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Ghrelin and Leptin increase voltage-gated Na^+ and Ca^{2+} currents in the RIN-m5f insulin-producing cell line

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β-pancreatic islet cells generate action potentials (AP) spontaneously or in response to different stimuli. This is orchestrated by the activity of diverse voltage-gated ion channels in the plasma membrane. Therefore, metabolic signals that modify the functional expression of these channels may alter β-cells excitability and in consequence the secretion of insulin. In particular, two hormones that regulate the voluntary food intake, Ghrelin and Leptin, may affect glucose serum concentrations by altering plasma insulin release. In this work, we use the insulin-producing RIN-M5f cell line to study the effects of the chronic treatment with Ghrelin or Leptin on the functional expression of voltage-gated Na^+ and Ca^{2+} channels. Cells were kept in culture and then subjected to patch-clamp electrophysiology. Our results show that chronic incubation with Ghrelin or Leptin (10 nM, 72 h) increased the frequency of the spontaneous APs and the macroscopic current density through Na^+ and Ca^{2+} channels. The analysis of the components of the Ca^{2+} currents showed that Ghrelin and Leptin differentially increased low- and high-threshold channels without causing apparent changes in current kinetics. The study of the electrophysiological mechanisms in insulin-secreting cells associated with the effects of Ghrelin and Leptin may shed light on the molecular processes involved in the endocrine/metabolic balance.

Keywords: Ca_V channels, Ghrelin, Insulin, Leptin, Na_V channels

Insulin secretion by β-pancreatic cells is essential in the control of blood glucose levels. Insulin is secreted by the β-cells of the pancreatic islets of Langerhans in response to elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This is produced by an influx of extracellular Ca^{2+} *via* voltage-dependent Ca^{2+} channels, whose activity, in turn, is regulated by the β-cell membrane potential. This means that electrical activity is critically important for insulin release[1]. Glucose, the main physiological regulator of insulin secretion, enters the β-cells through the GLUT2 (*Slc2a2*) membrane transporter. After glycolysis, pyruvate enters cycling routes in the mitochondria to produce $NADH^+$ and $FADH^+$, which transfer electrons to the respiratory chain producing ATP and increasing the ATP/ADP ratio in the cytosol[2]. An important aspect of ATP production it that leads, *via* K_{ATP} channel closure and initiating action potential (AP) firing, and causing the opening of voltage-gated Ca^{2+} (Ca_V) channels that allow the entry of Ca^{2+} into the cells[1,3,4]. The increase in $[Ca^{2+}]_i$ favors the mobilization of insulin granules towards the membrane and the consequent secretion of insulin[1,4].

Pancreatic β-cells express three types of Ca_V channels, and those of the L-type appear to be involved in the initial phase of insulin secretion[5-11]. Insulin acts on hepatocytes, adipocytes and striated myocytes to promote glucose uptake, but also has an anabolic activity that promotes the synthesis of glycogen, lipids, and proteins[12]. When the production or release of insulin to the circulatory system decreases or ceases, the individuals develop diabetes mellitus.

The control of insulin secretion occurs mainly by glucose, but there are other molecules involved in the energy balance that also contribute to its regulation including hormone and growth factors, among others[13]. Of particular interest for this work are Ghrelin and Leptin, two hormones involved in the control of food intake and body weight. Ghrelin acts

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through an increase in the secretion of neuropeptide Y (NPY) and the protein related to the agouti gene[14-17], and its secretion is stimulated in response to a negative energy balance, while its release and synthesis decrease under conditions of positive energy balance[18]. Under pathological conditions, the circulating levels of Ghrelin are reduced, in obesity, but they increase in malnutrition, cachexia and anorexia nervosa[19]. Leptin, on the other hand, is expressed mainly in white adipose tissue and acts as an indicator of the body energy reserves. Leptin also contributes to the regulation of the energy balance, the appetite and the control of body weight, as well as in the metabolism of lipids and carbohydrates[20,21]. Leptin has been proven to increase with increasing adiposity in humans and rodents[22]. Given that, the presence of leptin reduces food intake and body weight, elevated levels of leptin in obese persons is viewed as leptin resistance[23-25].

Previous studies of our group have shown that chronic treatment (≥48 h) with Ghrelin or Leptin may affect current density by increasing the number of Na_V and Ca_V channels in the plasma membrane in endocrine cells. Hence, Ghrelin or its synthetic analog GHRP-6 increases whole-cell inward current density, enhances AP firing frequency and facilitates GH secretion from clonal and pituitary somatotropes[26-29]. In addition, we have reported that Leptin enhances the functional expression of Na_V and Ca_V channels and modulates the AP firing pattern and the secretory properties of isolated gonadotropes[30]. Therefore, the main aim of the present report was to assess the potential effects of Ghrelin or Leptin chronic treatment on the properties of the macroscopic currents through Na_V and Ca_V channels in the RIN-m5f insulin-secreting cells. The investigation of the mechanisms underlying the actions of Ghrelin and Leptin on insulin-producing cells may be fundamental for a better understanding the physiological processes that are affected in endocrine/metabolic imbalance conditions as is the case of diabetes mellitus.

Materials and Methods

Cell culture

The rat (*Rattus norvegicus*) insulinoma cell line RIN-m5f (ATTC CRL-11605) from pancreas/islet of Langerhans[31], was kept in culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (Bio Wets), L-glutamine and 1% penicillin-streptomycin under an atmosphere of 95% air and 5% CO2, at 37°C. The RIN-m5F cell line is a clone derived from the RIN-m rat islet cell line, produce and secrete insulin. Once confluence was reached, cells were harvested by treatment with trypsin-EDTA and re-seeded in 35 mm diameter Petri dishes for treatment with 10 nM Ghrelin[27-29] or 10 nM Leptin[30] (American Peptide Co); and Ghrelin 10 nM plus 1 μM of D-Lys_3 GHRP-6[28] (ENZO Life Sciences) an antagonist of GHS-R1a, the Ghrelin receptor.

