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The Lipidosterolic Extract from *Serenoa repens* Interferes with Prolactin Receptor Signal Transduction

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Abstract
The lipidosterolic extract from the saw palmetto *Serenoa repens* (LSESr) is commonly used for medical treatment of benign prostatic hypertrophia due to its ability to inhibit 5α-reductase which permits the conversion of testosterone to dihydrotestosterone, the active androgen on prostate cell proliferation. However, the complete action mechanism of LSESr is still unknown. Several lines of evidence suggest that, in addition to inhibition of 5α-reductase, it may interfere with the action of prolactin (PRL). We therefore investigated a possible interference of this plant extract with another hormone that controls prostate gland growth, PRL. As the action mechanism of PRL is now fully documented in Chinese hamster ovary cells expressing the PRL receptor, we have conducted our experiments on these cells. In this study, using electrophysiological (whole-cell recording and single-channel recording), microspectrofluorimetric and biochemical techniques, we show that LSESr (1–30 μg/ml) reduced the basal activity of a K^+ channel and of protein kinase C (PKC) in CHO cells. In addition, pretreatment of the cells with 1–10 μg/ml LSESr for 6–36 h abolished the effects of PRL on $[Ca^{2+}]_i$, K^+ conductance and PKC. LSESr may block PRL-induced prostate growth by inhibiting several steps of PRL receptor signal transduction. LSESr may also be useful for diseases implicating PRL.

The growth and function of several tissues and organs are under hormonal control. For example, it has been reported that PRL augments the growth and function of the prostate gland. However, the mechanism by which PRL acts on the prostate gland remains unclear and somewhat controversial. PRL is thought to be capable of stimulating the prostate alone [24, 34] or in synergism with androgens [12, 30, 35]. PRL may act on any of the steps in the androgen action mechanism. Prins [30] observed an increase in nuclear androgen receptor levels in response to PRL, but other action sites cannot be excluded.

Dihydrotestosterone (DHT), the active androgen [8, 9], results from the action of the 5α-reductase on testosterone. 5α-Reductase inhibitors, such as finasteride [3, 41] and extracts (LSESr) obtained from the fruit of the saw palmetto *Serenoa repens* [2], decrease prostate size in adult rats and dogs. Thus, they are widely used for a non-invasive medical treatment of the symptomatic disorders

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occurring in patients with uncomplicated benign prostatic hypertrophia.

We have investigated a possible interference between the action of PRL and LSESr. For this purpose, we used Chinese hamster ovary (CHO) cells stably transfected with cDNA encoding for the long form of the PRL receptor [17, 18]. Such a transfection was shown to result in the formation of functional PRL receptors [17, 18]. Moreover, we have recently shown that, in these cells, PRL increases cytosolic calcium concentration ($[Ca^{2+}]_i$) by two mechanisms, a mobilization from intracellular stores and a stimulation of calcium influx [39]. The latter is due to the activation of a K^+ conductance responsible for a hyperpolarization of the membrane potential that in turn stimulates Ca^{2+} entry through hyperpolarization-driven, second-messenger-operated Ca^{2+} channels [29]. In addition, we proposed that the action of PRL on K^+ channels was mediated by the activation of a protein kinase [29].

Recently, biochemical studies have shown that PRL stimulates a signal transduction from the cell surface to the nucleus through the phosphorylation of proteins by signal responsive protein kinases, and particularly protein kinase C [4] and tyrosine kinases [7, 32]. These kinase phosphorylations may then affect either the DNA-binding activity, the transcriptional activity or the subcellular localization of different transcription factors.

As LSESr was shown to reduce the growth of benign prostatic tumors with both PRL and DHT receptors, we wanted to check if one or more steps of the PRL signal transduction pathway are targets for the action of LSESr. In this study, we show that, independently of its action on 5α-reductase, LSESr antagonizes the effects of PRL on $[Ca^{2+}]_i$, K^+ conductance and PKC.

Materials and Methods

Cell Cultures

In these experiments we used CHO cells transfected with PRL-R-cDNA (CHO E32) as previously described [17]. The cells were grown in Ham's F12 medium (Seromed, Strasbourg, France) containing 10% (v/v) fetal calf serum (Gibco, Grand Island, N.Y., USA) on glass coverslides (diameter: 30 mm for single cell experiments, 10 mm for cell population experiments). The cells were transferred into a serum-free medium [18] 8–24 h before the experiments in order to avoid the occupancy of PRL receptors by lactogenic factors and the effects of steroids contained in the serum of the culture medium. This medium was derived from the GC3 medium described by Gasser et al. [11] and is a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (Seromed) supplemented with nonessential amino acids (Gibco), insulin (Sigma, St Louis, Mo., USA; 80 mU/ml), glutamine (Sigma, 2.5 n*M*) and transferrin (Gibco, 10 mg/ml). The medium was changed every 2 or 3 days. Cells were maintained at 37 °C in a humified atmosphere of 95% air and 5% CO_2.

Colorimetric Assay for Cellular Growth and Survival

Cytotoxicity of LSESr was evaluated using a tetrazolium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) as described by Mosmann [22]. MTT was dissolved at a concentration of 5 mg/ml in PBS, sterilized by filtration and stocked at 4 °C. CHO cells were cultured for 72 h in 96-well microculture plates containing control medium or LSESr-containing medium (100 µl). Then, stock MTT solution (10 µl) was added to all wells and plates were incubated at 37 °C for 4 h. Acid-isopropanol (100 ml of 0.04 *N* HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark-blue crystals. The plates were read on a micro-Elisa reader, using 570 nm as test wavelength and 630 nm as reference wavelength.

