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Ghrelin and GHRP-6 enhance electrical and secretory activity in GC somatotropes

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Abstract

It is well established that pituitary somatotropes fire spontaneous action potentials (SAP) which generate Ca^{2+} signals of sufficient amplitude to trigger growth hormone (GH) release. It is also known that ghrelin and synthetic GH-releasing peptides (GHRPs) stimulate GH secretion, though the mechanisms involved remain unclear. In the current report, we show that the chronic (96 h) treatment with ghrelin and GHRP-6 increases the firing frequency of SAP in the somatotrope GC cell line. This action is associated with a significant increase in whole-cell inward current density. In addition, long-term application of Na^+ or L-type Ca^{2+} current antagonists decreases GHRP-6-induced release of GH, indicating that the ionic currents that give rise to SAP play important roles for hormone secretion in the GC cells. Together, our results suggest that ghrelin and GHPR-6 may increase whole-cell inward current density thereby enhancing SAP firing frequency and facilitating GH secretion from GC somatotropes.

Keywords: Ca^{2+} channels; GC cells; Ghrelin; GHRP6; Growth hormone; Na^+ channels; Patch clamp; Pituitary; Somatotropes

Under basal conditions, the majority of pituitary cells *in vitro* including somatotropes display spontaneous action potentials (SAP) and intracellular Ca^{2+} transients that result from rhythmic Ca^{2+} entry through voltage-gated Ca^{2+} channels in the cell membrane [1,2]. The main function of these SAP is to maintain cells in a ''pacemaker mode''. In somatotropes, growth hormone (GH)-releasing hormone (GHRH) triggers the pacemaker mode in silent cells and amplifies it in spontaneously active cells [1,2]. Interestingly, homologous normal and tumoral cells display the same type of activity *in vitro* in the absence or presence of hypothalamic hormones [2].

The GC cell line, which is a subclone of the rat pituitary tumor GH_3 strain [3], represents a homogeneous *in vitro* model of somatotropes. Previous reports aimed to understand the mechanisms underlying the release of GH have shown that GC cells display cycles of regularly spaced SAP with concomitant intracellular Ca^{2+} transients [4] which has been proposed as an effective manner to maintain GH exocytosis. In parallel, the presence of voltage-gated Na^+ [4,5], L-type Ca^{2+} [4–6], and inward-rectifying K^+ channels [7] has been documented in this somatotrope cell line.

Likewise, previous studies have reported that in addition to GHRH, xenobiotic peptides derived from the Leu- and Met-enkephalins show GH secretagogue (GHS) activity [8]. Interestingly, the most potent peptide reported, GH-releasing peptide-6 (GHRP-6), has shown to increase GH secretion by a different pathway to GHRH. Signal transduction pathways activated by GHRH increase cAMP, whereas GHRPs increase intracellular Ca^{2+} concentration in pituitary somatotropes [9,10]. In addition, ghrelin, an acylated peptide hormone isolated from the gastrointestinal tract [11,12], has shown to stimulate GH release by somatotropes [10,13] owing to its ability to bind and

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activate the receptor of synthetic GHS. Experimental evidence suggests that ghrelin acts on somatotropes in a similar manner to that reported for GHRP-6 [10,12].

At present, there is a lack of information on the cellular mechanisms that mediate the stimulatory action of the synthetic and endogenous GHS-R ligands on GH release in native or clonal pituitary somatotropes. Therefore, in the current report the electrophysiological effects of GHRP-6 and the endogenous GHS-R ligand, ghrelin, were investigated using the rat pituitary GC somatotrope cell line as a model system. We sought to determine whether these peptides could influence the firing pattern of SAP and examine the contribution of voltage-gated channels (in particular Na^+ and L-type Ca^{2+} channels) to GHS-induced hormone release in these cells. Overall, our results suggest that GHRP-6 and ghrelin may influence GH secretion by enhancing whole-cell inward current density and increasing the capability of the GC cells to fire SAP.

