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Leptin increases L-type Ca^{2+} channel expression and GnRH-stimulated LH release in $\text{L}\beta\text{T2}$ gonadotropes

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Abstract

Leptin, a mediator of long-term regulation of energy balance, has been implicated in the release of adenohypophyseal gonadotropins by regulating gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. However, a direct effect of leptin on hormone release from gonadotropes remains virtually unexplored. In the current report, we assessed the long-term (48 h) actions of leptin on voltage-gated channel activity and luteinizing hormone (LH) production in mouse pituitary gonadotrope $\text{L}\beta\text{T2}$ cells. Electrophysiological recordings showed that leptin treatment significantly increased whole-cell patch clamp Ba^{2+} current through L-type Ca^{2+} channels. Quantitative RT-PCR analysis revealed increased levels of L-type (α_{1D}) Ca^{2+} channel mRNA. Likewise, radioimmunoassays using specific antibodies provided evidence that leptin alone had no effect on LH release but did enhance GnRH-induced secretion of the hormone. Leptin had no apparent effects on LH gene transcription in absence of GnRH, as measured by transient transfection assays using a LH promoter-reporter gene and real time RT-PCR. These observations suggest that leptin might affect LH release by acting directly on the gonadotropes, favoring hormone production by enhancing responsiveness to GnRH as a result of increased Ca^{2+} channel expression.

Keywords

Ca^{2+} channels; $\text{L}\beta\text{T2}$ cells; Leptin; Patch clamp; Pituitary; Gonadotropes

1. Introduction

The elucidation of the mutation that underlies the obese phenotype of the *ob/ob* mouse unveiled a 18 kDa peptide hormone named leptin (Zhang et al., 1994), which is mainly synthesized in

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adipose tissue and secreted into the bloodstream as a 16 kDa protein following cleavage of a 21 amino-acid signal sequence (Chehab, 2000; Friedman, 2000). Several studies have revealed that leptin plays an important role in regulating food intake, energy expenditure and adiposity (Friedman, 2000; 2002).

Although leptin receptors (OB-Rs) are mainly expressed in the central nervous system in afferent satiety centres of the hypothalamus (Funahashi et al., 2003), OB-R expression has been also documented in peripheral organs such as adipose tissues, skeletal muscles, pancreatic β -cells, and liver, thus suggesting autocrine and paracrine roles for leptin in diverse physiological events (Frühbeck, 2002). Interestingly, mutations in the genes coding either leptin (Strobel et al., 1998) or the OB-Rs (Clement et al., 1998) result in infertility. Consistent with this, it has been shown that leptin allows maturation of the hypothalamic-pituitary-gonadal axis and reconstitution of the defective reproductive system in the *ob/ob* mouse model (Chehab et al., 1996). Leptin may also act as a signal triggering puberty (Chehab et al., 1997; Yura et al., 2000). However, the mechanism(s) of leptin action in the reproductive system remain elusive. Studies have shown that intracerebroventricular (ICV) injections of leptin induce prepubertal secretion of LH (Dearth et al., 2000), whereas leptin antibodies inhibit the leptin-mediated stimulation of LH release (Carro et al., 1997), suggesting that leptin may exert a facilitative effect on the central networks that regulate pituitary gonadotropin secretion.

Likewise, diverse studies have documented leptin and/or OB-R expression in the adenohypophysis. Leptin mRNA and protein have been found in distinct cell types of the anterior pituitary (Popovic et al., 2001; Sone and Osamura, 2001). For example, leptin proteins have been found in subsets of corticotropes, somatotropes, gonadotropes, or thyrotropes in humans and rodents (Jin et al., 1999; Jin et al., 2000; Vidal et al., 2000), while the expression of leptin mRNA and protein has been detected in the rat pituitary (McDuffie et al., 2004; Morash et al., 1999). Likewise, mRNA transcripts coding OB-R have been found in several species and immunohistochemical studies have demonstrated OB-R protein expression in gonadotropes of the ovine anterior pituitary (Iqbal et al., 2000). Other investigations have described the presence of isoforms of the OB-R and leptin in normal and neoplastic human pituitary tissue (Jin et al., 1999). These findings indicate that leptin has the potential to regulate pituitary function via endocrine, paracrine and/or autocrine mechanisms. Indeed, *in vitro* studies have shown that incubation of rat anterior hemi-pituitaries or pituitary tumors with leptin leads to a dose-dependent release of gonadotropins (Yu et al., 1996). However, the mechanisms involved in the regulation of pituitary hormone secretion by leptin have not been investigated.