Electrophysiology

The RIN-m5F cells were subjected to electrophysiological recording using the patch-clamp technique in the whole-cell configuration in its two modalities, current and voltage clamp, as previously described[26-29]. In current clamp modality, control and treated cells were recording without current injection and two types of responses were observed: (i) Silent, a cell without spontaneous electrical activity, (ii) Spontaneous Action Potential (SAP), an active cell with spontaneous electrical activity firing. Recordings were carried out at room temperature (~22°C) and the micropipettes were made of borosilicate glass tubing. Recording micropipettes had resistances of ~4 MΩ once filled with the internal recording solution. Voltage and current-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices). After reaching the whole-cell configuration, currents were evoked by depolarizing pulses of variable amplitude and duration from a holding potential (Vh) of −80 mV applied at intervals of 1-10 s using a Digidata 1440A interface (Molecular Devices) controlled by a computer. Current signals were acquired at 10-100 μs and filtered at 5 kHz. Data was stored on the computer and analyzed using pCLAMP v10.1 and Sigma Plot v11.0 software. The leakage and capacitative currents were digitally subtracted offline. Series resistance was not compensated, but cells were excluded from further analysis if values changed by >20% during the course of the recording. Membrane capacitance (Cm) and Membrane resistance (Rm) was determined as described previously[26-29] and Cm was used to normalize currents. To block I_{Na}, 1 μM TTX was added to the external solution, to block I_{Ca2+} 0.5 mM $CdCl_2$ was added to the external solution and to block I_{K+} 100 mM CsCl was added to the internal solution. The composition of the recording solutions is given in (Table 1). The osmolarity was adjusted to 300 and 290 mOsmol/L for the bath (external) and pipette (internal) recording solutions, respectively.

Table 1 — Recording solutions

Solution	NaCl	KCl	$CaCl_2$	TEA-Cl	$BaCl_2$	CsCl	$MgCl_2$	ATP/GTP	$CdCl_2$	TTX	EGTA	Hepes	Glu	pH
$Total_e$	145	5	5									10	5	7.3 (NaOH)
Na_e	150		2						0.5			10	5	7.3 (NaOH)
Ba_e	133			10	10					0.001		10	5	7.3 (NaOH)
$Total_i$	30	100	1				2	2/0.05			10	10	5	7.3 (KOH)
Na_i	30		1			100	2	2/0.05			10	10	5	7.3 (CsOH)
Ba_i	30		1			100	2	2/0.05			10	10	5	7.3 (CsOH)

Note: $Total_e$, Na_e, Ba_e, bath solutions. $Total_i$, Na_i, Ba_i, pipette solutions. Units are in mM

Control and treated cells were rinsed with peptide-free culture medium and maintained in this medium for ~60 min before electrophysiological recording.

Insulin secretion

The amount of insulin secreted by the RIN-m5F cells was determined by the enzyme-linked immunosorbent assay (ELISA) using the rat insulin ELISA kit (Alpco) according to the manufacturer's recommendations. Cells were seeded in 24-well culture dishes at a density of $2x10^4$ cells per well and incubated for 72 h with a 10 nM Ghrelin or Leptin. Cells were then preincubated with Krebs-Ringer buffer containing (in mM) 115 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 mM $CaCl_2$, 24 $NaHCO_3$, 25 HEPES, and 0.1% BSA for 5 min at 37°C. The preincubation buffer was removed and replaced with Krebs-Ringer buffer containing a high KCl concentration (40 mM) for 10 min at 37°C.

Data analysis

Data were analyzed and plotted by the combined use of pCLAMP software (see above) and SigmaPlot software (SPSS, Chicago, IL). The One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means of the groups, followed by the Turkey multiple comparison tests for possible pairwise differences. A P value <0.05 was considered to be statistically significant. Data are presented as means ± SE unless otherwise specified. Curve fits were made using the nonlinear, least-squares fitting procedure included in the Sigma Plot program. For analyzing activation kinetics of I_{ca2+}, tail current decay was fitted according to the method of Matteson and Armstrong[32]. In brief, the first step consisted in fitting a least-squares exponential to the slow component of the tail current, and extrapolate this exponential to the beginning of the step. The fitted slow exponential was then subtracted out, and the remaining current was fitted with a least-squares exponential. To minimize possible errors, the initial amplitude of the tail current was taken as the amplitude of the fitted exponential measured 100 μs after the onset of repolarization. Conductance at each test potential was estimated by measuring tail current amplitude. Conductance-Voltage (G–V) curves for activation were fitted with a Boltzmann equation of the form: $G = G_{max}/(1 + \exp[(V_m - V_{1/2})/k]-1)$, where G_{max} is maximum conductance, V_m is the test potential, $V_{1/2}$ is the potential for half-maximal activation of G_{max} and k is a slope factor. For analyzing activation kinetics of I_{Na+}, conductance was calculated from I/V curves values and normalized to its maximal value, conductance in treated cells was normalized to their corresponding control. For analyzing voltage dependence of steady-state inactivation of I_{Na+}, currents were recorded after conditioning pulses of 1s duration, applied from a V_h of −100 mV in 10 mV steps, followed by a 20 ms test pulse to −10 mV. The normalized data were plotted against the conditioning potentials. Data points in the G-V and inactivation curves were fitted with Boltzmann equations. The number of analyzed cells is given in parentheses.

Results

Initially, we examined the SAP firing pattern in RIN-m5F cells under control conditions and after chronic treatment (72 h) with Ghrelin or the Leptin (10 nM). Figure 1 shows that ~69% of the control cells fire SAPs and that this was increased to ~78% in the cells treated with the two hormones without significant differences (P> 0.05) (Fig. 1A). In most control cells the firing frequency was relatively low (~1 Hz), while in treated cells this frequency was significantly (P< 0.05) increased to >3 Hz (Fig. 1A & 1B, Table 1). Figure 1C shows that the peak of the APs in the control cells was reached rapidly (~5 ms) and had amplitudes of ~57 mV. In contrast, in the cells treated with Ghrelin and Leptin, the electrical activity was characterized by SAPs significantly faster and larger (~3.4 ms and ~68 mV; Table 2).