Materials and methods

Chemicals. Ghrelin (Cat. 55-0-03A) and GHRP-6 (Cat. 52-1-80B) were purchased from American Peptide Company Inc. Tetrodotoxin (TTX; Cat. T-550) and BayK 8644 (Cat. #B-350) were from Alomone Labs, Ltd. All other reagents were purchased from Merck or Sigma.

Cell culture. The GC cell line (a generous gift of H. Martínez; UANL, Monterrey, Mexico) was maintained routinely as a monolayer in a complete MegaCell DMEM culture medium (Sigma–Aldrich, St. Louis, MO) supplemented with 3% fetal bovine serum (MP Biomedicals, Aurora, OH), and 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma–Aldrich). Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Once a week, cells were harvested by trypsin–EDTA (Sigma–Aldrich) treatment (0.05% w/v and 453 mM, respectively) and reseeded at densities of $2–2.5 \times 10^5$ cells per flask of 25 cm^2. For experimental purposes, cells were seeded into 35 mm culture dishes containing poly-L-lysine-coated glass coverslips (for electrophysiological recording) or into multi-well culture dishes (for immunocytochemistry and secretion assays) as detailed below. In these cases, the culture medium, with or without peptide additions, was replenished every day.

Immunocytochemistry. GC cells seeded in 24-well tissue culture plates were washed in PBS and subjected to immunocytochemistry using the Vectastain ABC Kit (Vector Laboratories Inc., Burlingame, CA), according to the manufacturers' instructions. Briefly, the attached cells were fixed with 2% glutaraldehyde in PBS for 30 min, washed, and quenched with 0.3% H_2O_2 for 30 min. After blockade with 10% bovine serum albumin for 120–180 min at room temperature (~22 °C), and permeabilization with 0.1% Triton X-100, GC cells were incubated with goat polyclonal anti-rat GH (1:500 dilution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA), or goat polyclonal anti-rat GHS-R antibodies (1:500 in PBS; Santa Cruz Biotechnology). The specificity of these antibodies was validated in our laboratory (data not shown). Next, cells were incubated for 30 min in presence of the secondary antibody (rabbit anti-goat biotinylated; Santa Cruz Biotechnology) diluted 1:500 in PBS. Finally, the cells were washed with PBS and the reaction was developed using the AEC (Red) horseradish peroxidase substrate kit (Zymed Laboratories Inc.).

Electrophysiology. Current- and voltage-clamp recordings were performed in GC somatotropes under control conditions or after 96 h treatment with ghrelin (10 nM) or GHRP-6 (100 nM), 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 10–100 kHz. Membrane capacitance (C_m) was determined as described previously [14] and used to normalize currents. The bath recording solution contained (in mM) 145 NaCl, 5 KCl, 5 $CaCl_2$, 10 Hepes, and 5 glucose (pH 7.3). The internal solution consisted of 100 KCl, 30 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 Hepes, 2 Na-ATP, and 0.05 GTP, and 5 glucose (pH 7.3). Experiments were performed at room temperature (~22 °C). Ghrelin and GHRP-6 treatment was designed to end 96 h after plating. After this time, control and treated cells were rinsed with peptide-free culture medium and maintained in this medium for ~60 min before membrane voltage or currents were measured.

GH detection. GC somatotropes were seeded in 6-well cell culture plates and incubated with ghrelin (10 nM) or GHRP-6 (100 nM) for 96 h. Release of GH was detected and quantified in the supernatant by means of a commercially available enzyme immunoassay (ELISA) kit (SPI Bio, Massy Cedex, France) utilizing the double-antibody sandwich technique [15], according to the instructions of the manufacturer. The color intensity of the reaction product (proportional to the GH concentration) was measured by spectrophotometry in a plate reader using a 450 nm filter (Stat Fax 2100, Awareness Technology Inc., Palm City, FL). Intensity values of the samples were compared with values in a standard curve using Sigma Plot 8.0 software package (Jandel Scientific). In each experiment we also studied whether or not the buffer used gave any non-specific reaction with the ELISA kit. None of these controls gave a positive result.