Microfluorimetric Assay of Cytosolic Calcium

These experiments were performed using the fluorescent probe Indo 1 as already described [21]. The cells were incubated with 5 µ*M* Indo penta-acetoxymethyl ester (Indo 1/AM) and 0.02% Pluronic F-127 (Molecular Probes) in Hank's solution for 30 min at 22 ± 1 °C, then washed and maintained at room temperature in the same saline solution prior to the fluorescence measurements. Loading at low temperature in the presence of the detergent Pluronic F-127 greatly enhanced the percentage of hydrolyzed dye and prevented Indo 1/AM from being trapped in endocytotic vesicles or accumulating in the nucleus. This procedure resulted in an intracellular Indo 1 concentration of between 20 and 60 µ*M*, estimated from comparison with injection via a patch pipette.

In some experiments $[Ca^{2+}]_i$ determination was performed on a single cell using a dual emission microspectrofluorimeter consisting of a Nikon Diaphot inverted microscope fitted with epifluorescence (× 100 oil-immersion fluorescence lense, numerical aperture 1.3). For excitation of Indo 1, a collimated light beam from a 100 W xenon arc lamp (Nikon, France) was filtered at 355 nm and reflected from a dichroïc mirror (380 nm). The emitted fluorescence signal was passed through a pinhole diaphragm slightly larger than the selected cell and directed onto another dichroic mirror (455 nm). Transmitted light was filtered at 480 nm, reflected light filtered at 405 nm and the intensities recorded by separate photometers (P1, Nikon). Single photon currents were converted to voltage signals which were divided on-line by a monolithic laser-trimmed two-quadrant divider (AD535, Analog Devices). Under these experimental conditions, the ratio R = F405/F480 was recorded on-line as a voltage signal and was expressed as $[Ca^{2+}]_i$ using the formula derived by Grynkiewicz et al. [13]. Ca^{2+} calibrations were obtained by means of simultaneous whole cell clamp and microspectrofluorimetric measurements. The patch pipettes were filled with internal solution containing 10 m*M* EGTA (solution A), 10 m*M* $CaCl_2$ (solution B), or 9.2 m*M* EGTA and 5.4 m*M* $CaCl_2$ (solution C). Solutions A and B allowed an evaluation of minimum and maximum values, R_{min} and R_{max}, respectively, whereas solution C allowed an evaluation of the product of the apparent dissociation constant (K_d) and the ratio of fluorescence of free Indo 1 divided by the fluorescence of Ca^{2+}-bound Indo 1 with 355 nm excitation and 480 nm emission (β). The latter solution had a calculated free Ca^{2+} of 300 n*M*.

In other experiments $[Ca^{2+}]_i$ was measured on cell populations using a Hitachi F2000 spectrofluorometer. The glass coverslide carrying the cells was positioned on a plastic holder in a quartz

cuvette. Drugs and reagents were added directly to the cuvette under continuous stirring. The Indo 1 fluorescence response to the intracellular calcium concentration was calibrated from the ratio of 405/480 nm fluorescence values after subtraction of the background fluorescence of the cells at 405 and 480 nm as described by Grynkiewicz et al. [13]. The dissociation constant for the Indo 1-Ca^{2+} complex was taken as 405 n*M*. The values for R_{max} and R_{min} were calculated from measurements using 25 μ*M* digitonine and 5 m*M* EGTA.

A microfluorimetric assay of cytosolic calcium was performed at room temperature (22 ± 1 °C). The cells were placed in modified Hanks' solution containing 142.6 m*M* NaCl, 5.6 m*M* KCl, 2 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 5 m*M* glucose, 10 m*M* HEPES (N-2-hydroxyethylpiperazine-N'-2-ethano-sulfonic acid), and buffered to pH 7.3 with NaOH.

Electrophysiological Recordings

The whole-cell and outside-out modes of the patch technique were employed. The electrodes were pulled on a L/U-3P (List-Medical, Germany) puller in two stages from borosilicate glass capillaries (Ki_{max} -51, USA) (1.5 mm in diameter) to a tip diameter of 1.5–2.0 μm. Patch electrodes were coated with sylgard and then fire polished. The pipettes had an average resistance of 2–4 MΩ.

The cultures were viewed under phase contrast with a Leitz-Diavert (Leitz, Germany) inverted microscope. Electrodes were positioned with Leitz (Germany) micromanipulators. Grounding was achieved through a silver chloride-coated silver wire inserted into an agar bridge (4% agar in electrode solution).

An Axopatch-1D amplifier (Axon Instruments, USA) was used for tight-seal, whole-cell, outside-out voltage clamping. A PC computer, Tandon AT-80386 (Tandon, USA) fitted with a Labmaster TL-1 interface, using Pclamp 5.5.1 software (Axon Instruments, USA – interface and software) was used for stimulus control as well as data acquisition and processing.

Electrode offset was balanced before forming a giga-seal. Seal resistances were typically in the range of 13–30 GΩ. Leakage and capacity current subtraction protocols were composed of four or five hyperpolarizing pulses, one-fourth or one-fifth pulse, respectively, and were applied from a holding potential before test pulses were applied to elicit active responses. During data analysis, leak data were sealed and subtracted from the raw data. Compensation was made for series resistance. Series resistance was calculated before and after compensation. The series resistance averaged 1.15 MΩ and ranged from 0.4 to 1.7 MΩ. Recordings where series resistance resulted in a 5-mV or greater error in voltage commands were discarded. Currents were low-pass filtered at 2 kHz (whole-cell) or 1–2.5 kHz (single-channel records) with an 8-pole Bessel filter (–3 dB) and digitized at 10 kHz for storage and analysis.