The present study was aimed at detecting OB-R expression in clonal gonadotrope L β T2 cells and to examine the direct effects of leptin on voltage-gated channel activity, as well as on luteinizing hormone (LH) secretion after stimulation by GnRH. Our findings suggest that leptin may directly and positively influence the functional expression of high-voltage activated L-type Ca^{2+} channels, which results in increased hormone release in gonadotropes through an amplification of the stimulatory actions of GnRH.

2. Materials and Methods

2.1. Chemicals

Leptin (Cat. # 300-27) was purchased from Peptotech (Rocky Hill, NJ) and GnRH (Cat. # 52-160) was from American Peptide Co. (Sunnyvale, CA). Tetrodotoxin (TTX; Cat. # Cat. T-550) was from Alomone Labs, Ltd. (Jerusalem, Israel). All other reagents, unless otherwise noted, were purchased from Merck or Sigma.

2.2. Cell culture and transfection

The L β T2 gonadotrope cell line (Pernasetti et al., 2001) was maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotic-antimycotic at 37 °C, in a 5% CO₂ atmosphere. Cells were harvested by trypsin-EDTA-treatment (0.05% w/v and 453 mM, respectively) and re-seeded in 24-well plates at a density of 1.5×10^5 cells per well. After 24 h, cells were switched to serum-free medium and transfected using FuGENE reagent (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. Each well was transfected with 160 ng of the reporter plasmid: -1800LH-luciferase. Additionally, as internal control, L β T2 cells were transfected with 4 μ g of a reporter plasmid β -galactosidase driven by the herpes virus thymidine kinase promoter. One day later, cells were incubated with leptin or GnRH for 6 h and then harvested in lysis buffer (100 mM KH₂PO₄, pH 7.8; 0.2% Triton X-100) to yield cell proteins. Equal volumes of the samples were assayed for luciferase activity (buffer contained 100 mM Tris-HCl (pH 7.8), 15 mM MgSO₄, 10 mM ATP, and 65 μ M luciferin) or β -galactosidase activity (Galacto Plus Kit, Tropix, Bedford, MA) (Pernasetti et al., 2001; Kelley et al., 2002). Activities of β -galactosidase and luciferase were measured using an EG&G Berthold microplate luminometer (Perkin Elmer Corp., Norwalk, CT). All experiments were performed in triplicate, repeated a minimum of three times, and reported as the ratio of luciferase to β -galactosidase activity.

2.3. Immunocytochemistry (ICC)

L β T2 cells were seeded at a density of $\sim 1 \times 10^5$ in 12-well culture plates and subjected to ICC using the Vectastain ABC Kit (Vector Labs Inc, Burlingame, CA) according to the manufacturer's instructions. Cells were fixed with 2% glutaraldehyde for 30 min, washed and quenched with 0.3% H₂O₂ for 30 min. After blockade with BSA (10%) for 2 h at room temperature ($\sim 22^\circ\text{C}$) and permeabilization with 0.1% Triton X-100, cells were incubated with goat polyclonal anti-mouse OB-R (1 μ g/ml) (Santa Cruz Biotechnology, Santa Cruz, CA). Next, cells were incubated for 30 min with the secondary antibody (0.66 ng/ml) (rabbit anti-goat biotinylated; Invitrogen). Finally, the cells were washed with PBS and the reaction was developed using the Vectastain ABC horseradish peroxidase substrate kit. Cells were visualized using a digital photcamera (Moticam1000) adapted to an inverted Leica microscope (Wetzlar, Germany).

2.4. Electrophysiology

Current- and voltage-clamp recordings were performed in L β T2 cells under control conditions or after 48 h treatment with leptin and/or GnRH, using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Foster City, CA) and acquired on-line using a Digidata 1322A interface with pClamp9 software (Molecular Devices). After establishing the whole-cell mode, capacitive transients were canceled with the amplifier. Leak and residual capacitance currents were subtracted on-line by a P/4 protocol. Current signals were filtered at 5 kHz (internal 4 pole Bessel filter) and digitized at 20-200 kHz. Membrane capacitance (C_m) was determined as described previously (Avila et al., 2004) and used to normalize currents. The recording solutions are given in Table 1. Experiments were performed at room temperature.