Data analysis. The data are given as means ± SE. Statistical differences between two means were determined by Student's t tests ($p < 0.05$).

Results and discussion

The cellular mechanisms by which ghrelin and GHRP-6 stimulate GH release are largely unknown. Given that basal and GHS-induced GH secretion in pituitary somatotropes is linked to elevations in the intracellular free Ca^{2+} levels (which is mainly determined by Ca^{2+} influx via voltage-gated channels that are activated by cell membrane depolarization and SAP firing) in the present study, using the GC cell line as a model, we have investigated whether ghrelin and GHRP-6 may be acting by modifying the functional expression of ionic currents that give rise to SAP. To this end, in an initial series of experiments we first examined the expression of GH and the GHS-R using well-characterized specific polyclonal antibodies. Immunocytochemical staining employing the ABC technique showed that the vast majority of cells (~99%) were GH and GHS-R positive (Fig. 1A), thus identifying the cells as somatotropes. Likewise, to evaluate the functional capacity of the GC somatotropes, we measured GH release from cells kept in standard conditions (control) and cells from cultures that were challenged with ghrelin (10 nM) for 96 h. As shown in Fig. 1B, secretion of GH was significantly higher in the cells chronically treated with ghrelin. This result provides evidence that ghrelin can indeed function as a GHS and may act by stimulating the secretion on the somatotrope GC cells directly. Interestingly, the presence of GHRH seems not to be necessary to mediate ghrelin effects on GH release.

Next, the pattern of SAP firing was compared between GC somatotropes in the control condition and cells treated for 96 h with the GHS. As can be seen in Fig. 2A, SAP firing was observed in ~76% of the control GC cells that were examined. Interestingly, this number was increased to ~85% in the treated cells. In most spontaneously active