For whole-cell voltage clamp studies the standard extracellular solution was similar to that used for microfluorimetry. In some experiments, tetrodotoxin (TTX, 1–5 μ*M*) was added to the bathing solution to prevent activation of the fast sodium current. The recording pipette was filled with an artificial intracellular saline containing (in m*M*):150 KGlu, 2 $MgCl_2$, 1.1 EGTA (ethylene glycol bis (b-aminoethyl ether-N,N,N',N'-tetraacetic acid), 5 HEPES (pH 7.3 + 0.01 with KOH), osmolarity 290 mosm/kg. We used the same solutions as for whole-cell reccordings to study single K^+ channels from outside-out patches. All experiments were performed at room temperature (20–22 °C).

Peak currents in whole-cell recordings were measured using the automatic peak detection function in the Clampan section of the Pclamp software. Late currents measured isochronally were taken before the end of the pulse to avoid capacitative transients spread out by digital filtering.

Single-channel data analysis was performed after elimination of capacity and leak transients by subtraction of record averages without channel activity from each current record. The opening and closing of the channel was detected using a criterion of a 50% excursion between fully open and fully closed states to determine the occurrence of an opening or closing event such as crossings of the line at a half distance between zero current level and a level corresponding to the average open channel amplitude. In this way, real current records were put into ideal form by settting all intermediate amplitudes to the level of the zero current line or to the level of the average open channel amplitude.

Assay of PKC Activity

PKC was assayed using the method reported by Parant and Vial [27]. Briefly, PKC was determined by measuring the incorporation of ^{32}P into histone H1 type III-S (Sigma) from [γ-^{32}P] ATP (Amersham, France). Cells were scraped and washed before homogenization by soft sonication at 4 °C in a buffer (20 m*M* Tris/HCl, 2 m*M* EDTA, 10 m*M* EGTA, 300 m*M* sucrose, 2 m*M* dithiothreitol DTT, 2 m*M* phenylmethylsulfonylfluoride, PMSF, 25 μg/ml leupeptin, 50 μl/ml E64, pH 7.5). After a 100,000 *g* centrifugation for 1 h, the supernatant cytosolic fractions were isolated. The cell pellets, resuspended in the above buffer containing Triton X-100 (1%) for 30 min and centrifuged, constitute the particulate fraction. After a DEAE-cellulose purification, samples were collected with a 100-m*M* NaCl elution. PKC was measured in a total volume of 100 μl containing 20 m*M* Tris/HCl at pH 7.5, 10 m*M* Mg acetate, 50 μ*M* [γ-^{32}P] ATP, 500 μg/ml histone H1 type IIIS, 1 m*M* $CaCl_2$, 1 m*M* DTT, 80 μg/ml phosphatidylserine (PS) and 8 μg/ml diolein (Sigma). The reaction was stopped after 8 min at room temperature by adding 100 μl of a 40% trichloroacetic solution and 20 μl of 50 m*M* ATP and 5 mg/ml BSA mixture. The precipitates were collected on filter glass fiber disks, and washed and dried automatically using a Multiwash 2000 cell harvester. Enzyme activity was defined by subtracting the amount of ^{32}P incorporated into histone in a reaction mixture containing Ca^{2+} and PS to that obtained in a reaction mixture containing 0.5 m*M* EGTA but no Ca^{2+} and no PS. Data were expressed as pmol of ^{32}P incorporated/min/mg protein. Protein concentration was measured by the Bradford method [1].

Pharmacological Reagents

PRL (o-PRL-19) was kindly provided by the NIDDK (National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, Md., USA). Indo 1 AM was provided by Sigma (St Louis, Mo., USA). The lipidosterolic extract from *S. repens* (LSESr) was kindly provided by Dr. H. Cousse (Pierre Fabre Médicament, Castres, France). Originally dissolved in hexane, LSESr was diluted to the final concentration with ethanol. Then, solvents were evaporated under nitrogen. Finally, LSESr was dispersed in culture medium by shaking. PRL and LSESr were applied by pressure ejection from micropipettes (tip diameter, 2–5 μm) positioned close to the recorded cell (within 10–30 μm).

Statistical Method

Results are expressed as means ± SD where appropriate. Student's t test was used for statistical comparison among means and differences and $p < 0.01$ was considered significant.

Results

Cytotoxicity of LSESr in CHO Cells

LSESr was used in the range of concentrations (1–100 μg/ml), previously determined in clinical and in vitro studies. However, to investigate a putative cytotoxicity of LSESr in our particular cell model, we measured viable cell number by using the colorimetric assay described by Mosmann [22]. As shown in table 1, no cytotoxic effect was observed in the concentration range we used (1–30 μg/ml). A cytotoxic effect was only observed at 1 mg/ml LSESr, as previously described for other cell models. Furthermore, note that cytotoxicity was investigated following 72 h treatment, whereras the effects of LSESr we describe below were obtained with 6- to 12-hour treatments.

LSESr Inhibited Ca^{2+}-Dependent K^+ Conductance

In a previous study, we characterized the ion conductances in CHO cells [33]. A charybdotoxin-sensitive, Ca^{2+}-dependent K^+ channel is present in these cells and is activated by PRL [29, 33]. It was concluded that the PRL receptor is probably associated in a regulatory complex with K^+ channel and a protein kinase, which phosphorylates this channel. We have investigated the effects of LSESr on the K^+ channel using whole-cell and single-channel recording variants of the patch-clamp technique.