2.5. LH secretion

L β T2 gonadotropes were seeded a density of $\sim 1.5 \times 10^5$ in 24-well cell culture plates and incubated with leptin (30 nM) and/or GnRH (10 nM) for 48 h. LH release was detected and quantified in the supernatant by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. LH values were normalized by the amount of protein measured in each sample.

2.6. Quantitative PCR

The mRNA levels of the mouse Ca_v ion-conducting subunit $\text{Ca}_v1.3$ (GenBank accession number NM_028981) and the luteinizing hormone β (NM_008497) were measured by quantitative PCR assay as described elsewhere (Thackray and Mellon, 2008). RNA was obtained with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Contaminating DNA was removed with DNA-free reagent (Ambion, Austin, TX) and 1 μg RNA was primed with random hexanucleotides and reverse transcribed using Superscript III First strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed with the iCycler (Bio-Rad; Hercules, CA) using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and specific primers (Table 2), under the following conditions: 94 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 54 °C for 30 s, and 72 °C for 30 s. The equivalent of 50 ng of starting RNA (as quantified prior to reverse transcription) was used in each reaction. The expression of the TATA binding protein was used as internal control. Each sample was assayed in triplicate. In each experiment, the amount of transcript was calculated by comparing the threshold cycle obtained for each sample with the standard curve generated in the same run. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction.

2.7. Data analysis

Data are given as the mean $\pm$ S.E.M. Differences between two means were determined by Student's t tests. Dose-Response curve was fitted using the Hill equation while tail currents were fitted using SigmaPlot software (SSI, SJ California, USA.) as described previously (Dominguez et al., 2008).

3. Results

The cellular mechanisms by which leptin stimulate adenohypophyseal gonadotropin secretion are largely unknown. However, it is well established that basal and GnRH-induced gonadotropin release from pituitary gonadotropes is linked to the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Stojilkovic et al., 1989), which is mainly determined by Ca^{2+} influx via voltage-gated membrane channels (Tse and Hille, 1993). These channels are activated by cell depolarization and spontaneous action potentials (SAPs) firing. Therefore, in the present study, we initially investigated whether leptin might act by modifying the functional expression of ionic currents that give rise to SAPs, using the $\text{L}\beta\text{T}2$ gonadotrope cell line as a model (Thomas et al., 1996). First, we examined the expression of the OB-R using specific polyclonal antibodies. Immunocytochemical staining (Figs. 1A to 1C) showed that the vast majority of $\text{L}\beta\text{T}2$ cells were OB-R positive. To our knowledge, these data are the first to demonstrate the expression of the OB-R in the $\text{L}\beta\text{T}2$ gonadotropes.

Having established the presence of leptin receptors, we evaluated the pattern of SAP firing between $\text{L}\beta\text{T}2$ cells in the control condition and cells treated with leptin (30 nM) for 48 h. SAPs, recorded using solutions B1 and P1 (Table 1), were observed in both conditions (Fig. 1D). In the great majority of cells, the spontaneous electrical activity was characterized by the firing of single spikes. After each SAP, a pacemaker potential slowly depolarized the membrane potential toward the threshold (~ -31 mV) for the next spike, resulting in a rhythmic firing with a relatively low frequency (~ 0.6 Hz). The spike upstroke and repolarization were quite rapid limiting its duration to a few tens of msec. Interestingly, the amplitude of the SAPs was significantly decreased and the time at 90% repolarization was increased in leptin treated cells (Table 3). These data are consistent with previous results showing that pituitary gonadotropes exhibit spontaneous rhythmic action potentials (Tse and Hille, 1993; Van Goor et al., 2001) and provide, what is to our knowledge, the first evidence that chronic treatment with leptin may modify SAP in the $\text{L}\beta\text{T}2$ gonadotropes.