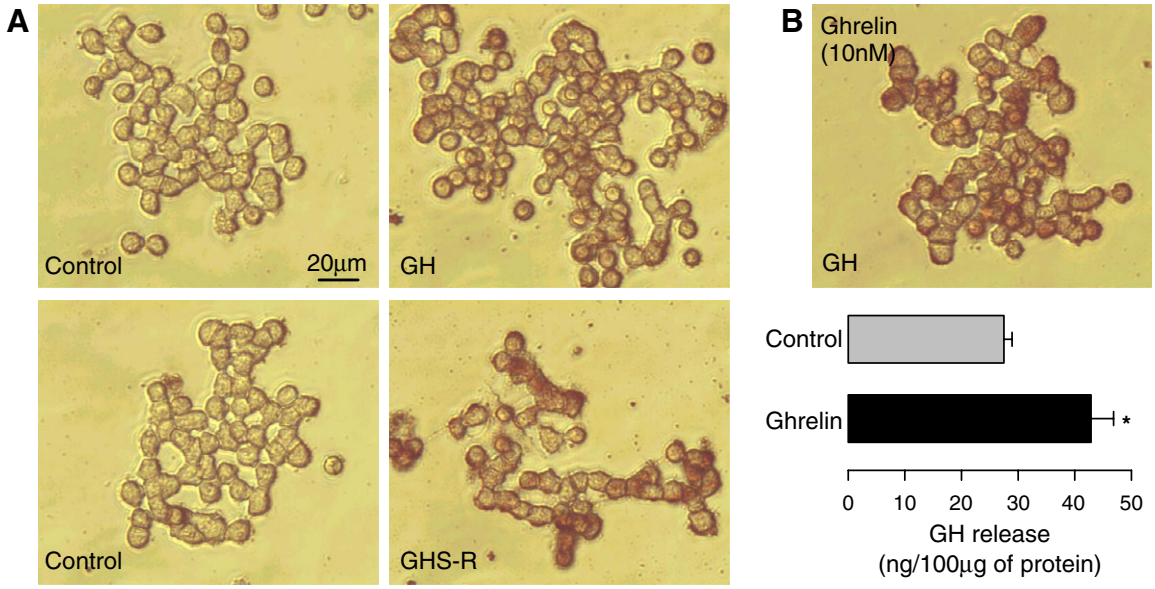

Fig. 1. GHS receptor expression and GH production in GC somatotropes. (A) Phase contrast photomicrographs showing detection of GH and GHS-R immunoreactivity revealed by the dark precipitate. (B) Phase contrast visualization of the immunohistochemical GH labeling in cells chronically treated with ghrelin (upper panel), and average increase of hormone release evoked by ghrelin treatment (10 nM) under control conditions. Bars represent averaged data (±SE) from 3 experiments. The asterisk denotes significant differences ($p < 0.05$) as compared with the untreated cells.

control GC somatotropes the electrical membrane activity was characterized by the firing of single spikes. After each SAP a pacemaker potential slowly depolarized the membrane potential toward the threshold (-30.9 ± 1.0 mV) for the next spike, resulting in a relatively low fire frequency (1.4 ± 0.2 Hz). Notably, the SAP frequency was increased to 2.2 ± 0.3 and 2.1 ± 0.3 in the cells treated with GHRP-6 and ghrelin, respectively (Fig. 2B and Table 1).

As illustrated in the representative traces shown in Fig. 2C, the spike upstroke in control GC cells was rapid (2.1 ± 0.3 mV/ms) and reached a total amplitude of 37.5 ± 1.8 mV. Spike repolarization was also relatively rapid (-1.4 ± 0.1 mV/ms), limiting its duration at one-half amplitude to 49.8 ± 4.0 ms. The interspike interval was characterized by a slow pacemaker depolarization from a baseline potential of -36.7 ± 2.7 mV and culminated in the initiation of another spike (Table 1). In contrast, in the treated GC somatotropes the electrical membrane activity was characterized by more frequent spikes with a threshold of -39.0 ± 1.5 and -36.9 ± 1.0 mV for GHRP-6- and ghrelin-incubated cells, respectively. Likewise, the amplitude of the SAP was significantly increased by ~20% in the GHS-treated cells (Table 1).

These data corroborate previous results indicating that pituitary GC somatotropes exhibited spontaneous rhythmic action potentials of relatively constant frequency in the absence of external stimulation [4,5], and provide what is to our knowledge the first evidence that chronic treatment with GHRP-6 or ghrelin can increase SAP amplitude, threshold, and firing frequency in GH-secreting cells.

The involvement of voltage-gated Na^+ and Ca^{2+} channels in controlling GH from somatotropes in primary culture has been extensively documented [16–21]. Therefore, we next examined whether there were differences in the ionic conductances between control and GHS-treated GC somatotropes that may account for the changes observed in the SAP firing patterns. Fig. 3A shows representative current traces recorded using the whole-cell configuration of the patch clamp technique in absence (control) and presence of ghrelin or GHRP-6. In response to 20 ms depolarizing steps ranging from −80 to +40 mV (with an increment of 10 mV between steps) from a holding potential of −90 mV, a rapid activating and inactivating inward current was observed. This current was followed by a non-inactivating outward component. The inward current measured at early times (2–5 ms) activated at membrane potentials >−40 mV (Fig. 3B, upper panel), whereas the outward current measured at late times (15–20 ms) became apparent at membrane potentials >−10 mV (Fig. 3B, lower panel). On the basis of the recording conditions (see Materials and methods) and the kinetics of voltage-gated ionic currents investigated previously in somatotropes [16–19], the inward currents measured at early time points after start of depolarizing pulses most likely reflect a mixture of both Na^+ and Ca^{2+} currents, whereas the current measured at the end of the depolarizing pulses may reflect K^+ currents. Interestingly enough, treatment of GC cells with ghrelin or GHRP-6 for 96 h induced a significantly increase in inward current density (Fig. 3B, upper panel). In contrast, there were no apparent differences in outward current density between cells treated with the GHS, compared with the controls (Fig. 3B, lower panel). The differences in inward current density observed in GHS-treated cells cannot be attributable to changes in cell size, since the mean cell capacitance (an index of the total plasma membrane area) was 11.65 ± 0.36 pF for control cells ($n = 73$),