To study the physiological action of LSESr on K^+ conductance, the cells were voltage clamped at −60 mV, which is close to the mean value of the resting membrane potential. Sustained K^+ outward currents were obtained by stepping up the membrane potential from the holding potential to a variety of potentials for 160 ms. Contamination of K^+ current recordings with Na^+ was avoided by the use of Na^+-free or TTX (1 μ*M*)-containing external solutions (see 'Materials and Methods'). Application of LSESr (30 μg/ml) induced a decrease in the K^+ current in 16 of 19 cells. K^+ currents were recorded 8 min after the application of LSESr. The amplitude of the steady state K^+ current was decreased by 65 ± 15% (at +30 mV) as compared to the control. Figure 1 presents the I-V relationships of K^+ current before (1, Io) and after (2, I) the application of LSESr (30 μg/ml). This LSESr-induced decrease was slow (time of inhibition to half the control value: 5 ± 1 min) with an incomplete return to control levels after 15 min (fig. 2Aa). The effect of 30 μg/ml LSESr was, at least in part, reversible.

The sensitivity of the K^+ channels to LSESr was also examined by using outside-out patches with internal free Ca^{2+} concentration of 0.2 μ*M*. Figure 3 presents recordings of the K^+ channels obtained from an outside-out patch during a depolarizing step to a membrane potential of +30 mV. LSESr (30 μg/ml), applied to the external face of the membrane patch, caused a decrease in the open probability of the K^+ channels but dit not affect the single-channel amplitude. The blocking of K^+ channels by LSESr displayed very slow kinetics. On average, activity was almost completely inhibited within 10 min, confirming the data obtained in 'whole cell' recordings (see above and fig. 2Aa). This inhibition of the K^+ channels by LSESr was seen in 7 of the 9 patches examined.

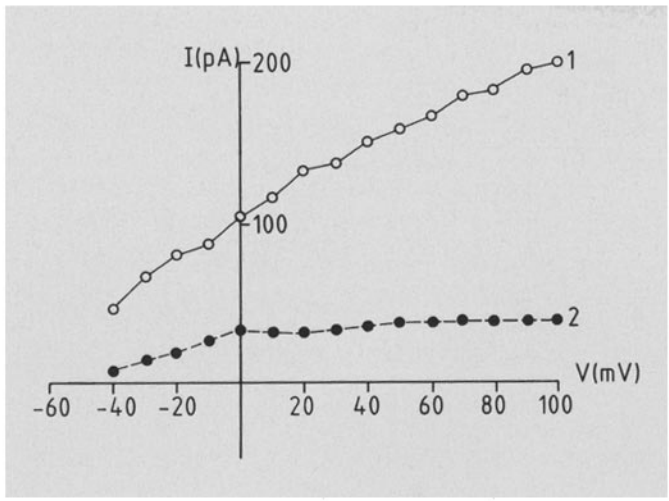
PRL Was Unable to Stimulate the K^+ Conductance in LSESr-Pretreated Cells

A series of experiments with K^+ channels was conducted with the cells pretreated for 4–6 h in LSESr (10 μg/ml) followed by application of PRL to investigate whether PRL was able to activate the K^+ conductance inhibited by LSESr and to check if LSESr acts on the same target as PRL. We have previously shown [29] that PRL (5 n*M*) in whole-cell voltage-clamp studies increased Ca^{2+}-dependent K^+ conductance and that, moreover, in cell-free patch-clamp experiments, it stimulates K^+ channel activity directly. Conversely, cells treated with LSESr revealed a considerable inhibition (over 80%) of the K^+ current amplitude in whole-cell recordings and almost complete

Table 1. Cytotoxicity of LSESr in CHO cells

Control	LSESr					
	1 μg/ml	10 μg/ml	30 μg/ml	60 μg/ml	100 μg/ml	1 mg/ml
100 (n = 8)	96 ± 5 (n = 8)	94 ± 4 (n = 8)	92 ± 5 (n = 12)	92 ± 6 (n = 8)	86 ± 6 (n = 8)	5 ± 4 (n = 8)

Cytotoxicity was evaluated using a colorimetric assay described in the 'Materials and Methods' section. Data are expressed as percent of control. No cytotoxicity was observed for the concentrations used in the other experimental procedures.