The next series of experiments were aimed at examining differences in the ionic conductances between control and leptin-treated cells that may account for the changes observed in the SAP. Figure 2A shows representative whole-cell patch-clamp current traces recorded in L β T2 cells, in response to 20 msec depolarizing steps from a holding potential (V_h) of -80 mV. Based on the recording conditions (solutions B1 and P1; Table 1) and on the kinetics of currents previously reported in pituitary and clonal gonadotropes (Tse and Hille, 1993; Van Goor et al., 2001; Bosma and Hille, 1992), the inward currents measured in L β T2 cells at early time points after start of depolarizing pulses most likely reflect a mixture of both Na $^+$ and Ca $^{2+}$ channel activity, whereas the outward current measured at the end of the depolarizing pulses may reflect K $^+$ currents. Interestingly, treatment of L β T2 gonadotropes with leptin induced a significant increase in inward current density (Fig. 2B) in a dose-dependent manner (Fig. 2C). These results suggest that treatment with leptin may increase functional expression of Na $^+$ and/or Ca $^{2+}$ channels. Increased expression of these proteins could be one mechanism by which leptin enhances hormone release in gonadotropes. To our knowledge, changes in Na $^+$ and/or Ca $^{2+}$ channel expression in response to leptin exposure have not been reported previously.

Under ionic conditions appropriate for recording of Na $^+$ channels (solutions B2 and P2; Table 1), depolarizing voltage steps from a V_h of -80 mV elicited fast transient inward currents in the L β T2 cells (Fig. 3A). These currents exhibited many of the characteristics of the Na $^+$ currents (I_{Na}) previously reported in pituitary and clonal gonadotropes (Tse and Hille, 1993; Van Goor et al., 2001; Bosma and Hille, 1992). Unexpectedly, treatment with leptin resulted in currents smaller than those recorded in control cells (Figs. 3A and 3B). The average peak amplitude of the I_{Na} normalized to cell capacitance decreased ~40% (at 0 mV) in relation to its control value (-18.22 ± 3.48 pA/pF to -10.47 ± 1.66 pA/pF). Changes in Na $^+$ channel functional expression may help to explain the alterations observed in SAP parameters after leptin treatment (Table 3). Likewise, under ionic conditions suitable for recording of K $^+$ currents (I_K ; solutions B4 and P4; Table 1) depolarizing voltage steps evoked long-lasting outward currents in the L β T2 cells (Fig. 3C). The properties of these currents remained unaltered after leptin treatment (Fig. 3D).

To assess whether the treatment with leptin is associated with a modification of voltage-activated Ca $^{2+}$ channel activity, we initially characterized whole cell currents using Ba $^{2+}$ as charge carrier (recording solutions B3 and P3; Table 1) in the L β T2 cells. When depolarizing pulses were carried out from a V_h of -80 mV, a sustained high voltage-activated (HVA) current was observed (Fig. 4A). This slowly inactivating Ba $^{2+}$ current (I_{Ba}) activated at >-40 mV and with a peak about 0 mV (Fig. 4B) and most likely corresponds to the activation of L-type Ca $^{2+}$ channels. Interestingly, the I - V relationship for the peak current shows that I_{Ba} density (at 0 mV) was significantly increased from -6.6 ± 1.3 in the control cells to -25.5 ± 6.1 pA/pF in the cells treated with leptin.

Likewise, it has been reported that the α T3-1 cell line (a less differentiated cell of the mouse gonadotrope lineage), in addition to HVA currents (through L-type channels), also expresses low voltage-activated (LVA) Ca $^{2+}$ currents that inactivate rapidly (Bosma and Hille, 1992). Therefore, we then investigated the potential role for leptin to regulate LVA and HVA currents in the L β T2 gonadotropes. To this end, we measured the amplitude of the maximum current (I_{peak}) and the current remaining at the end of the 200 msec step ($I_{sustained}$), to isolate the non-inactivating component, during a voltage step to 0 mV from a V_h of -80 mV (Fig. 4C). The result of this analysis showed that, other than a small reduction in magnitude, the effect of leptin on sustained current was similar to that of the peak current. To further investigate the effect of leptin treatment on "isolated" HVA currents, command pulses were delivered from a V_h of -40 mV. The rationale for evoking currents from a V_h of -40 mV is that LVA channels should be inactivated at this potential (Chen and Hess, 1990). Interestingly, leptin treatment resulted in a significant increase in the amplitude of I_{Ba} measured at its maximum (peak) value, which was very similar to that measured after pulses to 0 mV applied from a V_h -80 mV.