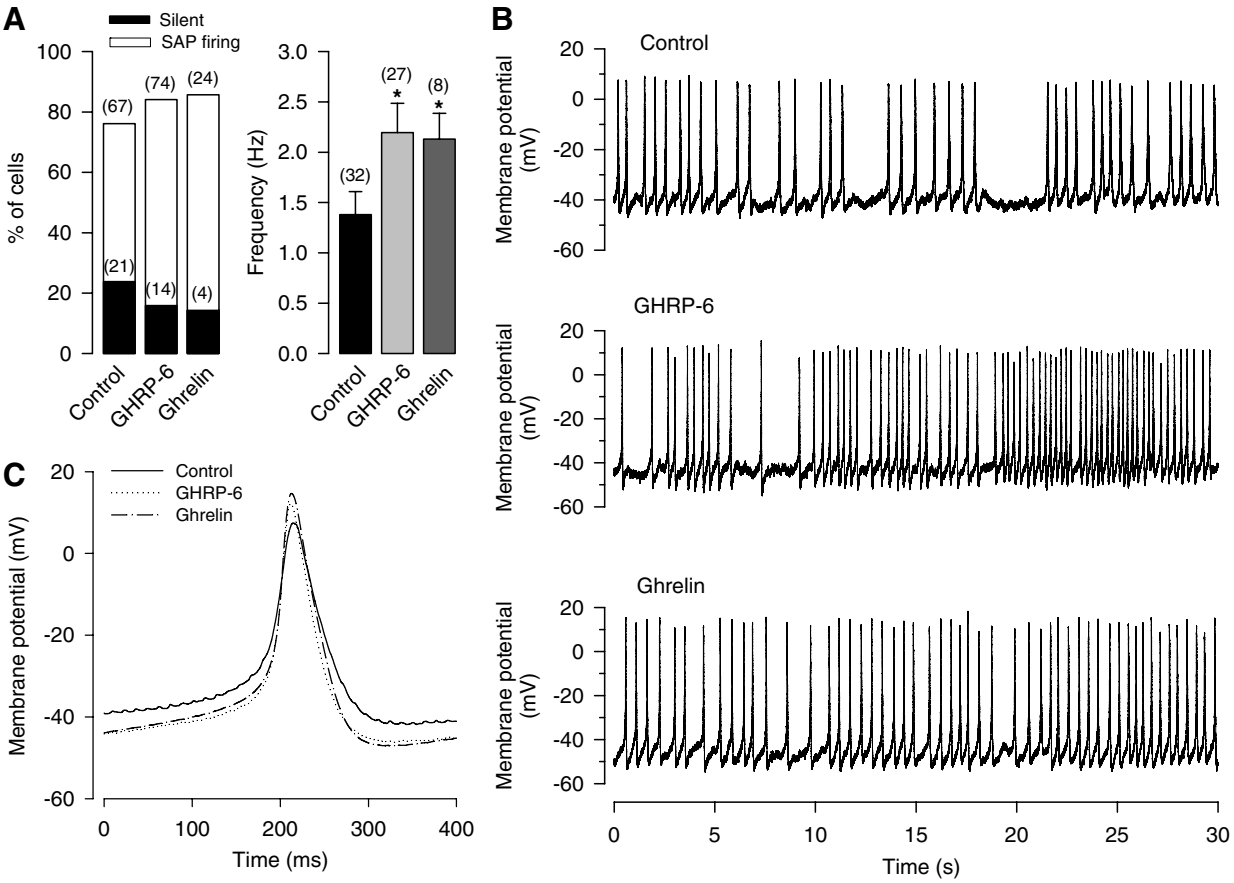

Fig. 2. Chronic treatment with ghrelin and GHRP-6 affects the spontaneous activity of GC somatotropes. (A) Left panel shows the comparison of the percentage of quiescent (solid bars) and SAP firing (open bars) cells. SAP were recorded using the whole-cell patch clamp technique in cells kept in culture in control conditions or treated with the GHS. The number of recorded cells is indicated in parentheses. The histogram in the right panel summarizes the frequency of SAP in control and GHS-treated cells. The number of recorded cells is indicated in parentheses, and the asterisks denote significant differences ($p < 0.05$). (B) Patterns of SAP in spontaneously active control and GHS-treated GC cells. Traces shown are representative of at least 6 recordings in six independent experiments. (C) Pattern of individual SAP (shown in expanded time scale) recorded in spontaneously active GC somatotropes in control (solid line), and ghrelin (dashed line) or GHRP-6 (dotted line) treated cells. The analysis of the SAP properties is given in Table 1.

Table 1
Effects of ghrelin and GHRP-6 on SAP properties of individual GC cells

Parameters	Control ($n = 10$)	GHRP-6 ($n = 27$)	Ghrelin ($n = 8$)
Membrane potential (mV)	-36.7 ± 2.7	-43.9 ± 1.1	-43.9 ± 1.6