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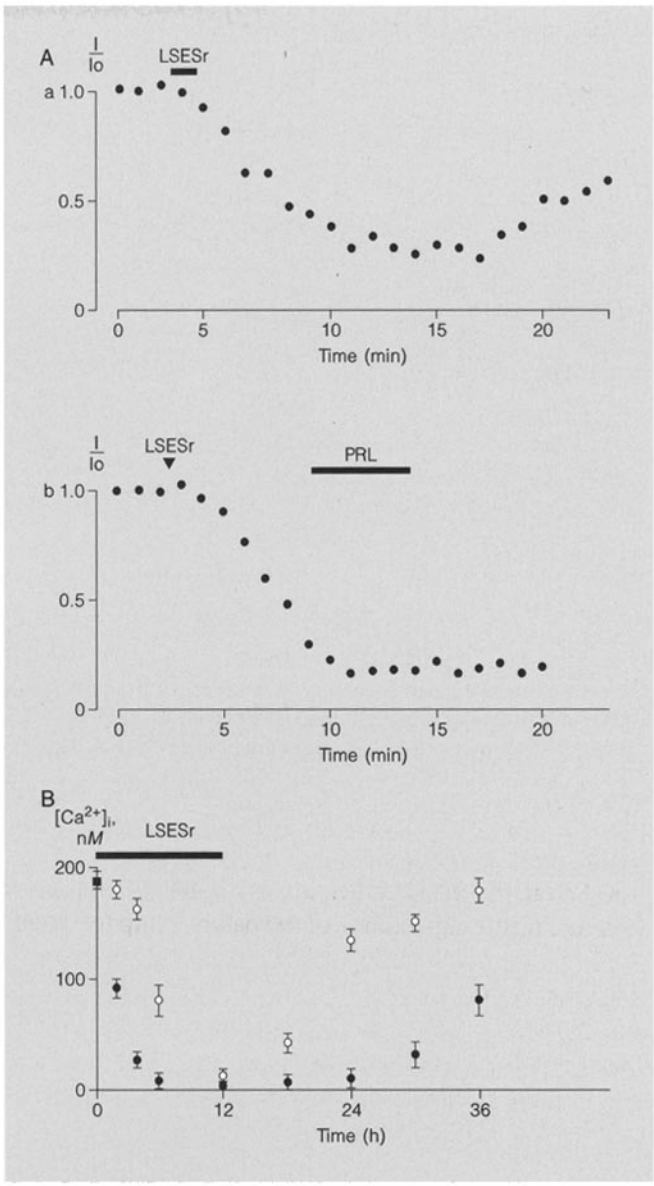

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Fig. 1. Effect of LSESr on K^+ current in CHO cells. I-V relationships for control current (1) and after application of 30 µg/ml LSESr (2). Membrane was depolarized from Vh of −40 to +50 mV for 160 ms.

Fig. 2. Inhibition of PRL responses by LSESr was time-dependent. Horizontal bars indicate the episodes of drug application. The filled triangle (▼) indicates the beginning of the continuous LSESr application. **A** Time course of LSESr action on K^+ current in CHO cells. **a** Plot of normalized current (I/Io) versus time before LSESr application, after 1 min application, and after washout. **b** Plot demonstrating the effect of 30 µg/ml LSESr addition and following 5 n*M* PRL (4 min) application. Currents were evoked by 160 ms test pulses from a holding potential of −40 to +50 mV test potential. **B** The time course of LSESr action on PRL-induced Ca^{2+} increases in CHO cells. $[Ca^{2+}]_i$ was recorded from cell populations loaded with Indo 1. Plot of PRL (5 n*M*) response amplitude versus time. ■ = Control; ○ = 1 µg/ml LSESr; ● = 10 µg/ml LSESr. Shown are the means ± SD (n = 5). Data were obtained in different cell populations.

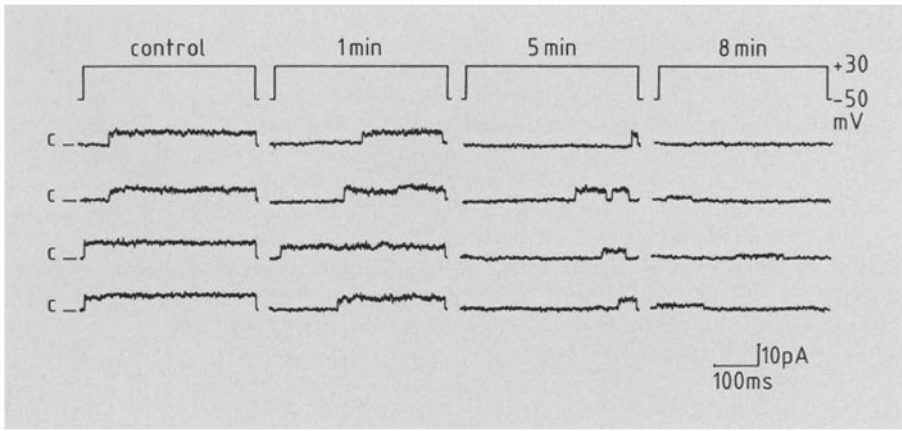

Fig. 3. Single-channel recordings of the effect of LSESr on K^+ channel activity. Recordings from excised (outside-out) membrane patch. K^+ channel activity in this patch was obtained with 0.2 µ*M* internal free Ca^{2+} at time of depolarizing step to a membrane potential of +30 mV. Records are shown for control solution and after 1, 5 and 8 min of 30 µg/ml LSESr application, respectively.

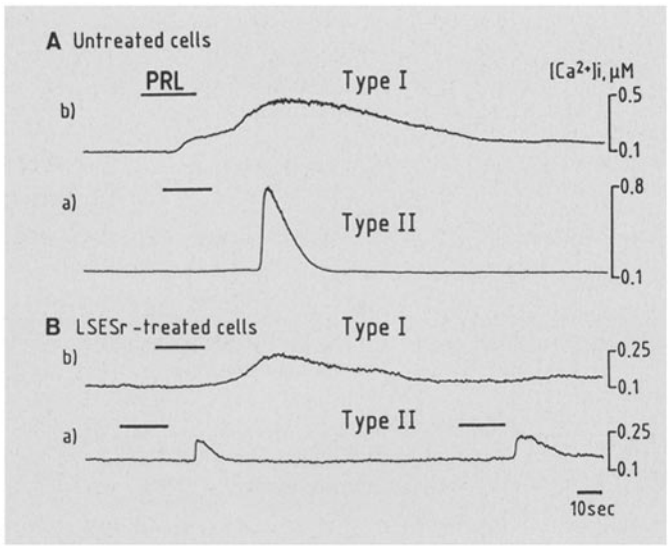

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Fig. 4. LSESr reduced both PRL-induced Ca^{2+} mobilization from intracellular stores and PRL-induced Ca^{2+} influx. $[Ca^{2+}]_i$ was recorded from individual cells loaded with Indo 1. PRL (5 n*M*) was applied continuously during the period covered by bars. **A** As we have previously shown [29, 39], PRL causes rises in $[Ca^{2+}]_i$ with distinct kinetics: (**a**) a fast transient corresponding to a release from intracellular stores, and (**b**) a slower, sustained Ca^{2+} increase due to Ca^{2+} influx. **B** In cells treated with 1 µg/ml LSESr for 6 h, PRL responses, Ca^{2+} release (**a**) and Ca^{2+} influx (**b**) were reduced.