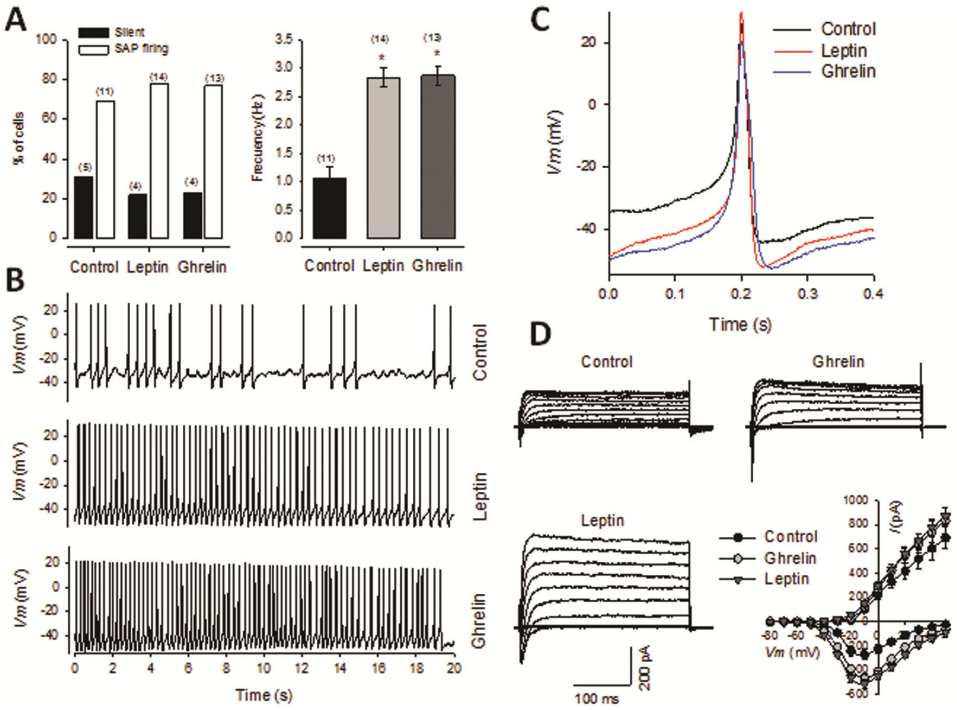

Fig. 1 — Chronic treatment with Leptin or Ghrelin affects the electrical activity of RIN-m5F cells. (A) Percentage of Silent and electrically active cells (left). The SAPs were recorded using the patch-clamp technique in modality Current Clamp without current injection (gap free) and the number of recorded cells is indicated in parentheses. The panel on the right shows the comparison of the SAPs firing frequency as in A; (B) Patterns of SAPs firing under control conditions and after hormone treatment (n=15-18); (C) Individual SAPs displayed on an expanded time scale recorded in control (black solid line) and treated with Leptin (red solid line) or Ghrelin (blue solid line). The analysis of the SAPs properties is shown in Table 2; and (D) Representative whole-cell patch clamp currents recorded in response to 300 ms command steps ranging −80 to +50 mV from a V_h of −80 mV in control and treated cells. *I-V* plots for total currents recorded from control (n=15) and treated with Leptin (n=17) or Ghrelin (n=16)

Table 2 — Membrane and action potential properties

	Control (10)	Leptin (12)	Ghrelin (11)
Membrane resistance (GΩ)	1.98 ± 0.41[a]	1.19 ± 0.19[b]	1.31 ± 0.33[b]
Membrane capacitance (pF)	17.01 ± 0.91[a]	18.06 ± 0.83[a]	17.02 ± 1.03[a]
Resting membrane potential (mV)	37.2 ± 3.4[a]	44.8 ± 2.53[b]	45.6 ± 2.41[b]
Peak amplitude (mV)	56.6 ± 0.4[a]	68.2 ± 0.4[b]	68.7± 0.5[b]
Time to peak (ms)	5.2 ± 0.05[a]	3.4 ± 0.06[b]	3.4 ± 0.06[b]
Half-amplitude (mV)	28.3 ± 0.2[a]	34.2 ± 0.2[b]	34.4 ± 0.2[b]
Maximum rise slope (mV/ms)	5.1 ± 0.1[a]	8.2 ± 0.1[b]	8.3 ± 0.1[b]
Maximum decay slope (mV/ms)	−3.9 ± 0.1[a]	−5.4 ± 0.1[b]	−5.5 ± 0.1[b]
Area (mV.ms)	558 ± 12[a]	460 ± 11[b]	466 ± 12[b]
Instantaneous frequency (Hz)	1.0 ± 0.2[a]	2.8 ± 0.1[b]	2.8 ± 0.1[b]

[a, b]Different literals between column of the same row are statistically significant ($P < 0.05$)
Note: Values expressed as mean ± SEM

We next investigated whether there were differences in the total ionic currents after the Leptin and Ghrelin treatments that could explain the changes in the SAP firing patterns in the RIN-m5F cells. Figure 1D upper panel shows a family of total currents in RIN-m5f cells under control conditions and treated with Leptin or Ghrelin where it is appreciated that the peptides increase the inward currents and outward currents. In lower right panel the current-voltage (*I-V*) relationships obtained using the whole-cell configuration of the patch-clamp technique in the absence and presence of the two hormones. In response to voltage steps of 20 ms ranging from −80 to +50 mV (in 10 mV step increments) from a V_h of −80 mV, an inward current of rapid activation and inactivation was observed, which was followed by a

non-inactivating outward component. The inward current was activated at membrane potentials (V_m) >−40 mV, while the outward component became evident at V_m >−10 mV. Based on the recording conditions (Table 1) and the kinetic analysis, it was inferred that the inward current corresponded to the activity of Na_V and Ca_V channels, while the outward current corresponded to the activation of K_V channels.

Interestingly, the chronic treatment of the RIN-m5F cells with Leptin or Ghrelin increased significantly (P< 0.05) the amplitude of the inward currents, without apparent effects on the outward current (not analyzed), in comparison with the controls (Fig. 1D). These results suggested that the hormone treatment may produce an increase in the functional expression of Na_V and Ca_V channels and might represent a plausible mechanism by which the hormones may alter SAPs firing. Although it has been shown that the effects of the acute treatment with Ghrelin on insulin release depend on $[Ca^{2+}]_i$ transient elevations with the participation of the L-type channels[28], to our knowledge, the increase in the functional expression of Na_V and Ca_V channels in response to the chronic treatment has not been reported previously.