SAP threshold (mV)	-30.9 ± 1	$-36.9 \pm 1.0^{*}$	$-39.0 \pm 1.5^{*}$
SAP amplitude (mV)	37.5 ± 1.8	$46.4 \pm 1.4^{*}$	$47.2 \pm 3.0^{*}$
SAP time-to-peak (ms)	58.3 ± 2.7	60.2 ± 3.2	62.7 ± 5.3
SAP depolarization rate (mV/ms)	2.1 ± 0.3	$3.3 \pm 0.3^{*}$	$3.1 \pm 0.6^{*}$
SAP repolarization rate (mV/ms)	-1.4 ± 0.1	-1.9 ± 0.2	-1.5 ± 0.1
SAP half-amplitude (ms)	49.8 ± 4.0	38.5 ± 2.8	39.8 ± 1.9
After hyperpolarization (mV)	-41.2 ± 1.0	$-45.2 \pm 1.2^{*}$	$-48.6 \pm 1.7^{*}$
SAP frequency (Hz)	1.4 ± 0.2	$2.2 \pm 0.3^{*}$	$2.1 \pm 0.3^{*}$

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 rates of depolarization and repolarization were calculated as the maximum positive and negative slop values of SAP, respectively. Time-to-peak was measured between threshold and peak. Duration was measured as the width at half-amplitude of SAP. After hyperpolarization measurement was made on visually identified subcomponents. Averaged data are expressed as means ± SE 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.

12.55 ± 0.57 ($n = 14$) and 11.45 ± 0.33 ($n = 58$) pF for ghrelin- and GHRP-6-treated cells, respectively. These results suggest that chronic treatment with ghrelin or GHRP-6 may lead to increased functional expression of Na^{+} and/or Ca^{2+} channels. Increased expression of these proteins could be one mechanism by which the GHS