Fig. 5. Inhibition of PRL responses by LSESr was dose-dependent. $[Ca^{2+}]_i$ was recorded from cell populations loaded with Indo 1. The dashed line (**A** and **B**) indicates the basal level of $[Ca^{2+}]_i$. **A** Under these experimental conditions, it was impossible to distinguish Ca^{2+} responses by their kinetics, but we have shown in figure 3 that both PRL responses are affected by LSESr. Applications of 0.5 n*M* PRL were repeated twice, both provoked an increase in $[Ca^{2+}]_i$. **B** A pretreatment of the cells with LSESr (1 µg/ml for 6 h) reduced the amplitude of the first and second responses to PRL. **C** Average values (mean + SEM of quadruplicate determinations) of $[Ca^{2+}]_i$ increases in response to various concentrations of PRL (from 10 p*M* to 50 n*M*) for untreated cells (control, ●), cells treated with LSESr (1 µg/ml) for 6 h (○), and cells treated with LSESr (10 µg/ml) for 6 h (□).

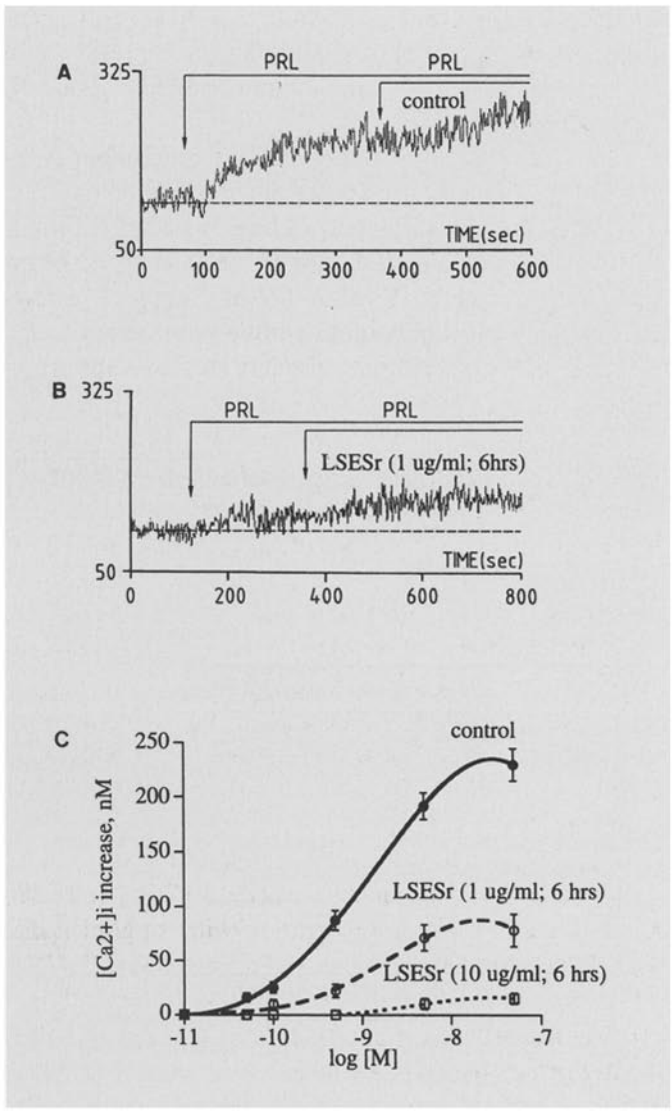

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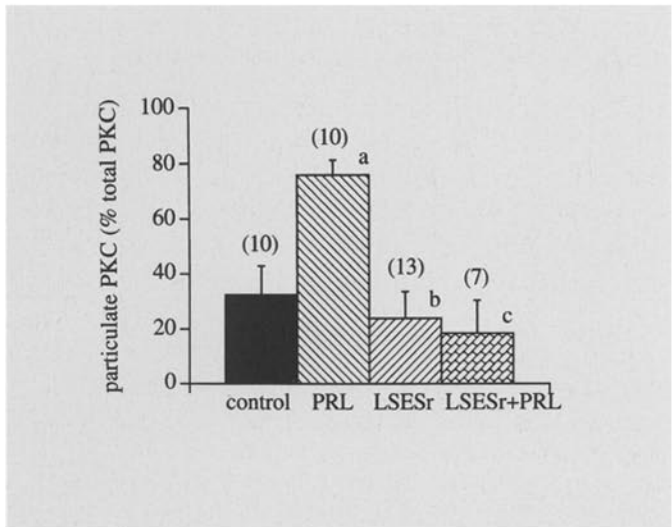
Fig. 6. The effects of LSESr on basal and PRL-stimulated protein kinase C activity. The values shown are the mean + SEM of n determinations (n being the number given in parentheses) using histone as the exogenous substrate, as indicated in the 'Materials and Methods' section. The total basal (2 h) and PRL-stimulated (2 h) PKC activities were 36.6 ± 5.7 p*M*/min/mg protein (n = 15) and 31.1 ± 4.6 p*M*/min/mg protein (n = 14), respectively. PRL (5 n*M*, 2 h) (a: PRL vs. control $p < 0.01$) increased the percentage of particulate PKC, and therefore of PKC activity, significantly since particulate PKC is the active form. Treatment with 1 µg/ml LSESr for 12 h did not affect PKC activity (b: LSESr vs. control $p > 0.05$). However, the same treatment completely blocked PRL-induced PKC activation (c: LSESr + PRL vs. PRL $p < 0.01$).



