might be involved in cell proliferation [26,49,53]. Furthermore, we have recently demonstrated that a new human gene KCNRG (potassium channel regulator) suppresses K^+ channel activity and therefore lymphocyte and LNCaP cell proliferation [54]. In the present work, we have demonstrated that PRL stimulates these K^+ channels by increasing their open probability. We have also tested the effect of androgens on activation, which failed to increase K^+ channel open activity. We have previously shown that the K^+ channel functioning in LNCaP cells does not appear to be ATP-dependent [27]. However, PRL failed to stimulate K^+ channel activity when ATP was absent from the internal solution, or when we used ATP[S] (a non-hydrolysable ATP analogue). This demonstrates that kinase phosphorylation is needed for channel stimulation by PRL. The effect of PRL on K^+ channels was dependent on tyrosine kinase activation, as it was inhibited by three distinct tyrosine kinase inhibitors. Therefore the stimulation of K^+ channels by PRL possibly occured through the phosphorylation of protein tyrosine residues. As mentioned by Clevenger and Medaglia [10], the protein kinase $p59^{fyn}$ is associated with PRL-R. We have shown that PRL induced a 2-fold enhancement of $p59^{fyn}$ phosphorylation without affecting its total amount. Furthermore, we have demonstrated that an antibody against $p59^{fyn}$ diffused in the cell abolished the PRL-induced K^+ channel activation. Thus $p59^{fyn}$ is a good candidate for K^+ channel phosphorylation induced by PRL in LNCaP cells. Our experiments, together with others using protein kinase C and protein kinase A activators and inhibitors, showed that these kinases are not involved in PRL effects.

Our findings have demonstrated that in LNCaP cells expressing the well-known long form of PRL-R, intracellular Ca^{2+} is not involved in PRL signal transduction. Indeed, PRL did not increase the cytosolic Ca^{2+} concentration in LNCaP cells as it did in CHO cells stably transfected with the long form of cDNA PRL-R [55]. For the epidermal growth factor receptor, which has intrinsic tyrosine kinase [56], the first ionic event is an activation of voltage-independent Ca^{2+} channels defined as direct receptor-operated channels [57]. This in turn causes the activation of Ca^{2+}-dependent K^+ channels, resulting in a delayed membrane hyperpolarization. In LNCaP cells, we did not observe Ca^{2+} conductance activation prior to K^+ channel stimulation. Conversely, our results showed that one of the first ionic events in PRL-R signal transduction is K^+ channel activation, since this activation is observed in excised patches. Our findings demonstrate that the effects observed in excised-patch experiments of PRL and protein kinase inhibitors were not controlled by cellular metabolism, but were direct, and that the protein tyrosine kinase remained closely associated with K^+ channel activity. Based on the observed inhibitory effects of protein tyrosine kinase inhibitors on the activity of PRL-stimulated K^+ channels, we propose that tyrosine kinase activity is involved in the positive regulation of these channels. Ion channels are often so closely associated with protein kinases and phosphatases, that complexes of these proteins remain intact even after reconstitution into artificial bilayers [58]. A variety of channels has been found to be biochemically and functionally associated with protein tyrosine kinases and protein tyrosine phosphatases [59–61]. These accessory proteins can modulate ion channel activity, both by catalysing changes in the phosphorylation state [43,62] and by allosterically regulating channel gating [59]. Tyrosine kinase regulation is obviously an essential link in PRL signal transduction, as the tyrosine kinase inhibitor herbimycin A was able to block a substantial portion of the PRL signal to the milk protein gene promoter, β-lactoglobulin [63]. In malignant Nb2 lymphocytes which express the PRL-R, it was shown that herbimycin A could also abolish tyrosine kinase and PRL-R phosphorylation [64]. Moreover, K^+ channel blockers inhibited PRL-induced proliferation in these cells [43].

In conclusion, the present work demonstrates for the first time that PRL stimulates the activity of the K^+ channels involved in cell proliferation via the protein tyrosine kinase phosphorylation in LNCaP cells. We suggest that tyrosine phosphorylation/dephosphorylation mechanisms can modify K^+ channels rapidly, producing flexible changes in PRL-R signalling leading to cell proliferation, which could provide a clue to understanding the regulation of cell proliferation by PRL. In the coming years, both pharmacotherapy with a tissue-specific K^+ channel modulator and an anti-prolactin approach could be new strategies to treat prostatic diseases such as cancer and hypertrophy.

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