Together, these findings strongly suggest that the contribution of LVA channels to the whole cell Ba^{2+} current in L β T2 gonadotropes is negligible. On the other hand, these observations indicate that L β T2 cells, as male rat gonadotropes (Tse and Hille, 1993), express predominantly HVA Ca^{2+} channels, and support the idea that the L β T2 gonadotropes kept in culture for 48 h in presence of leptin (30 nM) exhibit a significant stimulation of HVA current density in comparison to the cells maintained in control conditions.

Further supporting evidence for the stimulatory effect of leptin on functional expression of Ca^{2+} channels was obtained from tail current analysis. When the membrane is repolarized to -80 mV after a 20 msec activating pulse to 0 mV, the inward current initially increases in amplitude, due to an increase in the driving force for the permeant ions, and then decays as the channels close or deactivate (Fig. 5A). Tail current recordings in Figure 5, corroborate that leptin treated L β T2 cells exhibit larger current densities in comparison to the cells maintained in culture in absence of leptin (Fig. 5B, *left panel*). Interestingly, leptin treatment seemed not to have a significant effect on the deactivation rate of the channels (Fig. 5B, *right panel*). An augment in current density with no apparent changes in the kinetics or in the voltage dependence of the currents is suggestive of an increase in the number of functional Ca^{2+} channels in the cell membrane.

An accurate analysis of L-type Ca^{2+} channel expression was important for assessment of leptin effects on gonadotrope function. HVA L-channels are oligomeric complexes formed by a main α_1 subunit that is the permeation pathway as well as three auxiliary subunits ($\alpha_2\delta$, β and γ) that modulate the properties of the Ca^{2+} currents (Hille, 2001; Felix, 2005). Molecular cloning has identified 4 different genes coding for L-type Ca^{2+} channel α_1 subunits (Ca v 1.1-Ca v 1.4), and we therefore designed primers (Table 2) for the α_1 subunit (Ca v 1.3) that may underlie L-type Ca^{2+} currents in gonadotropes and quantitatively assessed their levels of expression using real-time RT-PCR. As shown in Figure 5C, Ca v 1.3 mRNAs are expressed in control L β T2 gonadotropes, and as anticipated from the observed leptin-induced increase in Ca^{2+} currents, the levels of the mRNA for Ca v 1.3 were significantly up-regulated (~46%) after leptin treatment (Fig. 5C).

To further investigate the direct effects of leptin on gonadotropes, we determined whether the leptin signaling pathway was capable of regulating LH expression at the transcriptional level. To pursue this objective, we used the LH promoter fused to a luciferase gene reporter and assayed the effect of leptin. These experiments showed that leptin had no apparent effects on LH gene transcription in absence of GnRH (Fig. 6A). Consistent with this, measurement of LH mRNA levels in L β T2 cells by quantitative real-time PCR showed that the relative expression of LH mRNA was not significantly altered in the L β T2 cells after leptin treatment (Fig. 6B). In contrast, a ~1.5-2-fold increase in LH gene transcription and in mRNA levels for LH was observed when cells were treated with leptin plus GnRH (Fig. 6A and 6B), though the combination was not statistically different than GnRH alone.

Having documented the actions of leptin on voltage-gated Ca^{2+} channel expression, we lastly evaluated the functional capacity of the L β T2 gonadotropes by measuring LH released from cells cultured in standard conditions (control) and from cells challenged with leptin to investigate the potential actions of leptin as a LH secretagogue. As shown in Figure 6C, secretion of LH was significantly higher in cells exposed to GnRH, the physiological secretagogue for the hormone. In contrast, leptin alone did not induce an increase in LH secretion. Instead, leptin treatment seemed to reduce hormone release, though this was not statistically significant. However, when applied in conjunction with GnRH, leptin appeared to potentiate the stimulatory effect of the secretagogue. These findings suggest that the leptin-induced increase in LH secretion may not be the result of a direct stimulation on the gonadotrope. Instead, leptin may act as a facilitator of GnRH signaling.

4. Discussion

We present here the first study of the spontaneous electrical activity and the subjacent ionic currents in individual L β T2 clonal gonadotropes. Using this model system we have demonstrated that leptin can modulate these two cellular responses.