Given that Leptin and Ghrelin increased the frequency of SAPs firing in the RIN-m5F cells, we next investigated whether the functional expression of Na_V channels was affected by the hormone treatment. As shown in (Fig. 2A), the application of voltage steps in the range of -80 to +50 mV (in 5-mV step increments) from a V_h of -80 mV resulted in inward currents that reached the peak in ~1 ms and that decayed also rapidly. After chronic treatment with Leptin or Ghrelin (10 nM, 72 h), the maximum Na^+ current (I_{Na}) amplitude increased significantly (30-42%) from a control value of 275 ± 46 to 392 ± 47 and 471 ± 60 pA, respectively (Fig. 2B). To eliminate the size of the cell as a variable source, the current value was divided by its respective capacitance value to obtain the current density (pA/pF), Leptin and Ghrelin increase significantly (P< 0.05) the current density (Fig. 2C).

After that, we investigated whether the effect of the Leptin and Ghrelin involved changes in the voltage dependence of the macroscopic I_{Na}. To this end, the

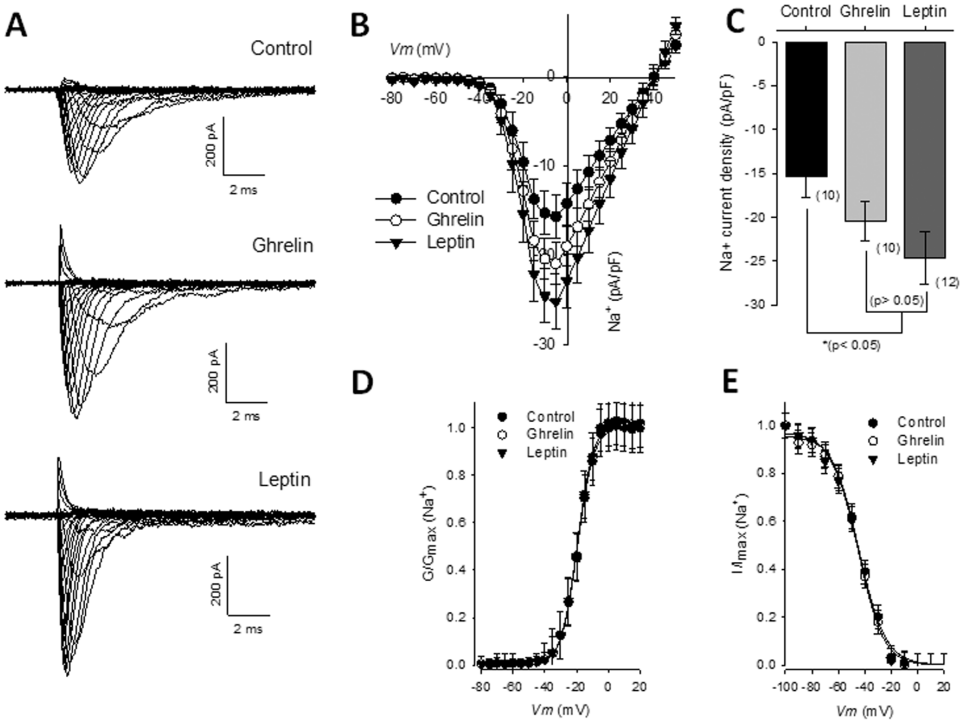

Fig. 2 — Chronic treatment with Leptin and Ghrelin increases the functional expression of Na_V channels in RIN-m5F cells. (A) Representative I_{Na} trace currents evoked by depolarizing pulses ranging −80 to +50 mV from a V_h of −80 mV in cells kept in culture for 72 h in the absence or presence of the hormones; (B) Comparison of the average *I-V* plots obtained in control (n=10) and cells treated with Ghrelin (n=10) or Leptin (n=12), as indicated; (C) Bar chart summarizing the effects of Ghrelin and Leptin on current densities, I_{Na+} were recorded at −10 mV from a V_h of −80; (D) Comparison of the normalized *G-V* plots estimated from the cells shown in B; data was fitted with Boltzmann equations; and (E) Comparison of normalized I_{Na+} steady-state inactivation plots in control and hormone-treated cells

currents in the three experimental conditions were normalized and the respective conductance-voltage (*G-V*) plots were generated. The results of this analysis showed that the position of the curves in the voltage axis was similar, suggesting that the activation of the channels was not altered by the hormone treatment (Fig. 2D). Here, it is worth recalling that alterations in G_{max} in the absence of changes in the voltage dependence or kinetics of the macroscopic currents are likely related to differences in the number of channels at the plasma membrane. In these experiments, the parameters describing the inactivation curves for the control condition were similar to the corresponding values obtained from cells treated with Leptin or Ghrelin (Fig. 2E).

After the analysis of the I_{Na}, a series of experiments were conducted to compare the Ca_V channel functional expression in the control condition and after chronic incubation with Leptin or Ghrelin. Figure 3A shows representative traces of the Ba^{2+} current (I_{Ba}) through Ca_V channels recorded in the control condition and in treated cells either with Ghrelin or its receptor antagonist [D-Lys3]-GHRP-6. The use of [D-Lys3]-GHRP-6 was to corroborate whether the effect showed by treatment of Ghrelin was to GHS-R1a activation and were used in combination with Ghrelin in a 1 μM concentration by the same exposition time. The analysis of the waveform and the *I-V* plots suggested the presence of two subtypes of channels (LVA and HVA) in the cell membrane of the RIN-m5F cells. Notably, in the presence of the Ghrelin receptor antagonist, the increase of I_{Ba} density was prevented (Fig. 3B).