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inhibition of the single-channel activity in outside-out experiments (fig. 3). No effect of the application of PRL on these cells was observed in either whole-cell (fig. 2Ab) or cell-free studies.

PRL No Longer Had Any Effect on $[Ca^{2+}]_i$ in LSESr-Treated Cells

We have shown that 5 n*M* PRL has complex effects on $[Ca^{2+}]_i$ in CHO cells. One or two sources of calcium ions are mobilized by PRL: extracellular medium and/or intracellular stores [38]. Two types of response to PRL were observed, type I is a slow and prolonged increase in $[Ca^{2+}]_i$ (fig. 4Ab) related to Ca^{2+} influx; type II is a rapid, transient increase in $[Ca^{2+}]_i$ (fig. 4Aa) related to Ca^{2+} mobilization from an intracellular store which remains to be determined. The amplitudes of type I and type II response were 375 ± 63 n*M* (n = 19) and 1,123 ± 86 n*M* (n = 16), respectively. $[Ca^{2+}]_i$ determination in the cell population (fig. 5A) did not show a biphasic response to PRL, probably because of the variability of the response delay observed in individual cell recordings (ranging from 15 to 120 s). In cell population recordings, type I and type II responses are not individualized but superimposed.

When CHO cells were pretreated for 6 h with 10 µg/ml LSESr 5 n*M* PRL was unable to increase $[Ca^{2+}]_i$ (60 cells). A treatment for the same duration with 1 µg/ml LSESr (fig. 4B) reduced the percentage of responding cells (10 of 30 cells vs. 35 of 50 cells in control) and the amplitude of calcium mobilization (type II: 166 ± 32 n*M*, n = 4) and calcium influx (type I: 142 ± 21 n*M*, n = 6). A treatment with the same concentration (1 µg/ml LSESr) but for a longer duration (12–36 h) completely abolished the PRL response in all the cells tested (n = 32).

The dose-dependence of PRL-induced Ca^{2+} increase is shown in figure 5. A concentration of PRL as low as 50 p*M* is effective on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increase was maximal in response to 50 n*M* PRL. The half-maximal values (ED_{50}) were 1 n*M* in control conditions, as compared with 2 n*M* in LSESr-treated cells (1 µg/ml; 6 h) and 4 n*M* in cells incubated with a 10-fold higher concentration of LSESr (10 µg/ml; 6 h). Preincubation with LSESr reduced (1 µg/ml) or abolished (10 µg/ml) the capacity of 0.5 n*M* PRL to increase $[Ca^{2+}]_i$, whatever the concentration used. Figure 5A, B shows the time course of the PRL-induced $[Ca^{2+}]_i$ increase in the absence (control) and the presence of LSESr (1 µg/ml; 6 h), respectively. The inhibition of PRL-induced Ca^{2+} increase by LSESr was reversible. The amplitude and the time-course of the reversibility are dependent on the concentration of LSESr (fig. 2B).

LSESr Inhibits PRL-Stimulated PKC Activity

DG-PKC is a well-known signal transduction pathway which is commonly used by several agonists to stimulate multiple cell lines. In particular, it was shown to be activated by PRL in Nb2 cells, a lymphoma cell line. Recently, we have shown that PRL, at 5 n*M*, the concentration used for microspectrofluorimetric and electrophysiological experiments, increases PKC activity in CHO E32 cells. We have investigated a possible effect of LSESr on the PKC pathway. Our data are expressed as a percentage of membrane PKC activity vs. total PKC activity. Indeed, PKC activation is due to the translocation of PKC from the cytosol to the membrane. Thus, the percentage we have calculated reflects PKC activation. PRL provoked a decrease in PKC activity in the cytosol and an increase in the particulate fraction leading to an increase in the percentage of membrane PKC activity vs. total PKC activity. The activation of PKC by 5 n*M* PRL is maximal (approximately a 3-fold increase, fig. 5) at 2 h. A treatment with 1 µg/ml LSESr for 12 h did not significantly influence basal PKC activity (fig. 6). However, the same treatment completely blocked the effect of PRL (fig. 6) on PKC.

Discussion

Several studies have suggested that many hormones can influence prostatic growth [15, 20]. PRL has emerged as the pituitary hormone most likely to account for prostate gland evolution [35]. The proliferative effects of PRL are closely associated with androgens. Indeed, PRL can act indirectly on the prostate by altering androgen biosynthesis at the testis [14], or directly in synergism with testosterone by an 'increase in androgen nuclear receptors' [10].

The lipido-sterolic extract of the dwarf palm tree, *Serenoa repens*, is commonly used for the treatment of human benign prostatic hypertrophy, because of its ability to inhibit 5α-reductase and the steroid binding to androgen receptors. In this study, we investigated a possible direct effect of LSESr on the signal transduction of PRL in a cell line that does not possess 5α-reductase activity.