Previous electrophysiological studies have shown that pituitary gonadotropes express tetrodotoxin-sensitive voltage-gated Na⁺ channels and high voltage-activated Ca²⁺ channels of the L-type (Mason and Sikdar 1988; 1989; Tse and Hille, 1993; Heyward et al., 1995). Currently, there is a general agreement that opening of Na⁺ channels accounts for the rise of the spontaneous action potentials (SAP) which allow a voltage-gated entry of extracellular Ca²⁺ through L-type channels (Tse and Hille, 1993; Heyward et al., 1995; Kwiecien and Hammond, 1998). The basic function of SAP is to keep cells in a readily activable responsive state called the “pacemaker mode”, since it persists in the absence of stimulation (Kwiecien and Hammond, 1998). When challenged by GnRH, gonadotropes exhibit two responses: amplification of pacemaker activity or shift to internal Ca²⁺ release mode. In the second mode, [Ca²⁺]_i oscillations are initiated by release of Ca²⁺ from intracellular stores. It has been suggested that the pacemaker activity allows long-lasting sequences of [Ca²⁺]_i oscillations and sustained periods of hormone secretion. In contrast, the internal Ca²⁺ release mode is tailored to trigger secretory peaks of short duration (Kwiecien and Hammond, 1998).

In this regard, it is worth mentioning that the [Ca²⁺]_i responses in L β T2 and α T3-1 clonal gonadotropes are qualitatively similar: neither of the two cell lines shows the characteristic, large-amplitude [Ca²⁺]_i oscillations observed in pituitary gonadotropes at low GnRH concentrations. However, at higher concentrations of GnRH (≥ 10 nM), the [Ca²⁺]_i responses observed are reminiscent of the biphasic responses of normal gonadotropes (Thomas et al., 1996).

Interestingly however, in the L β T2 gonadotropes treated with leptin the electrical membrane activity was characterized by SAP of smaller amplitude and significantly decreased repolarization rate with respect to the untreated cells. To understand the underlying ionic mechanisms mediating these differences, we compared the ionic channel functional expression from control and leptin-treated L β T2 cells. Our results indicated that a similar assembly of ionic channels is expressed in absence or presence of leptin, though there were differences in the expression levels: K⁺ channel activity did not change after treatment, Na⁺ channels were down-regulated and HVA Ca²⁺ channels were significantly up-regulated in the treated cells. Interestingly, increased Ca²⁺ channel activity is consistent with a decrease in the repolarization rate, the most prominent alteration observed in SAP wave form recorded after leptin treatment. This change in SAP properties might be attributed to modifications in HVA channel activity given that, as has been reported for pituitary gonadotropes (Tse and Hille, 1993; Heyward et al., 1995), low voltage-activated Ca²⁺ channels might not be expressed in the L β T2 cells.

Alterations in SAP may have relevant implications in the physiology of gonadotropes. Previous studies have shown that extracellular Ca²⁺ influx is one of the fundamental events underlying the receptor-activated release of LH. As mentioned earlier, episodic release of LH by pituitary gonadotropes may be controlled by GnRH through the mobilization of Ca²⁺ from intracellular stores. Although GnRH and inositol 1,4,5-trisphosphate (IP3) can initiate Ca²⁺ oscillations in gonadotropes bathed in Ca²⁺-free medium (Stojilkovic et al., 1992; Tse and Hille, 1992), Ca²⁺ entry through plasma membrane L-type voltage-gated Ca²⁺ channels may provide an important route for the replenishment of the endoplasmic reticulum stores (Stojilkovic et al., 1992; Kukuljan et al., 1997). In addition, transient Ca²⁺ entry, as observed during SAP, may shift the phase of the [Ca²⁺]_i spikes (Vergara et al., 1995). Stojilkovic and colleagues have evaluated the influence of sustained changes in the rate of Ca²⁺ entry on the pattern of GnRH/

IP3-induced Ca^{2+} spiking in pituitary gonadotropes. Their results indicate that $[\text{Ca}^{2+}]_i$ may play important roles in the control of the oscillatory mechanism and kinetics of Ca^{2+} release (Kukuljan et al., 1997).