We next explored whether the treatment with Ghrelin could differentially affect the two components of I_{Ba} in the RIN-m5F cells. Hence, we measured the amplitude of the remaining current at the end of a 300 ms step to isolate the non-inactivating component (HVA channels). Subsequently, this component was subtracted from the total current to thereby obtaining the rapidly inactivating component (LVA channels). Figure 3C shows that the density of the I_{peak} (at 0 mV)

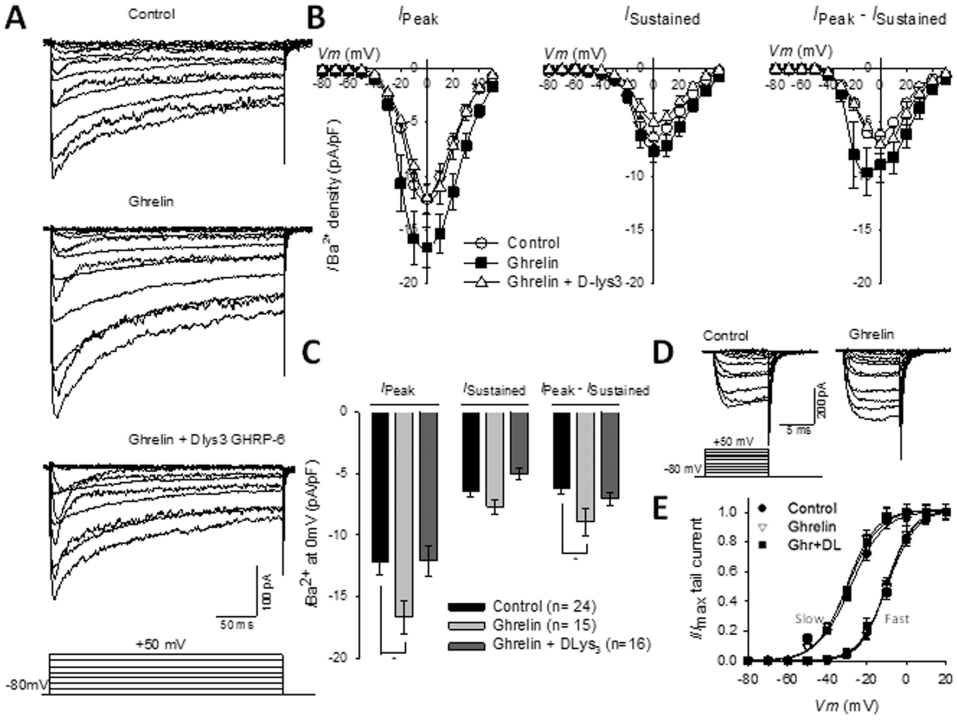

Fig. 3 — Ghrelin chronic treatment increases the functional expression of Ca_V channels in RIN-m5F cells. (A) Representative I_{Ba} traces in response to voltage steps of 300 ms ranging from −80 to +50 mV from a V_h of −80 mV in control and Ghrelin-treated cells; (B) Comparison of the *I-V* plots obtained from current (I_{peak}, $I_{sustained}$ and I_{peak}-$I_{sustained}$) recordings cells treated with the hormone as in A; (C) Comparison of the effect of the Ghrelin treatment on the total (I_{peak}), HVA ($I_{sustained}$) and LVA (I_{peak}-$I_{sustained}$) current densities obtained by applying depolarizing pulses to 0 mV from a V_h of −80 mV; (D) Representative I_{Ba} traces recorded in response to voltage steps of 10 ms ranging −80 to +40 mV from a V_h of −80 mV in control and Ghrelin-treated cells, in which the closing (deactivation) of the channels is observed immediately after returning V_m to the V_h level (tail currents); and (E) Comparison of the position of the normalized current plots in the voltage axis for the HVA and LVA currents (fast and slow components, respectively) estimated from the analysis of the tail currents. The data points were fitted with Boltzmann equations

increased significantly ($P< 0.05$) from –12.1 ± 1.2 pA/pF in the control cells to –16.6 ± 1.9 pA/pF in the cells exposed to Ghrelin. Likewise, the inactivating component was increased from –6.5 ± 0.6 pA/pF in the control cells to -7.9 ± 0.9 pA/pF in the cells incubated chronically with Ghrelin and the sustained component did not show changes significantly. We also corroborated the presence of the two components of I_{Ba} by analyzing tail currents. The traces in (Fig. 3D) confirm our observation that I_{Ba} is larger in the RIN-m5F cells incubated with the hormone in comparison to the controls. On the other hand, when the fast and slow components of the tail currents are normalized and plotted as a function of voltage, the position of the curves in the voltage axis was similar, corroborating that Ghrelin increases the amplitude of the currents without affecting the channel voltage dependence of activation (Fig. 3E).

The same voltage protocols were used then to investigate the effect of the chronic incubation with Leptin on Ca_V channel functional expression in the RIN-m5F cells. Figure 4A shows representative I_{Ba} traces recorded in control and Leptin-treated cells, where the presence of the two subtypes of the channels, LVA and HVA, was corroborated. As can be seen, the amplitude of the currents in cells treated with Leptin was significantly greater than those of the control cells at potentials ≥0 mV, suggesting a preferential effect of the hormone on the HVA channels (Fig. 4B). Consistent with this, the density of