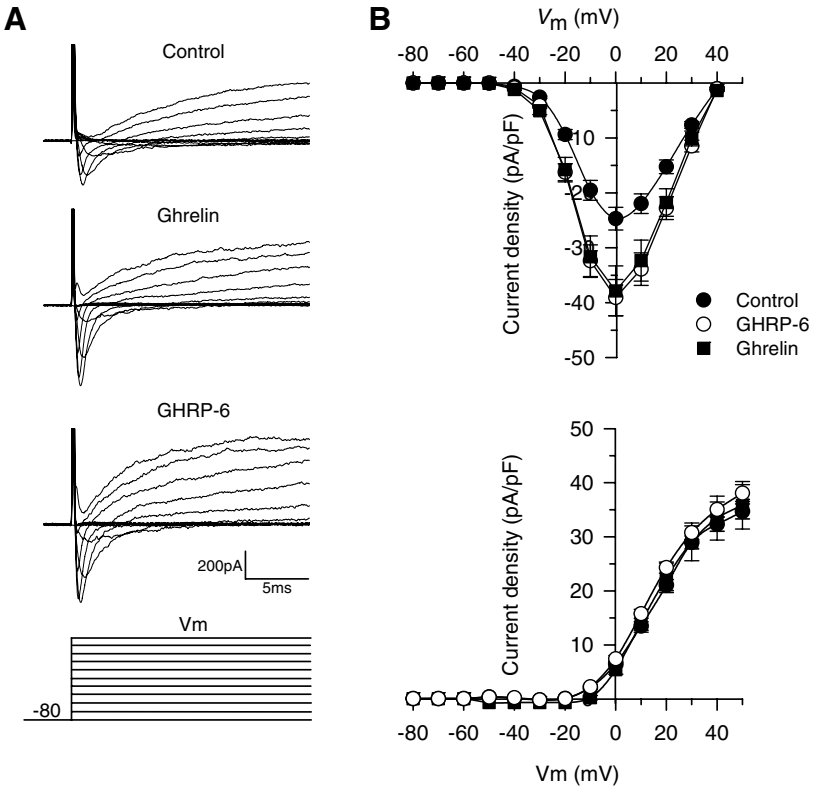

Fig. 3. Chronic treatment with ghrelin and GHRP-6 enhances membrane inward currents in GC somatotropes. (A) Representative whole-cell patch clamp currents recorded in response to 20 ms command steps ranging −80 to +40 mV from a holding potential of −90 mV in control and GHS-treated cells. (B) Current–voltage relations of the inward (upper panel) and outward (lower panel) currents recorded from 20 cells as in (A). Peak inward current was measured within 1–2 ms of the onset of the voltage command and outward current at the end of the voltage command.

enhances SAP firing and facilitates hormone release in GC somatotropes. Though the effects of acute treatment (1 min) with ghrelin on GH-release have been shown to depend on intracellular Ca^{2+} and extracellular Na^{+} and Ca^{2+} (with the possible participation of a voltage-gated L-type Ca^{2+} channel) [20], to our knowledge, increased Na^{+} and/or Ca^{2+} channel functional expression in response to chronic treatment with ghrelin or GHRP-6 has not been reported previously.

Lastly, to investigate the possible contribution of voltage-gated Na^{+} and Ca^{2+} channels to the GHS-mediated increase in hormone secretion by GC cells, we performed ELISA experiments using drugs that affect channel activity. In order to evaluate the role of Na^{+} channels, GC somatotropes were incubated for 96 h in control conditions and in presence of GHRP-6 alone or in combination with the selective channel antagonist tetrodotoxin (TTX; 1 μM). As expected, application of 100 nM GHRP-6 evoked an increase in GH release of ~32% (Fig. 4A). In contrast, hormone release in cultures treated with TTX was significantly smaller in response to the GHS compared with parallel run controls (20.3 ± 2.2 vs. 27.5 ± 1.5 ng of GH/100 μg of protein, respectively). Likewise, the presence of the toxin totally prevented the increase in GH secretion observed after GHRP-6 incubation (Fig. 4A) and has also an evident effect on basal hormone release. Qualitatively similar results were observed when cells were kept in culture in presence of ghrelin (data not shown). Though TTX-sensitive Na^{+} channels have been previously identified in somatotropes [19], our results extend this finding by showing that the expression of these channels is relevant for GHS-induced hormone secretion in GC cells.

Likewise, to investigate the contribution of voltage-gated Ca^{2+} channels in GH release evoked by GHS, experiments were performed in the presence of an antagonist (nifedipine), as well as of an agonist (BayK 8644) of L-type channels. As can be seen in Fig. 4B, application of 0.5 μM BayK 8644 in control cultures induced an increase of GH release of ~42%. In contrast, hormone release in nifedipine-treated cultures was significantly decreased compared with the control (14.9 ± 2.0 vs. 27.5 ± 1. 5 ng of GH/100 μg of protein, respectively). Chronic treatment with BayK 8644 had no apparent effects on the stimulatory action of GHRP-6, while nifedipine (0.5 μM) had a prominent effect and decreased the stimulatory action of the GHS (Fig. 4B), implying an involvement of the L-type Ca^{2+} channels in both basal and GHRP-6-induced GH release.