The mode of action of PRL is poorly understood. In Nb2 cells, a lymphoma cell line expressing a mutant form of the PRL receptor, it has been shown that the actions of PRL are associated with protein kinase C [4] and tyrosine kinase [32] activation, Ca^{2+} mobilization [4, 23], Na^+/H^+ antiporter activation [36, 37] and phospholipid metabolism [16]. More recently, we have shown that PRL at 1–25 n*M* caused an increase in $[Ca^{2+}]_i$ in CHO cells expressing a functional long form of the PRL receptor [39]. PRL effect on $[Ca^{2+}]_i$

was, at least in part, due to a protein kinase-dependent phosphorylation of a K^+ channel [29]. We have shown that activation of this conductance leads to hyperpolarization of the cell membrane which in turn facilitates calcium influx through voltage-independent Ca^{2+} channels, but according to the driving force for calcium ions.

For this reason, we looked for evidence that LSESr affected basal $[Ca^{2+}]_i$, ion channel activity, PKC activity and PRL signal transduction in CHO-PRL cells. The effect of LSESr on PRL-induced Ca^{2+} increase and K^+ channel activity was dose- and time-dependent. Indeed, treatment with 1 μg/ml LSESr for 6 h reduced both PRL-induced intracellular Ca^{2+} mobilization and Ca^{2+} influx, whereas a longer treatment (24 h) or a higher concentration (10 μg/ml) for 6 h completely blocked PRL effects. Thus, LSESr acts on the mechanisms by which PRL releases calcium ions stored in intracellular compartments and stimulates calcium influx. One of these mechanisms is the activation of a Ca^{2+}-dependent K^+ conductance [29]. By inhibiting K^+ conductance, LSESr blocked Ca^{2+} influx and perhaps intracellular Ca^{2+} mobilization since the latter may be due to Ca^{2+}-induced Ca^{2+} release [39]. PRL-, LSESr-sensitive K^+ conductance could be activated by phosphorylative reactions since intracellular application of ATP caused a strong activation of the conductance. On the other hand, a nonhydrolyzable ATP (adenylylimididiphosphate, AMP-PNP) had no effect [29]. Considering the previous biochemical studies made on Nb2 cells, showing stimulation of PKC and tyrosine kinase activity by PRL [4, 32], and our observations on CHO-PRL cells (activation of PKC; fig. 6) the effect of PRL and LSESr on the K^+ channel may be due to phosphorylation (PRL) or dephosphorylation (LSESr) of the channel on a serine/threonine or on a tyrosine by a PKC or a tyrosine kinase, respectively. The effects of inhibitors and activators of the PKC and tyrosine kinase pathways on basal and PRL-stimulated $[Ca^{2+}]_i$ and ion channel activity are under current investigation in our lab.

We cannot exclude an effect of LSESr at any other step of the signal transduction cascade of the PRL response. A decrease in the number of PRL receptors in LSESr-treated cells is unlikely, because in CHO-transfected cells the expression of the PRL receptor gene is controlled by a promoter which cannot be influenced by external factors. A more likely explanation is a direct effect of LSESr on the K^+ channel. LSESr is composed of several fatty acids, and fatty acids have been shown to modulate K^+ channel activity. We have shown that, in pituitary cells, arachidonic acid and other unsaturated fatty acids activated K^+ conductances, whereas lipoxygenated metabolites of arachidonic acid inhibited them [38]. In aplysia neurones, a lipoxygenated metabolite of AA, 12 HETE, was shown to increase the opening of a K^+ channel [28]. In addition, the fatty acids contained in LSESr would disrupt the lipidic bilayer by inserting among the phospholipids [25]. This action on plasma membrane would be reponsible for an inhibition of AA and prostaglandin E_2 production, probably because phospholipase A_2 was blocked [31].

It has been shown that, in T lymphocytes [19], fibroblasts [6], muscle cells [5], human breast carcinoma cells (MCF-7 [40]) and human melanoma cells [26], K^+ current regulation may be involved in controlling the driving force for a calcium influx, thereby interacting with Ca^{2+}-dependent cell cycle control proteins. Therefore, LSESr may also reduce cell proliferation by inhibiting the K^+ channel controlling the driving force for Ca^{2+} ions. Moreover, it was shown that K^+ channel inhibitors interrupted the growth of cells, preventing the entrance of the cells into the S-phase of the cell division cycle.

We have also shown that LSESr affected PKC activity. Since LSESr inhibited PRL-induced PKC activity, but not basal activity, it may be assumed that LSESr has no direct effect on PKC, but blocks a signal transduction pathway of the PRL response leading to PKC activation. The exact mechanism by which PRL stimulates PKC activity is still unknown. Activation of PKC requires the presence of three elements: diacylglycerol, calcium and phosphatidylserine. LSESr may block PRL-induced activation of PKC by inhibiting a PRL-induced increase in $[Ca^{2+}]_i$.

In summary, our results suggest that LSESr prevents the effects of PRL by acting on several steps of the PRL signal transduction pathway: LSESr inhibits Ca^{2+}-dependent K^+ channels; LSESr reduces both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx stimulated by PRL; LSESr inhibits PRL-stimulated PKC activity.

The role of K^+ channels, intracellular calcium and PKC in tumor growth is well documented. In addition, PRL is known to be involved in several pathologies such as auto-immune diseases, breast cancer, etc. Therefore, a possible effect of LSESr on these diseases could be investigated.

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