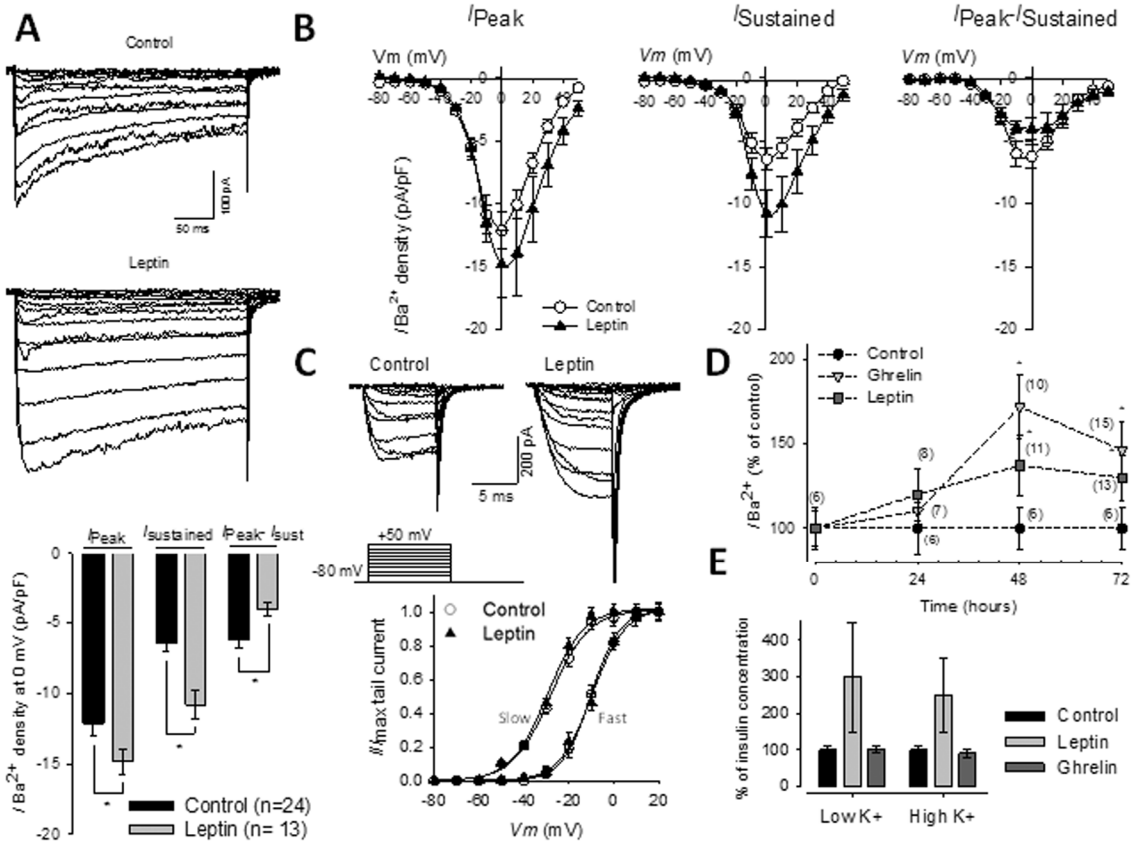

Fig. 4 — Chronic Leptin treatment affects the functional expression of Ca_V channels in RIN-m5F cells. (A) Representative I_{Ba} traces recorded in response to voltage steps of 300 ms ranging from −80 to +40 mV from a V_h of −80 mV in control and Leptin-treated cells. Lower panel, comparison of the effect of the Leptin treatment on the total (I_{peak}), HVA ($I_{sustained}$) and LVA (I_{peak}-$I_{sustained}$) current densities obtained by applying depolarizing pulses to 0 mV from a V_h of −80 mV; (B) Comparison of the *I-V* plots obtained from current (I_{peak}, $I_{sustained}$ and I_{peak}-$I_{sustained}$) recordings cells treated with the hormone as in A; (C) Representative I_{Ba} traces recorded in response to voltage steps of 10 ms ranging −80 to +50 mV from a V_h of −80 mV in control and Leptin-treated cells, in which the closing (deactivation) of the channels is observed immediately after returning V_m to the V_h level (tail currents); (D) Time course of the effect of Leptin and Ghrelin on I_{Ba} recorded at 0 mV from a V_h of −80 mV as a function of time in culture in the presence of the hormones. The asterisks indicate significant differences (P<0.05); and (E) Basal and high-K^+ induced insulin secretion in RIN-m5F cells after treatment with Leptin or Ghrelin. Cells were treated 48 h with the hormones and subsequently incubated with buffer containing 5 (low-K^+) or 40 mM (high-K^+) KCl. The insulin content in the supernatants was determined by ELISA. Data represent the mean ± SE of triplicates obtained in three independent experiments

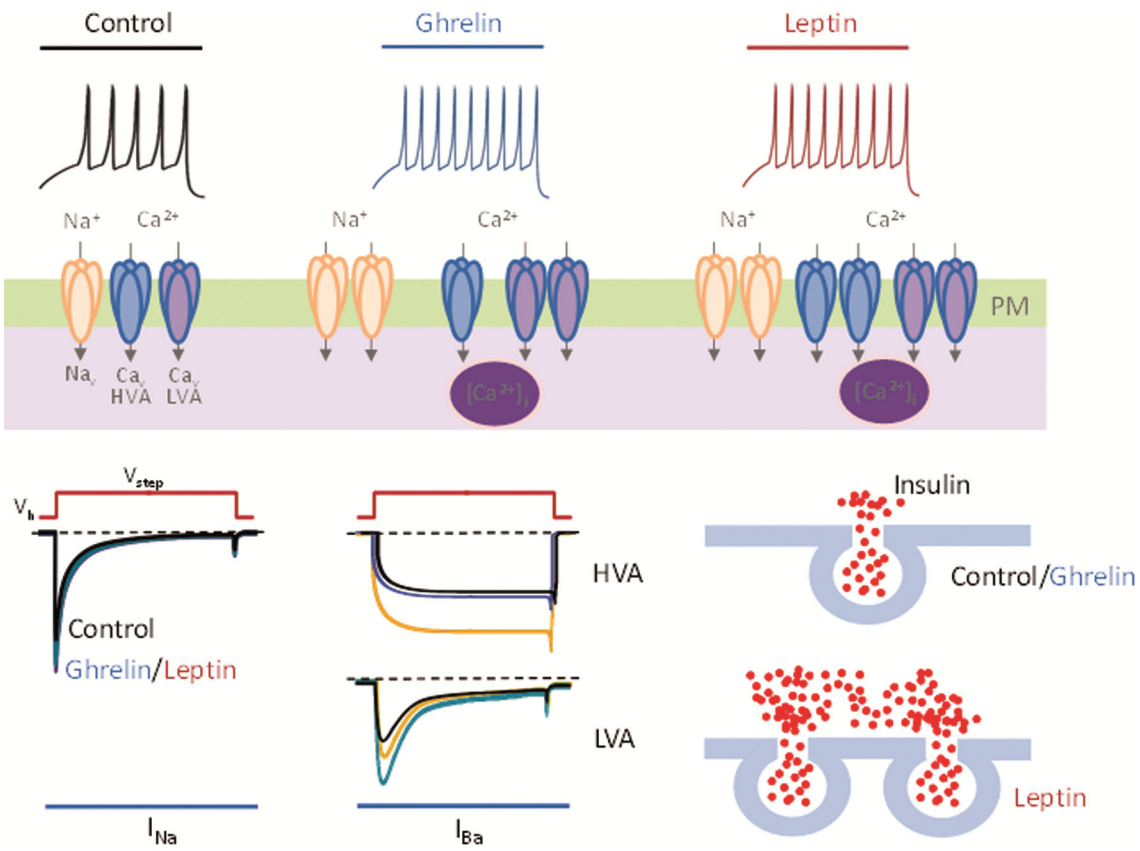

Fig. 5 — Action model of the effect of Ghrelin and Leptin on the electrical and secretory activity of β-pancreatic cells (RIN-m5F)