Although the possibility that leptin treatment has Ca^{2+} channel-independent, direct effects on other signaling pathways cannot be ruled out, based on our results and in the studies mentioned above we could speculate that the stimulatory action of leptin on HVA L-type Ca^{2+} current density may have a dual effect in LH secretion. First, enhanced Ca^{2+} channel activity might increase synthesis and release of LH associated to a larger entry of Ca^{2+} during SAP firing. In addition, increased Ca^{2+} channel activity might help to replenish the intracellular Ca^{2+} stores and act as a co-activator in the stimulation of intracellular Ca^{2+} release from the IP3-sensitive pools coupled with the response to GnRH (Kukuljan et al., 1997). It is worth noting however, that though the L β T2 cell line provides a very useful model of highly differentiated gonadotropes (Thomas et al., 1996), as mentioned earlier, some physiological responses might differ from that in pituitary gonadotropes.

Taken as a whole, our observations suggest that leptin might affect LH secretion acting directly on the gonadotropes favoring hormone release by enhancing responsiveness to GnRH most likely as a result of increased Ca^{2+} channel expression.

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Fig. 1.

Leptin receptor (OB-R) expression and alterations of spontaneous action potentials by leptin in LβT2 gonadotropes. A-C, Bright field visualization of the immunocytochemical OB-R labeling in LβT2 cells. The specific OB-R antibodies were omitted as a negative control (A), or applied at dilutions of 1 μg/ml (B) or 100 ng/ml (C). Bar scale represents 20 μm. D, Comparison of the SAP in control and leptin-treated cells as indicated. Traces shown are the average traces of recordings in 12 control and 10 treated cells. The analysis of the SAP properties is given in Table 3.

Fig. 2.

Leptin treatment enhances inward currents in L β T2 cells. *A*, Representative whole-cell patch clamp currents recorded in response to 20 ms command steps ranging -80 to +50 mV from a V_h of -80 mV in control (*upper panel*) and leptin-treated cells (*lower panel*). *B*, Current density-voltage relations of the inward currents recorded from L β T2 gonadotropes as in *A*. Peak inward current was measured within 1-2 ms of the onset of the voltage command. *C*, Inward current density values in response to increasing concentrations of leptin. Each data point is the mean $\pm$ S.E.M. and the number of recorded cells is shown in parentheses.

Fig. 3.

Chronic treatment with leptin differentially affects ion channels in L β T2 gonadotropes. **A**, representative whole-cell patch clamp Na⁺ currents recorded in response to 10 ms command steps ranging -80 to +40 mV from a V_h of -80 mV in control and leptin-treated cells. **B**, current-voltage relations of I_{Na} recorded from 10 control and 13 treated cells as in **A**. **C**, Representative voltage-gated I_K traces in gonadotropes elicited by 1.5 s voltage steps from -80 to +60 mV in 10-mV increments from a V_h of -120 mV. **D**, Comparison of I - V relationship of the peak (0-25 ms; *upper curves*), sustained (1.45-1.5 s; *lower curves*) and subtracted ($A - B$; *middle curves*) I_K in control and leptin-treated cells as indicated.

Fig. 4.

Leptin treatment enhances Ca^{2+} channel activity in $\text{L}\beta\text{T}2$ gonadotropes. **A**, Voltage-activated Ba^{2+} currents (I_{Ba}) through Ca^{2+} channels evoked by depolarizing pulses from a V_h of -80 mV with 10 mV intervals in cells kept in culture in absence or presence of leptin. **B**, Average current-voltage (I - V) relationships obtained from cells as in **A** and measured at the peak of each current trace. **C**, Bar chart summarizing the effect of the leptin treatment on I_{Ba} density determined in $\text{L}\beta\text{T}2$ cells by applying depolarizing pulses to 0 mV from a V_h of -80 or -40 mV, as indicated.

Fig. 5.

Leptin treatment increases HVA channel activity without altering the kinetics of the current. *A*, Ba^{2+} currents were elicited by 10 ms voltage steps from -80 to 0 mV. Tail currents were measured upon repolarization to V_h . The left trace shows the tail current obtained in a control cell in response to membrane repolarization, while the right trace corresponds to the current in a leptin-treated cell. *B*, Amplitudes of tail currents normalized by C_m (*left panel*) and mean deactivation time constants (*right panel*) obtained by fitting the tail currents with exponential functions. *C*, Quantitative real-time PCR results for $Ca_v1.3$ mRNA (Ca^{2+} channel mRNA levels were normalized to TBP mRNA).