It is worth noting that GHRP-6-evoked GH release in presence of TTX or nifedipine was slightly larger than in the control condition. Though the results of the statistical analysis showed that the control and the experimental values are not different, the possibility exists that other

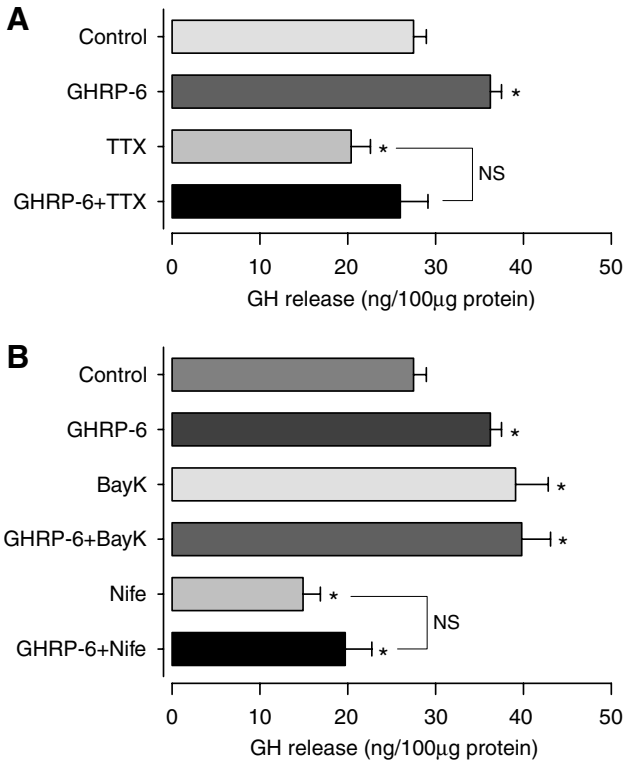

Fig. 4. Voltage-gated Na^+ and Ca^{2+} channel regulation affects the GHS-induced increase of GH release in GC somatotropes. (A) Bar graph illustrating the regulation of hormone release by GHRP-6 (100 nM) applied alone or in presence of the Na^+ channel selective antagonist TTX (1 μM). (B) Average increase of hormone release evoked by GHRP-6 treatment under control conditions and in presence of either an agonist BayK 8644 (BayK) or an antagonist nifedipine (Nife) of the L-type Ca^{2+} channels. Each value represents the mean ± SE of determinations performed in triplicate from three independent experiments. The asterisks denote significant differences ($p < 0.05$) as compared with the control untreated cells. NS, non statistically significant.

mechanisms (such as the release of Ca^{2+} from intracellular stores) may be involved. Though the role of Ca^{2+} release from intracellular stores in response to ghrelin may be more suited for acute effects, investigating the effects of ghrelin on intracellular Ca^{2+} channels is an interesting topic for future studies evaluating the long-term actions of the hormone.

Previous studies have revealed that acute incubation with GHS may regulate voltage-gated Na^+ [17] and Ca^{2+} [16,17,21–23] channel currents and may affect SAP frequency [17–19] in GH-secreting cells. However, the involvement of these channels in the long-term regulation of electrical membrane activity and GH release by GHS has not been explored. Therefore, our studies unveiling a role for Na^+ and Ca^{2+} channels in the ghrelin and GHRP-6-induced increase in GH release is also a novel finding.

Taking as a whole, the results presented allow us to speculate that the long-term regulation of Na^+ and Ca^{2+} channel functional expression induced by ghrelin and GHRP-6 may produce an enhancement of excitability that could in turn promote GH secretion by pituitary GC somatotropes. This mechanism may contribute to a better understanding of GHS-evoked hormone release in GH-secreting cells.

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