the sustained I_{Ba} component (at –10 mV) was significantly increased from –6.45 ± 0.59 pA/pF in the control cells to –10.79 ± 0.98 pA/pF in the Leptin-exposed cells. As observed from the results in the *I-V* plot, the inactivating component showed a decrease significantly in cells incubated with Leptin from −6.23 ± 0.49 pA/pF in the control cells to 4.02 ± 0.48 pA/pF in the Leptin-exposed cells. (Fig. 4A). We also corroborated the presence of the two components of I_{Ba} by analyzing tail currents as showed by Ghrelin. The traces in (Fig. 4C) confirm our observation that I_{Ba} is larger in the RIN-m5F cells incubated with Leptin comparison to the controls. The fast and slow components of the tail currents were normalized and plotted as a function of voltage, the position of the curves in the voltage axis was similar, corroborating that Leptin increases the amplitude of the sustained current component ($I_{sustained}$) without affecting the channel voltage dependence of activation (Fig. 4C).

The time course of I_{Ba} stimulation by Leptin, as well as by Ghrelin, was also investigated. Figure 4D shows that I_{Ba} amplitude, normalized with respect to the controls, was slightly increased within the first 24 h of treatment which became more evident gradually as a function of time, being statistically different and reaching a maximum at 48 h.

Last, we quantified the release of insulin from RIN-m5F cells kept in culture under control conditions and in presence of the hormones (10 nM) for 48 h. Figure 4E shows that Leptin -induced an increase in insulin secretion both basal (low K^+) and stimulated by the influx of Ca^{2+} evoked by membrane depolarization with high K^+ (40 mM for 30 min). Interestingly, insulin secretion was not affected in cells treated with Ghrelin.

In order to represent the effect of Ghrelin or Leptin on electrical and secretory activity of RIN-m5F cells, it was proposed to develop a model that showed the effect (Fig. 5.)

Discussion

The cellular and molecular mechanisms by which Ghrelin and Leptin may affect the function of insulin-producing cells are largely unknown. Given that the secretion of insulin is associated with elevations in $[Ca^{2+}]_i$ that are mediated by Ca^{2+} entry through Ca_V channels activated by cell membrane depolarization during SAPs firing, in the present report we investigated whether these hormones

modify the ionic currents that give rise to the SAPs in the insulin-producing RIN-m5F cells.

In the first instance, we examined the SAP firing pattern in RIN-m5F cells under control conditions and after chronic treatment with Ghrelin and Leptin. At non-stimulatory glucose concentrations, it has been reported that the mouse and human β-cell is hyperpolarized and electrically silent. The membrane potential can be as negative as −80 mV for mouse and −70mV for human, which is close to that predicted for a pure K^+ conductance. Increasing glucose to 5 mM depolarizes the β-cell to about −60 mV. In previous reports, electrical activity is not elicited until the glucose concentration exceeds 6 mM, which depolarises the membrane to between −60 and −50 mV[1]. In the present report, a concentration of 5 mM of glucose was used in the electrophysiological recording solutions (Table 1), which could result in modifications in the membrane potential of the cells in the different conditions. Control cells showed a resting membrane potential more depolarized than cells treated with the energy balance hormones, probably due to an increase in the potassium current observed in the total current data by treated cells.

The control cells fire SAPs and the frequency were increased in the cells treated. Rorsman and Ashcroft[1] have reported that in mouse and human β-cells have a highly distinctive glucose-dependent pattern of electrical activity. At glucose concentrations between 6-20 mM, this consists of short-lived bursts of action potentials, superimposed on a depolarized plateau and separated by repolarized electrically silent intervals, in human are shorter (~2 s) and more frequent than those of mouse. The action potentials observed in RIN-m5F did not show bursts of action potentials as shown by mouse and human β-cells, even though the record was maintained for 20 sec. Potential membrane values observed in the present report under control conditions are similar to observed in rat islets[33], in other reports indicate that rat β-cells generate oscillatory electrical activity very similar to that of mouse β-cells[34].

In most control cells, the firing frequency was relatively low while in treated cells this frequency was increased. The peak of the SAPs in the control cells was reached rapidly and had low amplitudes. In contrast, in the cells treated, SAPs significantly faster and larger characterized the electrical activity. These data indicate that RIN-m5F cells exhibit SAPs, and provide what is to our knowledge, the first evidence that the chronic treatment with Ghrelin or Leptin affects the properties of the APs in insulin-secreting cells, changing resting membrane potential to more negative values (Table 2).

In most excitable cells, the depolarizing phase of the action potential reflects activation of both voltage-gated Ca^{2+} channels and Na^+ channels, although the relative contribution of the Na^+ current varies between species and its contribution to control of insulin production has been previously documented[1]. Therefore, we investigated whether there were differences in the ionic conductances after the Leptin and Ghrelin treatments that could explain the changes in the SAP firing patterns in the RIN-m5F cells. The inward current was activated at membrane potentials (Vm) >−40 mV, while the outward component became evident at Vm >−10 mV. Based on the recording conditions and the kinetic analysis, it was inferred that the inward current corresponded to the activity of Na_V and Ca_V channels, while the outward current corresponded to the activation of K_V channels. Previous reports in mouse β-cells within intact pancreatic islets have shown TTX-sensitive voltage-gated Na^+ currents detectable at depolarizations above −50 mV, similar to values found in the present report. The maximum Na^+ current density is observed at -10 mV with a peak amplitude of 120 pA/pF[35]. Our data show that in RIN-m5F under control conditions has less Na^+ current density (15 pA/pF) at −10 mV than mouse β-cells, showing deference between species and the treatment with Ghrelin and Leptin hormones increase the Na^+ current density.