Fig. 6.

Effects of treatment with leptin on expression of LH β mRNA transcription, and release of LH. **A**, Average luciferase activity in the lysates of L β T2 cells transiently transfected with the LH promoter linked to a luciferase reporter. Thymidine kinase- β -galactosidase reporter was co-transfected as an internal control. The cells were treated with leptin (30 nM) and/or GnRH (10 nM) as indicated and the luciferase activity was measured and normalized to β -galactosidase. **B**, Quantitative real-time RT-PCR results of LH mRNA levels normalized to TBP expression in untreated (control) and in leptin-treated L β T2 cells in absence or presence of GnRH as indicated. **C**, Average increase of LH released in response to treatment with leptin in the absence or presence of GnRH. In all cases, bars represent averaged data ($\pm$ S.E.M.) from 3 independent experiments. The asterisks denote significant differences ($p < 0.05$) between the indicated bars and the control.

Table 1
Recording solutions. Units are in mM. The pH was adjusted to 7.3 with NaOH (B1-B4), KOH (P1, P4) and CsOH (P2, P3)

Bath recording solutions														
	NaCl	CaCl ₂	KCl	MgCl ₂	KH ₂ PO ₄	MgSO ₄	NaHPO ₄	NaHCO ₃	HEPES	Glucose	CdCl ₂	TEA-Cl	BaCl ₂	TTX
B1	140	5	5.4	0.5	0.4	0.4	0.3	4.2	15	5				
B2	150	2							10	5	0.5			
3	128								10	5		10	10	0.001
B4	140	5	5.4	0.5	0.4	0.4	0.3	4.2	15	5	0.2			0.001
Internal (pipette) solutions														
			K-Asp	KCl	MgCl ₂	HEPES	Glucose	Na-ATP	GTP	CsCl	NaCl	CaCl ₂		
		P1	120	20	2	10	5	5	0.05				EGTA	
		P2			2	10	5	2	0.05	100	30	1	10	
		P3			2	10	5	2	0.05	130		1	10	
		P4	120	20	2	10	5	5	0.05					

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Table 2

Sets of primers designed to amplify genes from L β T2 gonadotropes.

Gene	Forward Primer	Reverse Primer
<i>Cacna1D</i>	CGCCTCTCTGCTGTTACTCC	CGATGGCCAAGAAGACATTC
<i>Lhb</i>	CTGTCAACGCAACTCTGG	ACAGGAGGCAAAGCAGC
<i>GAPDH</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

Table 3Effects of leptin on SAP properties of individual L β T2 gonadotropes.

Parameters	Control (<i>n</i> = 12)	Leptin (<i>n</i> = 10)
Threshold (mV)	-31.5 $\pm$ 0.3	-31.9 $\pm$ 0.4
Amplitude (mV)	54.6 $\pm$ 0.7	50.6 $\pm$ 1.5 *
Overshoot (mV)	7.9 $\pm$ 0.5	2.2 $\pm$ 1.0 *
Depolarization rate (mV/ms)	1.6 $\pm$ 0.06	1.6 $\pm$ 0.1
Time at 90% repolarization (ms)	111.3 $\pm$ 10.6	143.0 $\pm$ 10.3 *
Afterhyperpolarization (mV)	-46.7 $\pm$ 0.3	-48.5 $\pm$ 0.7 *
Frequency (Hz)	0.6 $\pm$ 0.05	0.6 $\pm$ 0.02

Abbreviations: mV, millivolts; ms, milliseconds; Hz, hertz. SAP threshold was determined as the membrane potential at the point where the rate of depolarization started to increase (changed two standard deviations) above the baseline rate. Slope was determined from the first derivative of the SAP trace. Amplitude was measured from the threshold to the peak. The rate of depolarization was calculated as the maximum positive slope value of SAP. Afterhyperpolarization measurement was made on visually identified subcomponents. Averaged data are expressed as means $\pm$ S.E. and non-paired Student's *t*-tests used for statistical evaluation. The asterisks denote significant differences ($p < 0.05$) as compared with the control cells.