The chronic treatment of the RIN-m5F cells increased the amplitude of the inward currents and apparent effects on the outward current, in comparison with the controls. These results suggested that the hormone treatment may produce an increase in the functional expression of Na_V, Ca_V and probably Kv channels and might represent a plausible mechanism by which the hormones may alter SAPs firing. Although it has been shown that the effects of the acute treatment with Ghrelin on insulin release depend on $[Ca^{2+}]_i$ transient elevations with the participation of the L-type channels[36], to our knowledge, the increase in the functional expression of Na_V and Ca_V channels in response to the chronic treatment has not been reported previously.

Leptin and Ghrelin increased the frequency of SAPs firing in the RIN-m5F cells, an effect that is related to an increase in the density of the inward

macroscopic currents, we next investigated whether the functional expression of Na_V channels was affected by the hormone treatment. After chronic treatment with Leptin or Ghrelin, the maximum Na^+ current (I_{Na}) amplitude increased; in this same line of research, we investigated whether the effect of the Leptin and Ghrelin involved changes in the voltage dependence of the macroscopic I_{Na}. The results of this analysis showed that the position of the curves in the voltage axis was similar, suggesting that the activation of the channels was not altered by the hormone treatment. In mouse β-cells have been reported that the voltage dependence of activation is sigmoidal with a half-maximum at −20 mV[37], morphology and values similar to reported in this report. In the same way, it is worth recalling that alterations in G_{max} in the absence of changes in the voltage dependence or kinetics of the macroscopic currents are likely related to differences in the number of channels at the plasma membrane. Therefore, these results suggest a role for both hormones in the expression of Na_V channels at the cell surface of the RIN-m5F cells. The steady-state inactivation curves for I_{Na} provided support for this notion. Molecular mechanisms and cellular signaling pathways must be studied to determine how these hormones promote the upregulation Nav and Cav.

An increase in $[Ca^{2+}]_i$ is essential to trigger insulin release[38]. Ca_v is the primary conduit for extracellular Ca^{2+} entry. Their opening plays a critical role in the upstroke of the β-cell action potential and their blockade leads to inhibition of SAPs firing and insulin secretion[39]. For all this, a series of experiments were then conducted to analyze and compare the Ca_V channel functional expression in the control condition and after chronic incubation with Leptin or Ghrelin. These experiments were performed using a Na_V channel blocker (TTX 1 μM, Table 1) and Ba^{2+} as a charge carrier. The analysis of the waveform and the *I-V* plots suggested the presence of two subtypes of channels (LVA and HVA) in the cell membrane of the RIN-m5F cells. Interestingly, the peak current amplitudes in the cells treated with Ghrelin were greater than in those of the control cells at almost all voltages explored.

We next explored whether the treatment with Ghrelin could differentially affect the two components of I_{Ba} in the RIN-m5F cells. The procedure used for the separation of these current components was based on the fact that the LVA component inactivates faster than the HVA component[30-31]. Likewise, the inactivating component was increased in cells treated chronically with Ghrelin. That is, the I_{Ba} carried out by LVA Ca_v without significant changes in the non-inactivation component (HVA). When we corroborated the presence of the two components of I_{Ba} by analyzing tail currents. As occur in other endocrine cells, the slow and fast components indicate the closure of the LVA and HVA channels, respectively[28-30,40]. The results confirm our observation that I_{Ba} is larger in the RIN-m5F cells treated with the hormones in comparison to the controls, and Ghrelin increases the amplitude of peak component (I_{peak}) without affecting the channel voltage dependence of activation. It is important to note that there is no evidence of low threshold T-type I_{Ca} in mouse β-cells and T-type transcripts are only expressed at very low levels[41]. Our results show the existence of a component of Barium current with rapid inactivation reported previously[42]. To clarify is necessary to perform molecular biology experiments to determine LVA type calcium channel present in RIN-m5F cells membrane and analyze signaling pathways that explain the effect of Ghrelin on this protein. There is evidence that Ca^{2+} channel activity is regulated by glucose metabolism. Thus, the glucose metabolite glyceraldehyde increases Ca^{2+} channel activity by a protein kinase C (PKC)-dependent mechanism in RIN-m5F cells[43]. Probably the intracellular signaling pathway that uses Ghrelin[44].

The chronic incubation effect with Leptin on Ca_V channel functional expression in the RIN-m5F cells, suggest a preferential effect of the hormone on the HVA channels. Consistent with this, the density of the sustained I_{Ba} component was increased in the Leptin-exposed cells. Similar effects are reported in other cells[30].

There are different types of HVA calcium channels β-cells, they can be identified by their biophysical properties and the effects of selective inhibitors. L-type channels make the dominant contribution to the Ca^{2+} current of mouse β-cells (~50%)[1]. The presence of isradipine-sensitive L-type calcium channels in RIN-m5F cells has been reported[45]. Although other HVA calcium channels have been reported in RIN-m5F cells, L-type channels are primarily involved in insulin secretion[5-7,42]. Many are the intracellular signaling pathways that leptin uses to promote its effect in the tissues[25,46], exploring which

could be activated in pancreatic β-cells RIN-m5F is necessary to elucidate this point.

Respect to the time course of I_{Ba} stimulation by Leptin, as well as by Ghrelin, these data confirm that Leptin and Ghrelin regulate I_{Ba} density when chronically applied to RIN-m5F cells.

Last, the release of insulin from RIN-m5F cells kept in culture under control conditions and in the presence of the hormones to evaluate the functional repercussion of the treatment with Leptin and Ghrelin on the membrane currents and the electrical activity, showed different results between Ghrelin and Leptin. These findings suggest that Leptin, by altering both types of Ca_V channels, LVA and HVA, produces a stimulating effect on insulin secretion, in contrast to what is observed with Ghrelin that only increase the functional expression of the LVA channels. These data differ from previous findings indicating that Ghrelin increases $[Ca^{2+}]_i$ in β-pancreatic cells and thereby promotes insulin secretion[47], and with the idea that LVA channels may play a relevant role in the basal and glucose-stimulated insulin secretion[1,5-7,48]. It should also be mentioned that T-type channels not only contribute to the secretion of insulin but participate in determining the fluctuations in the membrane resting potential of endocrine cells that activate Na_V channels and generate APs[25,49-51].

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